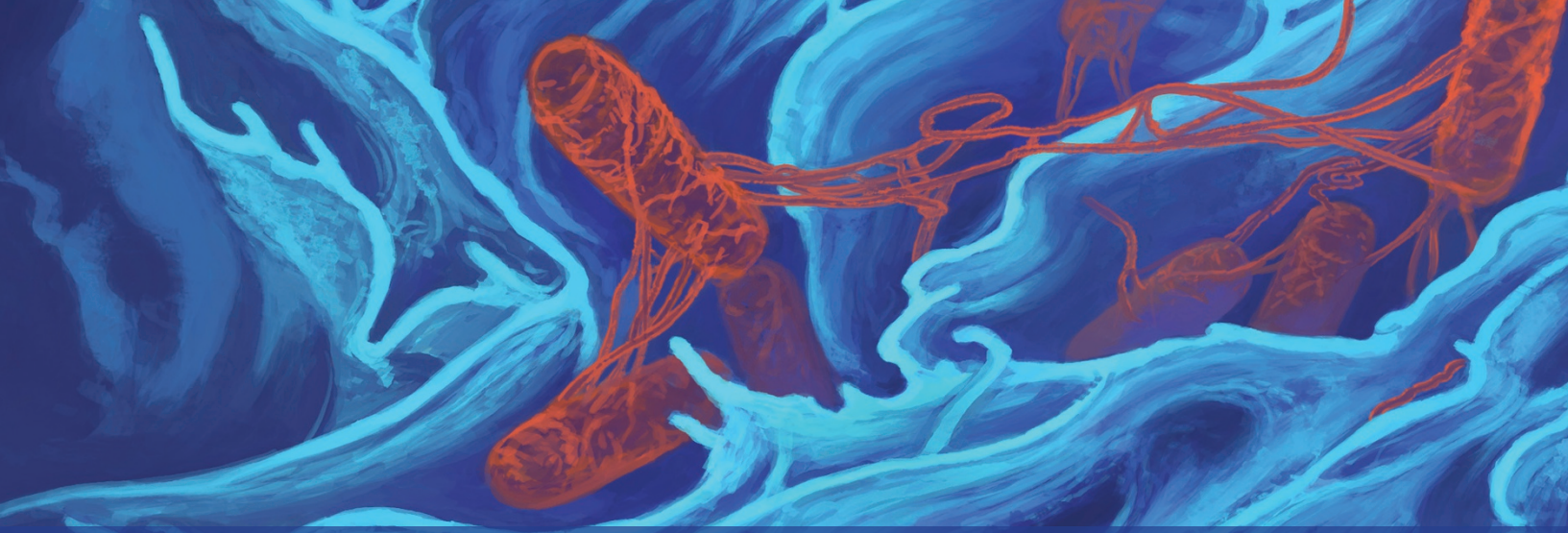


Fourth Edition

Veterinary Microbiology

Edited by **D. Scott McVey, Melissa Kennedy, M.M. Chengappa,** and **Rebecca Wilkes**

with website

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Veterinary Microbiology

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Fourth Edition

Editors

D. Scott McVey, DVM, PhD, DACVM
Director and Professor, School of Veterinary Medicine and Biomedical Sciences
Associate Dean, Iowa/Nebraska Program for Professional Veterinary Medicine
College of Agricultural Sciences and Natural Resources, Institute of Agriculture and Natural Resources
University of Nebraska Lincoln
Lincoln, NE, USA

Melissa Kennedy, DVM, PhD, DACVM
Professor Emeritus
Department of Biomedical and Diagnostic Sciences Department
College of Veterinary Medicine
University of Tennessee
Knoxville, TN, USA

M.M. Chengappa, BVSc, MVSc, MS, PhD, DACVM
University Distinguished Professor
Diagnostic Medicine Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, KS, USA

Rebecca Wilkes, DVM, PhD, DACVM
Associate Professor and Section Head, Molecular Section
Animal Disease Diagnostic Laboratory
Department of Comparative Pathobiology
College of Veterinary Medicine
Purdue University
West Lafayette, IN, USA

WILEY Blackwell

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Editorial Office
111 River Street, Hoboken, NJ 07030, USA

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List of Contributors

Mohamed A. Abouelkhair, DVM, MS, PhD, DACVM, CABMM
Assistant Professor of Virology and Immunology
and Director, Virology Diagnostic Laboratory
University of Tennessee Veterinary Medical Center
College of Veterinary Medicine
Biomedical and Diagnostic Sciences
University of Tennessee
Knoxville, Tennessee
United States

Michael D. Apley, DVM, PhD, DACVCP
Frick Professor
Department of Clinical Sciences
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Jonathan Arzt, DVM, MPVM, PhD, DACVP
Research Veterinary Medical Officer
Foreign Animal Disease Research Unit
Plum Island Animal Disease Center
Agricultural Research Service, USDA
Greenport, New York
United States

Udeni B.R. Balasuriya, BVSc, MS, PhD, FSLCVS
Professor of Virology, Department of Pathobiological Sciences
Director, Louisiana Animal Disease Diagnostic Laboratory (LADDL)
Director, LSU Biosafety Level 3 Core Facility
School of Veterinary Medicine
Louisiana State University
Baton Rouge, Louisiana
United States

Raul G. Barletta, PhD
Professor
School of Veterinary Medicine and Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Brian Bellaire, PhD
Assistant Professor
Department of Veterinary Microbiology and Preventative Medicine
College of Veterinary Medicine
Iowa State University
Ames, Iowa
United States

Paola M. Boggiatto, DVM, PhD
Veterinary Medical Officer
Infectious Bacterial Diseases Research Unit
National Animal Disease Center
USDA/Agricultural Research Service
Ames, Iowa
United States

Manuel V. Borca, DVM, PhD
Lead Scientist and Research Veterinary Medical Officer
USDA Agricultural Research Service
Plum Island Animal Disease Center
Foreign Animal Disease Research Unit
Greenport , New York
United States

Bruce W. Brodersen, DVM, PhD
Professor and Director
Nebraska Veterinary Diagnostic Center
School of Veterinary Medicine and Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Mariano Carossino, DVM, PhD, DACVM
Assistant Professor of Veterinary Pathology
Department of Pathobiological Sciences and Louisiana Animal Disease Diagnostic Laboratory
School of Veterinary Medicine
Louisiana State University
Baton Rouge, Louisiana
United States

Natalia Cernicchiaro, DVM, MS, PhD
Associate Professor of Epidemiology
Center for Outcomes Research and Epidemiology
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Kyeong-Ok Chang, DVM, MS, PhD
Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Christopher C.L. Chase, DVM, MS, PhD, DACVM
Professor
Department of Veterinary and Biomedical Sciences
South Dakota State University
Brookings, South Dakota
United States

M.M. Chengappa, BVSc, MVSc, MS, PhD, DACVM
University Distinguished Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Lee W. Cohnstaedt, PhD
Research Epidemiologist
USDA Agricultural Research Service
Foreign Arthropod-Borne Animal Diseases Research Unit
Manhattan, Kansas
United States

William B. Crosby, BS, DVM
Graduate Research Assistant
Department of Pathobiology and Population Medicine
College of Veterinary Medicine
Mississippi State University
Starkville, Mississippi
United States

Charles Czuprynski, PhD, DACVM (hon.)
Professor of Microbiology
Department of Pathobiological Sciences
School of Veterinary Medicine
University of Wisconsin
Madison, Wisconsin
United States

Joshua B. Daniels, DVM, PhD, DACVM
Associate Professor
Department of Veterinary Clinical Sciences
College of Veterinary Medicine and Biomedical Sciences
Colorado State University
Fort Collins, Colorado
United States

Gustavo A. Delhon, MV, MS, PhD
Research Professor
School of Veterinary Medicine and Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Barbara Drolet, MS, PhD
Research Microbiologist
USDA Agricultural Research Service
Center for Grain and Animal Health Research
Arthropod-Borne Animal Diseases Research Unit
Manhattan, Kansas
United States

Gerald E. Duhamel, DVM, PhD, DACVP
Professor of Anatomic Pathology
New York State Animal Health Diagnostic Center and Section of Anatomic Pathology
College of Veterinary Medicine
Cornell University
Ithaca, New York
United States

Bonto Faburay, DVM, MS, PhD
Section Head and Supervisory Veterinary Medical Officer
USDA Animal and Plant Health Inspection Service
Plum Island Animal Disease Center
Foreign Animal Disease Diagnostic Laboratory
Greenport, New York
United States

Bradley W. Fenwick, DVM, MS, PhD, DACVM
Senior Vice President for Open Science and Innovation
Taylor and Francis
London
United Kingdom

Timothy Frana, DVM, MS, MPH, PhD, DACVPM, DACVM
Boehringer Ingelheim Animal Health
St. Joseph , Missouri
United States

Jean-Pierre Frossard, BS (hons), MS, PhD
Emerging Virus Research Unit Head
Emerging Virus Research Unit
Animal and Plant Health Agency (APHA) Weybridge
Surrey
United Kingdom

Roman R. Ganta, MSc, PhD
University Distinguished Professor
Director, Center of Excellence for Vector-Borne Diseases
Fellow of the Conference of Research Workers in Animals Diseases (CRWAD)
Fellow of the Association of Biotechnology and Pharmacy (FABAP)
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Laurel J. Gershwin, DVM, PhD, DACVM
Distinguished Professor
Department of Pathology, Microbiology, and Immunology
College of Veterinary Medicine
University of California
Davis, California
United States

Yun Young Go, DVM, MS, PhD, DACVM
Assistant Professor
Department of Infectious Diseases and Public Health
Jockey Club College of Veterinary Medicine
City University of Hong Kong
Hong Kong SAR
China

Iman Mehdizadeh Gohari, DVM, MSc, PhD
Department of Microbiology and Molecular Genetics
University of Pittsburgh
Pittsburgh , Pennsylvania
United States

Nicholas J. Haley, DVM, PhD
Associate Professor
Department of Microbiology and Immunology
College of Graduate Studies
Midwestern University
Glendale, Arizona
United States

Seth P. Harris, DVM, PhD, DACVP
Professor
Nebraska Veterinary Diagnostic Center
School of Veterinary Medicine and Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Peter C. Iwen, MS, PhD, D(ABBM)
Director, Nebraska Public Health Laboratory
and University Professor and Biosafety Officer
University of Nebraska Medical Center
Omaha, Nebraska
United States

Megan E. Jacob, MS, PhD
Professor of Clinical Microbiology and Director, Clinical Microbiology Laboratory
Department of Population Health and Pathobiology
College of Veterinary Medicine
North Carolina State University
Raleigh, North Carolina
United States

Jobin J. Kattoor, BVSc & AH, MVSc, PhD
Post-Doctoral Research Associate
Animal Disease Diagnostic Laboratory and Department of Comparative Pathobiology
College of Veterinary Medicine
Purdue University
West Lafayette, Indiana
United States

Melissa Kennedy, DVM, PhD, DACVM
Professor Emeritus
Department of Biomedical and Diagnostic Sciences
College of Veterinary Medicine
University of Tennessee
Knoxville, Tennessee
United States

Yunjeong Kim, DVM, MS, PhD, DACVM
Associate Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Peter W. Krug, PhD
Research Molecular Biologist
USDA Agricultural Research Service
Plum Island Animal Disease Center
Foreign Animal Disease Research Unit
Greenport, New York
United States

Marilynn A. Larson, BS, MSc, PhD
University Assistant Professor and BSL-3 Core Facility
Laboratory Director
University of Nebraska Medical Center
Omaha, Nebraska
United States

Duan Loy, DVM, PhD, DACVM
Molecular Diagnostics Lab Manager
4040 East Campus Loop North
Nebraska Veterinary Diagnostic Center, University of
Nebraska -Lincoln
Lincoln, Nebraska
United States

John Dustin Loy, DVM, PhD, DACVM
Associate Professor of Veterinary Microbiology
Nebraska Veterinary Diagnostic Center
School of Veterinary Medicine and
Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Wenjun Ma, BVSc, MVSc, PhD
Associate Professor
Department of Veterinary Pathobiology
College of Veterinary Medicine, and Molecular
Microbiology and Immunology
School of Medicine
University of Missouri-Columbia
Columbia , Missouri
United States

Gabriele Maier, DVM, MPVM, PhD, DACVPM
Assistant Professor of Cooperative Extension
School of Veterinary Medicine
University of California
Davis, California
United States

Bethany L. McGregor, PhD
Research Entomologist
USDA Agricultural Research Service
Center for Grain and Animal Health Research
Arthropod-Borne Animal Diseases Research Unit
Manhattan, Kansas
United States

D. Scott McVey, DVM, PhD, DACVM
Professor and Director
School of Veterinary Medicine and Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Dana Mitzel, MS, PhD
Research Microbiologist and Molecular Biologist
USDA Agricultural Research Service
Foreign Arthropod-Borne Animal Diseases Research Unit
Manhattan, Kansas
United States

Susan M. Moore, BS, MS, PhD, HCLD(ABB)
Adjunct Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Rodney Moxley, DVM, PhD, DACVM (Hon.)
Charles Bessey Professor
School of Veterinary Medicine and Biomedical Sciences
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

T.G. Nagaraja, BVSc, MVSc, PhD, DACVM (Hon.)
University Distinguished Professor and Dr. Roy Walter
Upham Endowed Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Sanjeev Narayanan, BVSc, MS, PhD, DACVM, DACVP
Professor and Head
Department of Comparative Pathobiology
College of Veterinary Medicine
Purdue University
West Lafayette, Indiana
United States

Axel Neubauer, Dr. med. vet, Fachtierarzt für Mikrobiologie, DACVM
Boehringer Ingelheim Vetmedica GmbH
Ingelheim am Rhein
Germany

Stefan Niewiesk, DVM, PhD, DECLAM
Professor
Department of Veterinary Biosciences
College of Veterinary Medicine
The Ohio State University
Columbus, Ohio
United States

Leela Noronha, DVM, PhD
Research Veterinary Medical Officer
USDA Agricultural Research Service
Foreign Arthropod-Borne Animal Diseases Research Unit
Manhattan, Kansas
United States

Michael Oglesbee, DVM, PhD, DACVP
Professor and Director, Infectious Diseases Institute
Department of Veterinary Biosciences
College of Veterinary Medicine
The Ohio State University
Columbus, Ohio
United States

Ana R. S. Oliveira, DVM, MS
Research Fellow
International Livestock Research Institute (ILRI)
Addis Ababa
Ethiopia

Steven Olsen, DVM, PhD, DACVM
Veterinary Medical Officer
USDA Agricultural Research Service
National Animal Disease Center
Infectious Bacterial Disease Unit
Ames, Iowa
United States

Deepti Pillai, BVSc, MVSc, PhD, DACVM
Clinical Assistant Professor
Department of Comparative Pathobiology and Indiana Animal Disease Diagnostic Laboratory
College of Veterinary Medicine
Purdue University
West Lafayette, Indiana
United States

Pablo Piñeyro, DVM, MVSc, DVSc, PhD
Associate Professor
Department of Veterinary Diagnostic and Production Animal Medicine
College of Veterinary Medicine
Iowa State University
Ames, Iowa
United States

Lisa M. Pohlman, DVM, MS, DACVP
Associate Professor of Clinical Pathology
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

John F. Prescott, MA, Vet MB, PhD
University Professor Emeritus
Department of Pathobiology
University of Guelph
Guelph , Ontario
Canada

Sree Rajeev, BVSc, PhD, DACVM, DACVP
Professor of Infectious Diseases
Department of Biomedical and Diagnostic Sciences
College of Veterinary Medicine
The University of Tennessee
Knoxville, Tennessee
United States

Sheela Ramamoorthy, BVSc, MS, PhD
Associate Professor
Department of Microbiological Sciences
North Dakota State University
Fargo, North Dakota
United States

Kathryn E. Reif, MSPH, PhD
Associate Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine

Kansas State University
Manhattan, Kansas
United States

Donald L. Reynolds, DVM, PhD, DACVM
Professor and Poultry Veterinarian
School of Veterinary Medicine and
Biomedical Sciences
Nebraska Veterinary Diagnostic Center
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Juergen A. Richt, DVM, PhD, FAAAS
Regents and University Distinguished Professor
Department of Diagnostic Medicine / Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

Luis L. Rodriguez, DVM, PhD
Research Leader and Supervisory Veterinary
Medical Officer
Foreign Animal Disease Research Unit
Plum Island Animal Disease Center
Agricultural Research Service, USDA
Greenport, New York
United States

Andrew N. Rycroft, PhD, FRSB, FRCPath
Professor of Clinical and Veterinary Microbiology
The Royal Veterinary College
Hertfordshire
United Kingdom

Jishu Shi, DVM, PhD
Professor, Laboratory of Vaccine Immunology
and Director, U.S.-China Center for Animal Health
and Fellow, Biosecurity Research Institute
Department of Anatomy and Physiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas
United States

David J. Steffen, DVM, PhD, DACVP
Professor
School of Veterinary Medicine and Biomedical Sciences
Nebraska Veterinary Diagnostic Center
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

George C. Stewart, PhD
Professor Emeritus
Department of Veterinary Pathobiology
College of Veterinary Medicine
University of Missouri-Columbia
Columbia , Missouri
United States

Hiep Vu, BVSc, MS, PhD
Assistant Professor
Department of Animal Science
University of Nebraska–Lincoln
Lincoln, Nebraska
United States

Rebecca P. Wilkes, DVM, PhD, DACVM
Associate Professor
Department of Comparative Pathobiology and Animal
Disease Diagnostic Laboratory
College of Veterinary Medicine
Purdue University
West Lafayette, Indiana
United States

William Wilson, MS, PhD
Research Microbiologist
USDA Agricultural Research Service
Foreign Arthropod-Borne Animal Diseases Research Unit
Manhattan, Kansas
United States

Amelia R. Woolums, DVM, MVSc, PhD, DACVIM, DACVM
Professor
Department of Pathobiology and Population Medicine
College of Veterinary Medicine
Mississippi State University
Mississippi
United States

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Preface

This collection of chapters and supporting materials is intended to provide a very broad overview of veterinary microbiology and infectious diseases. The writings represent a combination of the biology of the organisms that cause or are associated with disease and the diseases themselves. The scope of this book is intended to be general to appeal both to students of veterinary sciences and to seasoned veterinary practitioners and scientists. Like many textbooks, this book will hopefully serve as a sound foundation for the study of veterinary infectious diseases as well as a good reference text. The content emphasizes diseases that occur in North America, but many global, transboundary diseases are included.

Part I of the book contains chapters that deal with basic microbiology and microbial virulence and parasitism. This chapter is intended to convey a basic knowledge of bacteria, viruses, and fungi to provide a better understanding of specific pathogens and the diseases they cause, which are described in Parts II, III, and IV. Part II describes bacterial pathogens. This section covers a very diverse set of bacterial pathogens and many diseases, but yet the similarities of pathogenesis, virulence properties, and host responses among these organisms are striking. Part III of the book describes in detail mycotic diseases and the fungal pathogens responsible for them. We have tried to emphasize the consequences of fungal infections and the host responses. Part IV deals with important viruses as well as diseases that they cause. We have described the veterinary significance of these diseases, along with methods of diagnosis, prevention and treatment. Part V, the last section of the book, deals with an overview of control of diseases and includes immune response to infectious agents, antimicrobial therapy and resistance, laboratory diagnosis, prophylactic measures with vaccines, and epidemiology and transmission of infectious agents. In the spirit of one medicine, the chapters take a comparative approach to describing both differences and similarities of diseases across many affected species. We have not included the clinical application section which exists in the third edition, as it did not fit well with the format as well as the scope of this book.

This edition contains numerous high-quality figures, which we believe will be very useful for veterinary students in their learning process. This edition will be very beneficial for veterinarians as they render their clinical services in a practice setting.

We have invited a group of outstanding microbiologists/experts/scientists to contribute to this edition. We believe the contents are accurate and up to date. However, we welcome any comments or suggestions that you may have regarding the contents of this book.

D. Scott McVey, Melissa Kennedy,
M.M. Chengappa, and Rebecca Wilkes

About the Companion Website

This book is accompanied by a companion website:

www.wiley.com/go/mcvey/microbiology4



The website includes

- Additional instructional materials for selected chapters
- PowerPoint slides of all figures from the book for downloading
- Videos

Part I

Introduction

1

Microbial Infections of Animals

D. Scott McVey, Melissa Kennedy, and Charles Czuprynski

Veterinary microbiology is the science of infectious agents that affect animals. These agents are categorized by their ecological associations with animals: (i) **parasites** live in permanent association with, and at the expense of, animal hosts; (ii) **commensals** are parasites that cause their host no discernible harm; (iii) **saprophytes** normally inhabit inanimate environments shared with animals; and (iv) **symbiosis,** or **mutualism,** usually refers to reciprocally beneficial associations of organisms. Pathogenic organisms can be either parasites or saprophytes and may cause disease in one or more animal species. The process by which organisms establish themselves in an individual host is termed **colonization** or **infection**. Some infections directly cause or induce deleterious outcomes in a host, whereas others do not and result in what might be called subclinical infection. The term **virulence** is sometimes used to express degrees of pathogenicity, often related to the severity of clinical illness and occurrence of deleterious outcomes (mortality or tissue damage) (Table 1.1).

Some Attributes of Host–Parasite Relationships

Many pathogenic microorganisms are host specific in that they parasitize only one or a few animal species. For example, the cause of equine strangles, *Streptococcus equi* subspecies ***equi***, is essentially limited to primary infection of horses. Other microorganisms – for example, certain ***Salmonella*** serotypes have a broad host range. The basis for differences in host specificity is often incompletely understood but may in part be related to the need for specific attachment mechanisms between hosts (receptors) and pathogens (adhesins).

Some agents infect several host species with varying effects. For example, the plague bacillus *Yersinia pestis* behaves as a commensal parasite in many small rodent species, but causes fatal disease in cats and humans. Evolutionary pressure may explain some of these differences. For instance, ***Coccidioides immitis***, a saprophytic fungus that requires no living host, infects cattle and dogs with equal ease. Yet it produces no clinical signs in cattle, but frequently causes progressive and fatal disease in dogs.

Potential pathogens also vary in their effects on different tissues in the same host. *Escherichia coli* strains that are commensals in the intestine can cause severe disease in the urinary tract and peritoneal cavity. Some microorganisms that are commensals in one habitat may be pathogenic in the same, or some other, habitat that is pathologically altered or otherwise compromised. For example, oral streptococci occasionally enter the bloodstream from which they can colonize a physically damaged heart valve and initiate bacterial endocarditis. In the absence of such a lesion, the streptococci do not colonize and are cleared uneventfully by the innate immune system. Similarly, the frequent translocation of intestinal bacteria across the intestinal mucosa, and into the vasculature channels, normally leads to their clearance by innate and adaptive defense mechanisms. However, in immunodeficient hosts or after overwhelming translocation of large numbers of bacteria, this translocation to the intestinal vasculature can lead to fatal septicemia.

Commensalism is a stable form of parasitic existence. But if a commensal gains entrance into a novel host or tissue, or there is a substantial reduction in host resistance, commensal parasites can become active pathogens that ensure the survival and multiplication of the microorganism. However, active disease can jeopardize pathogen survival by evoking an immune response that eliminates the microorganism, or by overwhelming defense mechanisms that kill the host and restrict further microbial

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Table 1.1 Degrees of pathogenicity.

Saprophytes	No disease – environmental microorganisms
Commensal organisms	Colonize host tissue – no disease
Symbiotic species	Beneficial relationship for the host; colonize host tissue – mutually and parasitic microorganisms
Opportunistic parasites	Colonize host tissue (usually saprophyte or commensal), but under favorable conditions cause disease with tissue damage
Pathogenic microorganisms	Infection directly causes disease (although this may be host specific)

multiplication and transmission (Table 1.1). In general, evolutionary selective pressure tends to select for commensalism and generally eliminates host–parasite relationships that threaten the survival of either partner. Over time, less virulent strains of a pathogen, which permit survival of the host, tend to arise and replace the more lethal strain. Evolutionary selection also favors establishment of a resistant host population by eliminating highly susceptible individuals. One example is in Africa, where regionally adapted livestock are resistant or partially resistant to the protozoal pathogen of theileriosis. Most agents that cause serious disease have alternative modes of survival as commensals in tissues in which they do not cause damage (e.g. uropathogenic *E. coli* in the intestine), hosts less susceptible to disease (e.g. *Y. pestis* in small rodents), or the inanimate environment (e.g. *Crabro immitis*). Some pathogens cause chronic infections lasting months or years (e.g. tuberculosis and glanders disease), which increases the time and opportunities for their dissemination to other hosts that ensures their survival.

Criteria of Pathogenicity – Koch's Postulates

The presence of a microorganism in diseased individuals does not prove its pathogenic significance. To formally demonstrate the causal role of an agent in a disease, the following qualifications or "postulates" of Robert Koch (1843–1910) should be satisfied:

1) The suspected agent is present in all cases of the disease.
2) The agent is isolated from such disease and propagated serially in pure culture, apart from its natural host.
3) Upon introduction into an experimental host, the isolate produces the original disease.
4) The agent can be reisolated from this experimental infection.

These postulates are ideals that cannot always be experimentally verified in all infectious diseases. The presence of some microorganisms cannot be demonstrated at the time of disease, especially in tissues affected by intoxication (e.g. tetanus and botulism). Some agents (e.g. *Mycobacterium leprae*) cannot be maintained in culture apart from their natural hosts. Other pathogens are difficult to isolate or die rapidly after isolation (e.g. ***Leptospira*** spp.). Still others, although clearly pathogenic, often require undetermined accessory factors to cause disease (e.g. ***Pasteurella***-related pneumonias). In addition, contemporary molecular microbiological methods suggest that some infections involve more than one microbial species via interactions that, at this time, might not be understood.

Elements in the Transmission and Production of Infectious Disease

Effective transmission of a microbial agent occurs by ingestion, inhalation, or inoculation of a mucosal or cutaneous surface. Airborne infection takes place largely via droplet nuclei, which are 0.1–5 mm in diameter. Particles of this size stay suspended in air and can be inhaled. Larger particles settle but can be resuspended in dust, which might also harbor infectious agents from nonrespiratory sources (e.g. skin squames, feces, and saliva). Arthropods can serve as mechanical carriers of pathogens (e.g. equine arteritis or African swine fever) or play an indispensable part in the life cycles of disease-producing agents (e.g. plague, ehrlichiosis, and viral encephalitides or hemorrhagic fevers) before inoculating the organism into the skin.

Attachment to host surfaces requires interaction between the agent's adhesins, which are usually proteins, and the host's receptors, which are most often protein or carbohydrate residues. Examples of bacterial adhesins include fimbrial proteins (*E. coli* and ***Salmonella*** spp.), P-1 protein of ***Mycoplasma*** **(***Mycoplasma pneumoniae***)**, and afimbrial surface proteins (some streptococci). Examples of host receptors include fibronectin for some streptococci and staphylococci, mannose for many *E. coli* strains, and sialic acid for *M. pneumoniae*.

Pathogen attachment can be inhibited by commensal organisms that occupy or block available receptor sites or discourage colonization by other microbes via excretion of toxic metabolites, bacteriocins, and microcins. This "colonization resistance" is an important defense mechanism and may be augmented by host-derived antibacterial substances (e.g. defensins, lysozyme, lactoferrin, and organic acids) or by mucosal antibodies that prevent attachment of or damage the invader.

Penetration of an epithelial or mucosal host surface is a variable requirement among pathogens. Some agents, having reached their primary target cell or tissue, do not invade further (e.g. enterotoxigenic *E. coli*). Others traverse epithelial cells by inducing cytoskeletal rearrangements that result in "ruffles," which entrap adhered bacteria or facilitate their passage between epithelial cells (e.g. ***Salmonella*** and ***Yersinia***). Inhalation of facultative intracellular parasites such as *Mycobacterium tuberculosis* results in ingestion by pulmonary macrophages, in which the bacilli may survive, multiply, and travel via lymphatics to lymph nodes and other tissues. Percutaneous penetration by pathogens occurs through injuries, including minor trauma (scratches, etc.) and insect or arthropod bites. Dissemination within tissues, or among adjacent tissues, might involve microbial invasion of host cells, aided in part by bacterial enzymes, such as collagenase and hyaluronidase, that degrade the extracellular matrix and facilitate microbial movement. Once microorganisms breach an epithelial surface (cutaneous or mucosal), they can spread via various "highways" in the host including lymphatic and blood vessels, the bronchial tree, bile ducts, and nerve trunks. Migration within the host can occur both as extracellular microbial cells and after entering and hitching a ride in mobile phagocytes (macrophages and neutrophils).

Except for foodborne pathogens that produce toxins in foodstuffs prior to ingestion (e.g. *Clostridium botulinum* toxins, Staphylococcal enterotoxins), growth in or on a host is a prerequisite for all pathogenic organisms. To do so microbes must circumvent host defense mechanisms. Microbial strategies include firm attachment to prevent mechanical removal, avoidance of phagocytosis, and impairment of phagocytic function by release of toxins or other components that prevent ingestion or intracellular killing of microbes. Some pathogens degrade antibodies or deplete complement components that are important for host defense. Other pathogens alter the vascular supply to tissue, thus creating an environment that restricts defensive resources and impairs antimicrobial activity in the affected area.

When host defenses are significantly inhibited, microbial growth can proceed if nutritional supplies are adequate and the pH, temperature, and oxidation–reduction potential are appropriate. Host control of these parameters is important in host defense. Iron is often a limiting nutrient for microbial growth. The ability to appropriate iron from host iron-binding proteins (transferrin and lactoferrin) is an important factor in microbial virulence. Gastric acidity makes the stomach a harsh environment for many pathogenic bacteria, although in response to acid stress some bacteria express alternative sigma factors that result in transcription of genes whose products help the pathogen survive in an acidic environment (e.g. ***Salmonella*** and **enterohemorrhagic** *E. coli*). The higher body temperature of birds may explain in part their resistance to some infectious diseases (e.g. anthrax and histoplasmosis). Requirements for a reduced oxygen environment allow anaerobic bacteria to multiply in tissues devitalized (i.e. nonoxygenated) as a result of injury, or in which prior or concomitant growth of facultative anaerobic organisms has sufficiently reduced the available oxygen.

Viral Infections

The outcome of any virus–host interaction will depend on various critical factors such as virus–cell interactions, susceptibility of the animal host, route of exposure, mode of virus dissemination, and host resistance. Although most virus-infected animals presented to veterinarians exhibit clinical signs, it is important to recognize that viral infections of animals often result in asymptomatic or subclinical infection. Nor can virulent viruses infect animals that are resistant to them. The potential consequences of virus–animal relationships are listed in Table 1.2.

Table 1.2 Consequences of virus–animal relationships.

No pathogenesis or disease	No disease – replication and transmission with no or minimal pathological changes
Some tissue damage and inflammation	Mild clinical disease with rapid viral clearance and minimal transmission, limited to localized tissues (no dissemination)
Moderate clinical disease	Significant replication and shedding of virus to sustain transmission, some tissue replication, viremia – inflammation associated with some clinical signs (fever, malaise, tissue-specific signs)
Severe clinical disease	Infect host tissues, significant viremia, and shedding – clinical signs associated with tissue damage (respiratory or neurological, for example) or systemic host responses (fever, malaise, muscle pain, and even shock) Some viral infections may be limited to specific tissues with little viremia and no broad cross-tissue dissemination (feline immunodeficiency virus). Manifestations of disease may be the result of a prolonged perturbation of cellular function or cellular loss (immunodeficiency)
Fatal or other serious outcomes	Infection that results in severe disease leading to death from mechanisms described above for severe clinical disease. This could include permanent tissue damage resulting from viral replication or consequent inflammation

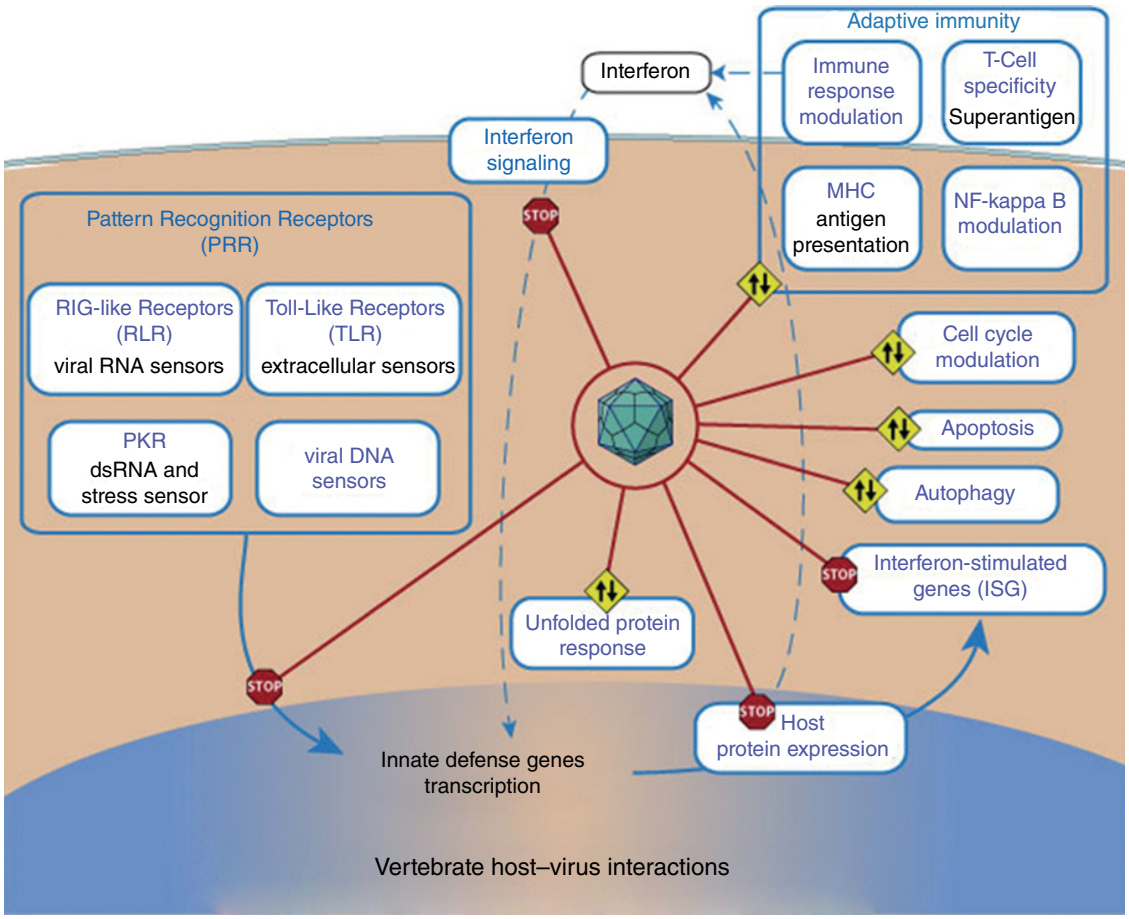


Figure 1.1 Interactions of viruses with host cells. *Source:* Courtesy of Creative Commons Corporation and ViralZone, 2021, https://creativecommons.org/licenses/by/4.0/#; https://viralzone.expasy.org/.

There are several major routes of viral entry into a host: respiratory, alimentary, urogenital, and direct transmission via an insect or animal bite. Successful establishment of viral infection depends on the presence of appropriate cell receptors for viral attachment and internalization, and the physicochemical nature of the viral agent (Figure 1.1). To successfully initiate infection, a virus must survive until a susceptible host is encountered and access is gained to cells in which it can replicate (permissive cells). This requires the virus to overcome the host defense mechanisms at these sites. For example, viruses that infect animals via the alimentary tract are typically resistant to the low pH and potent enzymes that occur in the digestive tract.

Mechanisms of Pathogenesis

Microbial disease manifests itself either as the result of direct damage to host cells and cellular functions by exotoxins or products of microbial growth, or via collateral damage due to host inflammatory or immune reactions that are triggered by the microbe or microbial components (e.g. endotoxin).

Direct Damage

Exotoxins are usually bacterial proteins that are freely excreted into the environment, whereas endotoxin (lipopolysaccharide) is an integral part of the gram-negative bacterial cell membrane that remains attached to the bacterial cell or is released in membrane vesicles. The different characteristics of endotoxins and exotoxins are described in Table 1.3 and Figure 1.2.

Exotoxins are encoded chromosomally, on plasmids or on bacteriophages. These toxins produce injury by destroying the cells in which they replicate or by altering cell function, appearance, and growth characteristics. There are several types of bacterial exotoxins. Some act extracellularly by damaging host cell membranes via enzymatic or

Table 1.3 Exotoxins and endotoxins compared.

Exotoxins	Endotoxins
Often spontaneously diffusible	Cell-bound as part of the cell wall
Proteins or polypeptides	LPS (lipid A is a toxic component)
Produced by gram-positive and gram-negative bacteria	Limited to gram-negative bacteria
Produce a single, pharmacologically specific effect	Produce a range of effects, largely due to host-derived mediators
Each is distinct in structure and reactivity according to its bacterial species of origin	Similar in structure and effect regardless of bacterial species of origin
Lethal in minute amounts (mice = nanograms)	Lethal in larger amounts (mice = micrograms)
Labile to heat, chemicals, and storage	Very stable to heat, chemicals, and storage
Convertible to toxoids (nontoxic, immunogenic toxin-derivatives); elicit antitoxin production	Not readily convertible to toxoids

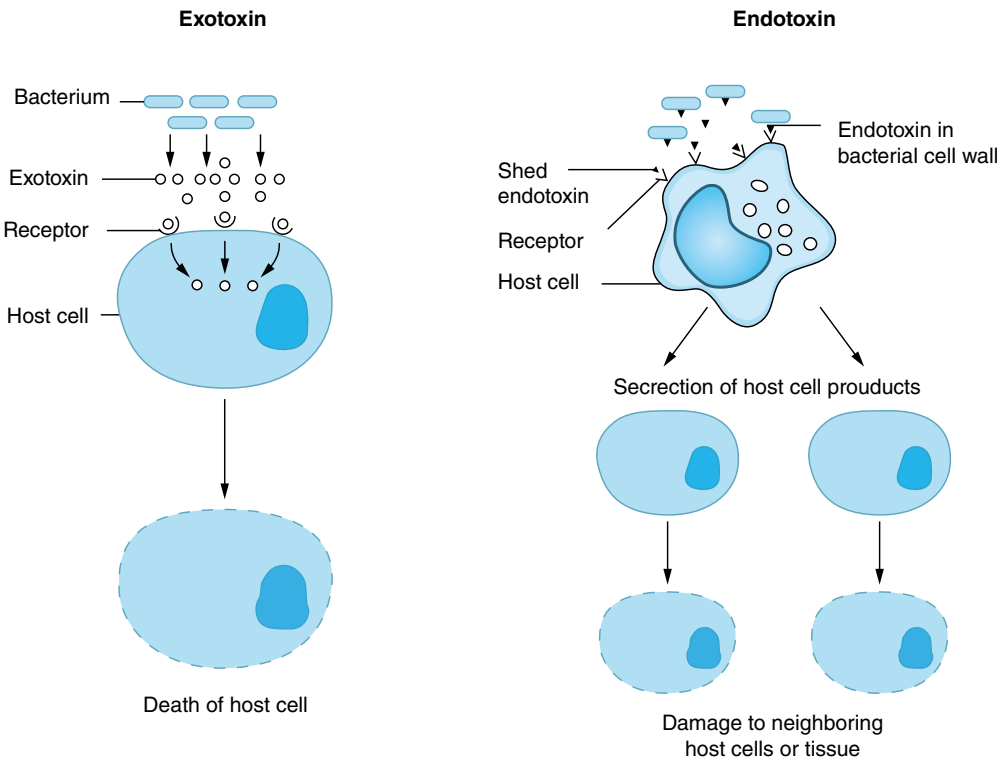


Figure 1.2 A comparison of endotoxins and exotoxins. *Source:* Used courtesy of the Creative Commons and @Read/Study, 2021.

detergent-like mechanisms. Examples of these toxins include bacterial hemolysins, and leucocidins. Some exotoxins act as enzymes that degrade the extracellular matrix (e.g. collagenases and hyaluronidases) and play an ancillary role in facilitating bacterial spread in host tissue. One group of exotoxins consists of proteins or polypeptides that enter cells and enzymatically disrupt intracellular processes. Many, but not all, of these are bifunctional polypeptides that consist of an A fragment with enzymatic activity and a B fragment that is responsible for toxin binding to target cells.

Endotoxins are lipopolysaccharides (LPS) that are part of the gram-negative cell wall and outer membrane. The LPS structure consists of a core polysaccharide, lipid A (which is the toxic moiety) and polysaccharide chains. The latter can act as an adhesin or virulence factor and contain

the somatic (O) antigens recognized by the host immune response. LPS can bind directly to leukocytes, or to LPS-binding protein (a plasma protein), which in turn transfers the LPS to CD14. The CD14–LPS complex interacts with other receptor proteins (e.g. Toll-like receptor 4) on the surface of macrophages and other cells, triggering the release of proinflammatory cytokines and other mediators that elicit the manifestations of endotoxemia. These can include fever, headache, hypotension, leukopenia, thrombocytopenia, intravascular coagulation, inflammation, endothelial damage, hemorrhage, fluid extravasation, and circulatory collapse. Many of these outcomes result from LPS or cytokine stimulating: (i) activation of the complement cascade; and (ii) production of arachidonic acid metabolites (prostaglandins, leukotrienes, and thromboxanes). The clinical signs of endotoxemia closely resemble those of gram-negative septicemias. Although mediated by different bacterial components (lipoproteins) and host receptors (Toll-like receptor 2), similar manifestations can be induced by the cell walls (peptidoglycans) of gram-positive bacteria.

Immune-Mediated Damage

Tissue damage due to immune reactions is considered in detail elsewhere in this volume (see Chapter 67). Innate immune and inflammatory responses are both essential to host defense and can cause substantial tissue damage and loss of function. For example, complement-mediated inflammation can occur in response to endotoxins or to peptidoglycan without preceding sensitization and can result in a vigorous local inflammatory response. Antigen-specific adaptive immune responses contribute to the pathogenesis of many infections, particularly those, such as tuberculosis, that elicit chronic granulomatous infection. Granulomas that form in response to infection with intracellular pathogens such as *M. tuberculosis* are the result of a cell-mediated immune response (Type IV delayed type hypersensitivity) by the host. These cell-mediated immune responses can damage tissue during the original infection, or upon subsequent encounters with the offending microorganisms or its antigens that stimulate T lymphocytes to release cytokines and other effector molecules. Other examples of immune-mediated damage include anemia seen in anaplasmosis, and during infection with the hemotrophic mycoplasmas. These occur as the result of an antibody response to the hemoparasites that targets infected host erythrocytes for phagocytosis and C-mediated hemolysis.

Viral Dissemination within the Infected Host

Viruses cause two basic patterns of infection: localized or generalized (Figure 1.3). In localized infections, viral multiplication and cellular damage remain localized near the site of entry (e.g. the skin or the mucous membranes of the respiratory, gastrointestinal, or genital tract). The infecting virus spreads only to neighboring cells immediately adjacent to the original site of infection. For example, rhinovirus infections of animals are often restricted to the nasal epithelium and do not spread to the lower respiratory tract. Other respiratory viruses, such as parainfluenza and respiratory syncytial viruses, can replicate within the lungs of infected animals, but tissue injury does not extend beyond the respiratory tract. Generalized infections develop through several sequential steps: (i) the virus undergoes primary replication at the site of entry and in regional lymph nodes, (ii) progeny virus spreads through blood (primary viremia) and lymphatics to additional tissues, where (iii) further virus replication takes place, (iv) the virus is disseminated to the other target organs via a secondary viremia, and (v) it multiplies further in these target tissues where it causes cellular degeneration and/or necrosis, tissue injury, and clinical disease.

After the initial viral invasion, there is an asymptomatic incubation period before clinical signs are observed. In generalized viral infections, overt disease begins after the virus is widely disseminated in the body and replicates to significant levels. It is at this stage that the veterinarian typically is first alerted. Canine distemper provides an example of a generalized viral infection of animals. Canine distemper virus initiates infection at the site of entry, and then disseminates through the blood or lymphatic system to produce generalized infection that involves a variety of target organs (Figure 1.4). The sequence of events during the incubation period and clinical signs that occur in individual animals depend on which organ systems are infected by the virus. The virus is disseminated to these organs during the viremic period, in which viral dissemination occurs both via free virus particles in the blood and infected blood cells that serve as carriers to transport the virus to target organs (cell-associated viremia). Cell-associated viremias typically involve blood leukocytes, but some viruses, such as bluetongue virus, hog cholera virus, or parvovirus, can associate with and be disseminated by red blood cells in the infected host. For viruses that infect the central nervous system, viral dissemination can occur by viremia or, in the case of rabies, by centripetal transmission along peripheral nerves.

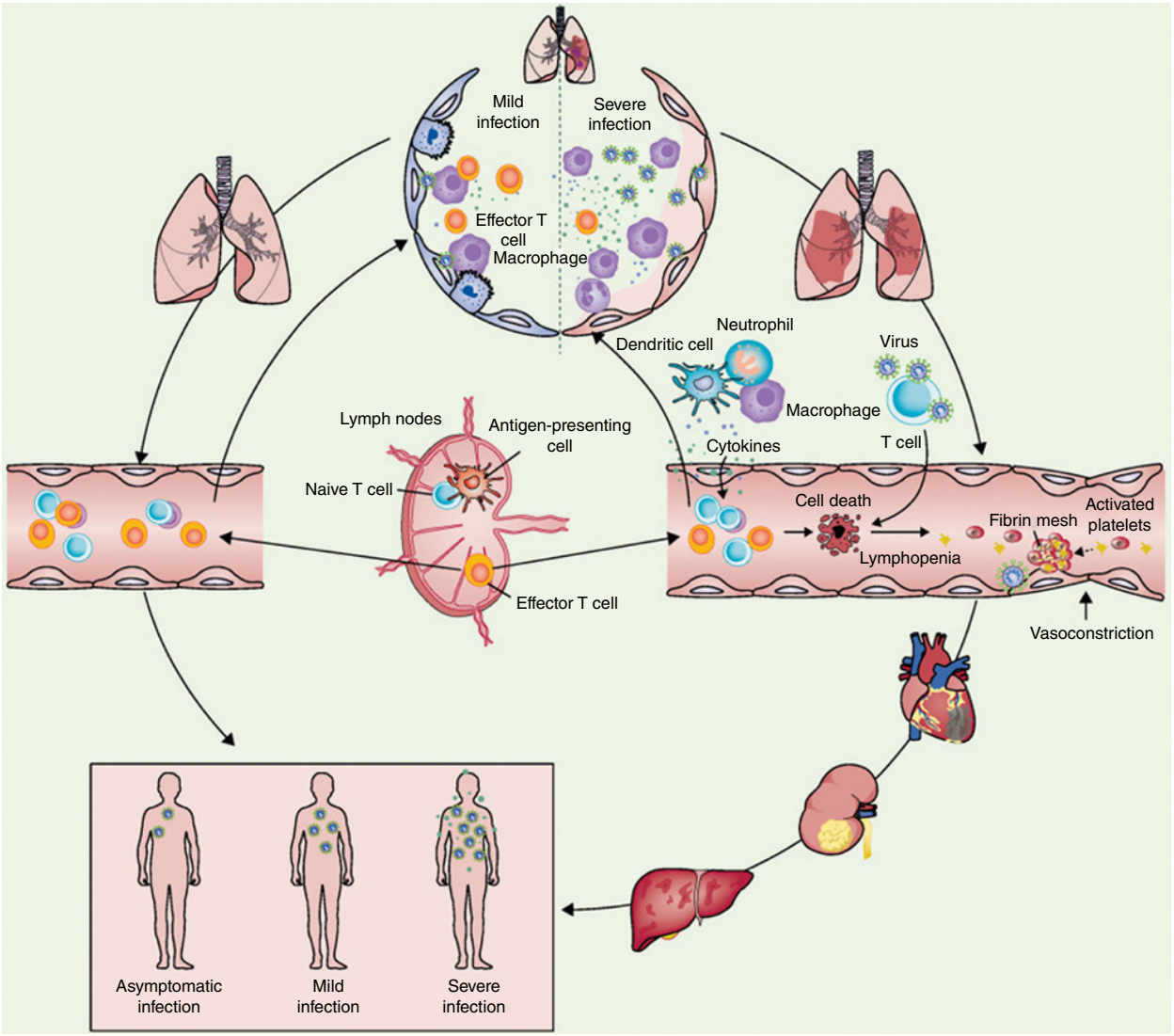


Figure 1.3 Infection, tissue distribution, and outcomes of severe acute respiratory syndrome coronavirus 2 viral sepsis. *Source:* Li et al. (2020).

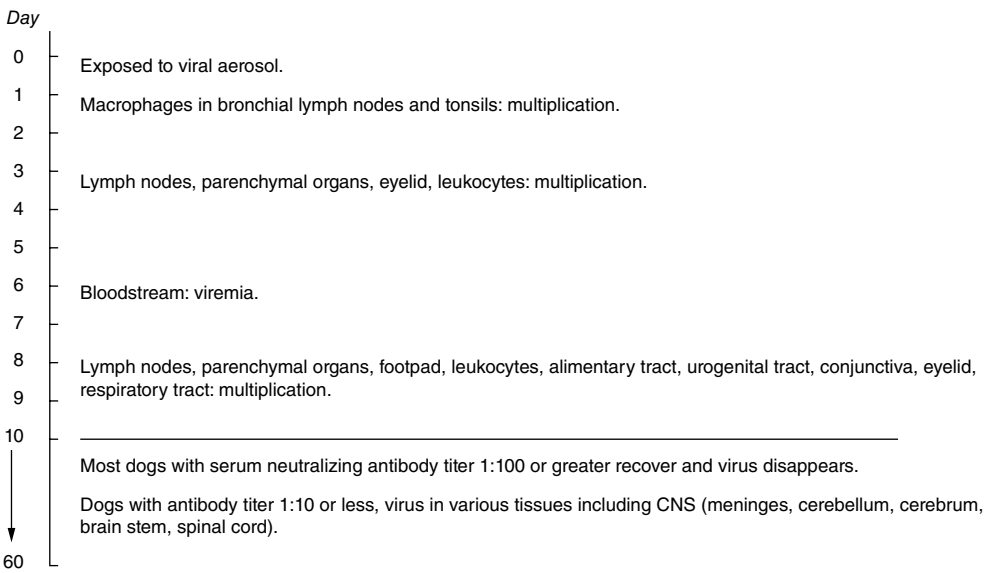


Figure 1.4 Primary and secondary tissue infections of canine distemper virus infections.

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2

Basic Bacteriology

Tiruvoor G. Nagaraja

In the early beginnings of biology (around 300 BCE), living organisms were categorized into two kingdoms, Plantae (plants) and Animalia (animals). As the science progressed, a third kingdom, Protista, was created to include microscopic organisms, bacteria, protozoa, algae, and fungi. As the structural differences became more apparent with the advent of light and electron microscopy, two distinct cell types were categorized: smaller and less complex Prokaryotes and larger and more complex Eukaryotes. In recent years, with the discoveries and developments in molecular biology and molecular techniques, three cell types have been recognized: two belonging to Prokaryotes (bacteria and a new cell type identified as Archaea) and all others (protozoa, fungi, plants, and animals) in Eukaryotes. The categorization was based on ribosomal RNA nucleotide sequences, which are present in all cells. In 1978, the three cell types were elevated to a level above the kingdom, called the domain.

Bacteria

Bacteria (singular: bacterium) are single-celled microscopic organisms, that is, too small to be seen by the unaided eye, that belong to the domain Bacteria (earlier Eubacteria or true bacteria). They are small and relatively less complex and exhibit typical prokaryotic cell structural features (Table 2.1). The other prokaryotic organism, which differs from bacteria in many respects, most notably in the 16S rRNA sequences, belongs to another domain called Archaea (singular: Archaeon). Archaea have unusual metabolic and physiological characteristics, such as methanogens that generate methane gas and organisms that live in extreme environments (extremophiles) of high temperature (thermophiles) and high concentrations of salt (extreme halophiles). None of the members of Archaea have been shown to be etiologic agents of diseases in animals or humans. Ribosomal RNA sequence analysis, particularly the 16S rRNA associated with the small subunit (30S) in prokaryotic cells or 18S rRNA with the small subunit (40S) in eukaryotic cells, has been a major breakthrough in the classification of microorganisms, and provides insights into the evolutionary relationships of all types of cells.

Bacteria, ubiquitous in nature, are abundant in soil, water, air, plants, animals, and humans. All the exposed body surfaces, the skin and mucous membranes, of animals and humans are colonized by bacteria. These bacteria constitute the normal flora of the body, and their relationship with the host is described as commensalism, a relationship in which bacteria are benefited and the host is unharmed. The bacteria that live in the gastrointestinal tract (the foregut in ruminants and the hindgut in nonruminants) help the host to digest food and produce energy, proteins, and vitamins for the benefit of the host. Such a relationship between bacteria and the host is described as mutualism or symbiosis. Most bacteria in nature are harmless or beneficial. Some, called pathogens, harm the host and cause diseases in animals and humans. Such an interaction where bacteria are benefited and the host is harmed is called parasitism.

Bacterial Anatomy: Structures and Functions

Bacterial cells are small and range from 0.2 to 2.0 μm in diameter and 2–8 μm in length. There are three basic shapes of bacterial cells: spherical, called coccus (plural: cocci); rod shaped, called bacillus (plural: bacilli); and spiral (called spirochete) (Figure 2.1). When bacterial

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Table 2.1 Characteristics of prokaryotic cells.

Characteristics	Prokaryotic cells
Members	Bacteria, Archaea
Size	Generally small (1–10 µm)
DNA	A single circular piece (single chromosome)
Nuclear membrane	Nucleus is not bound by a membrane
Nucleolus	Absent
Extrachromosomal DNA	Present as plasmids (small circular pieces of DNA in the cytoplasm)
Mode of reproduction	Asexual, binary fission
Cytoplasmic organelles: Endoplasmic reticulum, mitochondria, chloroplasts	Absent
Ribosomes	70S (30S and 50S)
First amino acid in protein synthesis	Formyl methionine
Membrane lipids	No sterols in the cytoplasmic membrane (except *Mycoplasma*)

cells divide, the cells can remain attached to one another, sometimes forming a characteristic arrangement. Cocci can be single, diplococci (attached in pairs), tetrads (groups of four), sarcinae (groups of eight), streptococci (in short or long chains), or staphylococci (in clusters). The arrangement of cells is helpful in the identification of certain cocci, as in species of the genera *Staphylococcus* and *Streptococcus* (Figure 2.1a). Bacilli generally occur as individual cells and some can be in pairs (diplobacilli), in short or long chains (streptobacilli), or in long rods (filamentous) (Figure 2.1b). Most bacilli are straight rods, but some are oval shaped; some almost look like cocci and are called coccobacilli; and some are curved and are called vibrios (comma shaped). An important genus with comma-shaped organisms is *Campylobacter*. The rods can also be spindle shaped (fusiform), as in the genus *Fusobacterium*. "Bacillus" refers to a bacterial shape, but there is also a specific genus named *Bacillus*. The ends of bacilli may be square, round, or tapered. Certain bacilli, such as *Escherichia coli* and *Salmonella enterica*, are slender; others such as *Bacillus anthracis* (causes anthrax) are rectangular with square ends; and still others, such as species of the genus *Corynebacterium*, are club shaped (one end of the rod is swollen) or, as in the genus *Dichelobacter*, dumb bell shaped (both ends are swollen). Spirals are rod shaped with multiple twists (Figure 2.1c). Spirochetes have a thin, flexible cell wall and have multiple twists with a unique internal flagellar arrangement. One of the genera of spirochetes, *Leptospira,* has a hook at one or both ends of the spiral. Spiral-shaped bacterial cells can be 1–100 µm in length. Helicobacter are long rods, but much shorter than spirochetes (5–10 µm in length) and have fewer twists.

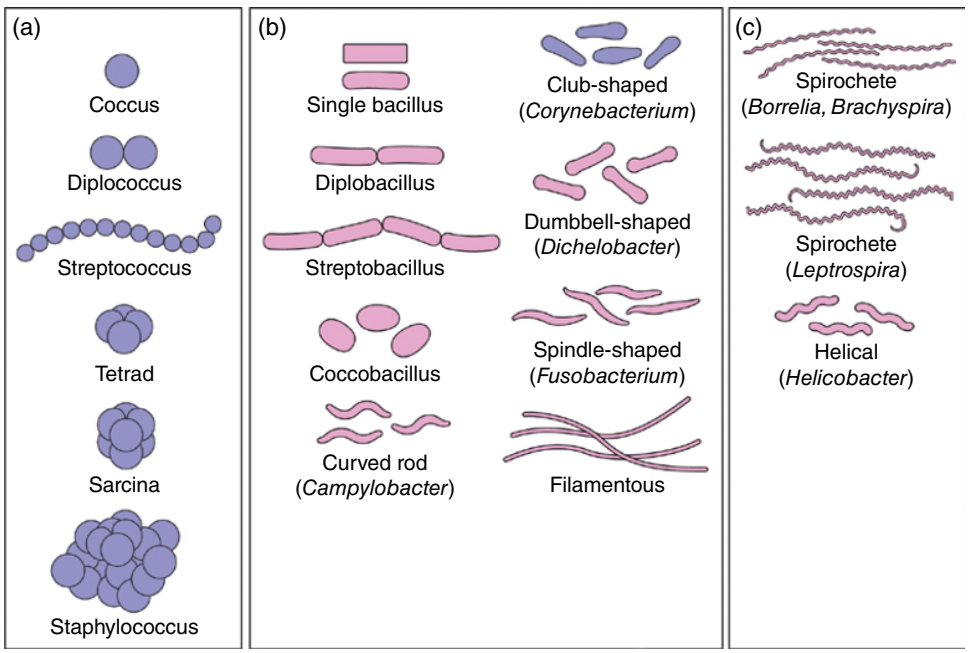


Figure 2.1 Basic shapes: (a) cocci, (b) bacilli, and (c) spiral-shaped and arrangement of bacterial cells.

The shape of the bacterium is genetically inherited, and most bacteria maintain a uniform shape and size. They are described as monomorphic. However, some bacteria have shapes that are not uniform and are variable. They are described as pleomorphic.

Structures of a Bacterial Cell

Understanding cellular structures, their organization, and functions is important in the study of bacteria, particularly pathogens, because they are the bases for identification of their genus and species, and for the diagnosis of diseases. More importantly, many of the structures contribute to the virulence of the organism. Some of the structures are targets for antibacterial actions of chemical agents (antibiotics, antiseptics, and disinfectants) and serve as antigens that induce protective antibodies (protective antigens) in vaccines (Table 2.2).

Bacterial structures can be grouped into three broad categories: (i) cell envelope, which are structures that envelop the bacterial cytoplasm; (ii) cytoplasmic structures, which are contained in the bacterial cytoplasm; and (iii) surface structures, which are on the surface of the cell wall (Figure 2.2).

Cell Envelope

The cytoplasmic membrane and the cell wall constitute the cell envelope of a bacterial cell.

Table 2.2 Bacterial cell structures, their chemistry, functions, and importance in pathogenic potential.

Cell structure	Chemistry	Functions and their importance in pathogens
Cell envelope structures		
Cytoplasmic membrane	Phospholipids and proteins	Semipermeable membrane; uptake of nutrients; transport of products; carry enzymes; target for antimicrobial agents (e.g. polymixin)
Cell wall	Polysaccharide and peptide (peptidoglycan); teichoic acid; phospholipids and lipopolysaccharide (LPS) in gram-negative bacteria	Confer shape, protection, antigenicity; contribute to virulence in some bacteria; LPS is a major virulence factor in gram-negative bacteria; target for antimicrobial agents (e.g. beta-lactams)
Cytoplasmic structures		
Nuclear body	DNA	Localization of essential genetic information; virulence genes, targets for antimicrobial agents (e.g. fluoroquinolones), antimicrobial resistance genes
Ribosomes	Proteins and RNA	Site of protein synthesis; targets for antimicrobial agents (e.g. tetracyclines)
Inclusions	Polysaccharides, polypeptides, polyhydroxybutyrate, polyphosphates, etc.	Store granules and maintain cell osmolality
Spores	Same as the cell (carbohydrates, proteins, lipids, and nucleic acids)	Cell survival in adverse conditions
Plasmids	DNA	Nonessential genetic information; carry genes that encode for virulence, antimicrobial resistance, and horizontal gene transfer (conjugation)
Actin-like cytoskeletons	Protein	Cell shape determination
Cell surface structures		
Capsule and slime	Polysaccharides, polypeptides	Mediate attachment to host cells; Resistance to phagocytosis; contribute to biofilm formation
Flagella	Protein (flagellin)	Motility toward attractants (nutrients) or away from repellants (adversity); contribute to virulence in some bacteria
Fimbriae or pili	Protein (pilin)	Mediate attachment to host cells; conjugative transfer of DNA (sex pili); responsible for twitching motility, a flagella-independent motility seen in some bacteria

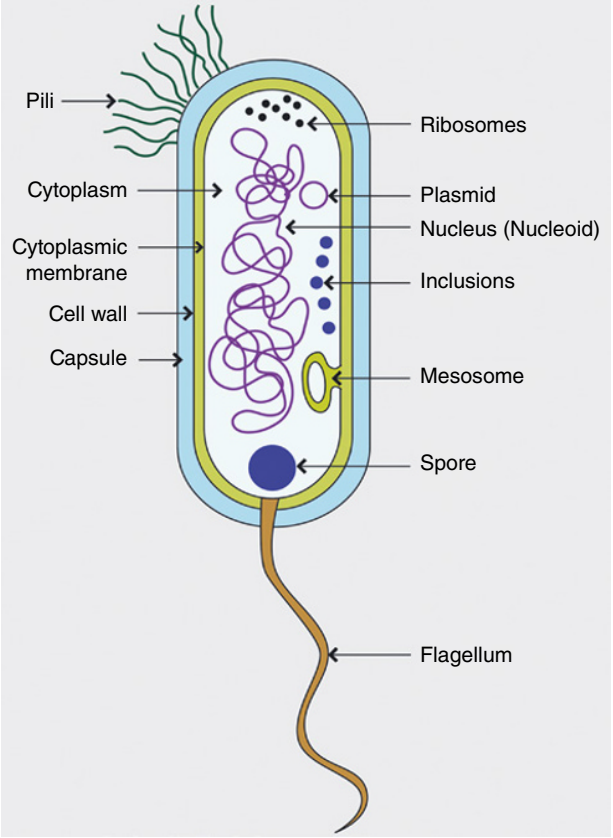


Figure 2.2 Structural features of a bacterial cell.

Cytoplasmic Membrane

The cytoplasmic membrane, also called the plasma membrane or inner membrane (in contrast to the outer membrane [OM] of gram-negative bacteria), is a thin layer that envelopes the cytoplasm of the cell. They are composed primarily of phospholipids and proteins. Eukaryotic cytoplasmic membranes have a similar composition, except that they also contain sterols, such as cholesterol. Bacterial cytoplasmic membranes do not contain sterols. An exception is *Mycoplasma*, which is characterized by sterols in the membrane.

The phospholipid molecules are arranged in parallel rows called a phospholipid bilayer. Each phospholipid molecule contains a polar head, composed of a phosphate group and a glycerol that is hydrophilic and soluble in water, and nonpolar tails, composed of fatty acids that are hydrophobic and insoluble in water. The polar heads are on the two surfaces of the bilayer, and the nonpolar tails are in the interior of the bilayer. Under an electron microscope, cytoplasmic membrane appears as two dark lines with a light space between them. The protein molecules lie scattered in the membrane (Figure 2.3). Some proteins are peripheral (easily removed by mild treatment), whereas others are integral (removed only by disrupting the membrane). Some integral proteins form channels or pores, called porins, through which solutes enter or exit the cell. The cytoplasmic membrane acts as a selective barrier that allows certain molecules to enter or exit the cell. The membrane contains enzymes involved in the catabolism of nutrients and production of adenosine triphosphate (ATP) (the site of electron transport phosphorylation). Antimicrobial agents, such as polymyxins, act on the cytoplasmic membrane to inhibit the cell. In addition, certain disinfectants such as alcohols and quaternary ammonium compounds act on the cytoplasmic membrane. The disruption causes leakage of intracellular contents and eventual cell death.

Cell Wall

The cell wall is outside the cytoplasmic membrane and protects the interior of the cell from adverse environmental changes. It is a complex, semirigid structure that provides structural integrity to the cell, maintains cell shape (cocci, bacilli, and spirals), and protects the cytoplasm from osmotic shock. The cell wall determines the Gram reaction, which categorizes bacteria broadly into gram-positive or gram-negative bacteria. Gram staining (named

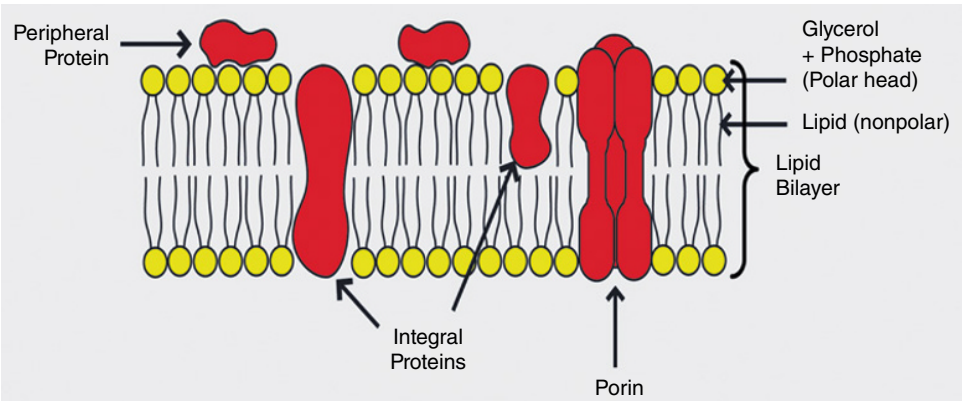


Figure 2.3 Cytoplasmic membrane of a bacterial cell. The phospholipid bilayer is interspersed with proteins.

after the Danish Microbiologist, Dr. Hans Gram) is the most widely used staining procedure in bacteriology. The staining depends on the ability of some bacteria (gram-positive) to retain a complex of a dye, crystal violet (purple) and iodine (a mordant, not a dye), when decolorized with alcohol. Others (gram-negative) are unable to retain the complex and are then counterstained with a different dye, safranin (pink or red). In pathogens, the cell wall contributes to their virulence and ability to cause disease. The cell wall is also a major target for some antibiotics because of the unique macromolecule, called peptidoglycan, which is present only in bacteria and is absent in all other cell types.

Peptidoglycan (also called murein) consists of a repeating disaccharide attached to a peptide to form a lattice. The disaccharide portion is made up of two modified monosaccharides, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) linked by β-1, 4 glycosidic linkage. Alternating NAG and NAM are linked in rows of 10–65 sugars to form a backbone (the glycan portion of the peptidoglycan). Adjacent rows are linked by a short peptide, tetrapeptide, the peptide portion of peptidoglycan, to NAM (Figure 2.4). The tetrapeptide is composed of alternating D and L amino acids, including a unique amino acid called, D, L, or DL (*meso*) diaminopimelic acid. The D amino acids are non-protein amino acids because only L isomers of amino acids are present in natural proteins. Parallel tetrapeptides may be directly bonded to each other or linked by a peptide cross-bridge consisting of a short chain of amino acids. The cross-linkage results in a thick interconnected lattice of peptidoglycan strands in gram-positive bacteria. The extent of cross-linking and the number of strands are considerably less in gram-negative bacteria. Because peptidoglycan is unique to bacteria, the enzyme necessary to synthesize the cross-bridge, called transpeptidase, is a target for certain antibiotics, such as penicillins, cephalosporins, carbapenems, bacitracin, and vancomycin. Therefore, the transpeptidases are called penicillin-binding proteins. When peptidoglycan synthesis is inhibited, the cell wall is weakened and the cell is lysed.

Cell Walls of Gram-Positive Bacteria

In gram-positive bacteria, the cell wall consists of a thick peptidoglycan (20–80 nm) with multiple layers. Therefore, the cell envelope is a two-layered structure: the cytoplasmic

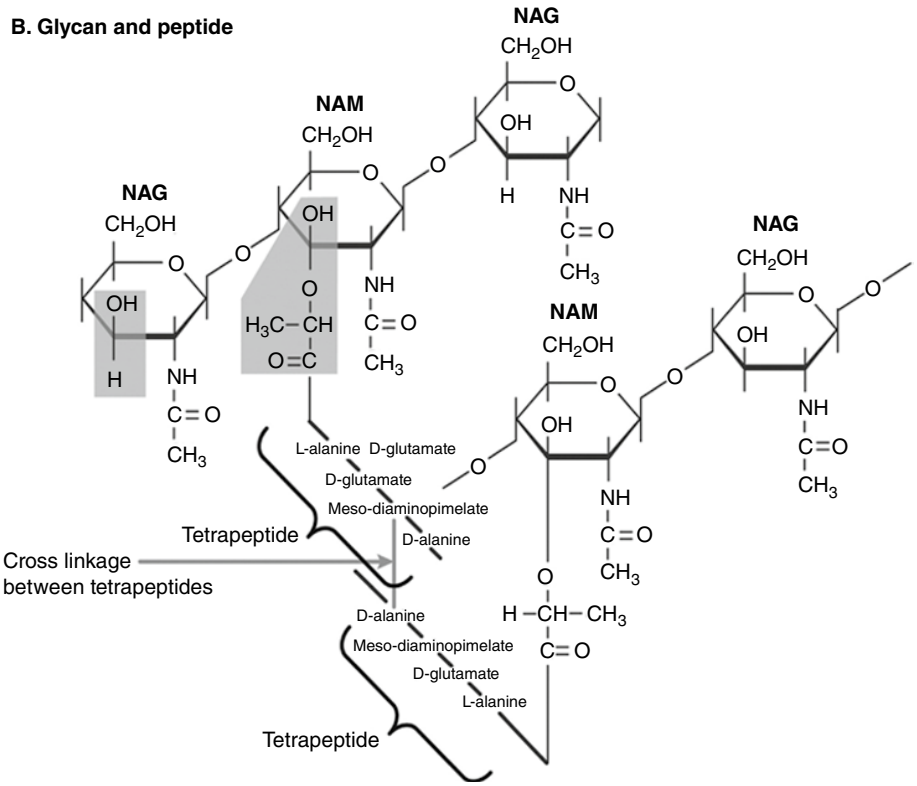


Figure 2.4 Chemical components of peptidoglycan. The glycan portion is composed of alternating units of *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM), and the peptide portion is composed of a tetrapeptide and the cross-bridge peptide.

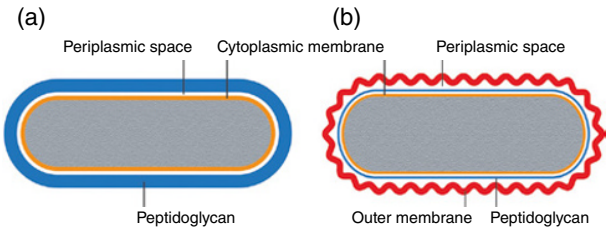


Figure 2.5 The cell envelope of a gram-positive and a gram-negative bacterial cell.

membrane and peptidoglycan (Figure 2.5a). The space between the two layers, the periplasmic space, contains enzymes. In addition to the proteins embedded in the periplasmic space, certain gram-positive bacteria contain surface proteins that are involved in interactions of the cell with its environment. These proteins are covalently or noncovalently linked to teichoic acid, peptidoglycan, or other polymers, such as capsular polysaccharide. These proteins have a role in the virulence of some pathogenic gram-positive bacteria (for example, *Staphylococcus* and *Streptococcus*) by binding to host proteins and interfering with host defenses. In addition, the cell walls of gram-positive bacteria contain another macromolecule called the teichoic acids, which are composed of glycerol (a 3-carbon alcohol) or ribitol (a 5-carbon alcohol) and phosphate. There are two types of teichoic acid: lipoteichoic acid, which spans the peptidoglycan layer and is linked to the cytoplasmic membrane, and cell wall teichoic acid, which is linked to peptidoglycan (Figure 2.6). The phosphate groups carry a negative charge and hence bind and regulate the movement of cations (positive charge) into and out of the cell. Teichoic acids provide much of the wall's antigenic specificity and are used in bacterial identification.

Cell Walls of Gram-Negative Bacteria

The cell walls of gram-negative bacteria have a thin layer of peptidoglycan (2–4 nm in thickness) and an OM; the cytoplasmic membrane is the inner membrane (Figure 2.5b). The inward-facing OM consists of a single layer of phospholipids, which are linked to peptidoglycan via lipoproteins. The outward layer of OM, the LPS, provides structural rigidity in place of the thicker peptidoglycan layer. The OM is attached to peptidoglycan via lipoproteins. The space between the cytoplasmic membrane and the OM is called the periplasm, which contains enzymes and transport proteins. The thin peptidoglycan lies in the periplasmic space. The periplasmic space is much wider than in gram-positive bacteria. Gram-negative bacteria do not contain teichoic acids (Table 2.3). The OM has several specialized functions. The strong negative charge is an important factor in evading phagocytosis and the actions of the complement system, two components of the host defense. The OM provides a barrier to certain antibiotics (penicillin), digestive enzymes (lysozyme), and chemicals (detergents, etc.). The OM also has proteins that form pores or channels (porins), which permit the passage of molecules such as nucleotides, sugars, peptides, and amino acids.

Although the major function of the OM is structural, one of its important biological properties is its toxicity to mammalian cells. The toxicity is due the LPS component, also known as endotoxin. The LPS of gram-negative bacteria has three components (Figure 2.7):

a) *Lipid A*: The fatty acids of the lipid component are linked to the phospholipid layer of the OM and are responsible for many of its biological activities, including toxigenicity.
b) *Core polysaccharide*: The core is composed of sugars, including two characteristic sugars, ketodeoxyoctanoic acid and heptose. The core attaches lipid A to the O-polysaccharide.
c) *O-polysaccharide*: The polysaccharide extends outward from the OM surface and is composed of a number of sugars, including some unusual sugars. The composition varies between species and strains within a species. The O-polysaccharide is antigenic (somatic or O antigen), which is the basis of serotyping in many gram-negative bacteria, particularly in the genera of *Escherichia* and *Salmonella*.

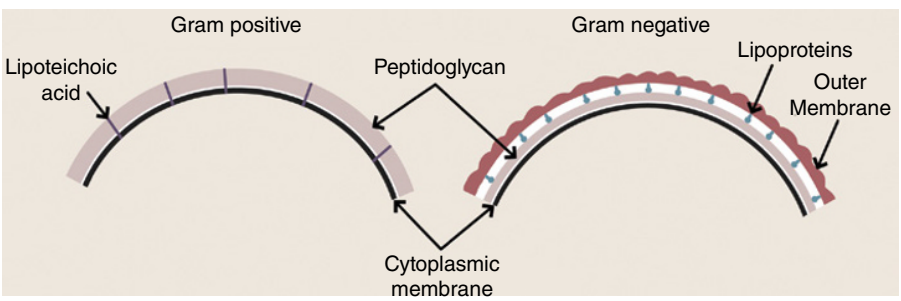


Figure 2.6 Cell envelope of gram-positive (bilayer) and gram-negative bacteria (trilayer).

Table 2.3 Comparative characteristics of gram-positive and gram-negative bacteria.

Characteristics	Gram-positive bacteria	Gram-negative bacteria
Gram reaction	Resist alcohol decolorization and retain the primary stain, crystal violet, and appear purple	Get decolorized by alcohol and take the counter stain, safranin, and appear red
Peptidoglycan layer	Thick (multilayered)	Thin (single or few layers)
Teichoic acid	Present in many	Absent
Outer membrane	Absent	Present
Lipopolysaccharide	Absent	Present
Lipid and lipoprotein content	Low	High
Resistance to physical disruption	High	Low
Susceptibility to penicillin	High	Not or low

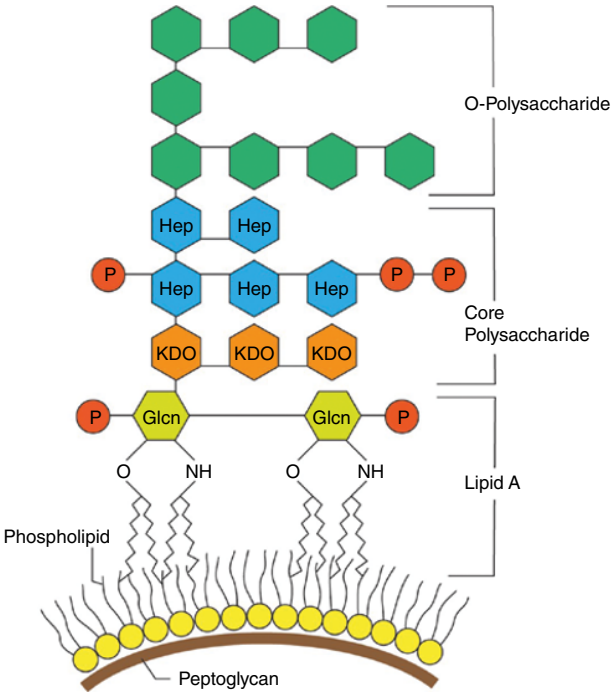


Figure 2.7 A simplified diagram of lipopolysaccharide of gram-negative bacteria composed of lipid A, core polysaccharide, and O-polysaccharide units.

The major components (polysaccharide, core, and lipid A) are common to all gram-negative bacteria, but the precise chemistry varies among species of bacteria. Endotoxins are a major virulence factor responsible for a number of animal diseases caused by gram-negative bacteria (pasteurellosis, salmonellosis, colibacillosis, etc.). Interestingly, LPS from nonpathogenic bacteria have the same three components and do have endotoxin activity, but for the most part, their potency is lower than that of pathogenic gram-negative bacteria. The basic differences between gram-positive and gram-negative bacteria are outlined in Table 2.3.

Bacteria that Lack Peptidoglycan

Peptidoglycan is unique to bacteria because no other cell contains this macromolecule. However, some bacteria do not contain peptidoglycan. Species of the genus *Mycoplasma* do not contain a cell wall and contain only cytoplasmic membrane. Therefore, *Mycoplasma* are spherical, tend to be pleomorphic and are somewhat osmotically sensitive. Species of bacteria in the phylum *Chlamydiae,* which includes obligate intracellular pathogens (*Rickettsia, Ehrlichia, Chlamydia,* etc.), do not contain the peptidoglycan, but do contain the OM with LPS, similar to gram-negative bacteria.

Bacteria with Complex Cell Wall

Complex cell walls are present in species of *Mycobacterium, Mycobacterium tuberculosis*, and *Mycobacterium avium* subsp. *paratuberculosis,* which cause tuberculosis and Johne's disease, respectively. The mycobacterial cell wall includes a thin peptidoglycan linked to chains of galactose and arabinose, called arabinogalactan. The ends of the arabinogalactan are linked to unusual and complex waxy lipids called mycolic acid. The mycolic acids, which are hydrophobic, accounts for 60% of the cell wall constituents in mycobacteria. The mycolic acids and peptidoglycan are held together by the arabinogalactan. The lipid layer causes *Mycobacterium* to clump to form bundles of cells in broth cultures. It also does not permit dyes to enter, and hence is not stained by Gram's stain procedure. Therefore, a special staining procedure called an "acid-fast stain" is used, in which carbol fuchsin is used to stain the organisms. *Acid fast* refers to the ability of the cell to retain the dye even after washing with acid alcohol. The waxy layer slows down nutrient uptake, which is why mycobacterial species are slow-growing organisms. Further, mycolic acids provide

protection against host defenses, which allow them to persist in the host for a long period and confer resistance to many antibiotics. Mycolic acids are also present in other bacterial species, such as *Nocardia asteroides, Corynebacterium pseudotuberculosis*, *Rhodococcus equi*, and species of actinomycetes, a large and diverse group of soil bacteria

Cytoplasmic Structures

Cytoplasm is the substance of the cell enclosed within the cytoplasmic (plasma) membrane. Cytoplasm is about 80% water and is composed primarily of proteins (enzymes), carbohydrates, lipids, inorganic ions, and low-molecular-weight compounds. Cytoplasm is thick, aqueous, and semitransparent. Cytoplasm contains the following major structures: nuclear material, ribosomes, inclusions, spores, plasmids, and actin-like cytoskeletal proteins.

Nuclear Material

The nuclear material is called nucleoid (meaning not a true nucleus) and contains a single, long, tightly coiled, circular double-stranded DNA. Although there are exceptions, DNA in most bacteria is circular. Bacterial DNA, which is longer than the length of the cell, is packaged densely in the cytoplasm because it is twisted into supercoils. Supercoiling is facilitated by certain proteins (nucleoid-associated protein) using the action of enzymes called topoisomerases. DNA gyrase, one of the topoisomerases that introduces supercoiling, is the target for fluoroquinolones, a widely used broad-spectrum antimicrobial agent in animals and humans. DNA contains genetic information, and chromosomes carry the genes required for the structures and functions of the bacterial cell. Unlike the chromosomes of eukaryotic cells, bacterial DNA are not enclosed in a nuclear membrane and do not include histones. Bacteria, with some exceptions (e.g. *Borrelia burgdorferi*, the causative agent of Lyme disease in humans and animals), contain a single chromosome.

In some bacterial species, the chromosome may contain an integrated bacteriophage DNA, called a prophage, from phage infection. The host cell is unharmed, and the phage DNA replicates as part of the host DNA replication, and both daughter cells carry the prophage. Such bacteria are called lysogenic bacteria or lysogens. A prophage can also spontaneously excise and initiate a lytic cycle. Prophages may carry genes that encode for virulence factors and contribute to the pathogenicity. For example, Shiga toxin-producing *E. coli,* which causes edema disease in swine, carries genes that encode for Shiga toxins, specifically a subtype called Shiga toxin 2e.

Ribosomes

The cytoplasm of bacterial cells is packed with ribosomes, which appear as small granules and give the cytoplasm a granular appearance. Bacterial ribosomes are smaller and less dense than the ribosomes of eukaryotic cells. Ribosomes are 70S and are composed of two subunits, small and large. The small subunit is 30S, and the large is subunit is 50S. The "S" refers to the Svedberg, the unit of the sedimentation coefficient, which indicates the relative rate of sedimentation during ultra-centrifugation. The sedimentation coefficient depends on the size, weight, and shape of the ribosome. Each subunit has a number of proteins and a type of RNA called ribosomal RNA (rRNA). The small subunit contains 16S rRNA, and the large subunit contains 5S and 23S rRNA.

Ribosomes function as the site of protein synthesis, and cells that have high rates of protein synthesis have more ribosomes. Several major antibiotics (aminoglycosides, macrolides, lincosamides, macrolides, tetracyclines, etc.), widely used in human and veterinary medicine, exert their antibacterial actions on ribosomes and inhibit protein synthesis. Because animal cells do not have the 30 or 50S subunit, these antibiotics inhibit only bacteria.

Inclusions or Storage Granules

Bacterial cells have several kinds of reserve deposits, either inorganic or organic, known as inclusions. Cells tend to store certain nutrients when they are plentiful and use them when needed. The amount of stored inclusions depend on the nutritional status. Some inclusions (carbon) are common to a wide variety of bacteria, whereas others (phosphorus or sulfur) are specific to certain bacterial species. Most bacteria store polysaccharides, such as glycogen and starch. A few store lipids (poly-ß-hydroxybutyrate), phosphorus, sulfur granules, and so on.

Plasmids

Plasmids are extrachromosomal DNA that lie in the cytoplasm and replicate independent of the chromosome (Figure 2.8). Many bacteria contain one or more plasmids. Plasmids are usually 1–5% the size of the host chromosome and range in size from a few thousand to a few million base

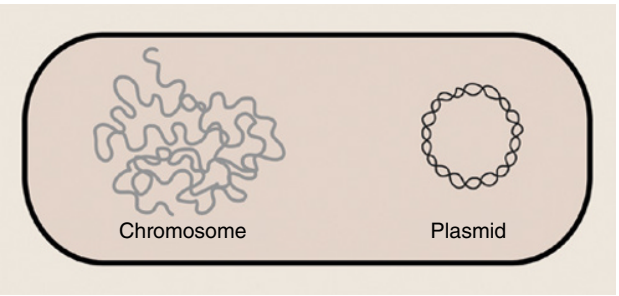


Figure 2.8 Plasmid (extrachromosomal DNA) in a bacterial cell.

pairs. Most plasmids are double-stranded DNA, most are circular, but a few are linear. The number of plasmids per cell, called the copy number, varies widely. Some plasmids replicate in a stringent fashion with only one to two copies per cell, and some plasmids have a copy number that ranges from 30–40 to over 100, due to the relaxed mode of replication. Typically, housekeeping genes essential for normal metabolism, growth, or cell division are not carried on plasmids. However, genes carried on plasmids can confer selective advantages to cells, such as genes for antimicrobial resistance, chemical resistance, sex pilus, and virulence factors. The elimination of plasmids, which can occur spontaneously or can be induced by treatments with chemicals, is called curing.

Spores

Spores are cytoplasmic structures formed in certain bacteria, generally in gram-positive bacteria. Spores are the dehydrated, highly durable, resistant, and dormant form of the bacterial cell. They can survive for a long time and resist extreme heat, lack of water, chemicals, radiation, and so on. The resistance is due to the thick spore coat formed during sporulation. Among pathogens, *Bacillus* and *Clostridium* are the two important genera that are spore formers. *Coxiella burnetii,* a gram-negative species, also forms spore-like structures that resist heat and chemicals. The diameter of the spore may be smaller than, equal to, or larger than the diameter of the vegetative cell. They may be located centrally, terminally (end of the cell), or subterminaly, which are the morphological features of spore-forming bacteria that are useful in identification.

The process of spore formation within a cell (vegetative cell) is called sporulation or sporogenesis. The process begins whenever a key nutrient (carbon or nitrogen) is depleted or deficient or when cells are exposed to adverse conditions. During the process, a thick spore coat consisting of proteins is synthesized outside the cell wall that confers resistance to the harsh environment. Spores may be released freely into the environment by the rupture of the vegetative cells (Figure 2.9). The clinical significance of spores is that they are resistant to processes, such as heat, disinfectants, and radiation, that normally kill vegetative cells. Most pathogenic bacteria are killed by heating to 70 °C or boiling (100 °C), but spores can survive. Therefore, increasing the temperature above boiling achieved by steam under pressure to 121 °C (autoclaving) is essential to kill spores. Spores contain all the essential components (DNA, proteins, and small molecules) necessary to grow into vegetative cells. The process by which spores become vegetative cells is called germination.

Actin-like Cytoskeletons

Similar to eukaryotic cells, bacteria contain specific proteins that define cell shape. Peptidoglycan, which was earlier thought to determine cell shape, is now known to play only a minor role. The shape-determining proteins show significant homology to the eukaryotic cell protein actin. Hence, bacterial cytoskeletal proteins are called actin-like proteins. A number of cytoskeletal proteins have been identified, and one of the best studied is MreB and its homologues. The MreB protein is present in rod-shaped and spiral-shaped bacteria, but is absent in cocci.

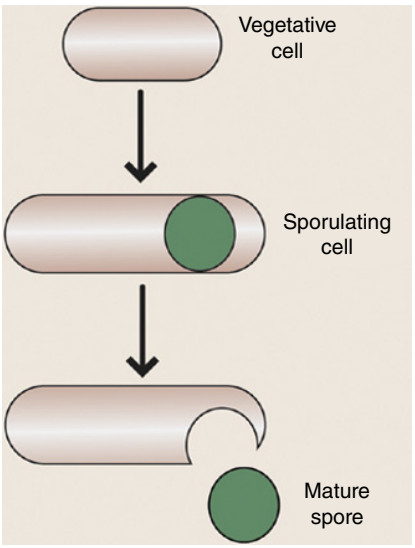


Figure 2.9 Sporulation and release of a spore.

Surface Structures of Bacteria

The structures present on the surface and external to the cell are the capsule, biofilm, flagella, fimbriae, or pili.

Capsules/Slime

The capsule is a well-organized structure, firmly attached to the cell wall layer. The substance is synthesized by the cell, secreted, and deposited on the cell surface. Capsules are not stained by Gram stain, although in some situations they appear as a clear halo surrounding the cell. A special staining procedure, called negative staining, is used to visualize the capsule. The procedure uses black dyes such as nigrosin or India ink to color the background surrounding encapsulated bacteria, and not the capsule or the cell. A second dye, usually safranin, is used to color the vegetative cell. The capsules appear as a clear halo surrounding a pink cell in a black background.

The capsules of most bacteria are composed of polysaccharide, but in others, it is made of proteins, glycoproteins, or other materials. The term *glycocalyx* (meaning "sugar coat") is used for capsular polysaccharide. In *B. anthracis,* a species that causes anthrax in animals and humans, the capsule is poly-D-glutamic acid. If the substance is unorganized, loosely attached to the cell wall, and often shed into the

surrounding environment, the capsular material is called a slime or slime layer. The composition of the polysaccharide varies widely with the species. The thickness of the capsule may increase under certain growth conditions (carbohydrate-rich medium). Although capsules are not essential for growth, they do confer some advantages. The functions of the capsule are to protect the cell against dehydration, chemicals, bacteriophages, and so on; nutrition; possibly serve as a carbon reserve; and mediate attachments to host cells and other surfaces. The capsule is an important virulence factor for certain pathogens. Besides mediating attachment to host cells, capsular bacteria resist phagocytosis and allow the bacteria to colonize and infect the host. For example, only capsulated *B. anthracis* strains can cause anthrax, whereas non-capsulated strains are avirulent. Another example is *Streptococcus pneumoniae,* a human pathogen that causes pneumonia. The capsule is also antigenic (K antigen) and is one of the cell surface antigens used in serotyping certain bacterial species, such as *E. coli* and *Salmonella*.

Biofilms

In some cases, a group of cells (of single or multiple species) become embedded in a common extracellular matrix composed of polysaccharide, which is referred to as a biofilm. The material, extracellular polymeric substance (EPS), is composed of polysaccharides, proteins, glycoproteins, glycolipids, and lipids, depending on the bacterial species. Bacterial cells within biofilms are physiologically different from their corresponding free cells (planktonic). The cells within a biofilm function cooperatively because of cell-to-cell communication and in some ways mimic the behavior of multicellular organisms. Bacteria within a biofilm interact in a variety ways, including metabolic interaction, horizontal gene transfer, and so on. Biofilm formation occurs on a range of animate or inanimate objects and organic or inorganic surfaces and includes distinct stages: initiation, maturation, maintenance, and dissolution. The initiation is triggered by various environmental signals (pH, temperature, ionic concentration, oxygen, amino acids, etc.) depending on the bacterial species. Biofilms play critical roles in bacterial pathogenesis as well as in the environment quality (equipment damage, product contamination, etc.). Formation of biofilm aids attachment and survival because it provides protection against phagocytosis, antibodies, or antibiotics. Examples of biofilms in bacterial pathogens and pathogenesis include colonization of heart valves (valvular endocarditis), teeth (dental caries), and of indwelling catheters, medical devices, and prostheses.

Flagella

These are structures that confer motility to the organism. Flagella (flagellum is singular) are filamentous appendages, 10–20 μm in length (longer than the bacterial cell), attached to the cytoplasmic membrane at one end and free at the other end. The rotation of the flagella, which requires energy (ATP), propels the cell to become motile. Flagella are too thin to be resolved by light microscopy unless specialized staining techniques that increase their thickness are employed. Most often, flagella are present in rod-shaped bacteria. Flagella are composed of protein called flagellin and therefore are antigenic. The flagellar antigen is called H antigen and is used in serotyping certain gram-negative bacterial species such as *E. coli* and *Salmonella*. The number (1, 2, or more) and arrangement of flagella are useful in bacterial identification (Figure 2.10). The arrangements of flagella include a single flagellum at one (monotrichous; *trikhos* means "hair" in Greek) or both ends (amphitrichous), tufts of flagella at one or both ends (lophotrichous), or present on the entire surface of the cell (peritrichous). The peritrichous flagella may allow the bacteria to move in unison, resulting in a type of growth on agar surface called swarming, as seen in some species of *Proteus*, an animal and human pathogen. Although the main function is motility, in some species they mediate attachment to the host cell and in others they play a role in virulence. An advantage of motility is that it enables the organism to move toward a favorable environment, for example, to seek nutrients, or to move away from harmful substances, for example, waste products and an adverse environment. The movement of a bacterium toward or away from a stimulus is called taxis, which includes chemicals (chemotaxis) and light (phototaxis).

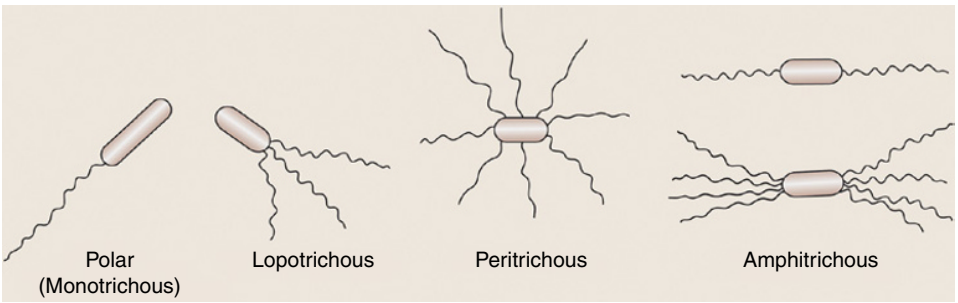


Figure 2.10 Flagellar arrangement in bacterial cells.

Spirochetes (spiral-shaped organisms such as *Borrelia, Brachyspira, Leptospira*) have flagella, which distinguish them from other bacteria. They have multiple flagella originating from each end of the spirochete, and do not extend outside the cell but remain within the periplasmic space between the inner membrane and the OM. These periplasmic flagella contribute to a type of motility of spirochetes called corkscrew motility.

Fimbriae and Pili

These are hair-like appendages that are shorter, thinner, and straighter than flagella. They are ultramicroscopic structures and are visualized only by electron microscopy. The number of fimbria or pilus (singular) varies from one to a few to several hundreds per cell. They are also composed of a protein called pilin. Both terms are used interchangeably, although the term *pilus* is always used for structures involved in bacterial conjugation, in which sex pilus mediates the joining of two bacterial cells which allows DNA transfer. Sex pili are larger than the other pili and are encoded by genes located on the conjugative plasmids. The major function of fimbriae is to facilitate attachment to host cells. For example, enterotoxigenic *E. coli* (causes diarrhea in animals and humans) are able to colonize intestinal epithelial cells because of pili. Blocking the attachment (pili-based vaccine) prevents the infection. A type of fimbria called type IV fimbria is involved in motility and confers a type of flagella-independent motility called twitching motility, which is a gliding type of motility. The type IV fimbriae are present in bacterial species such as *E. coli, Pseudomonas aeruginosa,* and *Dichelobacter nodosus*.

A summary of bacterial structures, their functions, and roles in bacterial virulence and antimicrobial susceptibility is presented in Table 2.4.

Table 2.4 Summary of the structural features and their functions in bacterial cells.

Structure	Chemical composition	Function	Comments
Cell envelope			
Cell wall	Peptidoglycan: Thick in gram-positive and thin in gram-negative bacteria	Shape determination Protection	Basis of Gram staining
Outer membrane	Lipid, polysaccharide, phospholipids	Antigenicity Endotoxin	Permeable to nutrients, contribute to pathogenicity, basis of serogrouping (O antigen)
Cytoplasmic membrane	Phospholipids, Proteins	Selective permeability Site of enzymatic reactions	Transport of nutrients
Cytoplasmic structures			
Nucleoid	DNA	Chromosome (single). Contain essential genes	Exists as a single, closed loop structure
Plasmids	DNA	Contain nonessential genes	Present only in some bacteria
Spores	Resistant form	Spore coat provides protection	Present in some bacteria
Ribosomes	RNA and protein	Site of protein synthesis	Target for antibiotics
Inclusion bodies	Carbohydrate, lipids, sulfur, etc.	Used as nutrients	Nutrient storage. Used during starvation
Actin-like cytoskeletons	Protein	Shape determination	Significant homology to eukaryotic actin
Cell surface structures			
Capsule	Polysaccharide in most cases. In some, polypeptides	Protection against phagocytosis Mediate attachment to epithelial cells Antigenicity	Contribute to pathogenicity Basis of serotyping (K antigen)
Flagella	Protein (flagellin)	Motility Antigenicity	Contribute to pathogenicity Basis of identification and serotyping
Fimbriae/pili	Protein (pilin)	Mediate attachment to epithelial cells Genetic transfer (conjugation) Antigenicity	Contribute to pathogenicity; conjugation

Bacterial Physiology: Metabolism, Growth, and Nutrition

Understanding the metabolic, growth, and nutritional requirements of bacterial pathogens is important because physiology and pathogenesis are closely related. The presence or absence of nutrients and ability to metabolize the nutrients often determines whether the organism can survive, colonize, and initiate infection. In addition, knowledge of the physiology, such as the substrates used, metabolic pathways, and products, are essential for cultivation of the organism, for identification, for vaccine production, and in industrial microbiology for commercial production of enzymes, antibiotics, etc. In addition, metabolic pathways can provide targets for antibacterial chemotherapy, as in folic acid biosynthesis for sulfa drugs.

Metabolism

Metabolism is the sum of all biochemical reactions that occur in a cell. Biochemical reactions either release or require energy. Therefore, metabolism is divided into two types of processes: catabolism and anabolism. Catabolism is the process of breaking complex molecules (degradative reactions) into smaller and simpler molecules with concurrent release of energy ATP and reducing electrons or cofactors, such as reduced nicotinamide adenine dinucleotide (NADH), flavin dinucleotide (FADH), and ferredoxin. The ATP generated in catabolic pathways is utilized in anabolic pathways.

These energy-yielding reactions are generally hydrolytic (reactions that use water to break chemical bonds) and are exergonic (they produce more energy than they consume). For example, breakdown of sugars into the final products of CO_2 and H_2O will yield ATP, and the intermediates produced may be used in biosynthetic pathways of sugars required to synthesize nucleotides and amino acids, which in turn are required to synthesize nucleic acids and proteins, respectively. The process of synthesis of complex molecules (biosynthetic reactions) from simpler molecules is required to form cell components and structures. The process is endergonic (requires energy) and often utilizes reducing power or electrons. The energy is frequently supplied by hydrolysis of energy-rich compounds, primarily ATP. Anabolic pathways start with catabolic intermediates and use them to form the building blocks of macromolecules. For example, amino acids produced during catabolism of proteins will be used to make proteins required by the cell.

Generation of ATP

Energy released during oxidation and reduction reactions is captured within the cell by the production of ATP. Specifically, a phosphate group is added to ADP with the input of energy to form ATP. The addition of phosphate to a chemical compound is called phosphorylation. Bacteria use three mechanisms of phosphorylation to generate ATP from ADP: (i) Substrate-level phosphorylation, (ii) oxidative phosphorylation, and (iii) photophosphorylation, only in photosynthetic bacteria (in which energy from sunlight is used). In substrate-level phosphorylation, ATP is produced by the direct transfer of a phosphorylated compound (a substrate) to ADP. In oxidative phosphorylation, electrons are transferred from organic compounds to electron carriers, generally NAD and FAD. The electrons are then transferred sequentially through a series of different electron carriers to molecules of oxygen, inorganic molecules other than oxygen, or organic molecules. The transfer of electrons from one electron carrier to the next releases energy, which is used to form ATP from ADP. The sequence of electron carriers used in oxidative phosphorylation is called an electron transport chain or system. The process occurs in the cytoplasmic membrane of bacteria.

Energy Production Mechanisms

Bacteria that use chemicals, either inorganic or organic, to derive energy are called chemotrophs. All bacterial pathogens are chemotrophs. Inorganic compounds, such as nitrogen, sulfur, and iron, are used to derive electrons and energy. There are two mechanisms of energy production: respiration and fermentation (Table 2.5). In each mechanism, the synthesis of ATP is driven by the energy released in oxidation-reduction reactions. Respiration is an energy-deriving process in which chemicals are oxidized and the

Table 2.5 Comparison of aerobic respiration and fermentation.

Characteristics	Aerobic respiration	Fermentation
Growth condition	Aerobic	Aerobic or anaerobic
Electron donor	Inorganic or organic molecule	Organic molecule
Final electron acceptor	Oxygen	Organic molecule
Type of phosphorylation used for ATP production	Substrate-level and electron transport	Substrate-level only
No. of ATP produced per mole of glucose	36 or 38	2–4

electrons are transferred to inorganic molecules. If the electron acceptor is oxygen, the respiration is called aerobic respiration. If the final electron acceptor is an inorganic molecule other than oxygen (nitrates, sulfates, etc.), the process is called anaerobic respiration. An essential feature of respiration is the involvement of the electron transport chain. The electrons released during respiration are transferred via carriers such as NAD, FAD, and ferredoxin to an electron transport chain. In contrast, fermentation does not use the electron transport chain. Fermentation is an energy-deriving process in which organic molecules are both electron donors and electron acceptors. Electron carriers such as NAD, FAD, and ferredoxin are involved in the electron transfer from donors to acceptors. An essential requirement of fermentation is that the reduced cofactors (NADH, FADH, FDH, etc.) are converted back to oxidized cofactors to ensure that the process to generate energy from organic substrates continues. The oxidation of an organic molecule (electron donor) is coupled to the reduction of an organic molecule (an electron acceptor) derived from the electron donor. The electron donors are substrates such as carbohydrates, proteins, and lipids. The electron acceptor is derived from within the cell and is an intermediate of the catabolic pathway, most commonly pyruvate. Therefore, no exogenous electron acceptor is involved, and the products are organic. Although oxygen is not involved, fermentation can take place in the presence of oxygen. During fermentation, ATP is generated by substrate-level phosphorylation in which a phosphate from a high-energy compound is transferred to ADP. Therefore, only small amounts of ATP and much of the energy remains in the organic end products (acetate, ethanol, lactate, etc.). Fermentative bacteria can use a variety of organic substrates, and the end products depend on the substrates and bacterial species. Bacteria that use fermentative metabolism live in habitats rich in organic molecules, such as the gastrointestinal tract and soil. A bacterium such as *E. coli,* a member of the normal flora of the gut of animals and humans, can oxidize glucose to carbon dioxide and water (aerobic respiration), reduce nitrate to nitrite (anaerobic respiration), and ferment glucose to produce acetic acid (fermentation), based on the availability of substrates and oxygen in the ecosystem.

Glucose is by far the most common substrate used by bacterial pathogens. Therefore, the two most common biochemical pathways in its metabolism are glycolysis (Embden-Meyerhof pathway) and tricarboxylic acid cycle (Krebs cycle). The Embden-Meyerhof operates either in the presence or in the absence of oxygen, whereas Krebs cycle is an oxidative process. One other pathway that is important in bacterial catabolism is pentose phosphate or hexose monophosphate pathway that can function aerobically or anaerobically permits interconversion of pentose to hexose or hexose to pentose. All three pathways are important not only because of ATP production, but also because many of the reactions are reversible and provide intermediates and reduced cofactors for the biosynthetic purposes of cellular constituents.

Bacterial pathogens that enter an animal's body have access to, in addition to saccharides and polysaccharides, other complex organic molecules, mainly proteins and lipids, that can be used to generate the energy required for growth. The metabolic mechanisms used by carbohydrates, lipids, and proteins to generate ATP are interrelated (Figure 2.11). Similar to polysaccharides, proteins, and lipids are too large to cross the cytoplasmic membrane of bacterial cells. Bacteria produce extracellular proteases and lipases to break down the polymer into oligomers or monomers. Proteins are polymers of amino acids, and proteases break peptide bonds to release peptides and amino acids. Most lipids are composed of glycerol and fatty acids, which are released by the action of lipases. Monomers, amino acids, glycerol, fatty acids, and sometimes oligomers (e.g.

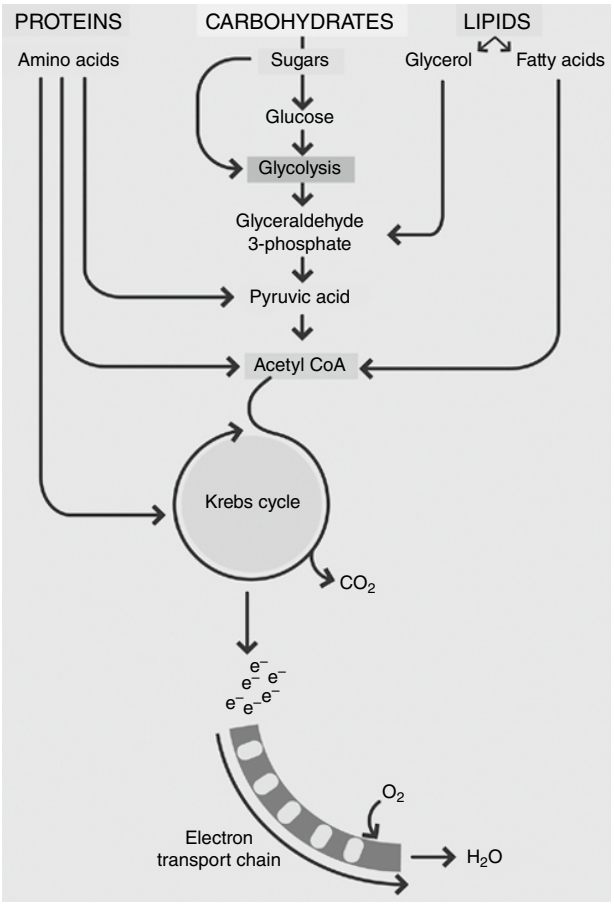


Figure 2.11 Interrelationships between carbohydrate, protein, and lipid metabolism in bacteria.

short peptides) can pass through the cytoplasmic membrane and are metabolized either to generate energy or to be assimilated for biosynthetic purposes. Glycerol is phosphorylated, converted to dihydroxyacetone phosphate, and enters the Embden-Meyerhof pathway to become pyruvic acid. Fatty acids are oxidized to become two carbon acetyl Co-A, which can then enter the Krebs cycle to generate energy or be used for biosynthesis of bacterial lipids. Amino acids that are transported into the cytoplasm can be used to synthesize cellular proteins or may be deaminated or transaminated to remove ammonia so that the rest of the carbon skeletons can be fed into the Krebs cycle to generate energy.

Growth and Nutrition

Bacterial growth means an increase in the number of cells and not in their size. Bacteria reproduce asexually by a process called binary fission. Typically, binary fission is initiated with elongation of the cell, followed by DNA replication, formation of a partition called the septum, and separation of two daughter cells. Each daughter cell will have genetic information identical to the parent cell. The time required for one cell to divide into two cells is called the generation time. The generation time varies considerably among bacterial species and is dependent on the growth conditions, such as temperature, pH, and availability of nutrients. It could range from 20 minutes to several hours to several days. Most pathogenic bacteria have a generation time of one to three hours. When one cell divides into two cells, one generation has occurred. Therefore, the number of cells in each generation is expressed as a power of 2, the exponent indicating the number of doublings or generations that have occurred (2^{10} means 10 generations). In an organism with a generation time of 20 minutes, 30 generations (<10 hours) will allow one cell to increase to 10^9 cells (1 billion cells), and in 24 hours the number would be 10^{21}, which is 1 followed by 21 zeros.

Growth Curve and Phases

Bacterial growth and concentrations achieved during growth are best studied in a liquid or broth culture. This type of culture in a single batch in which nutrients are not renewed and waste products accumulate is called batch culture. When a few bacterial cells are inoculated into a suitable liquid culture medium contained in a tube or flask, growth occurs. A number of methods can be used to measure the growth. Broadly, the methods are direct or indirect. In the direct method, the counts are determined under a microscope, called microscopic counts, or by diluting the samples and plating on to a suitable culture medium, called culture counts. Microscopic counts are a quick method, and the counts include all cells in a sample, live or dead, and hence they are called total cell counts. In the culture method, only live cells are counted because the method requires cells to divide and grow on an agar medium. The growth results in visible colonies, which are counted. Therefore, the viable count is also called the plate count or colony count. In the plate count method, the sample is diluted several times (usually 10-fold), a procedure called serial dilution, so that a reasonable number of colonies develop on a plate. When too many colonies are present, some cells are overcrowded and do not develop separate clearly, which causes inaccurate counts. The convention is to have plates between 30 and 300 colonies. In plate counts, the assumption is that each viable cell grows and divides to produce a single colony. This is not always true because cells are linked in pairs, chains, or clusters, which grow to become a single colony. Therefore, the counts are reported as colony forming units (CFUs) because the counts are not actual cell numbers. There are two ways of performing a plate count: spread plate and the pour plate methods. The former is used more often than the latter. The indirect method is rapid; it is an easier method to estimate bacterial growth in a liquid medium. The method measures the turbidity because when the bacterial numbers increase, the medium becomes turbid. More cells in suspension means higher turbidity. The turbidity is measured in a spectrophotometer, in which a beam of light is transmitted through a bacterial suspension to a light-sensitive detector. As the bacterial numbers increase, less light will reach the detector. The change in light is measured as the absorbance or optical density.

When bacteria are grown in a batch culture and growth is monitored and plotted against the incubation time, a characteristic growth curve is obtained. In a typical growth curve, there are four basic phases: lag, logarithmic or exponential, stationary, and death (Figure 2.12). In the lag phase, there is no measurable growth because there is no cell division. In this phase, cells are dormant, but metabolically active. The lag phase may be a few minutes to several hours depending on the organism, medium, and growth conditions. In the logarithmic phase, cells begin to divide to achieve an exponential growth phase, where one cell becomes two, two cells become four, four become eight, and so on. Because cell division is most rapid and cells are metabolically most active, they are also most susceptible to adverse conditions, such as antibiotics. In the stationary phase, the exponential growth begins to cease and the population density stabilizes. The reasons for the cessation of exponential growth include exhaustion of nutrients, accumulation of toxic waste products, and changes in the culture conditions (oxygen level, pH change, etc.). In this phase, the number of cells dying is approximately equal to

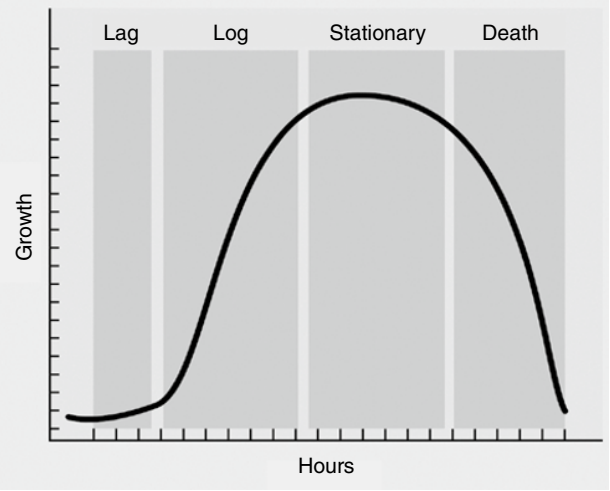


Figure 2.12 Growth phases in bacterial cell growth.

the newly formed cells, resulting in a flat line in the growth curve. The metabolic activities of surviving cells also slow down in this period. In the death phase, the numbers of dead cells exceed the numbers of cells formed, and the population enters the phase of decline. The death phase is almost exponential, but typically, it is slower than the log phase. In some species, all cells will die within a few days, and in other species, some cells will survive for a long period, almost indefinitely. The duration of each phase in the growth cycle is dependent on the organism and growth conditions, which include physical and chemical factors.

Physical and Chemical Requirements for Bacterial Growth

Understanding the requirements for bacterial growth helps to explain the habitat in which bacteria live and makes it possible to devise methods to grow, enhance their activities, or to control them. The requirements for growth include physical requirements, such as oxygen, carbon dioxide, temperature, and pH, and chemicals, which include nutrients and growth factors.

Oxygen

Oxygen is a critical requirement for many bacteria; it dictates the type of metabolism they carry out to generate energy for growth. Bacteria vary in their need and tolerance for oxygen. They are divided into two major groups, aerobes and anaerobes, depending on their need for oxygen and how they are affected by oxygen (Table 2.6):

a) Aerobes are species that require oxygen for growth (oxic condition). They utilize oxygen in their metabolism. There are three types of aerobes.
   - *Obligate*: Bacteria that cannot grow without oxygen.
   - *Facultative*: Bacteria that can use oxygen and grow when present, but can also grow when oxygen is not available. Growth is higher in the presence of oxygen because they use oxidative phosphorylation to generate ATP. Most pathogenic bacteria are facultative.
   - *Microaerophilic*: Bacteria that can grow only in oxygen concentration lower than that in air. Air has 20% oxygen, and these bacteria require only 2–10% oxygen.

b) Anaerobes are species that can grow only in the absence of oxygen (anoxic condition) and do not utilize oxygen in their metabolism. In many instances, oxygen is toxic to anaerobic bacteria. Based on the degree of oxygen tolerance, anaerobes are further categorized into two groups:
   - *Aerotolerant anaerobes*: These will not grow in the presence of oxygen, but survive exposure to oxygen for a period of time, which varies from a few minutes to several hours. Most anaerobes of clinical importance are aerotolerant.

Table 2.6 Categorization of bacteria based on their oxygen requirements.

Group	Relationship to oxygen	Type of metabolism	Examples of animal pathogens
Aerobes			
Obligate	Required	Aerobic respiration	*Brucella*
Facultative	Not required, but better growth with O_2	Aerobic respiration and fermentation	*Escherichia coli* *Salmonella enterica*
Microaerophilic	Required, but better growth with less O_2	Aerobic respiration	*Campylobacter, Helicobacter*
Anaerobes			
Aerotolerant	Not required	Fermentation	*Fusobacterium necrophorum*
Obligate	Not required and O_2 is harmful	Fermentation	*Bacteroides, Porphyromonas, Prevotella*

– *Obligate anaerobes*: These bacteria are inhibited or even killed by exposure to oxygen. However, obligate anaerobes can survive in ecosystems exposed to the air or oxygen (as in the oral cavity) because there are microenvironments in the habitat where there is no oxygen. In addition, facultative bacteria in the same habitat use oxygen and thus protect strict anaerobes.

Reasons for Oxygen Toxicity

There are several reasons for oxygen being inhibitory or toxic to anaerobes. Certain enzymes, which are key to their survival, may be inactivated because of the oxidation of sulfhydryl groups (SH to S) contained in the structure. Oxygen is an oxidant, and certain oxygen derivatives, called reactive oxygen radicals or reactive oxygen species, are more powerful oxidants – and hence also toxic – than molecular oxygen. The reactive oxygen radicals or species include singlet oxygen ($^1O_2$), superoxide (O_2^-), peroxide anion (O_2^{2-}), and hydroxy radical (OH^*). These oxygen radicals are toxic to bacteria and inhibit growth or cause cell death by disrupting nucleic acids and protein structures of the cell. These radicals are also present in phagocytic cells that kill bacteria following phagocytosis. Bacteria that grow in the presence of O_2 have enzymes that can detoxify the reactive forms of oxygen. The most common enzyme is catalase, which catalyzes conversion of hydrogen peroxide to water. Another enzyme that catalyzes hydrogen peroxide to water is peroxidase. A key enzyme that protects bacteria from oxygen radicals is superoxide dismutase, which, in concert with catalase or peroxidase, detoxifies two molecules of superoxides to form hydrogen peroxide and then water. Aerobes and facultative anaerobes generally contain superoxide dismutase and catalase or peroxidase, although there are many exceptions (*Streptococcus* is catalase negative). Anaerobes, particularly obligate anaerobes, generally produce neither catalase nor superoxide dismutase. However, some aerotolerant anaerobes do exhibit some superoxide dismutase activity, which in fact may be the reason for their aerotolerance.

Carbon Dioxide

Carbon dioxide (CO_2) is required for normal biosynthetic reactions (e.g. fatty acid biosynthesis) and hence is required for growth of bacteria. The CO_2 levels in air are sufficient for many bacteria. However, some require elevated levels of CO_2 for growth and are incubated in a 5% CO_2 incubator (e.g. *Brucella abortus* and *Trueperella pyogenes)*. Bacteria that grow better at higher CO_2 concentration are called capnophiles or carboxyphiles.

Temperature

Temperature is an important environmental factor affecting the growth and survival of bacteria. Generally, too low or too high a temperature will inhibit the growth of bacteria. The likely reason is the temperature requirement for optimal enzyme-catalyzed metabolic reactions. The minimum and maximum temperatures vary greatly among bacteria. Based on the optimal temperature for growth, bacteria are categorized into three broad groups:

a) Psychrophiles are bacteria that grow best at 10–15 °C (range 0–25 °C).
b) Mesophiles are bacteria that grow best at 30–37 °C (range 15–45 °C).
c) Thermophiles are bacteria that grow best at 55 °C (range 35–70 ° C).

Most pathogenic bacteria are mesophiles because the body temperatures of animals and humans are in that range. Certain foodborne pathogens are psychrophiles that are able to grow in foods stored in the refrigerator. The two best examples are *Listeria monocytogenes* (the usual sources are unpasteurized milk, milk products, and deli meats), which causes listeriosis, and *Yersinia enterocolitica* (the source is usually pork meat), which causes gastroenteritis in humans. Nonpathogenic psychrophiles can also grow and contaminate refrigerated drugs and biologicals, such as vaccines or intravenous solutions.

pH

The pH (negative log of the hydrogen ion concentration) of the bacterial environment (natural habitats or bacterial culture media), which reflects the concentration of acidic or basic molecules, has a major impact on bacterial growth and even survival. Each bacterial species has a minimum, maximum, and optimal pH requirements. Bacterial habitats in nature range from extremely acidic (pH of 1–2) to extremely alkaline (pH of 10–12) conditions. Most pathogenic bacteria grow best (optimal pH) at neutral pH (pH 7.0) with a range of 6.8–7.2. Certain pathogens are acid tolerant and can survive the harsh conditions of the stomach.

Ionic Strength/Osmotic Pressure

Pathogenic bacteria usually grow best at osmotic pressures equivalent to physiological saline (150 mM or 0.65% NaCl). Any change in the osmotic concentration of the surrounding medium, either hypotonic or hypertonic concentration, will affect the bacteria, although the cell wall will provide

some protection. Some bacteria have adapted to tolerate or grow at high salt concentrations. The capacity to tolerate elevated ionic strengths/or osmotic pressures (e.g. 5–7.5% NaCl) can be useful in the cultivation or identification of certain pathogens, notably *Staphylococcus aureus*. Certain bacteria, called halophiles, are adapted to live in hypertonic environments, such as salt-water lakes and seawater. A couple of species of *Vibrio, Vibrio parahemolyticus*, and *Vibrio vulnificus,* which live in seawater, are foodborne pathogens, with seafood being the major source of foodborne illnesses.

Chemical Requirements for Growth

Bacteria require nutrients for growth. Some nutrients are required in large amounts (macronutrients), others in less or even trace amounts (micronutrients). Different bacteria require different nutrients, often in specific forms. The two major macronutrients are carbon and nitrogen, which are the two major elements of bacterial cells. The major source of carbon is organic (mainly carbohydrate). Nitrogen sources are organic (proteins, urea, etc.) or inorganic (ammonia, nitrates, etc.). Other macronutrients are phosphorus, sulfur, potassium, magnesium, calcium, and sodium. Next to carbon and nitrogen, phosphorus and sulfur are important macronutrients required to synthesize sulfur-containing amino acids. Although most bacteria use glucose as an energy source, pathogens that enter an animal body are equipped to utilize complex carbohydrates, proteins, and lipids. These complex polymers are initially hydrolyzed by extracellular enzymes (glycoside hydrolases, proteases, and lipases) to oligomers and then to monomer constituents (monosaccharides, amino acids, glycerol, and fatty acids). The monomers are then transported into the cell by various transport mechanisms and utilized to generate energy and for the biosynthesis of cellular constituents. Most bacterial pathogens can be cultured in complex general-purpose media (blood agar, tryptone soy broth, brain-heart infusion broth, etc.); however, some require special or specific nutrients for optimal growth. Such bacteria are called nutritionally fastidious.

Micronutrients are required in small amounts, but are critical to cell function. They are vitamins and metals (copper, iron, manganese, molybdenum, nickel, zinc, etc.) and hence often called trace elements. Many vitamins and trace elements are important components of various enzymes and cofactors, and therefore vital for many biochemical reactions of the bacterial cell. Many bacteria synthesize their own vitamins from simpler substances, whereas others assimilate them from exogenous sources. Many bacterial pathogens require preformed vitamins, which are obtained from the animal cells and tissues, and complex general-purpose media (blood agar and brain-heart infusion) contain sufficient vitamins to meet their requirements. One of the B vitamins, folic acid, is part of a cofactor called tetrahydrofolate that is required for syntheses of nucleic acids. Most bacteria make their own folic acid by the action of dihydropteroate synthetase, which combines pteridine, *p*-aminobenzoic acid, and glutamic acid. Sulfa drugs, which are structural analogues of *p*-aminobenzoic acid, exert their antibacterial activities by inhibiting folic acid synthesis. Sulfa drugs have broad-spectrum activity (including effects on protozoa, such as coccidia) and are bacteriostatic, but in combination with diaminopyrimidines (another class of antibiotics that inhibits folic acid synthesis), they become bactericidal. Trace elements such as sodium, potassium, magnesium, chloride, copper, iron, zinc, and cobalt are required by bacteria, generally as ions. These elements serve as cofactors in many enzymatic reactions. Among the trace elements, many bacterial pathogens have a special requirement for iron.

Iron

In almost all bacteria, iron plays a major role in cellular respiration. Iron is a key component of cytochromes, and iron-sulfur proteins are involved in electron transport. The oxidized form of iron, Fe^{+++} (ferric form), is the available form of iron for bacteria. The reduced form of iron, Fe^{++} (ferrous form), is relatively soluble. Iron is in limited supply in blood and tissues because it is not freely available and is tied up with specific proteins, such as transferrin, ceruloplasmin, ferritin, and lactoferrin. Bacteria have developed strategies to acquire iron from the host when there is a limited supply of iron in vivo. Pathogenic bacteria produce iron-binding agents called siderophores that bind iron with great avidity and transport it into the cell. Without the siderophores, many pathogens would be unable to initiate infection. Siderophores are small-molecular-weight organic molecules secreted by bacteria. The molecules form a complex with iron and then bind to the siderophore-receptor protein on the cell surface to transfer the iron into the cell. Bacteria such as *E. coli* and *Salmonella* produce complex siderophores called enterobactins that have an extremely high binding affinity for iron.

Growth Factors

Some organic compounds are required in small amounts by only some bacteria, not by all bacteria. They include vitamins, amino acids, purines, and pyrimidines. Although most bacteria synthesize these compounds, some require them preformed (in the environment or culture medium). These compounds stimulate the growth of bacteria. *B.*

abortus, which causes abortion in cows, requires erythritol, a sugar alcohol, as a growth factor. This substance is found in reproductive organs or tissues (testis, uterus, mammary gland, etc.), including the placenta. The presence of erythritol explains the preference of *B. abortus* for settling in and causing infections in those organs. This predilection for a particular site or tissue is called tissue tropism. Other examples of bacteria with tissue tropism include species of *Yersinia*, *Yersinia pestis, Yersinia pseudotuberculosis,* and *Yersinia enterocolitica* for lymph nodes. The possible mechanisms for tissue tropism include tissue-specific nutrients (as in erythritol for *Brucella*) or specific receptors for bacterial adhesins (as in the cilia of respiratory epithelial cells in *Bordetella*).

3

Basic Mycology

Charles Czuprynski and M.M. Chengappa

General Characteristics of the Fungi

For the most part, the fungi are heterotrophic, aerobic, nonmotile, and possess complex cell walls composed of various carbohydrate polymers (chitin, glucans, and mannans). Fungi are not photosynthetic and consequently are restricted to a saprophytic or parasitic existence. They are abundant and widespread in the soil, on vegetation, and in water, where they subsist on decaying vegetation and wood. Digestion of organic substrates is accomplished by the production of various proteolytic, lipolytic, and glycolytic enzymes. The fungal cell wall prevents osmotic lysis and protects fungi against mechanical injury and entrance of harmful macromolecules.

Fungi grow as two distinct morphological forms: as molds that produce masses of multicellular filaments that form fuzzy or powdery colonies, or as yeasts that grow as single cells that reproduce by budding (Figure 3.1). The main element of the vegetative or growing form of a mold is the hypha, a tubular structure 2–10 μm in diameter that varies in length and can undergo extensive branching. The hyphae of many molds are divided by cross-walls called septa. As growth continues, multiple hyphae become intertwined to form a mycelium. The vegetative mycelium consists of the hyphae on or in the substrate, whereas hyphae that arise above the surface are referred to as the aerial mycelium. Under certain conditions, some of the hyphae in the aerial mycelium produce stalk-like structures referred to as conidiophores or sporangiophores. These in turn give rise to asexual spores referred to as conidia or sporangiospores, respectively. The stalk-like structures and associated spores are collectively referred to as fruiting bodies, and the specific components within them are referred to by a variety of names (see Glossary below). The asexual conidia and sporangiospores generally are more resistant to physical and chemical agents than hyphae. Release of conidia promotes the dissemination of fungi and expansion of fungal growth.

In contrast to molds, yeasts are oval or spherical cells ranging in diameter from 3 to 5 μm that reproduce by budding. In this process, the cell wall of the mother yeast cell begins to bulge out and form a daughter cell. The latter remains attached until it grows to a similar size, upon which it can enzymatically cleave the cell wall and separate from the mother cell, leaving a bud scar on the latter. If the daughter cells fail to separate, one can see chains of irregular yeast cells that are referred to as pseudohyphae.

It is important to note that some of the most important fungal pathogens are dimorphic. That is, they grow as hyphae (mold) in the environment or at room temperature in the laboratory and convert to a yeast or yeast-like form when in the tissues of animals and people, or when incubated in rich media at elevated incubator temperature (37 °C).

There are two independent classification systems for the fungi. One is based on the asexual spores and structures (anamorph phase) and the other on the sexual spores and structures (teleomorph phase). In general, the fungal forms seen in the diagnostic laboratory are asexual (anamorphs). Sexual reproduction takes place when fungi of two different mating types join together (plasmogamy), fuse their haploid nuclei (karyogamy), and undergo meiotic division of the diploid nucleus. The sexual state of some of the medically important fungi has not been demonstrated. Apparently for those fungi asexual reproduction rarely occurs, or only occurs within as yet unidentified specific conditions for these fungi.

Because the asexual and sexual stages of fungi may form under different conditions, and look quite different from one another, a given fungal species may have a common name for the asexual phase and a second name for the

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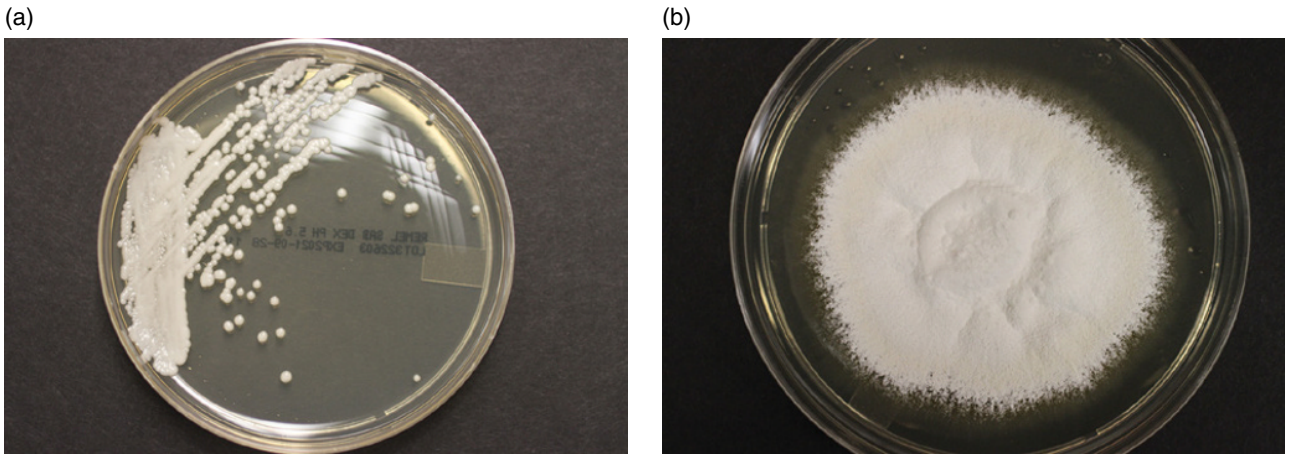


Figure 3.1 Yeast colonies on a Sabouraud dextrose agar plate (a). Mold colony on a Sabouraud dextrose agar plate (b).

sexual stage. One example is the important pathogenic yeast *Cryptococcus neoformans,* whose sexual stage is called *Filobasidiella neoformans.* Curiously, *F. neoformans* grows as a mold, not a yeast, and is in the phylum Basidiomycota, unlike the majority of pathogenic fungi, which are in the phylum Ascomycota. Another example is the dermatophyte (ringworm) fungus *Microsporum canis*, which exists as asexual stage in infected skin and whose sexual stage is named *Arthroderma otae*.

For the above reasons, clinical mycology largely focuses on asexual reproduction. The three mechanisms of asexual reproduction are (i) sporulation followed by germination of the spores (e.g. *Aspergillus fumigatus*); (ii) fragmentation of hyphae (e.g. *Coccidioides immitis, Geotrichum candidum*); and (iii) budding of yeast cells (e.g. *Candida albicans*, *C. neoformans*).

The various reproductive structures are defined below and referred to later under specific chapters.

Commonly Used Mycologic Terms

Arthrospore: An asexual spore formed by fragmentation of hyphae, as can be seen in *C. immitis* and *G. candidum*.

Ascospore: A sexual spore characteristic of the true yeasts and molds in the phylum Ascomycota. Ascospores result from the fusion of two nuclei and are contained within a sac-like structure called an ascus (see below).

Ascus: The specialized sac-like structure characteristic of the true yeasts (i.e. *Saccharomyces* spp.) in which ascospores are produced.

Chlamydospores: Thick-walled, resistant spores formed by differentiation of hyphae or pseudohyphae, as seen in *C. albicans* and *Histoplasma capsulatum*.

Clavate: Club-shaped, as seen in *Microsporum nanum*.

Columella: The persisting dome-shaped upper portion of the sporangiophore, which can be seen in *Mucor* spp.

Conidium: An asexual spore formed along hyphae or at the terminal end of hyphae, as seen in *Aspergillus* spp. and *Penicillium* spp.

Conidiophore: A stalk-like branch of hyphae on which conidia develop either singly or on a specialized structure, as found in *Aspergillus* spp. and *Penicillium* spp.

Dematiaceous: A term used to denote the darkly pigmented brown or black fungi such as *Alternaria* spp.

Dimorphic: Having two forms or phases, growing as a mold at ambient temperature in the environment and as a yeast-like structure at the elevated temperature in the body as seen in *Blastomyces dermatitidis*.

Echinulate: Spiny structure on the surface of macroconidia of *M. canis*.

Ectothrix: Dermatophyte growth on the external surface of the hair shaft, as seen in *Microsporum* spp.

Endogenous: Originating or produced from fungi within the body. *C. albicans* is part of the normal microbiota, hence infections it causes are usually considered endogenous.

Endothrix: Dermatophyte growth inside the hair shaft as seen in some *Trichophyton* spp.

Exogenous: Originating from outside the body, such as infection by pathogenic molds or dimorphic fungi (e.g. *Aspergillus fumigatus*, *H. capsulatum*) found in the environment.

Geophilic: Fungi whose natural habitat is the soil, such as *Coccidioides immitis* or *Microsporum gypseum*.

Germ Tube: Tube-like structures produced by germinating spores or yeast cells. These can develop into hyphae, as in *C. albicans*.

Glabrous: Smooth, for example the glabrous colonies of *G. candidum*.

Hyphae: The filaments that together compose the mycelium (colony) of a mold.

Macroconidia: Large, multinucleate conidia; they may be fusiform (spindle-shaped) or clavate (club-shaped). If divided by transverse and longitudinal septations, they are termed muriform (having walls) as seen in *Alternaria*.

Microconidia: Small, single-celled conidia borne laterally on hyphae. They may be spherical, elliptical, oval, pyriform (pear-shaped) or clavate as seen in *Trichophyton mentagrophytes*.

Mycelium: A mat made up of intertwining, branching, thread-like hyphae that forms the fungal colony.

Nodes: The points on the stolons from which rhizoids arise, as in *Rhizopus* spp.

Pseudohyphae: Chains of elongated budding yeast cells that have failed to detach, as seen in *C. albicans*.

Rhizoid: Root-like, branched hyphae extending into the medium, as seen in *Absidia* spp.

Septate: Having cross-walls or septa in the hyphae, as found in the hyphae of *Aspergillus* spp.

Sessile: Denotes attachment of conidia directly to the hypha without a stalk.

Sporangiophore: Within the Zygomycetes, a specialized hypha bearing a sporangium as seen in *Rhizopus* spp.

Sporangium: A spherical sac-like structure in which asexual spores are produced by internal cleavage, as seen in *Rhizopus* spp.

Sterigmata: A short structure from which chains of conidia form, as seen in *Penicillium* spp. Also referred to as phialides.

Stolon: A horizontal hypha that sprouts where it touches the substrate. It may form rhizoids in the substrate, as observed in *Absidia* spp.

Vesicle: The swollen terminus of the hyphae that form a conidiophore, as seen in *Aspergillus* spp.

Yeast: Unicellular fungi that do not form hyphae or mycelium and reproduce asexually by budding, or occasionally by sexually produced ascospores. Those that form the latter are considered the true yeasts, such as *Saccharomyces* spp.

Zygospore: A thick-walled, sexual spore of that results from the fusion of two aseptate hyphae as in the *Absidia* spp.

Fungal Infections: General Considerations

In general, fungi (excepting the dermatophytes) seldom cause disease in healthy immunocompetent animals. Disease usually results when there are debilitating conditions that favor the growth of fungi (e.g. immunosuppression) or when fungi accidentally penetrate host barriers. In general, fungi can be considered to cause the following types of disease in animals and humans:

1) Systemic Mycoses – This can be divided into a small number of serious pathogens (e.g. *Blastomyces, Coccidioides, Histoplasma*, and *Cryptococcus*), and a much larger number of opportunistic pathogens (e.g. *Aspergillus, Mucor,* and *Rhizopus)* that are widely distributed in nature but seldom cause serious disease in most animal species.
2) Subcutaneous or Deep Mycoses – Fungal infections of the epidermis, dermis, and associated lymphatics that seldom cause infection of internal organs (e.g. *Sporothrix*).
3) Cutaneous Mycoses – Infections of the epidermis, hair, hooves, and nails that seldom cause deeper infection (e.g. *Microsporum* and *Trichophyton*).
4) Mycotoxicoses – Toxins produced by a variety of molds that vary in their structure, characteristics, and target organs (e.g. aflatoxins). Although intoxication usually results from ingestion of preformed toxin, some can also have an adverse effect following inhalation or skin contact.

In general, animals and people are resistant to fungal infection. A number of innate and adaptive immune mechanisms are effective in preventing fungal attachment, spore germination (e.g. transition into hyphae), colonization, and multiplication. The complex bacterial microbiota found in and on the body generally inhibits extensive fungal growth, especially by endogenous agents such as *Candida*. If this microbiota is disrupted, as after systemic antibiotic administration, fungal multiplication can increase.

Innate immune mechanisms include the production of various inhibitory molecules (peptides, lipids) by epithelial cells, and the ability of phagocytic cells (macrophages and neutrophils) to ingest and inhibit or kill spores and other fungal elements. However, the effectiveness of the latter is limited and can be overcome by exposure to a large number of spores or following physiological or pharmacological experiences that impair host defense mechanisms. Examples of the latter include cancer and cancer therapy (radiation, chemotherapy), immunosuppressive drugs given to transplant patients, treatment of autoimmune or other inflammatory disease with chemical or biological immunomodulatory agents (corticosteroids, TNF inhibitors), and certain physiological or endocrine disturbances (e.g. poorly controlled diabetes, pregnancy).

Pathogenesis of Fungal Infections

With the prominent exception of dermatophytosis (ringworm), fungal infections are not contagious. That is, an infected animal or person is not likely to directly transmit infection to another individual. A practical clinical implication of this point is that a companion animal with a systemic infection like blastomycosis is not contagious for other dogs

or to human family members. The reason for the lack of transmission usually relates to the fungal element that initiates fungal infection (i.e. small inhaled spores) not being present in the tissue of an infected animal. Of course, fluid from any draining lesion should be treated with caution to prevent introduction of fungal elements into a break in the skin that might lead to a localized infection.

Fungal infections with dimorphic fungi usually result from inhalation, inoculation, or ingestion of an exogenous fungal element (typically spores from soil or vegetation). Although large outbreaks of fungal infection can occur (e.g. histoplasmosis), these result from exposure of multiple individuals to a localized or point source of fungal spores in the environment. Infection does not reflect direct transmission from one infected individual to the next.

A major exception to the above is represented by the dermatophytes, which should be considered contagious. Numerous fungal elements (spores, hyphal fragments) are shed on infected hairs and skin squames of an animal with dermatophytosis. If these land on the skin of another susceptible individual (animal or human), either via direct contact or fomites, infection can occur.

The microbiota of animals and people usually contain some yeast cells (*Candida*, *Malassezia*). Resistance to endogenous infection with yeast is generally effective, but if something happens to upset the natural balance of the microbiota (antibiotic therapy), or host defense mechanisms (immunosuppression), these yeast cells can proliferate and invade tissue.

Although the pathogenic mechanisms used by fungi are not as well understood in comparison to bacterial pathogens, some general comments can be made. The fungal cell wall provides protection against certain host defense mechanisms (e.g. complement-mediated cell lysis). Constituents of the cell wall in turn act as pathogen-associated molecular patterns (PAMPS) that can interact with cognate pattern recognition receptors (PRRs) to trigger inflammatory mediator release from leukocytes and other host cells. One example is binding to and activation of the Dectin-1 receptor on leukocytes by glucans in the fungal cell wall. This interaction triggers release of inflammatory mediators that both focus host defense against the invading pathogen and might cause localized collateral damage to other cells. The relatively large size of some fungal elements (hyphae) prevents effective phagocytosis and presents a challenge to host defense mechanisms. Conversely, some fungi are facultative intracellular pathogens (i.e. *Histoplasma capsulatum*) and have evolved mechanisms by which they induce host macrophages to ingest them, thus providing access to their preferred site for intracellular multiplication.

Several fungi produce and release proteases and other enzymes that damage host cells and tissue and facilitate spread of the fungal cells. Examples include enzymes (collagenase, elastase) produced by opportunistic systemic pathogens such as *Aspergillus* and *Mucor* and keratinase produced by dermatophyte fungi such as *Microsporum*. As indicated above, some fungi produce and release toxins (mycotoxins). There is ample evidence that ingestion of critical amounts of these mycotoxins can have an adverse effect on various host functions, including immunity. However, there is limited evidence for their direct role as virulence mechanisms during fungal infections.

Diagnosis of Fungal Infections

Diagnosis of fungal infections is generally more complicated than for bacterial infections. The clinical signs of the animal, and cytologic examination of representative fluids or tissues, are important tools in making an accurate diagnosis (Figures 3.2 and 3.3). Enzyme-linked immunoassay (ELISA)

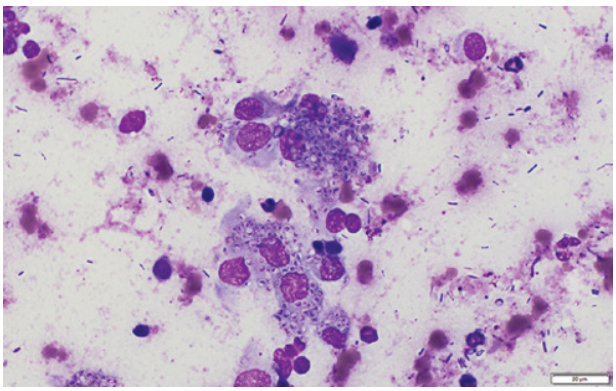


Figure 3.2 Direct examination of rectal scraping of a dog with systemic histoplasmosis revealing intraphagocytic yeast cells. Modified Wright's stain 1000×. Image provided by courtesy of Dr. Lisa Pohlman.

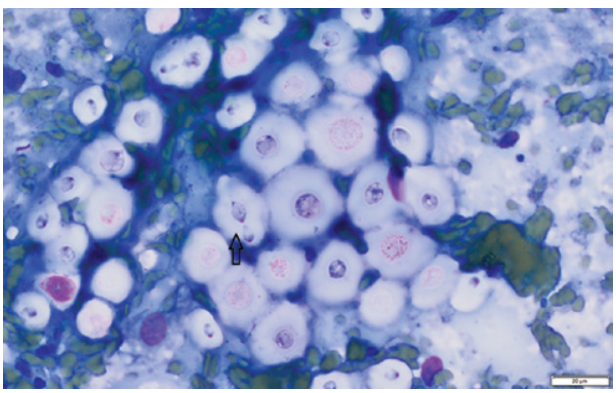


Figure 3.3 Direct examination of nasal mass of a cat with *cryptococcosis* revealing budding yeast cells (arrow) within the capsule of *Cryptococcus neoformans*. Modified Wright's stain 1000x. Image provided by courtesy of Dr. Lisa Pohlman.

and other immunologic tests are available, but interpretation of results may be confounded by prior environmental exposure that is not linked to active disease. For some infections (blastomycosis, histoplasmosis, cryptococcosis), fungal antigen detection in plasma and urine is a powerful tool for both diagnosis and prognostic evaluation of antifungal therapy.

Relatively few laboratories are able to conduct fungal isolation. It should be noted that isolation of the mold form of dimorphic pathogens can present a serious zoonotic risk and should not be attempted by laboratory personnel without proper training and biosafety equipment (certified biosafety cabinet). Unlike clinical bacteriology, biochemical characteristics are of limited importance for identifying many fungi. In general, identification of molds relies on examination of mycelial growth and microscopic identification of characteristic hyphae and spores. New diagnostic tests that are being developed by diagnostic laboratories include PCR-based assays for some fungal pathogens, and **M**atrix-**A**ssisted **L**aser **D**esorption **I**onization –**T**ime **o**f **F**light (MALDI-TOF).

Most fungi grow well aerobically on simple media at room temperature (22–25° C). The most commonly used media are variations of Sabouraud dextrose agar, which contains peptone and dextrose, and has a pH of 5.6 to inhibit the growth of bacteria. It is important to note that clinical samples may be contaminated with environmental fungi. A commonly used media to inhibit saprophytic fungi is Mycobiotic agar, which is Sabouraud agar to which cycloheximide is added to inhibit environmental fungi and chloramphenicol to inhibit bacterial contaminants. If one expects to isolate yeast, samples may be plated on richer media, such as brain heart infusion agar, and incubated at 37° C. For suspected cases of dermatophytosis, suspected hairs, nails, or skin scrapings are plated on dermatophyte test medium, a selective agar in which the pH indicator (phenol red) turns red when dermatophytes use protein for growth, rather than the sugars favored by most other fungi. There are a variety of other specialized media that can be used by diagnostic laboratories for fungal identification.

For suspected cases of mycotoxicosis, there are commercial ELISAs to detect some mycotoxins in extracts from suspected contaminated feed or other products. However, it is important to note that mycotoxin distribution is often focal in nature, so a negative result may not indicate the absence of toxin. Similarly, isolation of a potential toxin producing fungal species from feed or environmental samples by itself is not confirmatory evidence of toxin contamination.

Immune Response to Fungal Infections

As described earlier, innate immunity is important in preventing initiation of fungal infection and limiting fungal multiplication and spread. Macrophages are important for phagocytosing and inactivating spores and preventing their germination into hyphae. This approach generally protects animals and people against the many fungal spores encountered on a daily basis. Because fungal hyphae are too large to be phagocytosed, neutrophils and macrophages must attack them externally. Clinical and experimental studies in which macrophage and neutrophil activity is impaired clearly demonstrate the importance of these cells in preventing and reducing the severity of fungal infection.

Fungi and fungal products also elicit adaptive immune responses (antibody and cellular immunity). Although antibodies are frequently produced as a result of subclinical or clinical fungal infection, their role in protective immunity is thought to be limited. The thick cell wall of fungi makes them resistant to lysis by the classical or alternative complement pathways. Some experimental studies provide evidence that antibodies can opsonize and enhance the antifungal activity of macrophages and neutrophils. Although antibodies may not offer robust protection against fungal infection, their presence can be a diagnostic tool for fungal infection. Various assays to detect antifungal antibodies (ELISA, agar gel immunodiffusion, etc.) have been used as a tool of diagnosis. However, the widespread nature of many opportunistic fungi can confound the diagnostic value of antibody-based detection systems for opportunistic molds, or yeasts that are part of the microbiota.

Animals and people who have defects in cellular immunity are prone to more frequent and severe fungal infections. The innate and adaptive immune responses to fungal infections frequently result in pyogranulomatous inflammation (mixed infiltrates of neutrophils and macrophages). Some long-standing fungal infections can lead to granuloma formation. Fungi and fungal elements often trigger a T helper 1 cell-mediated immune response that results in effector cells such as cytotoxic T cells and activated macrophages. Although we have limited understanding of the precise roles these cells play in host defense against many fungi, there are various lines of evidence that they contribute to protection against serious fungal infections. This type of immune response can result in delayed type hypersensitivity that contributes to lesions seen during some fungal infections (e.g. "id" response in dermatophytosis). More recent experimental evidence suggests that a T helper 17 response, which results in recruitment and activation of neutrophils, contributes to host defense against some fungal pathogens.

Treatment of Fungal Infection: General

In general, we need more and better antifungal drugs. In contrast to antibiotic therapy of bacterial infections, antifungal therapy in veterinary species usually requires

prolonged treatment (perhaps for months), can be costly, and may present side effects. Historically, systemic fungal infections relied on the polyene drug amphotericin B. However, the poor solubility of this drug requires it to be administered intravenously as a colloidal suspension. Although this can be an effective approach for human infections, it poses challenges to use in domestic animals. The renal toxicity of amphotericin B is an additional limitation to its veterinary use. Two other polyene drugs (natamycin and pimaricin) are used topically for some superficial fungal infections.

Development of the azole and triazole antifungal drugs revolutionized antifungal therapy in domestic animals. Some of these drugs can be given orally (itraconazole, fluconazole, and voriconazole), which is a big advantage for animal patients, and they have less severe side effects than the polyene drugs. As a result, they have become the mainstay for treatment of systemic fungal infections, and some cutaneous infections, in domestic animals. Although often effective, until recently these drugs were quite expensive, which can be a significant factor for clients. Toxic effects include elevated liver enzymes and skin hypersensitivities.

Iodides, usually given orally, are effective in the treatment of sporotrichosis. Their mechanism of action is not clear as they do not inhibit growth of *S. schenckii* in vitro. Their low cost, and limited and reversible toxicity, makes them attractive for treatment of chronic granulomatous infections in large animal species, where the cost of alternatives like triazole drugs is prohibitive.

Acknowledgment

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4

Basic Virology

Mohamed A. Abouelkhair and Melissa Kennedy

Viruses are unique: they often consist of nothing more than a nucleic acid molecule packaged into a protein; they parasitize the cellular machinery to produce thousands of progeny. This simplicity is deceptive: viruses can infect all known life-forms and have a wide range of structures and genomes. Viral pathogenesis can be defined as the processes by which a virus produces disease in the host. The term **virulence** is used to describe the degree of pathogenicity of a virus – a virulent virus being one that causes a significant disease. The pathogenesis of viral infections is a multifactorial process and involves both the status of the host and properties of the infecting virus. The outcome of viral infection (i.e. the severity of disease or death) is therefore determined by the virulence of the virus and the susceptibility of the host. The susceptibility of a host to a viral infection is determined by many factors including the genetic background of the host, species of the host animal affected, level of immunity, immunocompetence of the host, nutritional status, age, and presence of concurrent infection(s). Some of the viral virulence-related properties include production of cytotoxic effects by the virus (direct or indirect), replication strategies, tissue tropism, dose of the infecting virus, and route of exposure. The host–virus interaction, as determined by these factors, can be examined at the cellular level and at the level of the host animal. The former relates to the ability of a virus to infect and replicate in a cell and the effects of virus replication on that cell, whereas the latter is related to the cumulative effects of cellular infection on the animal host. Thus, what is seen at the host level reflects the cellular effects of the virus.

Virus–Host Relationships

The outcome of any virus–host interaction can differ, depending on critical factors such as virus–cell interactions, the species of the animal host, route of exposure, mode of virus dissemination, and host resistance. Most virus-infected animals that present to veterinarians do so because they exhibit clinical signs of their infection. However, it is very important to recognize that microbial infections of animals often do not result in clinical disease. In fact, the majority of virus–animal interactions result in asymptomatic or subclinical infection, and viruses, however virulent, will not infect animals that are resistant to them. The potential consequences of virus–animal relationships are shown in Table 4.1.

There are several major routes of viral entry into a host: the respiratory, alimentary, and urogenital routes and direct transmission (such as by an insect or animal bite). Successful establishment of viral infection depends on the presence of appropriate cell receptors and the physicochemical nature of the viral agent. In order to successfully initiate infection in the host, the virus must be able to survive until a susceptible host is encountered and gain access to cells/tissues in which it can replicate (permissive cells). This requires the virus to overcome the host's defense mechanisms at these sites. For example, viruses that infect animals through the alimentary tract are typically resistant to the low pH and potent enzymes that occur in the digestive tract.

Modes of Dissemination of Viruses within the Hosts

Viruses cause two basic patterns of infection: localized and generalized (Figure 4.1). In localized infections, viral multiplication and cellular damage remain localized near the site of entry (e.g. the skin or the mucous membranes of the respiratory, gastrointestinal, or genital tract), so that the infecting virus spreads only to neighboring cells immediately adjacent to the original site of infection. For example,

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Table 4.1 Potential consequences of virus–animal relationships.

1) Animal is resistant to viral infection – no relationship established
2) Asymptomatic or subclinical infection – recovery or persistent infection
3) Acute viral infection – death, recovery, or persistent infection
4) Chronic viral infection – recurrent clinical disease, or persistent infection
5) Tumor formation

rhinovirus infections of animals are often restricted to the nasal epithelium and do not even spread to the lower respiratory tract. Other respiratory viruses, such as parainfluenza and respiratory syncytial viruses, replicate within the lungs of infected animals, but tissue injury induced by these viruses typically remains restricted to the respiratory tract. Generalized infections develop through several sequential steps: (i) the virus undergoes primary replication at the site of entry and in regional lymph nodes, (ii) progeny virus spreads through blood (primary viremia) and lymphatics to additional tissues, where (iii) further virus replication takes place, (iv) the virus is disseminated to the other target organs via a secondary viremia, and (v) it multiplies further in these target tissues, where it causes cellular degeneration and/or necrosis, tissue injury, and clinical disease.

The incubation period is the asymptomatic period after infection and prior to expression of clinical disease. In generalized viral infections, overt disease begins only after the virus becomes widely disseminated in the body and has attained maximum titers. It is at this stage of the infection that the veterinarian is usually first alerted. Canine distemper illustrates a generalized viral infection of animals. The canine distemper virus (CDV; *Canine morbillivirus*) initiates the infection at the site of entry, but then disseminates through the blood or the lymphatic system to produce generalized infection with involvement of a variety of target organs (Figure 4.2). The sequence of events during the incubation period and development of signs of disease in experimental canine distemper infections indicate that the different clinical signs that occur in individual animals depend on which of the various organ systems are infected by the virus. The virus is disseminated to these organs during viremia, which may be characterized by the presence of free virus particles in the blood or, as with CDV, blood cells can also serve as carriers to disseminate the virus to target organs (cell-associated viremia). Cell-associated viremias typically involve blood leukocytes, but some viruses, such as bluetongue virus or classical swine fever virus, can associate with the red blood cells of the infected host. The dissemination of virus to the central nervous system can occur by viremia or, in the case of rabies, by transmission along peripheral nerves.

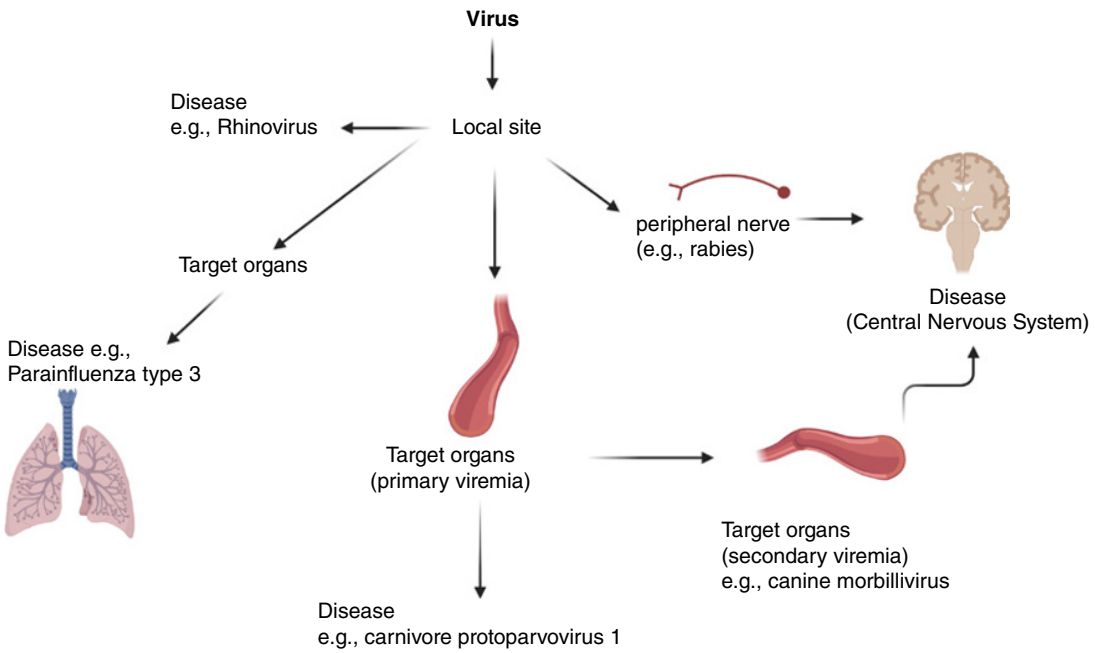


Figure 4.1 Modes of viral dissemination within the host.

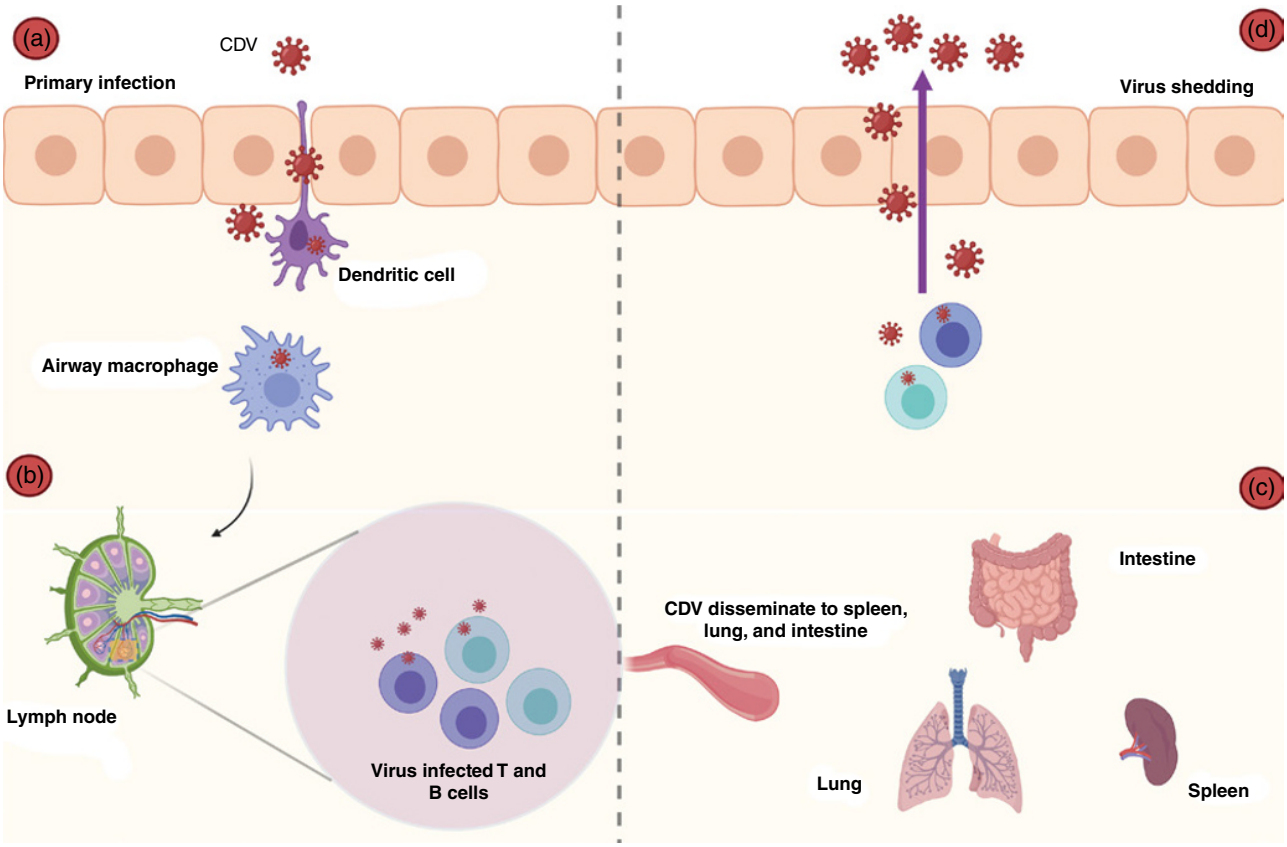


Figure 4.2 Pathogenesis of canine distemper viral infection in dogs. (a) Canine distemper virus (CDV) infects macrophages and dendritic cells of the respiratory epithelium; (b) immune cells of the lymph nodes are infected with the virus (c) CDV disseminates to the spleen, lung, and intestine; (d) CDV further infects epithelial tissues and is released from the apical surface of epithelial cells.

Viral Tropism

The restriction of viral infection and/or replication to certain cells or tissues is referred to as tropism. Some viruses have a very broad spectrum of tropism, infecting a variety of cell types, organs, and even host species. Pseudorabies (*Suid alphaherpesvirus 1*) can infect the respiratory tract, central nervous system, and fetus in swine. In addition, this virus causes fatal infection in most mammals except primates. The virus of malignant catarrhal fever, also a herpesvirus, infects only a subpopulation of T-lymphocytes in some ruminant species, including cattle and wildebeests. Viral tropism is integral to understanding the disease that results from infection with a particular virus.

The tropism of a virus, though it manifests at the host level, is largely determined at the cellular level. The presence or absence of certain cellular factors governs whether a virus can infect and/or produce infectious progeny in a cell. The cell must express an appropriate cellular receptor for virus attachment, the first step in virus replication. Viruses exploit these normal cell surface molecules to enter the cell. A particular cell surface molecule/receptor may be utilized by more than one virus. Conversely, a virus may be able to utilize multiple cellular receptors for attachment. The expression of an appropriate cell receptor is a major factor in determining the susceptibility of cells to a specific virus. In order for a virus to complete its replicative cycle after entry into a cell, the cell must provide certain factors. The absence of these factors prevents production of infectious progeny by the virus. The cellular factors required by viruses vary. Some of these cellular factors are available only during certain stages of the cell growth cycle. For example, parvoviruses require cellular DNA polymerase for synthesis of their genome. This enzyme is expressed in abundance in actively dividing cells (i.e. cells in the S phase of their growth cycle). Thus, parvoviruses replicate in cells with high mitotic index, such as hemopoietic cells in the bone marrow and intestinal crypt cells where the cell turnover rate is very high. Viruses that infect cells of the immune system are especially dependent upon the stage of differentiation of the cell. Activated lymphocytes and macrophages are usually better able to support productive virus replication than resting cells, though the mechanisms are unclear. The progeny of papillomaviruses is produced only in the differentiated keratinocytes.

Local environmental factors can also determine the tissue tropism for a given virus. These include temperature and pH. *Felid alphaherpesvirus 1* (FeHV-1) prefers temperatures of 33–35 °C for replication – the temperature of the upper respiratory tract. Thus, FeHV-1 is associated with disease of the upper respiratory tract, rhinotracheitis. The enteroviruses of the family Picornaviridae are quite resistant to acidic pH and can survive transit through the stomach to the intestine, the target organ. Rhinoviruses, which are also members of the Picornaviridae, are degraded by the acidic pH of the stomach and therefore remain confined to the upper respiratory tract.

The severity of the outcome of a viral disease in an animal is determined largely by organs targeted. Infection of organs that do not tolerate even minimal tissue damage, such as nervous tissue, can be life-threatening. In other tissues, particularly those that are capable of regeneration, such as the intestine, the infection may result in mild or short-lived, self-limiting disease. In pregnant animals, viruses that may cause little disease in adult animals may be lethal to the gestating fetus.

Viral infections that occur without producing overt disease are very common and are potentially important in the dissemination of viruses. Significantly, inapparent infections can confer protective immunity against subsequent challenge with virulent strains of the same agent. Several factors are involved in producing inapparent infections: (i) the nature of the virus (e.g. virulent or attenuated strains), (ii) degree of host immunity, (iii) appearance of viral interference, and (iv) failure of the virus to reach the target organ (e.g. due to the blood–brain barrier).

Host Responses to Viral Infections

The resistance of animals to viral infection is dependent in part on factors that act indiscriminately on most viruses and are therefore called nonspecific or innate resistance factors. These include anatomical barriers, physiological conditions (e.g. pH and temperature), hormonal factors, inhibitors other than antibodies, and phagocytes. Phagocytosis is an important defense mechanism in bacterial infections. However, many viral agents are capable of infecting lymphocytes and/or monocytes/macrophages, and thus these cells can actually serve as a vehicle to spread the virus through the host.

Interferon

An important component of viral defense is the production and secretion of interferon. Interferons are a group of cell proteins (cytokines) that can modulate the immune system, regulate the differentiation of certain cells, confer antiviral resistance on sensitive cells, and exert anticancer effects. Many viruses will induce interferon synthesis in infected cells, and many cell types have the ability to synthesize interferon after appropriate stimulation. At least three different types of interferon can be produced in the course of a viral infection: α, β, and γ. Two distinct mechanisms have been identified in interferon-treated cells (Figure 4.3). The first involves the production in interferon-treated cells of a protein kinase (P1/eIF2α kinase), which, in the presence of double-stranded RNA, blocks initiation of protein synthesis by phosphorylating the protein synthesis initiation factor eIF-2. The other mechanism involves an enzyme, 2–5A synthetase, which, in the presence of adenosine triphosphate and dsRNA, synthesizes a group of oligoadenylates collectively known as 2–5A. In turn, 2–5A activates a specific endonuclease that degrades viral and cellular RNA and so inhibits protein synthesis.

Besides its action on virus replication, interferon exerts other effects on cells, including effects on cell multiplication and regulation of such cellular functions as phagocytosis, production of antibodies and lymphokines by lymphocytes, expression of cell surface antigens, and cytotoxicity of cellular immunity. Interferon plays an important role in host resistance to viral infection.

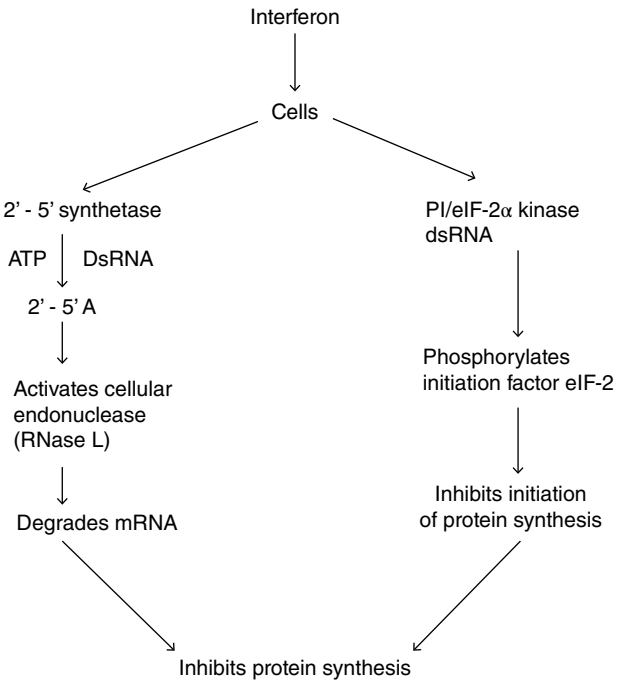


Figure 4.3 Mechanisms of interferon action on protein synthesis.

Humoral and Cellular Immunity

Viruses are antigenic and typically induce a strong specific immune response after infection. Humoral immune responses involve the production of antibodies that can be demonstrated by the usual serological procedures, such as complement fixation, agglutination, precipitation, and gel diffusion techniques. Antibodies, when bound to a virus or viral protein, mediate several antiviral events, including viral neutralization, virus agglutination, activation of complement, and antibody-dependent cell-mediated immune responses. These effects play an important role in terminating primary viral infection, limiting viremia, and preventing disease and reinfection. The neutralizing antibody is of particular importance in viral infections – when preparations of viruses are mixed with appropriate antisera and the mixtures are inoculated in susceptible hosts, infection will not occur if the antisera contain the virus-neutralizing antibody. Three classes of immunoglobulins – IgG, IgM, and IgA – can serve as neutralizing antibodies. The interaction of a virus and an antibody, particularly antibodies specific to the viral antigens responsible for attachment to specific cell receptors, results in a virus–antibody complex formation that prevents attachment of the virus to cell receptors, and to a lesser extent prevents the penetration of the virus into the susceptible cell. It is possible to recover the infectious virus from such apparently inert virus–antibody mixtures by simple dilution or centrifugation, suggesting that the virus and the antibody may be linked in a loose combination in the initial stages of reaction. The interaction between the virus and the antibody does not physically alter viral structure; however, the complement system and antiviral antibody can induce lysis of enveloped viruses as well as destroy virus-infected cells.

Cellular immunity is another important factor in host resistance to some viral infections. The destruction of virus-infected cells by cytotoxic lymphocytes can limit the infection and dissemination of the virus, particularly in instances where the virus is transmitted from infected to noninfected cells. Recent evidence also indicates that macrophages play a role in host resistance to viral infections. Macrophages are key participants in the inflammatory response, and they can be activated either by interaction with viruses or by the soluble products produced by the virus reacting with lymphocytes. Activated macrophages have been shown to participate in a wide range of host responses to viral infections, including phagocytosis of virus–antibody complexes, production of interferon, cytotoxicity for virus-infected cells, and immunoregulatory functions.

In viral infections, cellular and tissue damage result not only directly from the viral infection but also from the host's immune response generated against the invading pathogen as well. Cellular and humoral responses target the virus as well as virus-infected cells. In addition, "bystander" destruction of nearby uninvolved cells occurs. Cytotoxic mediators released from neutrophils, cytotoxic T-lymphocytes, and natural killer cells may affect uninfected cells in close proximity. In most cases, this damage is minimal and is a necessary component of the recovery process in viral diseases because virus-infected cells must be destroyed. However, in some instances such as persistent infections, the immune response may be responsible for the disease. The prolonged presence of antigens continually stimulates immunocompetent cells, leading to an excessive inflammatory response. The excessive response can be humoral, and cell mediated. The end result is cellular, tissue, and organ damage that manifests as disease.

Viral Immunosuppression

Several viruses of veterinary importance can infect lymphocytes, including CDV *(Canine morbillivirus)*, feline panleukopenia virus *(Carnivore protoparvovirus 1)*, feline leukemia virus, bovine viral diarrhea virus *(Pestivirus A and B)*, hog cholera virus, Newcastle disease virus *(Avian orthoavulavirus 1)*, and infectious bursal disease virus of chickens. The destruction of lymphocytes and the resultant atrophy of lymphoid tissues by the viruses can suppress or compromise immune response, predisposing the affected host to other opportunistic bacterial or viral infections. Less commonly, a variety of inherited primary immune deficiencies can affect animals and predispose them to infectious diseases. A good example is the fatal respiratory tract infection of Arabian foals with combined immunodeficiency disorder (lack of production of functional T- and B-lymphocytes) by equine adenovirus.

Persistent Viral Infection

Persistent and latent viral infections are characterized by the fact that the virus is not eliminated from the host. Disease may or may not occur in such chronically infected animals. Potential mechanisms of viral persistence include noncytocidal infection of host cells, destruction of immune effector cells or growth within these cell types, evasion of protective host responses including cytokines and antibodies, and integration of the viral genome into that of the host cell. Persistent infections are those in which the virus is continuously present, with or without expression of

disease. Disease, when it does occur in persistently infected animals, is often a result of immunopathologic mechanisms. Latent infections are those in which the virus is demonstrable only when reactivation (recrudescence) occurs; this is highly characteristic of herpesvirus infections.

Viral Shedding

The final stage of a viral infection is the shedding of the virus by the host into the environment. This may occur from the same body surface where the virus gained entrance into the host, even if systemic dissemination occurs. For example, many viruses infecting the respiratory tract are shed via respiratory secretions. Viruses that do not disseminate within the host are generally shed from the site of entry. Papillomaviruses enter through traumatic openings in the skin, replicate in the layers of epidermis, and are shed from the differentiated keratinocytes on the skin surface. Viruses that infect multiple tissues may be shed from a variety of locales. CDV *(Canine morbillivirus)*, which infects many epithelial cells throughout the host during generalized disease, may be shed in feces, respiratory secretions, ocular secretions, and urine. Viruses that infect the gastrointestinal tract are usually shed in the feces. Semen and milk can be the source of some viruses, particularly those that infect leukocytes. Viruses that infect hematopoietic cells or that cause a significant viremia can spread via blood. Blood is the source of infection for the arthropod-borne viruses as well as for viruses that spread iatrogenically via contaminated needles, through blood transfusions, and so on.

In certain viral infections, no shedding of virus may occur from the host. This happens most often in viruses infecting an aberrant or a "dead-end" host. For example, *Eastern equine encephalitis virus*, a togavirus, normally cycles between birds and mosquitoes. When horses, which are not the natural host of this virus, are bitten by infected mosquitoes, a transient viremia leads to the infection of the central nervous system. Because the viremia is so transient, horses are not normally a source of virus for mosquitoes and there is no virus shedding.

Further Reading

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Part II

Bacteriology

5

Family *Enterobacteriaceae*

Rodney A. Moxley

The family *Enterobacteriaceae* currently includes 33 genera and 140 species, validly published and correctly named (Table 5.1). Since the last edition of this text, the family includes 17 new genera and 46 new species with 29 former genera moved into the families *Budviciaceae*, *Erwiniaceae*, *Hafniaceae*, *Morganellaceae*, *Pectobacteriaceae*, *Thorselliaceae*, and *Yersiniaceae*. Species in the *Enterobacteriaceae* family are ubiquitous, colonizing humans, animals, insects, plants, water, and soil. Some species are significant causes of intestinal or extraintestinal disease in food and companion mammals, poultry, other avian species, reptiles, and humans. Extraintestinal infections often involve the urinary tract, respiratory tract, bloodstream, and wounds. Important pathogens of humans and animals include species of *Citrobacter*, *Cronobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Raoultella*, *Salmonella*, and *Shigella*. Less frequently occurring, opportunistic pathogens of humans and animals include species in the genera *Cedecea*, *Kosakonia*, *Leclercia*, *Lelliottia*, *Plesiomonas*, *Pluralibacter*, *Pseudescherichia*, *Pseudocitrobacter*, *Scandinavium*, *Siccibacter*, and *Yokenella*. Some genera predominantly include pathogens or commensals of plants (*Gibbsiella*, *Mangrovibacter*, *Phytobacter*, *Rosenbergiella*, and *Saccharobacter*) and insects or nematodes (*Buttiauxella*, *Enterobacillus*, *Intestinirhabdus*, *Izhakiella*, *Shimwellia*, and *Trabulsiella*).

Descriptive Features

Morphology and Staining

These organisms are gram-negative straight rods (Figure 5.1), most ranging from 0.3 to 1.0 μm wide by 0.6–6.0 μm long (Figure 5.2). Members of the family *Enterobacteriaceae* are indistinguishable from one another based simply on morphology.

Cellular Structure and Composition

The cell wall is consistent with that of other gram-negative bacteria, containing both inner (cytoplasmic) and outer membranes (OMs), a thin peptidoglycan layer, and periplasm (Figure 5.3). The OM is an asymmetric bilayer with phospholipids on its inner surface, and lipid A (endotoxin), the hydrophobic anchor of lipopolysaccharide (LPS) on the outside. The OM contains proteins, (Braun's) lipoproteins, and an acidic polysaccharide known as the enterobacterial common antigen (ECA). OM phospholipids are similar in composition to those in the cytoplasmic membrane. Outer membrane proteins (OMPs) vary considerably, but OmpA and other porins are major components. Porin proteins are assembled as trimers, forming pore-like structures with hydrophilic lumens, allowing ions and aqueous solutions to pass through the otherwise hydrophobic lipid bilayer. ECA contains *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosaminuronic acid, and 4-acetamido-4,6-dideoxy-D-galactose.

The proximal portion of the LPS macromolecule consists of the hydrophobic lipid A region. This region is a polar lipid in which a backbone of glucosaminyl-β-(1 → 6)-glucosamine is substituted, usually with six or seven saturated fatty acid residues. Distally, the lipid A backbone is connected to the inner core polysaccharide region, and both the inner core and the lipid A backbone contain many charged groups, most of which are anionic. The outermost region of the LPS consists of the hydrophilic, O-antigen polysaccharide region. Core polysaccharide is positioned between the lipid A region on the proximal side (inner core) and the O-antigen region on the distal side (outer core) (Figure 5.4). The lipid A component is a polar lipid in which a backbone of glucosaminyl-β-(1 → 6)-glucosamine is substituted usually with six or seven fatty acid residues, all of them saturated. In bacteria that produce smooth LPS, the core polysaccharides are divided into two regions: inner core (lipid A proximal)

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Table 5.1 Taxa of the family *Enterobacteriaceae*.

Genus[a]	Species and subspecies	Hosts and associated diseases and occurrences
Buttiauxella	*agrestis* *brennerae* *ferragutiae* *gaviniae* *izardii* *noackiae* *warmboldiae*	Mollusks (slugs and snails) and annelids (earthworms). Soil, drinking water, foods (raw salmon, beef, milk), plants (e.g. chrysanthemums), and humans (e.g. sputum). *B. agrestis*, as an endophyte acquired from soil, promotes growth of some plants (e.g. banana). *B. agrestis* was associated with a post-cesarean surgical infection in a woman. *B. gaviniae* was isolated from a urine sample of a patient with cystitis. Strains may carry antibiotic resistance genes, e.g. bla_{VIM}.
Cedecea	*davisae* *lapagei* *neteri*	Colonizes vegetables and midguts of insects (e.g. mosquitoes and fruit flies). *C. davisae* degrades diesel and may have use in bioremediation. Human infections most often with *C. davisae*, with isolation from blood and sputum of immunocompromised patients or those with comorbidities. *C. neteri* caused a urinary tract infection in a pregnant woman, and *C. lapagei*, pneumonia and septic shock in a man with diabetes mellitus.
Citrobacter	*amalonaticus* *braakii* *cronae* *europaeus* *farmeri* *freundii* *gillenii* *koseri* *murliniae* *pasteurii* *portucalensis* *rodentium* *sedlakii* *werkmanii* *youngae*	Human and animal infections. *C. rodentium* is "host-restricted" (mice and gerbils). Other species are commensal inhabitants of the intestinal tracts of humans and animals. *Citrobacter* spp. also recovered from water, sewage, and soil. Opportunistic pathogens of humans, causing urinary tract and wound infections, gastroenteritis, pneumonia, brain abscesses, septicemia, meningitis, and endocarditis, mainly in neonates and immunocompromised hosts. *C. koseri* causes sepsis, meningitis and brain abscesses in human neonates. Antibiotic-resistant *C. freundii* isolated from farmed goldfish.
Cronobacter	*condimenti* *dublinensis;* subspp. *dublinensis*, *lactaridi*, *lausannensis* *malonaticus* *muytjensii* *sakazakii* *turicensis* *universalis*	Environmental organism, plant endophyte, foodborne and opportunistic pathogen. Contaminant of powdered infant formula, spices, nuts, fruits, meats, fish, and other foods. Important cause of necrotizing enterocolitis, bacteremia, meningitis and brain abscesses in neonates. Sporadic cause of urinary, biliary and wound infections in adults.
Enterobacillus	*tribolii*	Isolated from gut of red flour beetle (*Tribolium castaneum*).
Enterobacter	*asburiae* *bugandensis* *cancerogenus* *chengduensis* *chuandaensis* *cloacae*; subspp. *cloacae*, *dissolvens* *dykesii* *hormaechei*; subspp. *hoffmannii*, *hormaechei*, *oharae*, *steigerwaltii*, *xiangfangensis* *huaxiensis*	Environmental organism, isolated from soil, water, sewage, plants, vegetables and fruits. *E. ludwigii* is a growth-promoting bacterium of plants; *E. mori* is a plant pathogen. *E. soli*, found in soil, degrades lignin. Different species isolated from intestinal tracts of humans and animals occur as commensals, but also constitute significant pathogens of humans and animals. *Enterobacter* spp. cause bovine mastitis and omphalophlebitis, equine metritis, canine cystitis, and wound infections in many animal species. *Enterobacter* spp., e.g. *E. cloacae* and *E. hormaechei* are important nosocomial bloodborne pathogens in humans with increasing prevalence of multidrug resistance. Multidrug-resistant *Enterobacter* spp. are included among those known as ESKAPE organisms (*Enterococus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.). Antibiotic-resistant *E. cancerogenus*, *E. cloacae*, and *E. ludwigii* are isolated from farmed goldfish. *E. aerogenes* was moved into the *Klebsiella* genus in 2017, and *E. oryzae* and *E. radicincitans* into the *Kosakonia* genus in 2013.

Table 5.1 (Continued)

	kobei	
	ludwigii	
	mori	
	oligotrophicus	
	quasihormaechei	
	quasimori	
	quasiroggenkampii	
	roggenkampii	
	siamensis	
	sichuanensis	
	soli	
	vonholyi	
	wuhouensis	
Escherichia	*albertii*	*Escherichia* is the type genus of the *Enterobacteriaceae* family, and *E. coli* is the type species of the genus and includes some of the most important pathogens of the family. *E. albertii*, added to genus in 2003, is an emerging human pathogen (gastroenteritis), isolated from wild and domestic animals, and drinking water. *E. fergusonii* is isolated from blood, gallbladder, wound, urine, and diarrheic stools of human patients. *E. marmotae and E. ruysiae* were added to the genus in 2015 and 2021, respectively. *E. marmotae* is isolated from feces of *Marmota himalayana*, and *E. ruysiae* from feces of humans. *E. vulneris* was reclassified as *Pseudescherichia vulneris* in 2017.
	coli	
	fergusonii	
	hermannii	
	marmotae	
	ruysiae	
Franconibacter	*daqui*	Isolated from dried fruit powders, spices, herbs, and powdered infant formula; previously misidentified as *Cronobacter zurichensis* (*Siccibacter turicensis*). Type species *helveticus* was originally placed into *Enterobacter* genus in 2007, then into *Cronobacter* genus in 2013, and then into *Franconibacter* in 2014.
	helveticus	
	pulveris	
Gibbsiella	*dentisuri*	Primarily an inhabitant and pathogen of plants, but may have a more diverse habitat. *G. quercinecans* and *G. greigii* cause acute oak decline of oak trees and bacterial canker of Russian olive trees. *G. dentisuri* was isolated from the oral cavity of a bear, and the intestinal tract of a butterfly.
	greigii	
	quercinecans	
Intestinirhabdus	*alba*	Isolated from gut of plastic-eating larvae of *Coleoptera* insect *Zophobus atratus*.
Izhakiella	*australiensis*	*Izhakiella* established and designated a genus in *Enterobacteriaceae* in 2016, although a formal proposal was made in 2020 to move it into the family *Erwiniaceae*. *I. capsodis* was originally isolated from the mirid bug *Capsodes infuscatus*. *C. infuscatus* deposits eggs inside inflorescence stalk of host (*Asphodelus aestivus*). By feeding on *A. aestivus* plants, bugs suppress development of inflorescence and significantly reduce nectar and fruit production. *I. australiensis* was originally isolated from Australian desert soil.
	capsodis	
Klebsiella	*aerogenes*	Found in a variety of sources including soil, water, plants, humans, and other animals. *Klebsiella* isolated from human intestinal tract, nasopharynx, and range of infections. Significant cause of both nosocomial and community-acquired infections. *K. pneumoniae* is most common species causing urinary tract infections, pneumonia, septicemias, and soft tissue infections. *K. granulomatis* is human-only pathogen causing granuloma inguinale (donovanosis) as sexually transmitted infection. *K. pneumoniae* causes endometritis and infertility in mare and mastitis in cattle. *K. alba* first isolated from heavily polluted soil sample in China. *K. michiganensis* isolated from toothbrush holder in Michigan. *K. singaporensis* (now named *K. variicola*) originally isolated from soil in Singapore. *K. variicola* also isolated from inside banana tissues, leaves, stems, and banana plantlets obtained from tissue culture. Antibiotic-resistant *K. aerogenes* has been isolated from farmed goldfish.
	africana	
	granulomatis	
	grimontii	
	huaxiensis	
	indica	
	michiganensis	
	oxytoca	
	pasteurii	
	pneumoniae	
	quasipneumoniae	
	spallanzanii	
	variicola; subspp. *tropica* and *variicola*	

(*Continued*)

Table 5.1 (Continued)

Kluyvera	*ascorbata* *cryocrescens* *georgiana* *intermedia* *sichuanesis*	Isolated from sewage, water, soil, vegetables, insects, mollusks, fish, humans, animals and food products from animals. Naturally colonizes the respiratory, gastrointestinal, and urinary tracts of humans and causes urinary tract and soft tissue wound infections, pancreatitis, cholecystitis, peritonitis, bacteremia and sepsis. Infections are prone to abscess formation. Multidrug-resistant strains have been isolated from various sources.
Kosakonia	*arachidis* *cowanii* *oryzae* *oryzendophytica* *oryziphila* *pseudosacchari* *quasisacchari* *radicincitans* *sacchari*	Type species *cowanii*, and species *radicincitans*, *oryzae*, and *arachidis*, formerly classified as *Enterobacter*, reclassified to form new genus *Kosakonia* in 2013. In 2020, *K. cowanii* identified as rare cause of acute cholecystitis in humans, and strain of *K. cowanii* isolated from digestive tract of a White Sea fish reported to exhibit bioluminescence. *K. radicincitans* is diazotrophic, plant growth-promoting endophyte, but associated with bacteremic illness in human patients.
Leclercia	*adecarboxylata*	*Leclercia* created as genus in 1986 by reclassification of *Escherichia adecarboxylata*. *L. adecarboxylata* isolated from food, drinking water, and other environmental sources, and human infections including sputum, blood, urine, feces, and wounds.
Lelliottia	*amnigena* *jeotgali* *nimipressuralis*	*L. amnigena* associated with urinary tract infections in humans. *L. jeotgali* isolated from drinking water and Korean fermented clam. *L. nimipressuralis* isolated as endophyte of cactus (*Euphorbia trigonas*).
Limnobaculum	*parvum*	*L. parvum* was isolated from freshwater lake.
Mangrovibacter	*phragmitis* *plantisponsor* *yixingensis*	*M. plantisponsor* fixes nitrogen and isolated from roots of mangrove-associated wild rice (*Porteresia coarctata*). *M. phragmitis* is endophyte isolated from roots of *Phragmites karka* (invasive weed growing in the Chilika Lagoon, Odisha, India). *M. yixingensis* isolated from farmland soil in Yixing, China.
Phytobacter	*diazotrophicus* *massiliensis* *palmae* *ursingii*	*P. palmae* is endophytic, nitrogen-fixing, plant growth promoting isolated from oil palm (*Elaeis guineensis* Jacq.). Extended-spectrum beta-lactamase-producing (bla_{TEM}) strain of *P. diazotrophicus* isolated from lake in Brazil.
Plesiomonas	*shigelloides*	The only oxidase-positive member of the *Enterobacteriaceae* family, with *P. shigelloides* the only species. The main environmental source is fresh water (lakes, rivers, estuaries) and commonly colonizes the guts of fish. Human infections are mainly associated with fresh water contact (e.g. swimming), foodborne illness (especially seafood), and foreign travel. Gastroenteritis (acute diarrheal disease), and extraintestinal infections (wound contamination, sepsis, abortion, arthritis, pneumonia, cellulitis, peritonitis, and osteomyelitis reported in humans. Causes localized infections (e.g. skin with punctate hemorrhages, anal reddening) and peritonitis/septicemia in fish. Abortion in an otter has been reported.
Pluralibacter	*gergoviae* *pyrinus*	*P. gergoviae* isolated from packed fish paste. *Pluralibacter* sp. isolated from tuna salad. *P. gergoviae* associated with acute cholecystitis in human patient. *P. gergoviae* (formerly *Enterobacter gergoviae*) isolated from many plants: maize, grape bay, various vegetables, coffee beans, and spring water. Also isolated from insect gut (*Anastrepha*), fruit flies, and gut of pink bollworm (*Pectinophora gossypiella*), a moth pest on cotton.
Pseudescherichia	*vulneris*	*E. vulneris* was reclassified as *Pseudescherichia vulneris* in 2017. This organism has been associated with wound infections, osteomyelitis, septic arthritis, intravenous catheter-associated bacteremia, urosepsis, meningitis, peritonitis, and diarrhea in humans. It has been isolated from hospital wastewater.

Table 5.1 (Continued)

Pseudocitrobacter	*faecalis* *vendiensis*	*P. faecalis* isolated from fecal samples from hospitalized patients in Pakistan, and from sugarcane (*Saccharum* sp.) as endophyte. *P. vendiensis* (positive for bla_{KPC}) isolated from perineum of hospitalized patient.
Raoultella	*electrica* *ornithinolytica* *planticola* *terrigena*	*Raoultella* genus created in 2001 by reclassification of *Klebsiella ornithinolytica*, *K. terrigena*, and *K. planticola*. *R. planticola* and *R. terrigena* share pathogenicity characteristics with and difficult to distinguish biochemically from *K. pneumoniae* without special tests. Environmentally, *Raoultella* isolated from soil, water, vegetables, chicken eggs, fish, and cattle rumen and manure. *R. planticola* more frequently isolated than other two species from aquatic environments, whereas *R. electrica* isolated from eggs. *Raoultella* acquired nosocomially in humans, and associated with infections of the biliary, urinary, and respiratory tracts, soft tissues, and blood (sepsis). Infections caused by *R. planticola*, *R. ornithinolytica*, or *R. terrigena R. terrigena* frequently multidrug resistant (MDR) with high case-fatality rate (44%). Cases of bovine MDR *R. ornithinolytica* infections (neonatal calf sepsis and mastitis) reported.
Rosenbergiella	*australiborealis* *collisarenosi* *epipactidis* *nectarea*	Common in floral nectar from insect-pollinated plants. Type species *R. nectarea* isolated from floral nectar originally reported in 2013. Other species added to genus in 2015.
Saccharobacter	*fermentatus*	*S. fermentatus* first isolated from squeezed leaf juice of agave in Wuhan, China.
Salmonella	*bongori* *enterica*; subspp. *arizonae*, *enterica*, *diarizonae*, *indica*, *salamae*, *houtenae* *subterranea*	Important veterinary and human pathogen causing diarrhea and sepsis with human infections frequently foodborne. *S. enterica* subsp. *enterica* is the main pathogen with >2500 serotypes (or serovars). Human infections broadly divided into typhoidal (*S.* Typhi) and non-typhoidal (all others). Some serotypes are host-adapted, e.g. *S. choleraesuis* in swine, and some are non-host-adapted, e.g. *S.* Typhimurium. Refer to Chapter 7 for more information.
Scandinavium	*goeteborgense*	*S. goeteborgense* was isolated from a wound infection of an adult patient in Gothenburg, Sweden. Initial phenotypic and genotypic analyses identified the strain as a member of the *Enterobacteriaceae*, but could not assign it to any previously described species. The strain carried a novel functional variant of *qnrB* conferring resistance to ciprofloxacin.
Shigella	*boydii* *dysenteriae* *flexneri* *sonnei*	Obligate pathogens of humans and non-human primates causing large intestinal infections with bloody diarrhea (bacillary dysentery, shigellosis). Refer to Chapter 9 for more information.
Shimwellia	*blattae* *pseudoproteus*	Isolated from brewery yeasts and guts of cockroaches and *Anopheles* spp. mosquitos. *S. blattae* used in industrial microbiology: naturally synthesizes cobalamin (vitamin B_{12}); genetically engineered to convert waste products of biodiesel production (e.g. glycerol) to polyhydroxyalkanoates, a component of biodegradable plastics.
Siccibacter	*colletis* *turicensis*	Isolated from fruit powders, spices, herbs, and powdered infant formula. Infections in infants and immunocompromised adults; *S. turicensis* associated with angular cheilitis in adult. Species frequently misidentified as *Cronobacter*. *S. turicensis* isolated from livestock manure and kangaroo feces; frequent host of antibiotic resistance genes.
Trabulsiella	*guamensis* *odontotermitis*	Commensal microbiota of gut, skin, and urogenital tract, although may potentially contribute to gut dysbiosis in survivors of natural disasters, e.g. floods, causing food contamination. *T. guamensis* originally isolated from vacuum cleaner on island of Guam; antibiotic-resistant strains of *T. guamensis* have been isolated from gallbladders of pigs. *T. odontotermitis* naturally colonizes guts of *Anopheles* sp. mosquitos and termites (e.g. *Heterotermes* sp.), and via production of cellulase, degrades cellulose, hemicellulose and lignin. Industrial microbiologists exploit *T. odontotermitis* to degrade cellulose in control of agricultural residues. An unidentified species of *Trabulsiella* was capable of degrading polyaromatic hydrocarbons, toxic environmental pollutants.

(*Continued*)

Table 5.1 (Continued)

Yokenella	*regensburgei*	Originally named Enteric Group 45 and then *Koserella trabulsii*; phenotypically resembles and frequently misidentified as *Hafnia alvei*. Isolated from environmental samples, e.g. well water, insect guts, mollusks, and sporadically from human clinical samples, as an opportunistic pathogen, namely, feces, sputum, urine, abscesses, joint fluid, diabetic foot infections, necrotizing fasciitis, finger osteitis, otitis externa and media, and wounds. A multidrug resistant strain of *Y. regensburgei* was isolated from sewage from an animal farm in China.

Source: Adapted from the LPSN – List of Prokaryotic Names with Standing in Nomenclature database, https://lpsn.dsmz.de (accessed December 9, 2021).
[a] Total: 33 genera, 140 species, and 23 subspecies (validly published and correctly named).

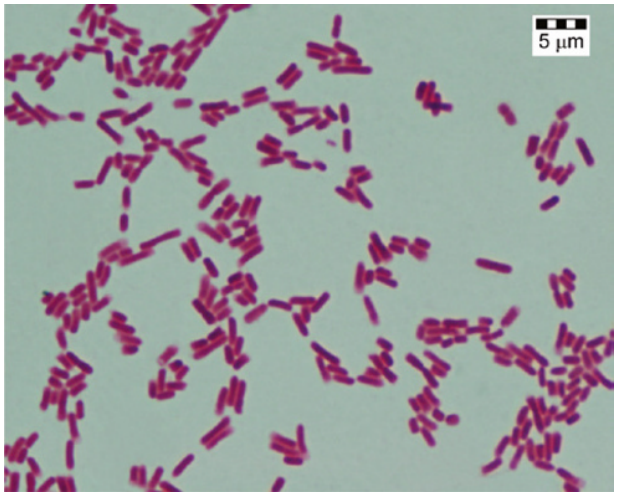


Figure 5.1 *E. coli*, gram-negative rods, magnification 1000 ×. *Source:* Courtesy of Hans Newman, Bacteria in Photos, Copyright 2011, www.bacteriainphotos.com, accessed August 18, 2021.

and outer core. The outer core region provides an attachment site for O-polysaccharide (O-antigen). Within family and genus, the structure of the inner core tends to be well conserved. The inner core typically contains residues of Kdo and L-glycero-D-manno-heptose (Figure 5.4).

The somatic antigens (O-antigens) are composed of repeat units that can differ in the monomer glycoses, the position and stereochemistry of the O-glycosidic linkages, and the presence or absence of noncarbohydrate substituents (Figure 5.4). O-repeat units from different structures may comprise varying numbers of monosaccharides, may be linear or branched, and may form homopolymers or more frequently, heteropolymers. Extensive heterogeneity in the sizes of molecules occurs due to variations in the chain length of the O-polysaccharides, giving rise to the classical "ladder" pattern in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, where each "rung" in the ladder

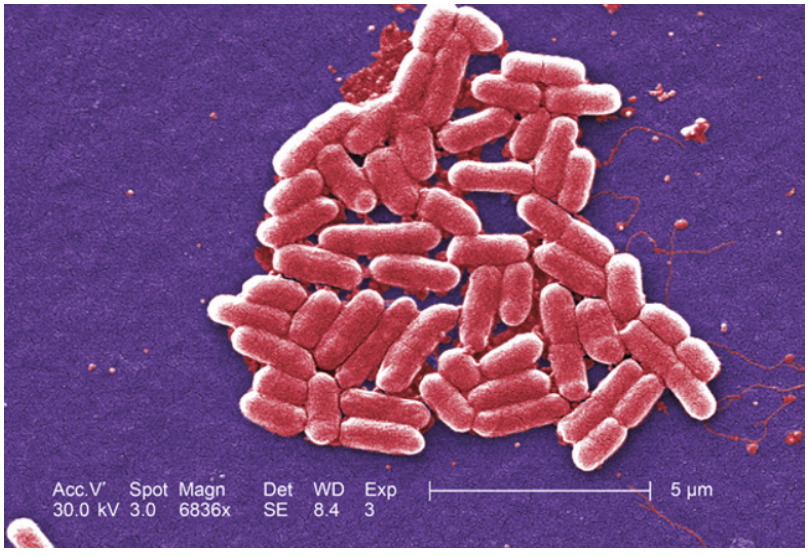


Figure 5.2 Digitally-colorized scanning electron microscopic image (magnification 6836×) of a growing cluster of *E. coli* O157:H7 rod-shaped, bacterial cells. *Source:* US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID# 10068, accessed August 18, 2021.

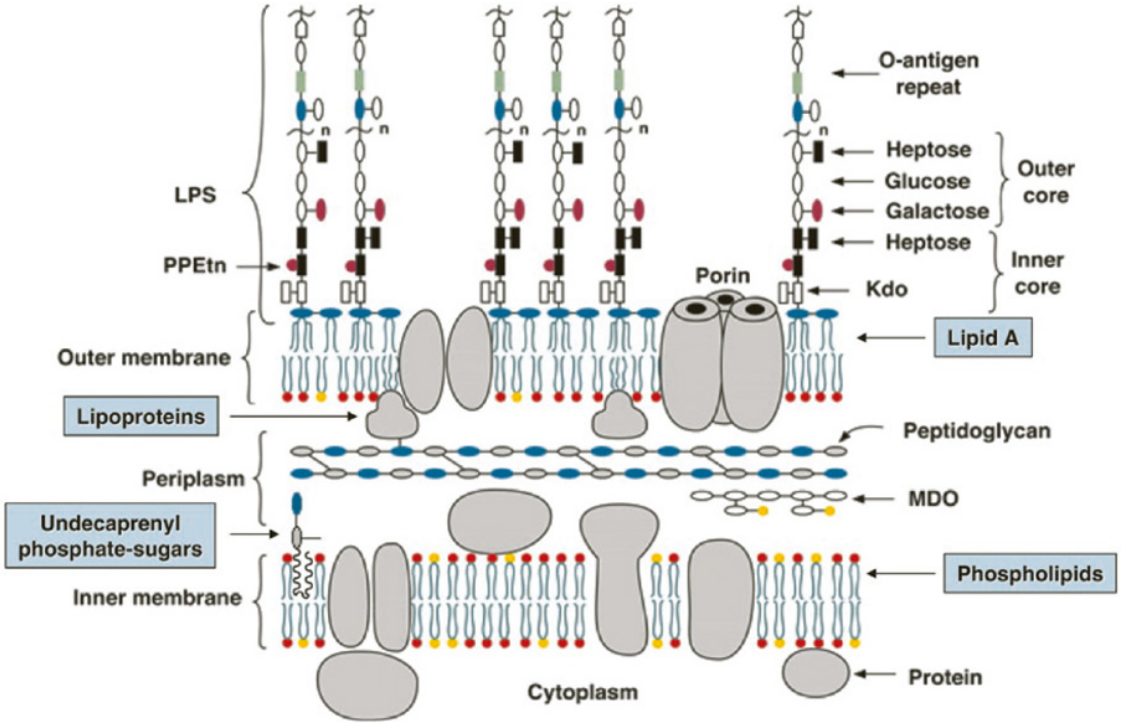


Figure 5.3 Molecular model of the inner and outer membranes of *E. coli* K-12. Ovals and rectangles represent sugar residues, as indicated, whereas circles represent polar head groups of various lipids. PPEtn, ethanolamine pyrophosphate; LPS, lipopolysaccharide; Kdo, 2-keto-3-deoxyoctulosonic acid; MDO, membrane-derived oligosaccharide. *Source:* Reproduced by permission of Raetz and Whitfield (2002); original publication in black and white, Raetz et al. (1991).

represents a lipid A-core molecule substituted with an increment of one additional O-unit.

The O-antigen serological specificity of an organism is defined by the structure of the O-polysaccharide; however, there is great variability in the numbers of unique O-antigens within a species. The O-antigen portion of the LPS molecule provides protection against phagocytosis. Further, loss of the more proximal part of the LPS core ("deep rough" mutants) makes the strains exceptionally sensitive to many different hydrophobic compounds. These include dyes, antibiotics, bile salts, other detergents, and mutagens; thus, this area is involved in maintaining the barrier property of the OM. The lipid A component is essential for the assembly of the OM.

Enterobacteriaceae often expresses a capsule that consists of an acidic polysaccharide. Two types of capsular polysaccharides may be produced. The first type, known as M (mucous) antigen, consists of colanic acid and is produced by most strains. M antigen is thought to provide protection against desiccation. The second type, known as K antigen (K for *kapsel*; capsule in German), is a component of the serotype and may provide antiphagocytic, serum resistance, and mucosal adherence properties depending on the chemical composition.

Many members of this group express adhesins that generally behave as lectins, recognizing oligosaccharide residues of glycoproteins and glycolipids. These adhesins consist of proteins embedded in the outer cell membrane that are composed of subunits and assembled into organelles, or consist of a single OMP. Those assembled into organelles include fimbria (pili) and afimbrial (nonfimbrial) adhesins.

Fimbriae are hair-like appendages that are arranged diffusely on the surface of the bacterial cells. Fimbriae are thinner and typically shorter and more numerous than flagella; they range from 2 to 8 nm in diameter, and usually number 100–1000 per cell. The fimbriae bind to receptors on the surfaces of host cells, and different types of fimbriae vary in their binding specificities. A single bacterial isolate can express multiple fimbrial types. The term pili (plural) is used interchangeably with fimbriae, although some authors reserve "pili" for those structures (F-pili) that are involved in conjugation.

The fimbrial structure consists of a shaft and an adhesin tip, with the latter conferring binding specificity to the host cell. The construction of fimbriae requires the coordinated folding, secretion, and ordered assembly of multiple distinct protein subunits. Both shaft and adhesin tip are

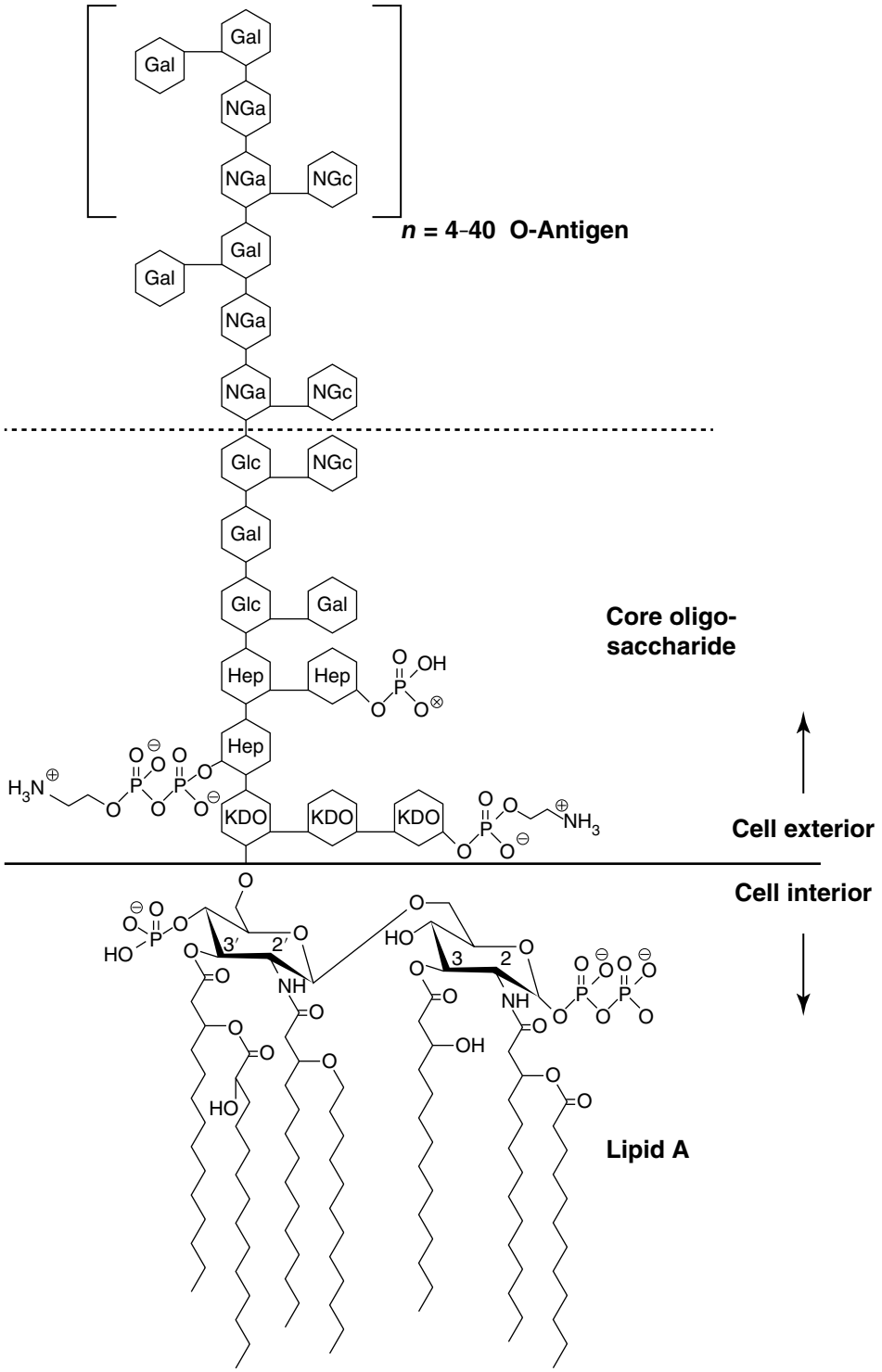


Figure 5.4 Chemical structure of endotoxin from *E. coli* O111: B4 according to Ohno and Morrison (1989). Hep, l-glycerol-d-manno-heptose; gal, galactose; Glc, glucose; Kdo, 2-keto-3-deoxyoctonic acid; NGa, N-acetyl-galactosamine; NGc, N-acetyl-glucosamine. *Source:* Magalhães et al. (2007). Licensed under CC BY.

made up of repeating protein subunits called fimbrins. The fimbrin composition of the shaft and the adhesin tip may differ or be the same. The fimbrin proteins are antigenic, and these properties have been used to group them into F-types. The adhesive properties of fimbriae have also been used for their classification, for example, whether or not they bind to mannose-containing host cell receptors. The binding of fimbriae to a mannose-containing receptor

can be inhibited by exposing the fimbriae to mannose prior to exposure to the receptor; those that are inhibited are called "mannose-sensitive" (MS) and those that are not are called "mannose-resistant" (MR). The type 1 fimbria of *Escherichia coli*, also known as F1, is an example of an MS adhesin. Mannose-containing receptors are found on the surfaces of red blood cells of different species; hence, hemagglutination reactions have been used to test for the presence of MS and MR fimbria. Molecular-based methods that are more practical and specific are now more commonly used for diagnostic purposes, for example, assays such as polymerase chain reaction, which may be used to detect the genes that encode fimbrin proteins.

Afimbrial adhesins are associated with an amorphous capsule-like outer membrane-associated structure, and do not form visible fimbriae on the bacterial surface. The first afimbrial adhesin discovered was found on certain uropathogenic *E. coli* strains and named AFA (for afimbrial adhesin). Afimbrial adhesins were later found on the surfaces of some pathogenic *E. coli* in calves and lambs (see Chapter 6).

Most wild-type strains of the *Enterobacteriaceae* family are motile, a property imparted by flagella. The number of flagella expressed varies, but typically there are about 5–10 per cell, and they are arranged in a peritrichous pattern. Each flagellum is typically about 5–10 μm in length and 20 nm in diameter. The extracellular portion includes, from proximal to distal, the following structures made up of specific proteins (in parentheses): a curved hook region (FlgE) that extends from the surface of the outer cell membrane; a hook–filament junction (FlgK and FlgL); a long helical filament (FliC); and a filament cap (FliD). The filament consists of flagellin (FliC) subunits arranged in a cylindrical or tubular pattern, giving it a hollow lumen. Despite having a hollow structure, the filament is rigid, making it suitable for its function as a propeller. The flagellum is anchored in the cell envelope by its basal body. The basal body also has an approximately cylindrical symmetry, and consists of a rod and a set of five rings. The rings – L, P, M, S, and C – are named for the plane in which they are found – LPS, peptidoglycan, membrane (cytoplasmic), supramembrane, and cytoplasm, respectively.

The mol% guanine-plus-cytosine (G + C) content of the DNA is 38–60.

Cellular Products of Medical Interest

Cellular products of medical interest common to all or most of the members of the family are endotoxins and various siderophores. Products produced by many include serine protease transporters of *Enterobacteriaceae* and extended-spectrum β-lactamases.

Endotoxin

Endotoxin (lipid A) elicits severe toxic reactions in animals due to its effects on the innate immune and coagulatory systems. Lipid A binds to LPS-binding protein (a serum protein), which converts oligomeric micelles of LPS into a monomer for delivery to CD14. CD14 concentrates lipid A for binding to the TLR4–MD2 complex, found on the surfaces of macrophages, dendritic cells, and endothelial cells. TLR4 is a transmembrane protein that complexes with MD2 and CD14. Binding of lipid A to CD14 triggers a signal transduction cascade that results in expression of proinflammatory cytokines. This binding results in the association of the cytosolic domain of TLR4 with myeloid differentiation factor 88 (MyD88) adaptor protein, which is necessary for the recruitment of the protein kinases interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK6 and TNF receptor-associated factor 6 (TRAF6) to the complex. Ultimately, the IκB kinase is phosphorylated, which phosphorylates IκB, which allows nuclear factor (NF)-κB to translocate to the nucleus and activate the transcription of TNF-α, IL-1β, and IL-6. In addition, expression of tissue factor by endothelial cells and B7 costimulatory molecules by macrophages and dendritic cells is activated. These proinflammatory and procoagulatory responses are responsible, in part, for the clinical signs associated with endotoxemia. However, the elicitation of this pathway is also necessary for host resistance to the bacteria. Diversity in lipid A molecules among bacterial species prevents the innate immune systems of some hosts from responding. This is especially true with some of the most virulent gram-negative bacteria, for example, *Yersinia pestis* (now in the *Yersiniaceae* family, see Chapter 8), and host species that are susceptible to sepsis with the respective pathogen. In contrast to *E. coli*, whose lipid A is hexa-acylated with five 14-carbon and one 12-carbon fatty acids, *Y. pestis* is tetra-acylated with four 14-carbon fatty acids. Consequently, *Y. pestis* lipid A is poorly recognized by the TLR4s of some host species, thereby preventing, among other functions, costimulatory molecule expression, which is needed for activation of T helper cells and ultimately macrophages, which interferes with the host's ability to clear the infection.

Siderophores

Iron is an important inorganic cation micronutrient as it is present in almost all bacterial cells. Iron is a cofactor for many different metalloproteins needed for bacterial growth and survival. These include heme-containing proteins such as cytochromes; iron–sulfur proteins, for example, some enzymes involved in amino acid and pyrimidine biosynthesis, the electron transport chain, and the

tricarboxylic acid cycle; and non-iron–sulfur proteins, for example, some enzymes required for DNA synthesis, amino acid synthesis, and antioxidant activity. Although animal hosts contain abundant amounts of iron, it is not readily available to bacteria, because it is tightly bound to such carrier proteins as transferrin and lactoferrin. In culture conditions, iron availability is affected by the pH of the media and aeration. In aerobic conditions, iron is present in the ferric (Fe^{3+}) state and forms insoluble oxyhydroxide polymers at neutral pH. In contrast, ferrous (Fe^{2+}) iron is relatively soluble and accessible to bacterial cells growing under anaerobic conditions. To obtain ferric iron that is tied up in insoluble complexes, bacteria synthesize and release small iron-chelating molecules into the environment called siderophores. Siderophores have a molecular mass generally less than 1 kDa, are synthesized only under iron-deficient conditions, and have an affinity and specificity for iron in the ferric state. More than 100 different siderophores have been described in bacteria; however, all are broadly categorized as either catecholates or hydroxamates. Enterobacterin (also known as enterochelin) is the prototype catecholate siderophore and is commonly found among members of the *Enterobacteriaceae* family. Some strains also produce the hydroxamate siderophore aerobactin. When in low-iron conditions, the bacteria synthesize and release siderophores that bind to Fe^{3+} ions and in turn bind to a receptor on the outer cell membrane, which then transports the ferrisiderophore complex into the cell. A given bacterial cell may also utilize siderophores synthesized by other bacterial species and even fungi. When in relatively high environmental concentrations of ferric iron, bacteria utilize a low-affinity system that functions without a siderophore. Iron is taken across the outer cell membrane by several different transport systems. In *E. coli*, six Fe^{3+} transport systems can function in aerobic conditions, and another, for Fe^{2+} transport, functions under anaerobic conditions. Five of these transport systems utilize siderophores and include enterochelin, aerobactin, citrate–Fe^{3+}, ferrichrome, and ferrioxamine B hydroxamate. These five systems have separate outer membrane receptors but share a dependency on an inner membrane protein called TonB for functioning of the outer membrane receptor proteins. In addition, there is a low-affinity system that functions at high concentrations of Fe^{3+} without a siderophore transport cofactor. The genes governing aerobactin uptake may be on the chromosome in some organisms, or on a plasmid, such as the ColV class plasmids in enteric bacteria. Once accumulated within the cell, the iron cations are either incorporated into iron-specific proteins (both heme- and non-heme-containing) or stored in either of two *E. coli* iron storage proteins, namely, ferritin and bacterioferritin.

Growth Characteristics

Members of this group of microorganisms are non-spore-forming, non-acid-fast facultative anaerobes that grow rapidly under aerobic or anaerobic conditions. Their ability to grow in the presence or absence of oxygen reflects both a respiratory and fermentative metabolism, although fermentation is the more common method of utilizing carbohydrates, and often with production of acid and gas. Almost all members of this group ferment glucose to pyruvic acid via the Embden–Meyerhof of pathway. Some produce succinic acid, acetic acid, formic acid, and ethanol by way of the mixed acid fermentation pathway, whereas others produce butanediol from pyruvic acid. These microorganisms utilize a variety of simple substrates for growth, although most have simple growth requirements. Some are capable of utilizing D-glucose as the sole source of carbon. Under aerobic conditions, the range of suitable substrates includes organic acids, amino acids, and carbohydrates. Members of this family are usually motile with peritrichous flagella, catalase positive, and oxidase negative (due to a lack of cytochrome *c*), and reduce nitrate to nitrite. Most grow well on MacConkey medium, and on peptone or meat extract without supplements or sodium chloride; however, some require vitamins and/or amino acids. Most grow well at 22–35 °C.

Resistance

Members of this group are susceptible to killing by sunlight, drying, pasteurization, and common disinfectants, for example, chlorine-, phenol-, and quaternary ammonium-based compounds. They can survive for weeks to months in moist, shaded environments, such as pastures, manure, litter, and bedding. Although many are susceptible to broad-spectrum antimicrobial agents, their susceptibility is not accurately predictable and can change rapidly through acquisition of R plasmids, or resistance-encoding DNA cassettes, which may insert into numerous integrons located in the genome and in plasmids. Persistent use of antibiotics to treat infections (e.g. swine herds with enzootic enteric colibacillosis) may potentially select for and result in an increased herd-level prevalence of pathogens resistant to the antibiotics being used.

Variability

Variability of one isolate of enteric organism as compared to another within the same species or genus depends upon the genetic basis for the trait under consideration. Differences in the capsular, somatic, or flagellar antigens account for variability among isolates of the

same genus and species. Some variation among members of the same genus and species in the family, as well as among members of different genera and species, is accounted for by the presence of genes residing on plasmids encoding certain phenotypic traits. Such traits as resistance to antimicrobial agents, production of toxin, or secretion of hemolysin may be plasmid encoded and will vary depending on the presence or absence of a particular plasmid.

Transition from the smooth to the rough phenotype occurs with all members of the family, and is often the result of phase variation. Likewise, change in the O-antigens has been shown to occur following lysogeny by certain bacteriophages (lysogenic conversion).

Susceptibility to various bacteriophages (phage typing) is sometimes useful in demonstrating differences in isolates (strains) of the same genus and species. Phage typing is a useful epidemiological tool.

Laboratory Diagnosis

The family is composed of a large number of related, facultatively anaerobic, oxidase-negative (with *Plesiomonas* as an exception), nitrate-reducing, gram-negative rods. Differentiation within the family is accomplished by some or all of the following: culture, biochemical tests, immunological tests (i.e. serotyping of O : K : H, or more commonly O:H antigens and detection of virulence products), and PCR. PCR detection of serotype-specific genes has largely replaced antigenic testing for serotyping of isolates. 16S rRNA sequencing is beginning to be used in some clinical laboratories to identify organisms that cannot be cultured or phenotypically classified. Whole genome sequencing is becoming more increasingly used for more thorough characterization of isolates for different purposes.

A number of manuals deal exclusively with this family, and because of the extreme clinical importance and prevalence of these organisms, an increasing number of programmed and/or computerized identification schemes are becoming commercially available.

Morphology and Staining

All are gram-negative rods with similar morphological features (Figures 5.1 and 5.2).

Cultural Characteristics

Methods used to isolate members of the *Enterobacteriaceae* family vary depending upon whether the sample normally contains intestinal or other background microbiota, or is normally sterile. When the source is normally sterile and proper aseptic procedures during sample collection (e.g. extraintestinal specimen) are used, isolation of any member of the *Enterobacteriaceae* family is significant. The medium for this purpose is sheep blood agar, and incubation is at 35–37 °C (Figure 5.5). In the case of intestinal samples, commensal strains of *E. coli* will be present and cannot be differentiated from pathogenic strains of *E. coli* simply by colony phenotype on such standard culture media as blood agar or MacConkey agar. In the case of some targeted serotypes (e.g. *E. coli* O157:H7), commercially available chromogenic media are available, and these media are designed to allow for presumptive differentiation of the pathogen from other microbiota on the plates.

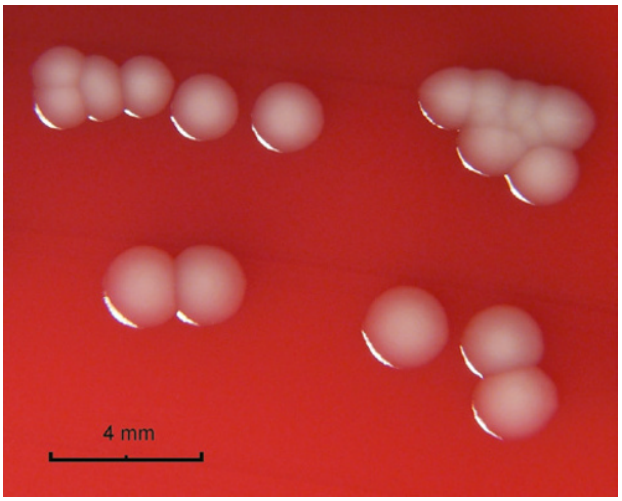


Figure 5.5 Colonies of *E. coli* growing on blood agar culture medium. *Source:* Courtesy of Hans Newman, Bacteria in Photos, Copyright 2011, www.bacteriainphotos.com, accessed August 18, 2021.

Different enteric media have been devised to favor the identification of *Salmonella*, *Shigella*, *Yersinia*, or *E. coli* O157:H7 or other Shiga toxin-producing *E. coli*. The bases of the media are as follows:

1) An inhibitory substance, for example, bile salts, a dye, or antibiotics. These substances inhibit gram-positive or other competing enteric bacteria from growing.
2) A substrate, whether utilized or not, by the pathogen of interest.
3) A pH indicator to tell if the substrate has been changed, for example, utilization of a carbohydrate with production of fermentation products.

The following are useful selective media for isolating enteric pathogens.

Brilliant Green Agar

Inhibitor. Brilliant green dye (suppresses the growth of most members of the family except nontyphoidal *Salmonella*).

Substrate. Lactose and sucrose – *Salmonella*, and some strains of *Proteus*, do not ferment these sugars. Neither does *Shigella*, but *Shigella* grows poorly (if at all) on this medium.

Phenol Red. If sugars are not fermented (alkaline), colonies will be red; if sugars are fermented (acid), colonies will be yellow-green (due to the color of the background dye).

Usefulness. Excellent for isolating *Salmonella*.

Cefsulodin–Irgasan™–Novobiocin (*Yersinia* Selective Agar)

Inhibitor. Bile salts, crystal violet, cefsulodin, Irgasan, novobiocin.

Substrate. Mannitol.

Neutral Red. If mannitol is fermented (acid), colonies will be colorless with a red center ("bulls-eye" appearance); if mannitol is not fermented (alkaline), they will remain colorless, translucent.

Usefulness. Excellent for isolating *Yersinia enterocolitica*.

Eosin Methylene Blue (*EMB*). Agar, Levine.

Inhibitor. Eosin Y, methylene blue (these dyes inhibit gram-positive bacteria to a limited degree).

Substrate. Lactose

Eosin Y and Methylene Blue. These dyes play a role in differentiating between lactose-fermenters and non-fermenters. Bacteria that ferment lactose, especially the coliform bacterium *E. coli*, appear as colonies with a green metallic sheen or blue-black to brown color. Bacteria that do not ferment lactose appear as colorless or transparent, light purple colonies.

Usefulness. Isolation of gram-negative enteric bacteria with good differentiation between *Escherichia* and *Enterobacter* species.

Hektoen Enteric Agar

Inhibitor. Bile salts.

Substrate. Lactose and salicin – *Salmonella*, *Shigella*, and some species of *Proteus* and *Providencia* are lactose-negative and salicin-negative.

Ferric Salt. Organisms producing H_2S will form black-centered colonies.

Bromothymol Blue. Fermenters of salicin and/or lactose will form yellow to orange colonies; isolates not fermenting these sugars will form green or blue-green colonies.

Usefulness. Excellent for *Salmonella* and *Shigella*.

MacConkey Agar

Inhibitor. Bile salts and crystal violet (inhibit gram-positive bacteria).

Substrate. Lactose – *Salmonella*, *Shigella*, and *Proteus* do not ferment lactose.

Neutral Red. If lactose is fermented (acid), colonies will be pink; if lactose is not fermented (peptides digested – basic), colonies will be colorless.

Usefulness. Very permissive medium. *Salmonella* and *Shigella* readily grow on this medium (as do most other *Enterobacteriaceae* and *Pseudomonas*).

Sorbitol MacConkey Agar

Inhibitor. Bile salts and crystal violet (inhibit gram-positive bacteria).

Substrate. Sorbitol. More than 98% of *E. coli* ferment sorbitol within 24 hours; *E. coli* O157:H7 is an exception.

Neutral Red. If sorbitol is fermented (acid), colonies will be pink; if sorbitol is not fermented (peptides digested – basic), colonies will be colorless.

Usefulness. Screening for *E. coli* O157:H7. The addition of 4-methylumbelliferyl-β-D-glucuronide(MUG)or5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) to the medium increases its specificity for *E. coli* O157:H7 (see Sorbitol MacConkey Agar BCIG, cefixime, and potassium tellurite).

Sorbitol MacConkey Agar BCIG, cefixime, and potassium tellurite

Inhibitor. Bile salts and crystal violet inhibit gram-positive bacteria. Addition of cefixime and potassium tellurite inhibits sorbitol non-fermenters (e.g. *Proteus* and *Providencia*).

Substrate. Sorbitol. More than 98% of *E. coli* ferment sorbitol within 24 hours; *E. coli* O157:H7 is an exception.

Neutral Red and BCIG. The non-sorbitol-fermenting and β-glucuronidase-negative *E. coli* O157:H7 will appear as straw-colored colonies. Organisms with β-glucuronidase activity will cleave the substrate, leading to a distinct blue-green coloration of the colonies.

Usefulness. Presumptive detection of *E. coli* O157:H7.

Xylose Lysine Deoxycholate Agar

Inhibitor. Bile salts.

Substrate. (1) Xylose – not fermented by *Shigella* (*Salmonella* ferments xylose). (2) Lysine – isolates that ferment xylose, but not lactose and sucrose, and are lysine decarboxylase–negative (*Proteus mirabilis*) produce amber-orange colonies. *Salmonella* decarboxylates lysine. The ratio of xylose to lysine is such that an alkaline pH predominates (more decarboxylation). *Shigella* does not decarboxylate lysine. (3) Lactose and sucrose – *Salmonella* and *Shigella* do not ferment these sugars rapidly.

(4) Ferric salt – colonies of organisms producing H_2S (*Salmonella*; *Proteus*) will have black centers (iron sulfide).

Phenol Red. Acid colonies (non-*Salmonella* or non-*Shigella*) will be yellow. Alkaline colonies (possible *Salmonella* or *Shigella*) will be red.

Usefulness. An excellent all-purpose medium for both *Salmonella* and *Shigella*.

Enrichment Media

At times, the numbers of *Salmonella* or *Shigella* in fecal samples may be too low ($<10^4$/g) to be detected on the primary plating media discussed above. Therefore, in addition to being plated directly to a selective medium, the fecal sample is placed in an enrichment medium. To detect *Salmonella* by utilizing enrichment methods, at least 100 salmonellae/g are needed.

For *Salmonella*, enrichment may be achieved by incubating feces for 12–18 hours in selenite F broth or tetrathionate broth. During this time, the growth of organisms other than *Salmonella* is suppressed, whereas the growth of *Salmonella* is not. After the 12–18 hours has elapsed, an aliquot of the broth is streaked onto a plate of selective medium (e.g. brilliant green agar).

Enrichment for *Shigella* is not easy because it is rather sensitive to commonly used inhibitory substances found in selenite F and tetrathionate broths. An enrichment broth called gram-negative broth (or GN broth) is used in the same manner as selenite is used for *Salmonella*. GN broth and trypticase soy broth with novobiocin are both excellent for enrichment of fecal samples for *E. coli* O157:H7.

Members of the genus *Pseudomonas* (especially *P. aeruginosa*) may be found in feces, but this is probably an insignificant finding. *Pseudomonas* (not a member of the family *Enterobacteriaceae*) will grow on enteric media. This microorganism does very little to the substrates other than the peptides and peptones and thus mimics *Salmonella* and *Shigella* on selective media. *Pseudomonas* is oxidase-positive, a useful distinction.

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6

Enterobacteriaceae

Escherichia

Rodney A. Moxley

The genus *Escherichia* contains six species: *albertii*, *coli*, *fergusonii*, *hermannii*, *marmotae*, and *ruysiae*, with the addition of *E. marmotae* and *E. ruysiae* in 2015 and 2021, respectively, and *Escherichia vulneris* reclassified as *Pseudescherichа vulneris* in 2017. *Escherichia* is the type genus of the *Enterobacteriaceae* family, with *E. coli* the type species of the genus. Heretofore, all species except *E. ruysiae* have been associated with animal and/or human disease, but *E. coli* is the only species of pathogenic importance for animals. Many *E. coli* are commensals of the intestinal tract, especially the large intestine; however, many are opportunistic or primary pathogens too. Pathogenic *E. coli* are broadly divided into diarrheagenic (DEC) and extraintestinal pathogenic (ExPEC) strains, and divided into further pathotypes based on main virulence factors and associated clinical illnesses (Table 6.1, Figure 6.1).

DEC are economically important pathogens of neonatal piglets, calves, and lambs. Post-weaning diarrheal infections are also important in swine. Extraintestinal infections commonly occur in the urinary tract, umbilicus, blood, lung, and wounds in any location, and these infections occur in most animal species. *E. coli* causes septicemia in neonates of most species, but especially calves, piglets, lambs, foals, puppies, and kittens, and causes opportunistic septicemia in older immunosuppressed animals. In avian species, *E. coli* is an important cause of air sacculitis, omphalitis, and many other systemic manifestations of infection. Zoonotic infections with enterohemorrhagic *E. coli* (EHEC) and host-specific DEC and extraintestinal infections are of major importance in human medicine.

Descriptive Features

Cellular Structure and Composition

Escherichia are straight, cylindrical, gram-negative rods with rounded ends that are approximately 0.5 μm in diameter and 1.0–3.0 μm in length (Figure 6.2). The cell wall contains lipopolysaccharide (LPS), outer membrane proteins, lipoproteins, porins, and a thin peptidoglycan layer (see Chapter 5, Figure 5.3). Cells with a complete LPS layer will typically express an O-antigen, although not all are typable. Cells may express a capsule (K-antigen), flagella (H-antigen), and adhesins (Figure 6.2).

Cellular Products of Medical Interest

Adhesins. Adhesins are composed of protein subunits assembled into organelles or consist of a single outer membrane protein, in either case forming a filamentous structure that projects from the surface of the cell. Those assembled into organelles include fimbria (pili) and afimbrial (nonfimbrial) adhesins. The construction of fimbriae requires the coordinated folding, secretion, and ordered assembly of multiple distinct protein subunits. In *E. coli*, fimbriae are assembled by several pathways, but the chaperone-usher pathway is the most frequent in pathogenic strains. Most *E. coli* produce type 1 (F1) fimbriae, and these bind to mannose. Thus, F1 are called mannose sensitive because their ability to bind can be blocked by pretreatment with mannose. Mannose-resistant fimbriae, which include many fimbrial types on pathogenic strains, are not inhibited by mannose. F1 plays a role in *E. coli* urinary tract

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Table 6.1 Pathotypes of diarrheagenic, extraintestinal, and other *Escherichia coli*.

***E. coli* Pathotype**	**Main virulence factors**	**Clinical manifestation**
Diarrheagenic *E. coli* (DEC)		
Shiga toxin-producing (STEC)	Shiga toxin (Stx); EHEC is a subset	Subclinical carriage or bloody diarrhea
Enterohemorrhagic (EHEC)	Stx, EscF, EscC, EspA, EspB, EspD, Intimin (Eae), Tir	Foodborne bloody diarrhea and hemolytic uremic syndrome (HUS)
Enteropathogenic (EPEC)	EscF, EscC, EspA, EspB, EspD, Eae, Tir, EAF plasmid (tEPEC), Bfp (tEPEC)	Watery diarrhea, infantile diarrhea, traveler's diarrhea
Enteroaggregative (EAEC)	pAA plasmid, aggregative adherence fimbria (AAF), AggR regulator and dispersin	Acute and chronic diarrhea
Enteroinvasive (EIEC)	pINV plasmid and invasins	Bloody diarrhea
Enterotoxigenic (ETEC)	Enterotoxins: heat-stable (ST) and heat-labile (LT)	Watery diarrhea, infantile diarrhea, traveler's diarrhea
Diffusely-adhering (DAEC)	Afa/Dr adhesins	Acute diarrhea to asymptomatic cases
Adherent-invasive (AIEC)	Type IV secretion system, type 1 fimbria, long polar fimbria	Chronic gut inflammation (granulomatous) and Crohn's disease
Cell detaching (CDEC)	K-hemolysin, pyelonephritis-associated pili, and cytotoxic necrotizing factor (CNF)	Infantile diarrhea, cell detaching, linked to Crohn's disease
Extraintestinal pathogenic *E. coli* (ExPEC)		
Uropathogenic (UPEC)	P fimbriae, other mannose-resistant adhesins and type 1 fimbria, K capsule, hemolysin, aerobactin	Urinary and bacteremic infections
Sepsis-causing (SEPEC)	Type 1, P, and S fimbriae, K capsule K1/K5, hemolysin, aerobactin, yersiniabactin, salmochelin, CNF1, secreted autotransporter toxin, serum resistance, colicin V	Bacteremia and sepsis
Neonatal meningitis-associated (NMEC)	OmpTp, HlyF, CvaC, EtsA, CvaA, EtsB, CvaB, Iss, IutA, Tsh	Infantile meningitis and bacteremia
Avian pathogenic (APEC)	Adhesins (Type 1, P, and S fimbriae), invasins (Ibe, Tia), hemolysins (HlyF), iron acquisition (aerobactin, salmochelin, yersiniabactin), protectins (Iss, OmpT),	Air sacculitis, perihepatitis, omphalitis, pericarditis, egg peritonitis, salpingitis, coligranuloma, osteomyelitis, arthritis, cellulitis in poultry and other avian species
Other pathogenic *E. coli*		
Necrotoxigenic (NTEC)	F17 fimbria, Afa adhesin, CNF1, CNF2, CNF3, CdtIII	Diarrhea and septicemia in calves and lambs

Source: Modified from Braz et al. (2020).

infections, in which it mediates adherence to uroepithelium by binding to uroplakin.

Fimbrial adhesins found on *E. coli* that cause disease (colibacillosis) in animals include F4 (with ab, ac, and ad subtypes), F5 (K99), F6 (987P), F11 (PapA), F17 (with a, b, c, and d subtypes), F18 (with ab and ac subtypes, formerly known as F107), F41, and F165 complex ($F165_1$ and $F165_2$). Several of the fimbriae on enterotoxigenic *E. coli* (ETEC) isolates from humans are called colonization factor antigens (CFAs), for example, CFA/I (also known as F2), CFA/II (also known as F3), and others. Some fimbriae in ETEC isolates from humans are called coli-surface (CS) antigens, for example, CS1, CS2, CS3, and others. Finally, other ETEC fimbriae from human isolates are called putative colonization factors (PCFs), also numbered sequentially. Approximately 75% of ETEC isolates from humans express CFA/I, CFA/II, or CFA/IV.

In animals, strains that express F4, F5, F6, F18ac, or F41 fimbria are ETEC and cause enteric colibacillosis manifested as diarrhea (Figure 6.3). Genes for expression of F4, F5, and F6 are usually carried on plasmids, whereas those for F41 are on the chromosome. F4, formerly known as K88, mediates adherence to brush border receptors on small intestinal enterocytes of piglets (Figure 6.4). In pigs, the resistant and susceptible phenotypes for F4ab and F4ac are inherited as a monogenetic trait, with the susceptibility

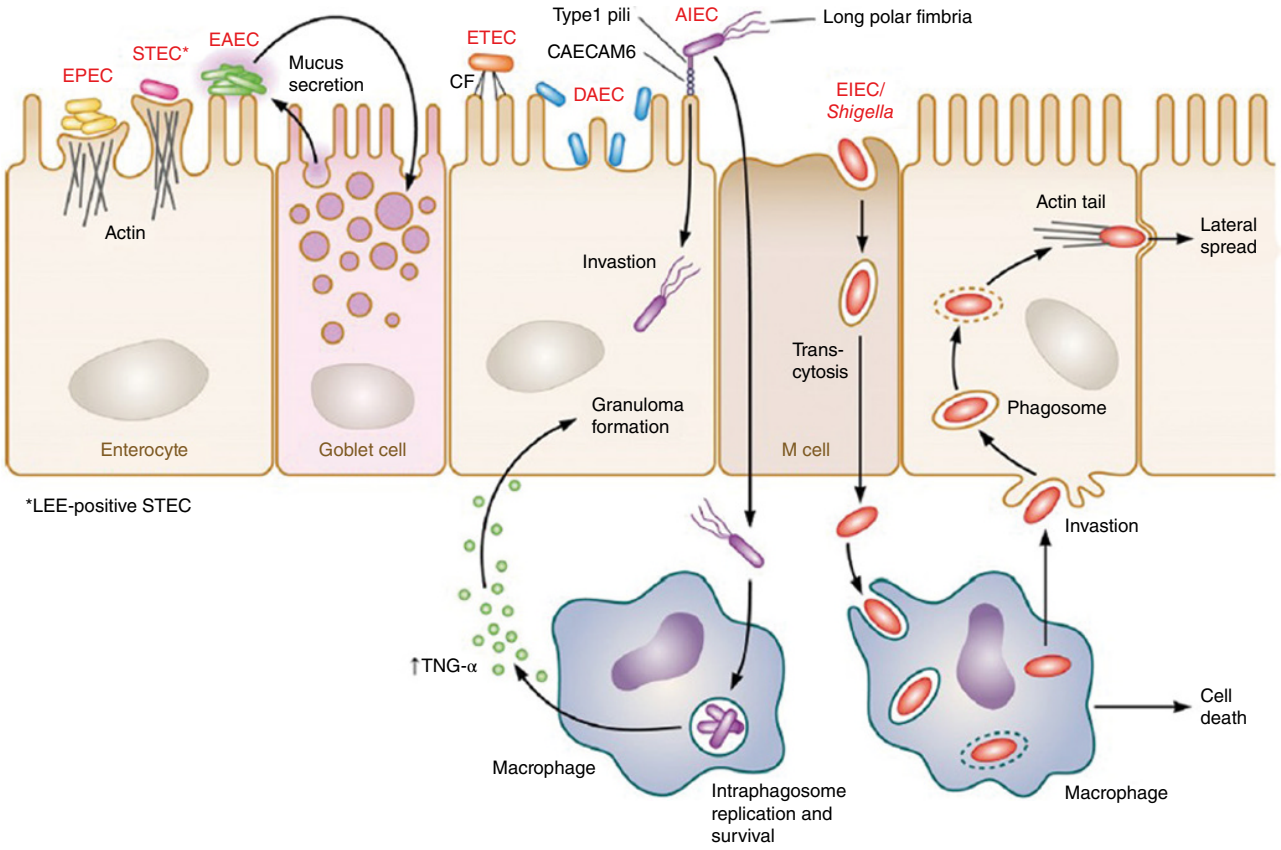


Figure 6.1 Adherence patterns of enteric *E. coli*. Pathogenic *E. coli* requires adherence to the host epithelium. EPEC (represented in yellow) and LEE-positive Shiga toxin-producing *E. coli* (STEC) (represented in pink) are extracellular pathogens that attach to the intestinal epithelium and efface microvilli, forming characteristic A/E lesions. Due to the presence of bundle-forming pili, EPEC is capable of forming microcolonies, resulting in a localized adherence (LA) pattern. ETEC (represented in orange) uses colonization factors (CFs) for attachment to host intestinal cells. EAEC (represented in green) forms biofilms on the intestinal mucosa, and bacteria adhere to each other as well as to the cell surface to form an aggregative adherence pattern (AA) known as "stacked brick." DAEC (represented in blue) is dispersed over the surfaces of intestinal cells, resulting in a diffuse adherence (DA) pattern. Adherent invasive *E. coli* (AIEC) (represented in purple) colonizes the intestinal mucosae of patients with Crohn's disease and is capable of invading epithelial cells as well as replicating within macrophages. AIEC uses type I pili to adhere to intestinal cells and long polar fimbriae that contribute to invasion. Enteroinvasive *E. coli* (EIEC)/Shigella (represented in red) are intracellular pathogens that penetrate the intestinal epithelium through M cells to gain access to the submucosa. EIEC/Shigella escape submucosal macrophages by induction of macrophage cell death followed by basolateral invasion of colonocytes and lateral spread. *Source:* Reproduced by permission of Croxen et al. (2013).

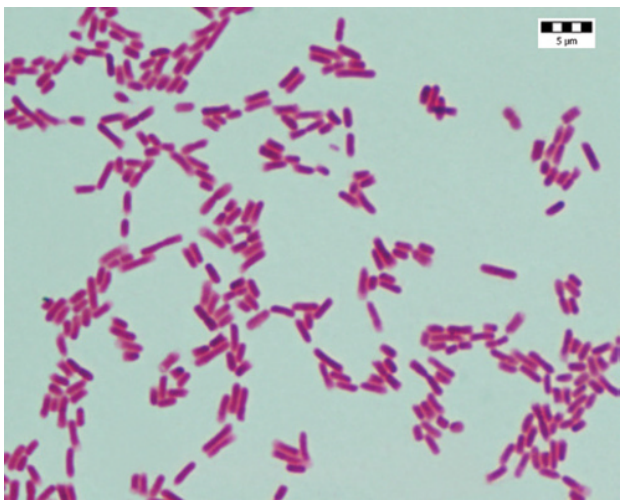


Figure 6.2 *Escherichia coli,* gram-negative rods, magnification 1000 ×. *Source:* Courtesy of Hans Newman, Bacteria in Photos, Copyright 2011, www.bacteriainphotos.com, accessed August 18, 2021.

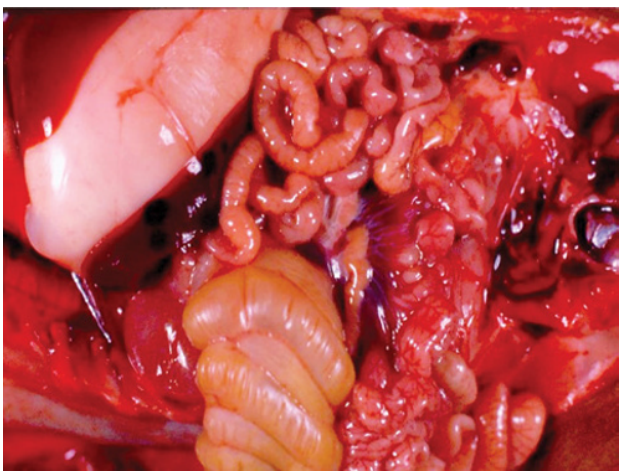


Figure 6.3 Piglet at necropsy with naturally occurring diarrheal disease caused by ETEC infection (enterotoxic colibacillosis). The small intestines are slightly hyperemic, and the mesenteric lymphatics contain chyle. The spiral colon contains yellow-tinged, clear fluid ingesta. *Source:* Photo courtesy of Dr. Alan R. Doster, University of Nebraska-Lincoln.

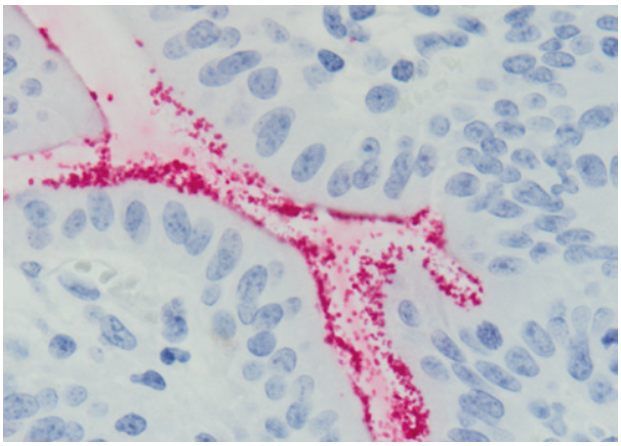


Figure 6.4 The photomicrograph taken at an objective magnification of 60× shows mucosal epithelium in the jejunum of an 11-day-old gnotobiotic piglet infected with porcine-origin enterotoxigenic *E. coli* (ETEC) strain WAM2317 (O8:K87:H^-, $F4^+$). Immunohistochemistry was used to demonstrate the bacteria that are adherent to the enterocytes. Rabbit anti-O8 was used as the primary antiserum. Alkaline phosphatase-labeled goat antirabbit serum and fast red were subsequently used in the staining procedures.

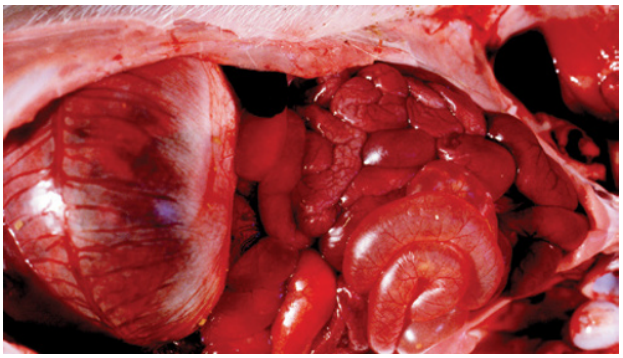


Figure 6.5 Piglet at necropsy with naturally occurring severe enterotoxic colibacillosis caused by $F4^+$ ETEC infection. There is diffuse hemorrhagic infarction of the greater curvature of the stomach and small intestines resulting from combined hypovolemic and endotoxic shock.

allele being dominant over the resistance one. The F4ab/ac receptor is a mucin-like sialoglycoprotein encoded on a locus distal to the mucin-4 gene on chromosome 13. Piglets expressing the F4ab/ac receptor are susceptible to F4ab- or F4ac-mediated fimbrial adherence.

Enterotoxic colibacillosis caused by F4-positive strains occurs in piglets only a few hours old to approximately eight weeks of age. The disease is often clinically severe, with combined hypovolemic and endotoxic shock manifested as hemorrhagic infarction of the intestines and greater curvature of the stomach (Figure 6.5). F4-positive ETEC bacteria adhere to the full length of the small intestine, a feature that greatly increases the severity of disease. F5-positive fimbria mediate adherence to receptors on the brush borders of enterocytes in the distal half of the small intestine. Strains expressing F5, formerly known as K99, cause diarrhea in piglets less than one week old and in calves and lambs during only the first few days of life. In pigs, resistance to F5-mediated fimbrial adherence results from a decrease in the number of receptors on the intestinal epithelium. Similar to F5, F6-positive fimbriae mediate colonization of only the distal small intestine. Strains expressing F6, formerly known as 987P, cause diarrhea only in piglets, and clinical disease is usually limited to piglets less than one week old. Age-mediated resistance to F6 results from shedding of fimbrial receptors into the intestinal lumen, facilitating bacterial clearance rather than colonization. F18ab- and F18ac-positive fimbriae mediate colonization of the small intestine in post-weaned swine. Strains that express F18ab typically produce Shiga toxin-2e and cause edema disease (see below). Strains that produce F18ac are typically enterotoxigenic. F18ab colonization may occur in either the proximal or distal part of the small intestine.

F17-positive *E. coli* are typically isolated from 4- to 21-day-old calves with diarrhea or septicemia. Approximately half of the F17-positive *E. coli* strains isolated from calves with diarrhea are resistant to complement and produce aerobactin, suggesting a role in septicemia. Approximately one-fourth of the F17-positive *E. coli* strains isolated from calves produce cytotoxic necrotizing factor-2 (CNF-2) and afimbrial adhesin (Afa), the findings also suggesting a role in extraintestinal infections. Most bovine strains expressing F17c subtype fimbriae also produce the afimbrial CS31A adhesin. Genes for expression of F17c fimbria are often found on a self-transmissible plasmid that also encodes for CS31A adhesin, aerobactin, and antibiotic resistance.

$F165_1$ and $F165_2$ fimbriae have also been found on *E. coli* isolates from piglets and calves with septicemia. F165-positive isolates from piglets are usually non-enterotoxigenic, serogroup O115, and express F11 (PapA) fimbriae, aerobactin, K "V165" O-antigen capsule, and additional virulence factors, many of which are found other in ExPEC strains. F165-positive strains colonize the distal small intestine of piglets, but cause septicemia and polyserositis instead of diarrhea. The bacteria are thought to enter extraintestinal tissues by translocation from the intestine and are known to resist phagocytic killing by porcine neutrophils. Both $F165_1$ fimbriae and the K"V165" capsule are required for complete resistance to killing by neutrophils.

Curli are thin, coiled, fibrillar fimbriae that promote adherence to extracellular matrix proteins, for example, fibronectin and laminin. Curli fimbriae play a role in

biofilm formation, which assists bacterial survival in the environment. Curli fimbriae are produced by both pathogenic and nonpathogenic strains of *E. coli*, as well as by other members of the *Enterobacteriaceae* family.

Aggregative adherence fimbriae (AAF/I and AAF/II) mediate adherence of enteroaggregative *E. coli* (EAEC) to HEp-2 cells *in vitro*. Following growth, adherent bacteria form an autoaggregative, "stacked-brick" pattern on the surfaces of the host cells. EAEC are an important cause of persistent diarrhea in children and adults in developing and developed countries. In humans, EAEC are thought to primarily colonize the colon. EAEC with ability to cause disease usually contain a large set of virulence genes regulated by the AggR transcription activator. These include plasmid (pAA) genes encoding the AAF, a protein coat secretion system (Aat), secreted dispersin protein (Aap), plasmid-encoded toxin (Pet; a serine protease autotransporter that targets spectrin), Shf (a protein involved in intercellular adhesion), and Aai (a putative type VI secretion system). EAEC also express virulence factors that are encoded by genes on the chromosome. Protein involved in colonization (Pic) (which is a type of serine protease autotransporter of *Enterobacteriaceae* [SPATE]) that functions as a secreted mucinase and Irp2 (yersiniabactin, a siderophore) are two important virulence factors encoded on the chromosome. A newly emergent EAEC clone of serotype O104:H4 is highly virulent in humans. It produces most of the aforementioned EAEC virulence factors, plus Shiga toxin (Stx)-2, aerobactin (*iutA*), *iha* (IrgA homologue adhesin), extended spectrum β-lactamases, and two additional SPATEs rarely found in EAEC (SepA; unknown function) and SigA (which cleaves the cytoskeletal protein spectrin). Based on detection of aggregative adherence, EAEC have been detected in piglets and calves, but these isolates contained almost none of the virulence factors found in human EAEC isolates, and consequently are not likely to be pathogens of animals or humans.

The bundle-forming pilus (Bfp) is a fimbrial adhesin produced by class I (typical) enteropathogenic *E. coli* (EPEC). These fimbriae form "bundles" and mediate localized adherence to HEp-2 cells in culture. *In vivo*, together with the type III secreted outer membrane protein known as EspA, they mediate the first stage of EPEC adherence to small intestinal epithelial cells. EPEC are one of the most important causes of infantile diarrhea in humans in the world. EPEC infections occur naturally in pigs, calves, dogs, and cats, but only a small fraction of the causative strains produce Bfp.

Afimbrial (nonfimbrial) adhesins in various pathogenic *E. coli* include CS31A, AfaE-VII, Afa-VIII, AIDA-I, LifA, Efa1, ToxB, Paa, Saa, OmpA, Iha, and TibA. CS31A is typically found on neonatal calf septicemia isolates that are also positive for F17c fimbria and aerobactin. CS31A is present as fine, wiry fibrillae that collapse onto the surface of the bacterium, forming a capsule-like structure. CS31A mediates adherence to *N*-acetylneuraminic acid receptors on host cells. Afa-VII and Afa-VIII are Afa's found on bovine isolates. Afa-VII has been found on isolates from diarrhea cases, and Afa-VIII has been found on isolates from diarrhea and septicemia cases in calves. Isolates expressing Afa-VIII usually also produce cytotoxic necrotizing factor (CNF)-1 or CNF-2. Afa-VIII is produced more often than Afa-VII by bovine isolates, as is CNF-2 than CNF1. AIDA-I (adhesin involved in diffuse adherence) is an autotransporter produced by diffusely adherent *E. coli* (DAEC) of humans, and mediates the diffusely adherent phenotype. AIDA-I has been found in isolates from pigs with edema disease and post-weaning diarrhea. Many porcine isolates that are genotypically positive for AIDA-I are also positive for heat-stable enterotoxin-b (STb) alone or in combination with enteroaggregative *E. coli* heat-stable toxin-1 (EAST1). LifA (lymphostatin) in EPEC and its homologue Efa1 (EHEC factor for adherence) in EHEC mediate enterocyte adherence. LifA also inhibits lymphocyte activation and the production of certain cytokines. Efa1 is produced by non-O157 EHEC strains and mediates adherence to epithelial cells *in vitro* and in the bovine intestine. ToxB is a homologue of LifA and Efa1 and is produced by EHEC O157:H7. Paa (porcine attaching and effacing associated) factor is found in porcine EPEC and EHEC isolates; its role in the pathogenesis is unknown. Saa (STEC-autoagglutinating adhesin) is found in non-O157 STEC strains that lack the locus of enterocyte effacement (LEE) and is much more highly prevalent in cattle than in human isolates. OmpA (outer membrane protein A) is a putative adhesin used by EHEC in adherence to large intestinal enterocytes. OmpA is also produced by meningitis-associated *E. coli* in human isolates. Saa is produced by EHEC. Iha (IrgA homologue adhesin) is produced by EHEC O157:H7, EAEC O104:H4 and many STEC isolated from cattle. TibA is produced by a subset of ETEC isolates of humans.

E. coli cell surface antigens are subject to phase variation. Phase variation is the reversible change of one or more cell surface antigens of a bacterium at a high frequency, and involves the regulation of genes at the transcriptional level. Phase variation of uropathogenic *E. coli* (UPEC) fimbrial *pap* genes involves the switching "on" or "off" of transcription in response to environmental signals. Similarly, ETEC F4 fimbriae are expressed *in vitro* when cultured on blood agar at 37 °C, but are suppressed at 18 °C. Hence, through phase variation, the bacteria sense environmental signals and expend energy and nutritional resources only to the extent necessary (e.g. they shut off fimbrial expression when it is not needed).

Capsule. The capsule consists of an acidic, high-molecular-weight polysaccharide that is anionic and hydrophilic. The negative charge of the capsule helps protect the bacteria against phagocytosis, because the phagocytes also have a negative charge on their cell surfaces. The capsule is usually antigenic, and if so, is listed as a K-antigen type. Some capsular antigen types (e.g. K87) confer "serum resistance" on the bacteria; that is, they protect the outer cell membrane from the membrane attack complex of the complement cascade (C5b-C9). This property is especially important for survival of pathogenic strains causing extraintestinal infections.

Cell wall. The cell wall is typical of the Enterobacteriaceae family (see Chapter 5, Figure 5.3). The LPS layer in the outer membrane is an important virulence determinant by virtue of the biological effects of lipid A (endotoxin) and the O-antigen repeat unit (Chapter 5, Figure 5.4). The effects of lipid A are described in detail in Chapter 5. A thick O-antigen layer may provide effects similar to a K-antigen capsule. These effects may include protection against phagocytosis and the membrane attack complex of the complement system. Thick O-antigen layers that mediate these protective effects are sometimes referred to as an O-antigen capsule.

Enterotoxins

Some pathogenic strains, especially those belonging to the DEC class known as ETEC, may produce one or more enterotoxins. These enterotoxins are protein exotoxins encoded by genes usually carried on transmissible plasmids. *E. coli* produces four different enterotoxins: heat-labile enterotoxin (LT), heat-stable enterotoxin-a (STa), STb, and EAST1. LT is labile when heated to 70 °C for 10 minutes, whereas STa and STb are stable at 100 °C for 15 minutes.

Heat-labile enterotoxin. LT is very similar structurally, antigenically, and functionally to cholera toxin. The genes for LT expression, *eltAB*, are organized in an operon and typically carried on a large, transmissible plasmid. Expression of the *eltAB* operon is enhanced by exposure of the cells to free glucose. This is due to derepression as a result of inhibition of the cAMP receptor protein (CRP). Enhanced expression upon exposure to glucose is opposite the effects of glucose exposure on expression of the genes for STa and STb, both of which are subject to catabolite repression. Because an individual ETEC strain often carries both LT and STb or LT and STa, expression of one or the other toxin is enabled in either condition.

LT is a rather large macromolecule with a molecular weight of approximately 86 000 Da. The LT holotoxin includes one 28-kDa A subunit and five 11.5 kDa B subunits. The A subunit contains a serine protease cleavage site that allows for separation into an A2 and an enzymatically active A1 chain. The A subunit is linked noncovalently to the B subunit via the A2 chain, and the A1 and A2 chains are bridged together by a disulfide bond. The B subunits are arranged in a homopentamer around the A subunit, and bind with greatest affinity to Galβ3GalNAcβ4(NeuAcα3)Glcβ1-ceramide, more commonly known as monosialoganglioside G_{M1}. The B subunits also bind with lower affinity to the disialoganglioside GD1b, and to terminal galactose units on other molecules, for example, intestinal glycoproteins. Lipid rafts in the apical cell membranes of intestinal epithelium are enriched in G_{M1}, and this serves as a route by which LT enters the host cell. While either in the intestinal lumen or upon binding to the apical membrane of the cell, the A subunit is proteolytically cleaved ("nicked") into A1 and A2 chains. Trypsin is able to cleave the A subunit, but the identity of the protease(s) involved in this step is not known. Following this nicking event, the two chains remain attached by the disulfide bond.

Based on studies with cholera toxin, after being bound to G_{M1}, the holotoxin is taken up by a variety of endocytic mechanisms, including clathrin- and caveolin-dependent, as well as caveolin- and dynamin-independent ones. Following uptake, the holotoxin traffics through early and recycling endosomes, and then enters the trans-Golgi network. From there it bypasses the Golgi cisternae and travels to the endoplasmic reticulum (ER), utilizing v- and t-SNARES, and other transmembrane vesicle fusion proteins. From the initial endocytic step to entry into the ER, the B subunit is bound to G_{M1}. While in the ER lumen, the A1 chain is unfolded by protein disulfide isomerase and dissociates from the A2 chain and the B subunits. The unfolded A1 chain is then recognized by the ER Hsp70 chaperone BiP and solubilized by the ER-associated degradation lumenal pathway. At this point, the A1 chain retrotranslocates into the cytosol by an as-yet-unidentified pathway; the Sec61 translocon and the Hrd-1 complex are hypothetically possible routes. Upon reentry into the cytosol, the A1 chain refolds into its native conformation, and catalyzes the ADP-ribosylation of arginine 201 of the α-subunit of the host G_s protein that regulates adenylyl cyclase. This modification leads to inhibition of the GTPase activity of Gsα of the host G_s protein. Adenylyl cyclase, located at the basal surface of the cell, is constitutively activated.

Constitutive activation of adenylyl cyclase results in a marked intracellular increase in cAMP, which causes activation of protein kinase A. Protein kinase A causes phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel in the

apical cell membrane. Phosphorylation opens the channel, resulting in excessive secretion of Cl^- and bicarbonate ion (HCO_3^-) into the gut lumen. Secretion mainly occurs by cryptal enterocytes, making the electric potential of these cells negative and their serosal potential positive, both relative to the lumen. The transmural potential drives the diffusion of Na^+ through the tight junctions into the gut lumen. The increased Na^+ concentration in the gut lumen results in an increased osmotic pressure and corresponding diffusion of water into the gut lumen, which presents clinically as diarrhea.

A second mechanism by which LT may cause secretory diarrhea is through stimulated synthesis of prostaglandins of the E series (e.g. PGE_2) and platelet-activating factor (PAF). Binding of the B subunit to G_{M1} activates phospholipase A_2, resulting in release of arachidonic acid and generalized enhancement of cyclooxygenase activity, that is, production of PGE_2. Fibroblasts, mast cells, and leukocytes are major sources of prostaglandins and leukotrienes. Binding of the B subunit to these cells could potentially stimulate electrolyte transport and motility in the intestine through induced synthesis of PGE_2. Prostaglandins, including PGE_2, have prosecretory and anti-absorptive effects on the intestine through induction of Ca^{2+} uptake, activation of protein kinase C, and increased intracellular cAMP in such target cells as neurons and epithelial cells. In addition, PGE_2 causes increased transcription of proinflammatory cytokines, for example, interleukin-6 (IL-6).

A third mechanism by which LT may cause secretory diarrhea is through stimulation of the enteric nervous system (ENS). The ENS has highly significant control over secretion and absorption in the intestine. Both cholera toxin and LT cause the release of vasoactive intestinal polypeptide (VIP), an ENS neurotransmitter that acts on epithelial cells and has prostimulatory and anti-absorptive effects. VIP binds to its receptor on secretory epithelial cells. The VIP receptor is a G-protein-linked activator of adenylyl cyclase; hence, VIP binding to its receptor has the same result on these cells as does the direct binding by cholera toxin or LT. Cholera toxin also causes release of two other prosecretory and anti-absorptive neurotransmitters that LT does not, namely, serotonin and substance P. This effect has been proposed as an explanation for why cholera toxin tends to be a more potent toxin than LT.

In addition to its pathogenic effects, LT is antigenic and a mucosal adjuvant. Antibodies against the B subunit neutralize the toxin by preventing its binding to $G_{M1.}$ Active and toxoided LT holotoxin have marked adjuvant activity, and both have been used to boost the immune responses to a large variety of antigens, especially those applied mucosally. The A subunit of the toxin provides the adjuvant effects.

Heat-stable enterotoxin-a. STa (STI, ST) is a small, nonimmunogenic polypeptide of approximately 2.0 kDa, and encoded by *estA*, which is carried on transmissible plasmids. Expression of *estA* is subject to catabolite repression; hence, exposure of cells to free glucose results in gene repression. ETEC strains isolated from pigs express a form of STa referred to as STp (STIa), which is an 18-amino-acid variant. Isolates from humans express either STp or a 19-amino-acid variant called STh (STIb). The receptor for both forms of STa is the transmembrane protein, guanylyl cyclase-C (GC-C), located in the apical cell membrane of intestinal epithelial cells. STa is active on enterocytes of a variety of species (e.g. humans, mice, pigs, and cattle) and causes fluid accumulation in ligated intestinal loops of neonatal mice and pigs, and in the natural disease mainly causes diarrhea in the neonatal animal.

The mature 18- or 19-amino-acid STa peptide is initially produced as a 72-amino-acid pre-pro form. The latter is cleaved by a signal peptidase to a 53-amino-acid pro-STa peptide. Another protease then cleaves this precursor to form the mature toxin. Upon binding to GC-C, this enzyme is activated to convert guanosine triphosphate (GTP) to cyclic 3′, 5′-monophosphate (cGMP), increasing the intracellular concentration of cGMP in the cell. This activates a cGMP-dependent protein kinase (protein kinase G), which phosphorylates the CFTR, opens the channel, causing secretion of Cl^- and accumulation of Na^+ and water in the intestinal lumen. Guanylin, a hormone produced by goblet cells, is the natural ligand for GC-C, and bears significant amino acid homology to STa. The enterotoxic domain of STa is homologous to the active site of guanylin and the enterotoxic domains of EAST1 and *Yersinia enterocolitica* heat-stable toxin. In the case of guanylin, its natural function is thought to be the hydration of mucus secreted by goblet cells.

Heat-stable enterotoxin-b. STb is synthesized as a 71-amino-acid precursor, and contains a 23-amino-acid N-terminal signal sequence that is cleaved during export to the periplasm. The mature STb (STII) enterotoxin is secreted as a nonimmunogenic 48-amino-acid peptide of approximately 5.2 kDa. The gene for Stb (*estB*) is carried on a transmissible plasmid and is often linked (i.e. in close proximity to the genes for LT). Similar to *estA*, *estB* is subject to catabolite repression; glucose represses gene expression.

STb is unrelated to STa in terms of its amino acid sequence and mode of action. It does not cause an increase in intracellular cAMP or cGMP, in contrast to LT and STa. STb is thought to be involved only in disease in pigs, because with few exceptions it is active only on enterocytes of pigs (rat enterocytes are an exception). STb is inactivated

in the gut lumen by trypsin. Diarrheic piglets less than one week old have a lower incidence of carriage of *estB*-positive ETEC strains. Studies with gnotobiotic piglets inoculated with isogenic and complemented *estB* mutants support the hypothesis that STb is less effective as a virulence factor in neonatal piglets. The high content of trypsin in the small intestines at this age may play a significant role in this regard. Studies with gut loop assays and carriage of *estB*-positive strains both provide evidence that STb is more important as a virulence factor in weaned pigs, that is, post-weaning colibacillosis. *E. coli* strains that produce AIDA as the adhesin and either STb alone or in conjunction with EAST1 have the potential to cause of diarrhea in pre- or post-weaned swine.

The receptor for STb on enterocytes is a glycosphingolipid known as sulfatide. STb is internalized and stimulates a pertussis toxin-sensitive GTP-binding regulatory protein. This results in an influx of Ca^{2+} through a receptor-dependent ligand-gated calcium channel, which activates calmodulin-dependent protein kinase II. This activates the opening of an intestinal ion channel, and may also activate protein kinase C and consequently, the CFTR. The increased calcium level regulates phospholipases A_2 and C activities and the release of arachidonic acid from membrane phospholipids, leading to the formation of PGE_2 and serotonin. As noted earlier, PGE_2 and serotonin both have prosecretory and anti-absorptive effects on the intestine. STb causes HCO_3^- and Cl^- (but especially the former) secretion from the enterocytes, which results in diffusion of Na^+ and osmotic accumulation of water into the gut lumen.

EAEC heat-stable toxin-1. EAST1 is a 38-amino-acid peptide of approximately 4.1 kDa. EAST1 is encoded by the *astA* gene, which may be carried on plasmids or the chromosome, in one or more copies. EAST1 shares 50% identity with the enterotoxic domain of STa, binds to the same receptor as STa, guanylyl cyclase C, and causes increased cGMP concentrations in target host cells. However, anti-STa antibodies do not neutralize EAST1, and EAST1 does not contain an N-terminal signal sequence region as does STa, and hence the two toxins differ significantly outside of their enterotoxic domains. EAST1 has been demonstrated to be freely secreted from the bacterial cell.

EAST1 was first detected in an EAEC strain isolated from a child with diarrhea, hence the name. However, besides EAEC, EAST1 has since been found to be highly prevalent among EHEC, atypical EPEC, ETEC, DAEC, and other bacterial genera such as *Salmonella*. In *astA*-positive *E. coli* isolates from diarrheic swine, *astA* is found most often as the sole enterotoxin gene. The second most common presentation is carriage of *astA* in conjunction with the STa gene (*estA*). Detection of *astA* in combination with genes for STb, LT, Stx2e, and fimbrial F4, F5, F6, or F18 genes is also seen. The *astA* gene is highly prevalent among DEC *E. coli* isolated from pre-weaned piglets, but also has been associated with post-weaning diarrhea and edema disease isolates. The *astA* gene has been found in *E. coli* bacteremia isolates from cattle. In these extraintestinal isolates, carriage of *astA* was significantly associated with carriage of the *clpG* gene encoding the major subunit of CS31A, and with *afa-8*. EAST1 has been detected in conjunction with STb in *E. coli* isolates from diarrheic pigs that express AIDA-I. Despite the *in vitro* evidence for activation guanylyl cyclase-C, no *in vivo* studies have shown that EAST1 by itself will cause diarrhea. In each study, diarrhea has been caused by *astA*-positive strains that produced at least one other enterotoxin. Hence, clarification is still needed on the role of EAST1 in diarrhea, in addition to other clinical manifestations of disease.

Other Toxins

Shiga toxin. The Shiga toxin family includes the prototype Shiga toxin (Stx) produced by *Shigella dysenteriae* serotype 1, and the closely related Stx types produced by *E. coli* strains present in reservoir hosts, food, the environment, or other sources, known as STEC. Those STEC strains isolated from human patients with hemorrhagic colitis and/or hemolytic uremic syndrome (HUS) are known as EHEC, and are discussed below.

Stx produced by *E. coli* strains were previously called Shiga-like toxins, and all members of the Stx family are also known as verotoxins or verocytotoxins. The Stx produced by *E. coli* strains fall into two major immunologically non-cross-reactive groups, Stx1 and Stx2. The prototypical Stx1 and Stx2 toxins have 55% and 57% sequence identity in the A and B subunits, respectively. Stx1 exists as two variants; one (called Stx1) is identical to or varies by only one amino acid from Stx produced by *S. dysenteriae* serotype 1. The other, Stx1c, has only 97.1% and 96.6% amino acid sequence identity to Stx1. Stx2 variants include Stx2, Stx2c, Stx2d, Stx2e, and Stx2f; the last four are 84–99% homologous to Stx2. Stx2e is the toxin that causes edema disease in swine.

E. coli strains acquire the genes for all Stx except Stx2e through infection with lambdoid bacteriophages. These phages become integrated in the chromosome, but in response to stress may be induced to excise from the chromosome and assume a cytosolic replicating form (lytic cycle). Treatment of patients with some antibiotics (e.g. quinolones) is contraindicated because it may cause induction of prophage with cytosolic replication the phage; this results in increased expression of Stx and bacterial lysis,

releasing a much greater amount of Stx products than before.

Stx family members have an AB_5 molecular configuration, in which the B subunits bind to receptors on the plasma membranes of target cells, and the A subunit mediates the toxic activity following endocytosis of the holotoxin. Functionally, the Stx belong to a larger family of ribosome-inactivating toxins that includes such other potent toxins such as ricin, produced by castor beans, although the activity of Stx is not limited to inhibition of protein synthesis. The host cell receptor for all Stx except Stx2e is the neutral glycosphingolipid globotriaosylceramide (Gb3, also known as CD77). The Stx2e B subunits bind to globotetraosylceramide (Gb4). The molecular mass of the A subunit is 32 kDa, whereas that of each B subunit is 7.7 kDa. Similar to LT, the holotoxin assembles in the periplasm and consists of a B subunit homopentamer surrounding and becoming noncovalently bound to the A subunit. Although some EHEC contain genes for a type II secretion system, to date there is no evidence that Stx is actively secreted by a classical secretion system. Instead, Stx is released by outer membrane vesicles in live cells, and through lysis of dying cells.

Trafficking of Stx in the target cell also bears many similarities with the mechanisms employed by LT, described earlier. Following contact with the receptor, the holotoxin molecule is endocytosed into the cell via clathrin-dependent and -independent mechanisms. During the early entry process, probably at the stage of the early endosomes, a protease-sensitive loop located in the C-terminal region of the A subunit is cleaved by the membrane-associated endoprotease furin. This "nicking" process divides the A subunit into the catalytic A1 fragment and a B-subunit-associated A2 fragment. The A1 fragment remains linked to the A2-B subunit complex via a disulfide bond. This disulfide bond is ultimately reduced in the ER lumen, releasing the enzymatic A1 fragment, which is thought to use the ER-associated protein degradation pathway to become subsequently retro-translocated to the cytosol. The N-terminal 27-kDa fragment of A1 acts as an *N*-glycosidase and removes an adenine from the 28S rRNA of the 60S ribosomal subunit. This alters rRNA and prevents elongation factor (EF)-1-dependent binding of aminoacyl tRNA to the ribosome.

The result of Stx intoxication of a cell depends, at least in part, on the cell type. Endothelial cells respond to Stx intoxication by apoptosis, the initial stage of vascular damage. Some cell types may primarily undergo actin and microtubular rearrangement. Some cells, such as circulating monocytes, are not killed by Stx but respond by upregulation of proinflammatory cytokines, for example, GM-CSF and tumor necrosis factor (TNF). TNF in the circulation induces the expression of Gb3 by endothelial cells, thus exacerbating the exposure of the latter cells to Stx. In those cells that respond to Stx intoxication by intracellular signaling, a ribotoxic stress response is generated that may activate kinases such as JUN N-terminal kinase and mitogen-activated protein kinase p38.

The clinicopathologic effect of Stx-mediated tissue damage is mainly seen in the human host and is manifested as bloody diarrhea caused by hemorrhagic colitis and HUS. Most of these effects stem from the direct and indirect effects of Stx (apoptosis of endothelium and upregulated cytokine expression by monocytes, macrophages, and other cells).

Cyclomodulins. Cyclomodulins are a family of bacterial toxins and effectors that interfere with the eukaryotic cell cycle. They are broadly divided into two groups: those that stimulate cellular proliferation, and those that inhibit the cell cycle. Pathogenic strains of *E. coli* are known to produce any of four different ones: CNF, cytolethal distending toxin (CDT), cycle-inhibiting factor (Cif), and colibactin. CDT, Cif, and colibactin inhibit the cell cycle, whereas CNF stimulates cellular proliferation. The effects of these toxins have mainly been studied *in vitro*; other studies have tried to assess the relationships between carriage of a respective virulence gene and natural disease. In general, these toxins cause cell death or interference with cell function, both of which arguably increase the potential for colonization or invasion of an epithelial surface. A study of *E. coli* isolates from calves and dogs with diarrhea, cystitis, or no disease revealed that the *E. coli* isolates positive for cyclomodulin genes were almost always from cases of disease (diarrhea or cystitis). In another study involving bovine isolates, the *cnf2* and *cdt*-III genes were found to be colocalized on the Vir plasmid. It is thought that the different cyclomodulins may be synergistic in their effects.

Cytotoxic necrotizing factor. CNFs are large (110–115 kDa) monomeric proteins that cause permanent activation of the Rho, Rac, and Cdc42 GTPases of target cells by deamidation of a glutamine residue. There are two types of CNF, CNF1 and CNF2, which are immunologically related and similar in size. The activation of Rho GTPases reorganizes the actin cytoskeleton with formation of stress fibers, membrane ruffles, and filopodia. The cells become flattened and multinucleated and are increased in their phagocytic behavior. CNF also activates NF-κB, resulting in increased expression of proinflammatory cytokines and protection of the cell against apoptotic stimuli. The gene encoding CNF1 is located on the chromosome on a pathogenicity island (PAI), whereas that encoding CNF-2 is located on a plasmid. CNF1 is produced by extraintestinal *E. coli* isolates from humans, especially

UPEC and *E. coli*, which cause meningitis in the human infant. The PAI carrying *cnf1* in these strains also contains genes that encode for α-hemolysin (*hlyA*) and fimbriae (*papC* and *sfa*). CNF-2 is produced by *E. coli* isolates from the intestines of calves with diarrhea, or the blood of calves or lambs with bacteria.

Cytolethal distending toxin. The CDTs are a family of related toxins that cause arrest at the GM2/M phase of the cell cycle, which ultimately leads to cell death. Three adjacent genes, *cdtA*, *cdtB*, and *cdtC*, are required for protein expression, and five CDT types (CDT-I to CDT-V) have been described. The CDTs have not been associated with any particular pathogenic type of *E. coli* except for a few isolates of EPEC, and a role in pathogenicity has not been determined. Strains that express CDT may produce a number of other virulence factors.

Cycle-inhibiting factor. Cif is mainly produced by EPEC and EHEC. *In vitro*, Cif causes irreversible cytopathic effects characterized by the progressive recruitment of focal adhesions, assembly of stress fibers, and arrest of the cell cycle at the GM2/M and G1/S phases. Cif requires a type III secretion system to be injected into the host cell. Cif triggers cell cycle arrest due to the accumulation of cyclin-dependent kinase inhibitors (CKI) p21 and p27.

Colibactin. Colibactin blocks the cell at the G2/M phase, similar to the effect exhibited by CDT-producing bacteria. Bacterial colibactin induces DNA and chromosomal damage in eukaryotic host cells, which leads to their senescence and immune cell apoptosis. The *clb* genes encode colibactin and other proteins needed for its synthesis and secretion. They reside on a 54-kb genomic island called the *pks* (polyketide synthase) island. The cytopathic effects are seen only upon direct bacterium contact with the host cell. The 54-kb island is highly associated with ExPEC strains. The proteins encoded by the *pks* island are classified into three groups of polyketide synthases, PKS (ClbC, ClbI, and ClbO), non-ribosomal peptide synthases, NRPS (ClbH, ClbJ, and ClbN), and hybrid PKS-NRPS (ClbB and ClbK). These enzymes synthesize pre-colibactin, which is hydrolyzed and transported to the site of action by the products of other genes.

Serine protease autotransporters of Enterobacteriaceae. The SPATEs are a subfamily of serine protease autotransporters that are produced by DEC *E. coli*, UPEC, and *Shigella* spp.

Plasmid-encoded toxin. Pet is a 104-kDa SPATE that cleaves spectrin (also known as α-fodrin, a part of the cytoskeleton) and is produced by EAEC. Fodrin cleavage disrupts the actin cytoskeleton in intestinal epithelial cells, causing loss of stress fibers and release of focal contacts. These changes also stimulate an inflammatory response. Diarrhea is thought to result from prostaglandin synthesis by the recruited PMNs and affected epithelial cells, as well as activation of various inositol-signaling pathways within affected host cells. The net result is the secretion of chloride ions and water. Pet is encoded on a large virulence plasmid in close proximity to the gene encoding AAF.

Protein involved in colonization. Pic is a SPATE produced by UPEC, EAEC, EIEC, and *Shigella* spp. It is a mucinase and a protease. The *pic* gene is encoded on the chromosome, in the same locus but on the opposite DNA strand from the gene encoding *Shigella* enterotoxin 1 (ShET1). Pic is thought to be involved in the early stages of the pathogenesis of EAEC infection, and most likely promotes intestinal colonization. It does not damage epithelial cells, cleave fodrin, or degrade host defenses (e.g. secretory IgA, lactoferrin, or lysozyme) embedded in the mucus layer. Pic also induces intestinal mucus hypersecretion, which is thought to enhance biofilm formation.

Hemolysins. *E. coli* produce at least three hemolysins: α--hemolysin, enterohemolysin (Ehx for EHEC toxin), and cytolysin A (Cly).

α-Hemolysin. α-Hemolysin (Hly) is the prototype of the RTX toxin family. RTX is an acronym for repeats-in-toxin, and was named for the repeats in glycine-rich sequences within the protein. Hly is a pore-forming protein exotoxin that is secreted by a type I secretion system. Hly is commonly produced by extraintestinal *E. coli* isolates in humans and animals and is also ubiquitous in F4-positive ETEC isolates from swine. Hly is encoded by the operon *hlyCABD*, and loss or gain of *hly* by genetic manipulation produces corresponding changes in virulence of extraintestinal *E. coli* strains. In human extraintestinal isolates, *hly* genes are carried on PAIs on the chromosome, in conjunction with *pap* and *prs* genes. In contrast, in porcine ETEC isolates *hly* genes are carried on transmissible plasmids. Hly causes hemolysis, and is detected by the presence of β-hemolytic zones surrounding isolated colonies on blood agar medium (Figure 6.6). Lysis of erythrocytes provides a source of iron, but perhaps even more important is its effects on granulocytes. Neutrophils exposed to sublytic concentrations of Hly have impaired capabilities of responding to chemotactic signals, phagocytosis, and bacterial killing. In addition, exposure of neutrophils to sublytic concentrations of Hly causes release of inflammatory mediators that can cause tissue damage and increase the ability of the bacteria to evade the epithelial barrier.

Enterohemolysin. Enterohemolysin (Ehx, for enterohemorrhagic *E. coli* toxin) also is an RTX toxin, and is produced by many STEC serotypes. Its virulence features are

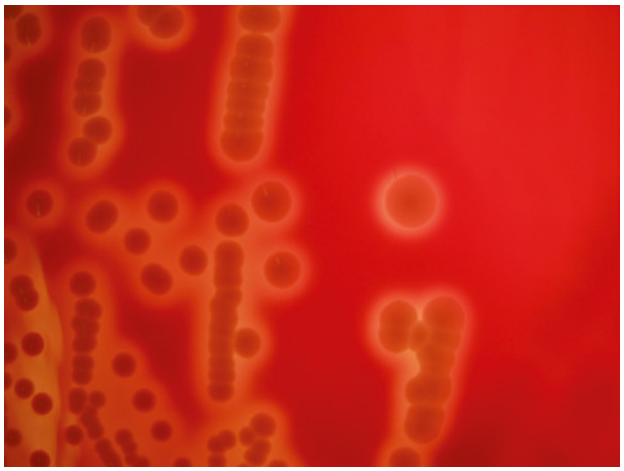


Figure 6.6 β-Hemolysis produced by α-hemolysin. Porcine-origin enterotoxigenic *E. coli* strain 3030–2 (O157:H⁻:F4) was streaked for isolation on tryptic soy agar containing 5% sheep red blood cells, and incubated at 37 °C for 24 hours. *Source:* Xing (1996).

essentially the same as those of α-hemolysin. The genes for *ehx* are carried on large plasmids along with other virulence genes for a catalase-peroxidase (KatP) and an extracellular serine protease (EspP).

Cytolysin A. A gene encoding a cryptic hemolysin called *clyA* (cytolysin A), *sheA* (for silent hemolysin), or *hlyE*, is found on the chromosome of essentially all *E. coli* strains. The hemolytic protein is 34-kDa and a pore former. Both activators and repressors regulate the transcription of the gene. Because the protein has hemolytic activity, it is presumed to have a potential *in vivo* role in iron release from erythrocytes.

Iron acquisition. Iron is an absolute growth requirement for most, if not all, living things. Siderophores (e.g. aerobactin) that remove iron from host iron-binding proteins are necessary if a microbe is to have invasive capabilities (see Chapter 5).

Acid tolerance. RNA polymerase containing RpoS (the sigma factor associated with stationary phase) preferentially transcribes genes responsible for acid tolerance (survival at pH 5), allowing safe transit through the stomach.

Locus of enterocyte effacement. The LEE is a PAI on the chromosome in EPEC and EHEC strains that contains genes needed for proteins that cause attaching-and-effacing (A/E) lesions in the host cell (see below).

Intimin and translocated intimin receptor. Intimin is a transmembrane, outer membrane protein encoded by *eae* located in the LEE5 operon. The N-terminus of intimin is in the periplasm, and the C-terminus projects above the surface of the bacterial cell. The N-terminus has a largely conserved amino acid sequence, whereas the C-terminus is variable and forms the basis of intimin types and subtypes. At least 17 different intimin types and subtypes have been described, and these are differentiated by Greek letter and number designations (e.g. α1, α2, β1, and β2). The C-terminus of the intimin molecule binds to another secreted bacterial protein, translocated intimin receptor (Tir), encoded by *tir*, also located in the LEE5 operon. The Tir protein is inserted into the host cell membrane with the C- and N-termini located in the host cell cytoplasm and the mid-region of the polypeptide external to the cell, forming a hairpin loop. It is this hairpin loop to which the C-terminus of the intimin molecule binds. Numerous adjacent intimin-Tir molecular complexes result in an intimate anchoring of the bacteria to host cell membrane, significantly enhancing colonization.

Type III secretion system. The genes encoding a type III secretion system (an assemblage of proteins – more than 20 – that form a hollow tube-like structure through which effector proteins are injected into host target cells) are also encoded on the LEE (see LEE).

***E. coli* secreted protein.** The genes encoding *E. coli* secreted protein (Esp) proteins are also located on the LEE. EspA forms the hollow, syringe-like structure through which Tir, EspB, EspD, and other effectors are injected into the host cell. EspB and EspD form the pore in the host cell. Several other effectors elicit cytoskeletal rearrangements leading to effacement of the microvilli. Other less well-defined effectors, such as Cif and Map, are thought to mediate toxic effects to the host cell, for example, cell cycle arrest and mitochondrial damage. Diarrhea occurs secondary to increases in intracellular calcium ions and activation of protein kinase C. Protein kinase C is responsible for phosphorylation of proteins composing the chloride channels resulting in loss of chloride and water into the intestinal lumen, as well as the phosphorylation of the membrane-associated ion transport proteins, resulting in blockage of NaCl absorption.

Variability

One measure of variability of *E. coli* resides in the antigenic makeup of the O-repeat units (type of sugar subunits, how the subunits are hooked together, and the length of the chain), the composition of the flagellar protein (flagellin), and the composition of the capsule. The O-, H-, and K-antigens are used in serotyping a particular isolate. There are 174 O-antigens, at least 80 distinct K-antigens, and 53 H-antigens in the international serotyping scheme. The O-antigens are numbered 1–181, but several numbers

have been deleted due to cross-reactivity with other antigens. For example, O8:K87:H19 describes an isolate with antigens of the O-antigen number 8, capsular antigen number 87, and flagellar-antigen number 19.

Ecology

Reservoir and Transmission

Strains of *E. coli* capable of producing disease reside in the lower gastrointestinal tract and are abundant in environments inhabited by animals. Transmission is through the fecal-oral route. The lower intestinal tract has been termed the "primary habitat" and the environment outside the animal, the "secondary habitat" of *E. coli*. This reflects the importance of the lower intestine in providing the necessary nutrients and warm temperatures for *E. coli* (a mesophile) to be in a positive growth state, and also the need for it to exit one host in order to enter a new one to complete its "life cycle."

Pathogenesis

Mechanisms and Disease Patterns

For *E. coli* to produce disease, it must have the necessary genes encoding the virulence factors needed to do so. If the genes are acquired (by transduction, conjugation, or transformation), the nonpathogenic strain may be changed to one with pathogenic potential. This lateral form of gene acquisition, often through bacteriophages or plasmids, is of paramount importance in the emergence of new pathogenic types. Also, the type of disease produced depends upon the genes acquired.

ETEC. This disease occurs in neonatal pigs, calves, and lambs, and in weanling pigs. It has been reported in dogs and horses.

Enterotoxigenic diarrhea is caused by strains of ETEC that produce adhesins that promote attachment to glycoproteins on the surface of epithelial cells of the jejunum and ileum, and an enterotoxin(s) that affects the epithelial cell (to which the enterotoxigenic strain of *E. coli* is adhered), resulting in fluid secretion and diarrhea. Both traits are necessary for disease to result, because unless the ingested strain adheres to these cells, peristalsis will move it into the large bowel. The cells of the jejunum and the ileum are susceptible to the action of enterotoxin; the cells of the large bowel are not.

As discussed above, at least four fimbrial adhesins may be found on ETEC: F4, F5, F6, and F41. They possess some host species specificity: F4 and F6 are almost always associated with isolates from swine; F5 with isolates from cattle, sheep, and swine; and F41 with those from cattle. The epithelial cell receptors for these adhesins regulate the age incidence of this disease as well. In calves and lambs, the receptors appear transiently during the first week or so of life. Analogous receptors are present in pigs throughout the first six weeks of life. There are many uncharacterized adhesins that probably play a role.

Also, as discussed above, some enterotoxigenic strains of *E. coli* express fimbria known as curli. Curli fimbria mediate adherence to glycoproteins on the surface of epithelial cells and to extracellular matrix proteins, and are important in the formation of biofilms. The presence of curli may explain the increase in the window of age susceptibility to enterotoxigenic disease in animals concurrently infected with rotavirus or *Cryptosporidium parvum*, two agents that may cause enough tissue damage to lead to exposure of extracellular matrix proteins.

In addition to adherence to the target tissues of the small intestine, enterotoxigenic strains must have the genetic capability of synthesizing enterotoxins. As noted above, four different enterotoxins are produced by ETEC strains, and although there are clones that have become highly prevalent throughout the world (e.g. porcine strains that produce the combination of F4, LT, and STb), with time and extensive testing (usually by polymerase chain reaction [PCR]), just about every possible combination is detected.

Some of the adhesins and enterotoxins are encoded on plasmid DNA. As a consequence, it is difficult to predict which strain of *E. coli* possesses the genetic information necessary to produce disease. Some adhesins are associated with certain serotypes. In particular, the genes encoding the protein for the F41 adhesin are almost always found within strains of *E. coli* of the O9 and the O101 serogroups. As might be expected, the genes encoding the proteins for F41 adhesin are located on chromosomal DNA.

Following ingestion by the host, ETEC adhere to target cells, multiply, and secrete enterotoxin (Figure 6.1). Fluid and electrolytes accumulate in the lumen of the intestine, resulting in diarrhea, dehydration, and electrolyte imbalances. In time, the infecting strain is moved distally away from the target cell, and the disease process stops, due probably in part to the cessation of expression of the adhesin along with a decrease in available substrate following the almost explosive multiplication of the strain in the small intestine. Unless steps are taken to correct the fluid and electrolyte imbalances, the disease has high mortality.

The diarrhea produced is watery and non-bloody. In uncomplicated infections, Bacteria will be observed histologically coating the villi of the mid-to-distal portions of the small intestine.

EAEC. As noted above, strains of *E. coli* that express the AAF and therefore defined as EAEC have been isolated from weaned pigs and calves with diarrhea. However, these isolates contained almost none of the other virulence factors found in human EAEC isolates, and consequently were not likely to be pathogens of animals or humans. EAEC are noted pathogens of humans and have a number of other virulence factors that were described earlier.

ExPEC and NTEC. In susceptible animals (usually a neonate that has received inadequate amounts of colostrum or colostrum of inadequate quality), ExPEC or NTEC strains may infect extraintestinal tissues, gaining access via the conjunctivae, umbilicus, or intestine. If these strains are acquired by ingestion, they first adhere to target cells in the distal small bowel. NTEC adherence is via Afa's, namely, CS31A, AfaE-VII, and AfaE-VIII. Likewise, the fimbrial adhesin F17c and the siderophore aerobactin originally described on a plasmid termed Vir (so-called because of its association with virulent *E. coli*) are prevalent on these *E. coli* strains. Following adherence, these strains may induce their own uptake by expression of either CNF1 or CNF2, resulting in an endocytotic process that allows them to enter the intestinal epithelial cells. Entry into the lymphatics and subsequently the bloodstream follows. The mechanism by which these strains gain access to lymphatics after uptake by the epithelial cell is unknown. Likewise, the mechanism of entry into lymphatics after association with conjunctivae or the umbilicus is unknown. Once the epithelial surface is traversed, expression of adhesins is repressed (otherwise adhesin-expressing bacteria could adhere to host phagocytic cells with disastrous consequences for the bacterium).

The infecting strain multiplies in the lymphatics and bloodstream, and endotoxemia develops. Death of the host occurs if antibacterial therapy, the immune system, or both do not remove the microorganism.

ExPEC express virulence factors that allow them to escape phagocytosis and/or complement-mediated lysis, and acquire iron in host tissues. Capsule and various outer membrane proteins confer resistance to complement-mediated lysis (serum resistance). How capsules protect the outer membrane from insertion of the membrane attack complex is not known. Certain capsules (such as K1) are chemically similar to the surface of host cells in that they are composed mainly of sialic acid. Complement components associating with surfaces composed of sialic acid are shunted to degradative pathways rather than amplifying and forming membrane attack complexes.

Escape from phagocytosis is also related to capsule and certain outer membrane proteins. How outer membrane proteins function as antiphagocytic factors is not known.

The genes encoding the adhesin (e.g. CS31A and F17) and those responsible for siderophore production reside on plasmids. As mentioned above, the genes encoding F17 have been associated with the plasmid Vir, as has the gene encoding CNF2; those responsible for siderophore production have been associated with the plasmid pColV. In the latter instance, the siderophore genes are linked closely with the genes for the production of colicin V. The siderophore, aerobactin, has a high affinity for iron.

Many of the strains with invasive capability, except those from foals, produce α-hemolysin (Hly) and are β-hemolytic on blood agar.

Histopathologically there are inflammatory changes in liver, spleen, joints, and meninges. There may be hemorrhages on the pericardium, peritoneal surfaces, and adrenal cortices.

EPEC. Enteropathogenic strains of *E. coli* produce diarrhea in all animal species, they infect including human beings. EPEC do not produce ST, LT, or other enterotoxins. Most notably, they cause A/E lesions in the small intestines in humans, which they colonize the small intestine. In animals, EPEC colonize and cause A/E lesions in both the distal small intestine and the large intestines. The characteristic lesion is named thus because the microvilli are effaced and at these sites of effacement, the bacteria are intimately attached to the apical plasma membrane. Cytoskeletal rearrangements induced by effector proteins (discussed earlier) cause the formation of pedestals, to which the bacteria attach (Figure 6.1).

STEC and EHEC. As described above, STEC that produce intimin and other LEE gene products are called EHEC. These strains have a greater virulence because they most likely have the ability to produce A/E lesions, and they produce Stx. *E. coli* O157:H7 is the prototype of the EHEC and is thought to have evolved from EPEC O55:H7, a human infantile diarrhea pathogen. *E. coli* acquire Stx genes via lysogeny with the bacteriophage(s) that encode Shiga toxin (Stx)-1 and/or −2. Cattle are reservoir hosts for STEC; for some reason, the bovine intestine has become a natural primary habitat for these organisms. Neonatal ruminants, especially cattle, and in some cases, older animals develop diarrheal disease that can be bloody in association with EHEC infection (Figure 6.7). EHEC bacteria infect the large intestine and cause A/E lesions and epithelial necrosis with resultant erosions. Occasionally, bacteria may be seen within cytoplasmic vacuoles of mucosal epithelial cells (Figures 6.8–6.10).

Many different serotypes of *E. coli* are now known to be STEC; however, a much smaller subset of these contain the LEE PAI. As noted above, some non-O157 STEC that lack the LEE genes produce an alternative adhesin known as

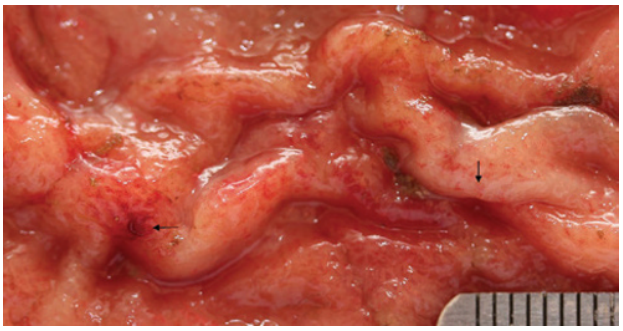


Figure 6.7 Photograph of mucosal surface of colon of 1-year-old feedlot heifer naturally infected with EHEC O165:H25 and clinically exhibiting bloody diarrhea. Acute erosions are hyperemic, some with adherent blood clots (horizontal arrow), whereas re-epithelialized erosions are paler and nearly the same color as that of surrounding mucosal epithelium (vertical arrow). Distance between each ruler mark is 1 mm. *Source:* Reproduced by permission of Moxley et al. (2015).

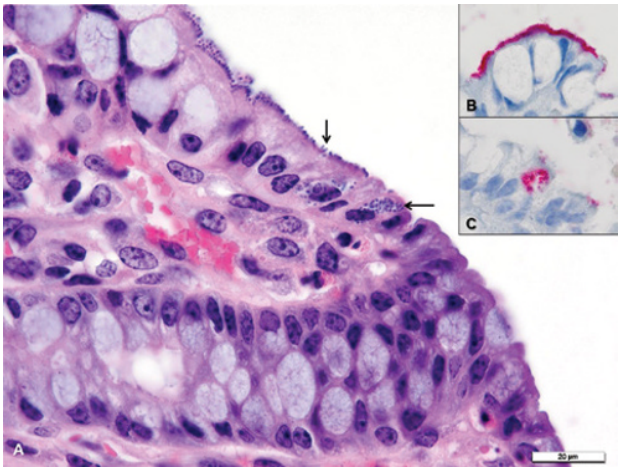


Figure 6.8 Light photomicrographs of colonic mucosa shown in previous figure with *E. coli* O165-positive bacteria adherent to apical surfaces and within cytoplasmic vacuoles of colonic mucosal epithelium. (a) Photomicrograph of colonic mucosa showing an area of colonization by bacteria intimately adherent to the apical surfaces of enterocytes (vertical arrow) and bacteria within vacuoles in the cytoplasm (horizontal arrow). Hematoxylin & eosin stain. (b, c) Subsequent tissue sections from the same block stained immunohistochemically with rabbit polyclonal antiserum against *E. coli* O165 antigen showing positive (red) staining of bacteria on apical surfaces (b) and in cytoplasmic vacuoles (c). Bar (20 µm) is representative for (a–c). *Source:* Reproduced by permission of Moxley et al. (2015).

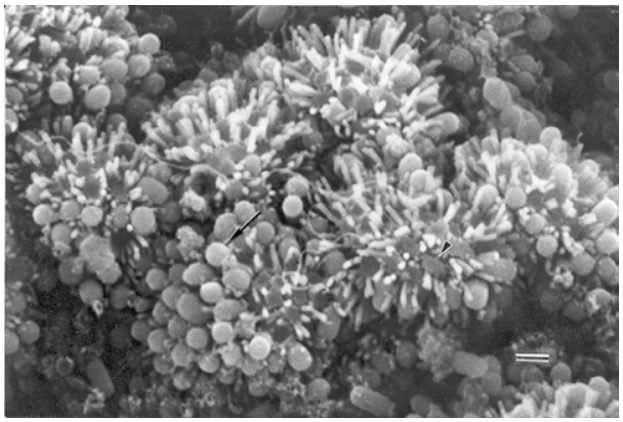


Figure 6.9 Scanning electron photomicrograph of colon of neonatal gnotobiotic calf orally inoculated with EHEC O5:K4:H⁻. EHEC bacterial cells (arrow) cover the apical cell membranes of enterocytes. Cuplike or pedestal-like distortions of the apical cell membranes (arrowhead) are present at sites where bacteria were detached during tissue processing. Microvilli between attached bacterial cells are prominent and elongated. Enterocytes are swollen and are in the process of detachment from the mucosal surface (original negative magnification 5000×; bar = 1 µm). *Source:* Moxley and Francis (1986). With permission from American Society for Microbiology.

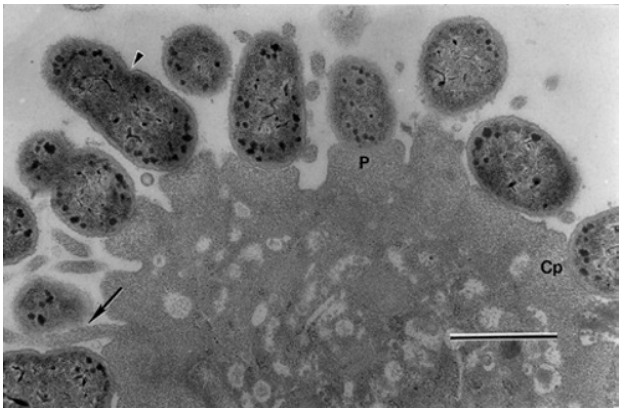


Figure 6.10 Transmission electron photomicrograph of rectum of neonatal gnotobiotic calf orally inoculated with EHEC O5:K4:H–. The attachment of bacteria to this enterocyte results primarily in pedestal-like cell membrane evagination (P), with occasional cuplike invagination (Cp). Microvilli between sites of bacterial attachment are elongated (arrow). Some bacteria are in the process of binary fission (arrowhead). The cytoplasm lacks a discernible terminal web and contains numerous vacuoles (original negative magnification × 15 000; bar = 1 µm). *Source:* Moxley and Francis (1986). With permission from American Society for Microbiology.

Saa. Human patients are highly susceptible to the effects of Stx, whereas cattle are not. Cattle lack the Gb3 receptor for Stx on their vascular endothelial cells; in addition, Stx that enters into the enterocytes of cattle ends in the lysosomes instead of the trans-Golgi and ER, which prevents toxic effects. Human patients are susceptible to vascular necrosis, thrombosis, and infarction of the large intestine, which is manifest as hemorrhagic colitis. Approximately 5–10% of these patients develop post-diarrheal sequelae, namely, HUS. Although the pathogenesis is complex, Stx plays a major role through the direct causation of

endothelial apoptosis and induction of cytokine production by leukocytes, which in turn increase the expression of Gb3 on the endothelial cells. Clinically, HUS is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and uremia.

In general, animals acquire EPEC and STEC by fecal-oral transmission. Both STEC and EPEC have zoonotic potential, but STEC strains are of greater concern due to the potentially life-threatening effects of Stx. Human beings primarily become infected following ingestion of contaminated food and water, or through direct contact with reservoir hosts (all ruminants). Petting zoos have become a source of infection. At slaughter, the surface of the carcass becomes contaminated with fecal microorganisms. The surfaces of cuts of meat derived from an infected carcass are usually adequately decontaminated by cooking. However, when meat is ground, the microorganisms on the surface are spread throughout the product. Though proper cooking will readily kill surface microorganisms, including STEC O157:H7, those inside may not be killed. Currently in the United States, non-O157 STEC cause more cases of illness than does O157:H7. In the United States, six serogroups, namely, O26, O45, O103, O111, O121, and O145, cause approximately 71% of the cases of non-O157 STEC-related illness in humans. Recently, the USDA-FSIS declared these serogroups, in addition to O157:H7, as adulterants in beef.

Edema disease. Edema disease is an acute, often fatal enterotoxemia of weaned pigs. The disease is characterized by edema in potentially many different parts of the body, for example, the eyelids, subcutis, stomach (Figure 6.11), and mesentery of the spiral colon. Death is most often caused by infarction of the brain, most often in the brainstem (Figure 6.12), usually presenting previously with neurological signs such as head tilt, nystagmus, and tonic-clonic convulsions. Edema in the tissues is caused by vascular damage resulting from the toxic effects of Stx2e on endothelial cells, and other cells of blood vessels, for example, medial myocytes in the case of arterioles. Stx2e is absorbed from the intestine and is spread systemically, hence the name enterotoxemia. Causative strains are usually of certain serotypes, for example, O141:K85, O138:K81, and O139:K82, and usually express α-hemolysin and F18ab fimbria. F18ab fimbrial expression enhances bacterial colonization of the distal small intestine. Stx2e is absorbed from the intestine into the bloodstream, and binds predominantly to the surfaces of erythrocytes, which are rich in Gb4. Erythrocytes are thought to then deliver the toxin to endothelial cells, which also express the Gb4 receptor. Stx2e enters the endothelial cells as described earlier and intoxicates the cells by inactivating the ribosomes, shutting down protein synthesis, and causing cell death. Vascular leakage and thromboses result in edema and infarcts, respectively, in different tissues and organs. Grossly, pigs typically exhibit subcutaneous edema on the forehead, in the eyelids, and also in the wall of the stomach, mesocolon, and other areas (Figure 6.12).

APEC. APEC cause colibacillosis of fowl, which is economically important to the poultry industry. APEC are invasive extraintestinal strains of *E. coli* that are usually of specific serotypes, and bear several virulence genes in common with UPEC of humans. One common serotype is O1:K1:H7. Notably, K1 is a capsular antigen that plays an



Figure 6.11 Edema in the wall of the stomach of a pig with edema disease. *Source:* Noah's Arkive, F01528, Stomach, Edema Disease, submitted by Crowell, The University of Georgia. Reproduced by permission of Noah's Arkive, F01528, Stomach, Edema Disease, submitted by Crowell, The University of Georgia. Accessible at: https://davisthompsonfoundation.org/image-detail?image=F01528

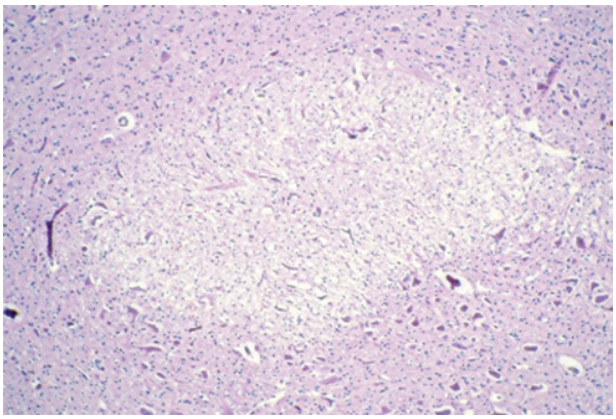


Figure 6.12 Photomicrograph of brainstem of post-weaned pig with neurological signs associated with natural edema disease. The pale-staining region in the center of the figure is an infarct resulting from arteriolar necrosis and thrombosis caused by Stx2e.

important role in the pathogenesis of infection of human infants, causing meningitis in these cases. In addition, O1 is notably an invasive serogroup. Virulence factors in common with human UPEC strains include the siderophores enterobactin, aerobactin, salmochelin, and yersiniabactin, as well as a hemin uptake system. In addition, both APEC and human UPEC strains produce Pap and type 1 fimbriae.

The disease takes many forms in fowl, depending upon the age of the host and mode of infection. In the case of egg infections, the egg surface may be contaminated with potentially pathogenic strains at the time they are laid. The bacteria penetrate the shell and infect the yolk sac. If the bacteria grow, the embryo dies, usually late in incubation. Embryos that survive may die shortly after, with losses occurring as late as three weeks after hatching. A very important clinical manifestation in poultry is respiratory and septicemic disease. The course may be rapidly fatal or chronic, manifested by debilitation, diarrhea, and respiratory distress. Air sacculitis and pneumonia are common presentations. Other clinical syndromes caused by APEC include cellulitis, synovitis, pericarditis, salpingitis, and panophthalmitis.

AIEC. AIEC were first described in human patients with Crohn's disease. They have been significantly associated with Crohn's disease, but a definite cause-and-effect relationship has yet to be determined. AIEC have also been found in dogs with histiocytic ulcerative colitis, and in this disease there is considerable scientific evidence for an etiological role. AIEC adhere to and invade intestinal epithelial cells. Entry into these cells is dependent upon induction of actin microfilament and microtubule recruitment. The bacteria then gain entrance into macrophages beneath the epithelial layer, within which they replicate in endocytic vacuoles without causing host cell death. Infection of the macrophages induces these cells to produce large amounts of TNF-α. This upregulation of cytokine production is thought to significantly exacerbate inflammation and tissue damage.

Immunologic Aspects

Immunologic defense against diseases produced by pathogenic *E. coli* occurs at two levels: at the site of attachment to the target cell and through destruction of the bacteria or neutralization of its products.

Enterotoxigenic diarrhea. Specific anti-adhesin antibodies (sIgA and sIgM) found in colostrum and milk prevent bacterial attachment to small intestinal enterocytes. Likewise, specific anti-LT neutralizes LT enterotoxin, although the importance of this effect is incompletely understood. LT has been shown to be delivered directly to the enterocyte by the bacteria, which may bypass the effect of luminal antibodies.

Extraintestinal disease. The neonate acquires immunity from the dam, and depending upon the isotype of the immunoglobulin (IgA, IgG, or IgM), the type of protection differs. For the first 36 hours or so of life, ingested IgG and IgM attach to receptors on the surface of epithelial cells of the small intestine. Transfer across the cell into the systemic circulation follows attachment. If the antibodies are specific for a virulence determinant, then disease may not result if the neonate encounters a pathogenic strain expressing that virulence determinant. For example, anticapsular antibodies acquired from the dam will protect the newborn from fatal invasive disease by strains of *E. coli* possessing that particular capsule.

EPEC and STEC infections. Specific antibodies to intimin and type III secreted proteins that mediate A/E lesions may provide some degree of protection. Studies with a type III secreted vaccine for STEC O157:H7 have shown efficacy in cattle. In the case of the neonate, sIgA and sIgM found in colostrum and milk are thought to prevent attachment to enterocytes.

Edema disease. Antibody specific for Stx2e prevent endothelial and vascular damage, further preventing ischemic lesions. It is imperative, therefore, that the dam be exposed either naturally or artificially to the microorganism and its virulence determinants before parturition. Such exposure allows for antibodies to be made for secretion into colostrum and milk.

Laboratory Diagnosis

Diagnosis of ETEC. The currently preferred diagnostic method for microbiological diagnosis of ETEC is detection of virulence genes, that is, fimbria and enterotoxin, in isolated *E. coli* colonies on culture plates by PCR. A multiplex PCR format is commonly used in diagnostic laboratories that targets a variety of virulence genes besides those of concern with ETEC, for example, Stx, intimin, and others. Bacterial isolates chosen for PCR should first be subcultured onto a nutrient medium (e.g. blood agar), both to ensure purity and to remove the possibility of inhibitors of the DNA polymerase.

Histopathology on animals submitted for necropsy should be conducted, preferably on an acutely affected animal that has been euthanatized. Selection of an acutely affected animal that has not undergone postmortem autolysis and postmortem bacterial overgrowth will greatly enhance the chances of detecting the pathogen among a background of commensal flora. Detection of bacterial adherence to enterocytes in the small intestine is

pathognomonic. Immunohistochemistry also could be conducted if the laboratory has antiserum that includes most of the common serogroups (Figure 6.1). However, culture is needed for detection of the specific pathogen involved from the standpoint of virulence factor production. Culture on blood and MacConkey agars are standard procedures for ETEC diagnosis, although detection of some fimbrial types may be enhanced by culture on additional media, namely, E medium for F4; Minca medium for F5 and F6; and E or Minca medium for F41. Detection of large numbers of *E. coli*, that is, 10^8 to 10^9 colony-forming units per milliliter of luminal contents, is supportive of a diagnosis of ETEC infection.

Immunologic approaches may still be used, for example, slide agglutination tests run on each colony using antiserum specific for the various adhesins. An enzyme-linked immunosorbent assay may be used to measure directly the presence of F4 and F5 adhesin-expressing bacteria in feces. Fluorescent-labeled antibody techniques still provide an easy method of detection of fimbria. Bacterial colonies or smears of scrapings taken from the small intestine are flooded with antisera that are specific for the various adhesins. After treatment with fluorescent-labeled secondary antiserum, preparations are examined for labeled bacteria adhering to the epithelial cells. STa or LT enterotoxin production by isolated strains of *E. coli* can be detected with an ELISA test. This test is reputed to detect 140 pg/ml of STa (>100 times more sensitive than the suckling mouse assay) and 290 pg/ml of LT.

Diagnosis of ExPEC and APEC. The microbiological diagnosis of extraintestinal disease is based upon the demonstration of *E. coli* in normally sterile sites or locations (joint, bone marrow, spleen, or blood). In fowl, the same sites are cultured, plus those grossly affected (lung, air sac). Dead in-shell embryos are cultured. Culture of the liver is to be avoided even though the Kupffer cells remove bacteria from the blood, because retrograde movement of enteric bacteria during the agonal stages of the disease complicates the microbiologic findings.

Diagnosis of EPEC, STEC and EHEC. Histopathology can allow for detection of A/E bacteria, but will not result in definitive identification of the pathogen. Specific protocols and special media for isolation of *E. coli* from fecal or intestinal samples are now available. Other media, for example, for non-O157 STEC, are also available, but have not been validated for all serogroups of interest. Some of the selective media for this purpose, for example, sorbitol-MacConkey, are described in Chapter 5. Once *E. coli* isolates that are suspected to be STEC (e.g. showing the proper phenotype on chromogenic media) are obtained in cases of animal infection, detection of Stx genes by PCR is the preferred method for confirmation of these organisms as STEC. The most important virulence gene targets are stx_1, stx_2, and *eae*; *ehx* is also a useful target for diagnostic purposes. Fecal isolates also may be obtained from a more general-purpose selective medium for *E. coli* (e.g. MacConkey agar), and these can be tested by PCR.

Diagnosis of edema disease. The microbiological diagnosis of edema disease depends upon the isolation and demonstration of certain serotypes that have been shown to play a role in this disease. The characteristic gross and microscopic tissue changes make this disease relatively easier to diagnose pathologically than microbiologically.

Treatment, Control, and Prevention

Treatment of an animal that has diarrhea due to an infectious cause centers on correcting fluid and electrolyte imbalances. If the animal is in shock due to cardiovascular collapse, then the fluid and electrolytes (sodium bicarbonate, KCl) are given IV; if not, oral electrolyte solutions are given. Because the animals are acidotic, sodium bicarbonate is included. Adding glucose to oral electrolytes will enhance the absorption of the sodium ions being excreted. The use of antimicrobials is controversial. Because the concentration of antimicrobic achievable (and available) in the lumen of the bowel is not known, the results of *in vitro* susceptibility tests to guide therapy are of doubtful reliability. Administration of nonabsorbable antimicrobics (such as neomycin) will sufficiently reduce the numbers of *E. coli* in the upper small bowel to allow correction of fluid and electrolyte imbalances. Such reduction occurs even though *in vitro* tests show that strains of *E. coli* commonly test "resistant" to neomycin. The fact that *in vitro* tests measure susceptibility to microgram amounts whereas milligram amounts may be available locally accounts for the discrepancy.

Antimicrobial agents, fluid, and electrolyte augmentation are necessary to successfully treat septicemic disease produced by invasive strains of *E. coli*. Invasive disease results in an endotoxemia progressing to a lactic acidosis because of decreased organ perfusion secondary to hypotension and disseminated intravascular coagulation. This should be taken into account when the electrolyte replacement is chosen. Antimicrobial agents should be chosen according to susceptibility trends in the practice area. *E. coli* isolated from farm animals are usually susceptible to gentamicin or amikacin, trimethoprim-sulfonamides, and ceftiofur. They are usually resistant to tetracyclines, streptomycin, sulfonamides, ampicillin, and kanamycin. The severity of the signs of endotoxemia has been reduced

experimentally by administering antibodies to the lipid A portion of the LPS.

Prevention and control of the enteric diseases produced by pathogenic strains of *E. coli* are one and the same. The key is sound husbandry practices. It is important that the dam be exposed to the antigenic determinants of the various virulence factors expressed on or by the infecting strains. Exposure can be provided naturally by placing the dam into the environment in which parturition will take place or artificially by vaccinating the dam with preparations containing the antigenic determinants perceived to be a threat to the newborn. Commercially produced preparations containing monoclonal antibodies to the adhesins (for ETEC) can be given orally to the neonatal animal. Although this practice will not significantly reduce the incidence of diarrhea, it will reduce the severity and mortality.

Other Coliforms

There are a few other medically important members of the family *Enterobacteriaceae*. Like *E. coli*, the bacteria usually ferment lactose, resulting in the traditional reddish-pink colonies on MacConkey agar. The genera *Klebsiella, Enterobacter*, *and Citrobacter* are also facultative anaerobes. These organisms are not hemolytic on blood agar, and although they produce some toxins, these bacteria are generally opportunistic pathogens.

Klebsiella

The most common pathogens in veterinary medicine are *Klebsiella pneumoniae* and *Klebsiella oxytoca*. These bacteria, like most coliforms, are commensals of the intestinal tract of animals. Therefore, fecal-contaminated environments are often the source of perinatal infections of young ruminants. However, bovine mastitis attributed to *K. pneumoniae* spp. *pneumonia*e can be a very serious condition with case-fatality rates ranging from 10% to 80%. This form of coliform mastitis is associated with cool, wet weather and various forms of sawdust bedding. Many of the affected cattle develop severe mammary gland infections with septicemia and endotoxic shock.

Klebsiella spp. have been associated with many forms of opportunistic infections. Contaminated obstetric equipment, surgical equipment, cleaning devices, and clinic surfaces may contribute to the likelihood of infection. Once tissue infection is established, the organism may spread and eventually cause a fulminating septicemia. *Klebsiella* will also rapidly acquire extended spectrum β-lactamase resistance. Virulence factors associated with *Klebsiella* are similar to those for other *Enterobacteriaceae*. The capsule is essential for resistance to host defense mechanisms (phagocytosis, opsonization, and cytolysis). Endotoxins, adhesins, enterotoxins, siderophores, and cell wall components are also important.

Klebsiella are easily identified through routine diagnostic laboratory culture at 37 °C. The colonies on blood agar are nonhemolytic, large and very mucoid. *Klebsiella* ferment lactose and are generally indole-negative (see Table 6.2).

Enterobacter and *Citrobacter*

Like *Klebsiella*, bacteria of the genera *Enterobacter* and *Citrobacter* are opportunistic pathogens. These bacteria often cause infections in contaminated wounds or the urogenital tract. Both *Enterobacter* and *Citrobacter* are identified using traditional colony morphology and biochemical testing (Table 6.2). *Enterobacter* are usually motile. The *Citrobacter* produce hydrogen sulfide in triple sugar-iron slant agar tubes; thus, sometimes these cultures may be confused with *Salmonella*. These bacteria are notably resistant to most penicillins and cephalosporins and rapidly acquire other forms of antibiotic resistance.

Table 6.2 Properties of *Klebsiella*, *Enterobacter*, and *Citrobacter*.

Properties	*Klebsiella*	*Citrobacter*	*Enterobacter*
Lactose fermentation	+	−	+
Urea hydrolysis	(+)	V	V
Citrate utilization	(+)	(+)	(+)
Hydrogen sulfide	−	(+)	−
Voges–Proskauer	+	−	+
Motility	−	(+)/weak	+
Common species	*pneumoniae oxytoca*	*freundii rodentium*	*cloacae aerogenes*

+, positive; (+), most positive; −, negative; V, variable.

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7

Enterobacteriaceae

Salmonella

Rodney A. Moxley

The genus *Salmonella* is a member of the family *Enterobacteriaceae*, and is composed of three species: *S. bongori*, *S. enterica*, and *S. subterranea*. *S. enterica* contains six subspecies (Table 7.1); these include *enterica* (also known as subspecies I), *salamae* (subspecies II), *arizonae* (subspecies IIIa), *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies VI). Subspecies V was reclassified as *S. bongori*. The type species is *S. enterica* ssp. *enterica*. The type strain is *S. enterica* ssp. *enterica* serotype Typhimurium strain LT2 (Lilleengen strain type 2). Subspecies I strains are commonly isolated from humans and warm-blooded animals. *S. enterica* includes more than 2600 serotypes, also known as serovars (ser.) or varieties (var.), and approximately 60% of these fall within subspecies I. In the United States, approximately 99% of the reported *Salmonella* isolates from humans belong to subspecies I. *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, and VI mainly infect cold-blooded vertebrates and live in the environment. *S. subterranea* is a recent addition to the genus and was isolated from low-pH subsurface sediment contaminated with nitrate and hexavalent to tetravalent uranium.

The *Salmonella* nomenclature has been problematic for many years due to the use of several different taxonomic schemes and the listing of serotypes as species. In 2005, Judicial Opinion 80 was published in the *International Journal of Systematic and Evolutionary Microbiology* and clarified many of these issues. Serotypes of *S. enterica* ssp. *enterica* are written in Roman letters in which the first letter is uppercase; others are denoted by antigenic formulae.

In this chapter, the subspecies designation will not be used unless it is important to the discussion. For example, *S. enterica* ssp. *enterica* serovar Typhimurium will first be denoted as *S. enterica* serovar Typhimurium, then simply *S. Typhimurium*.

Descriptive Features

Cellular Description and Composition

A capsular type that is a polysaccharide antigen has been named Vi (for virulence). Vi is produced by serovar Typhi and Paratyphi C strains, some *Citrobacter* strains, and occasionally by strains of serovar Dublin. The cell wall is typical of gram-negative bacteria, composed of lipopolysaccharide (LPS) and protein. As described in Chapter 5, the kind and number of sugars together with the linkage between them in the outermost portion of the LPS macromolecule determine the O-antigen serogroup of the particular isolate. The O-antigens, together with the antigenic determinants on the surface of the flagella (H-antigens), which are possessed by most salmonellae, define the serotype. This classification is called the Kauffmann–White scheme.

Cellular Products of Medical Interest

Adhesins. Depending on the serotype, *S. enterica* contains gene clusters for more than 10 different fimbriae. *S. Typhimurium* potentially encodes at least 13 fimbrial operons: *agf* (*csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *sth*, *sti*, and *stj*. Production of at least 11 of these fimbriae has been detected, with expression being dependent on growth conditions. The fimbria mediates adherence to mucosal epithelial cells in the gastrointestinal tract. Because of their relative hydrophobicity, fimbrial adhesins may also promote association with the membrane of phagocytic cells. Adhesins are important virulence factors when the microbes are on mucosal surfaces.

Of the aforementioned fimbriae, there is evidence for Pef, Agf, and Lpf as being important adhesins for the intestinal tract. Pef (plasmid-encoded fimbriae) mediate attachment

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Table 7.1 Subspecies of *Salmonella enterica*.

Numeral designation	Nomenclature
I	*enterica*
II	*salamae*
IIIa	*arizonae*
IIIb	*diarizonae*
IV	*houtenae*
VI	*indica*

to murine small intestinal epithelial cells. The genes for Pef are carried on the virulence plasmid of *S. Typhimurium*, pSLT. Mutations in *pef* increase the oral lethal dose 50 (LD_{50}) about 2.5-fold. Agf (thin aggregative fimbriae, or curli) are analogous to curli fimbriae of *Escherichia coli* (see Chapter 6). Agf promote autoaggregation, biofilm formation, and virulence. Agf also mediates attachment to small intestinal epithelial cells. Lpf (long polar fimbriae) mediates attachment of *S. Typhimurium* to M cells, but not to the absorptive enterocytes. Mutations in *lpf* increase the oral LD_{50} about fivefold, and delay death by about three days compared to the wild-type parent strain. The Pef, Lpf, and Agf fimbriae are thought to be functionally redundant. Inactivation of all three genes results in an about 30-fold increase in the oral LD_{50}.

Capsule. The Vi capsule is a linear homopolymer of α-1,4-2-deoxy-2-*N*-acetylgalactosamine uronic acid, which can be acetylated at the C-3 position. The role of the capsule (Vi) is unclear. Because salmonellae are primarily intracellular parasites, possession of a capsule does not seem to be a strategy that is consistent with the role this structure has in other microorganisms (i.e. antiphagocytic). However, Vi protects the outer membrane from effective interactions with membrane attack complexes generated by the complement system. This is useful in protecting extracellular salmonellae.

Cell wall. As described in detail in Chapter 5, the LPS in the outer membrane is an important virulence determinant. Not only is the lipid A component toxic (endotoxin), but the length of the side chain in the O-repeat unit hinders the attachment of the membrane attack complex of the complement system to the outer membrane. LPS binds to LPS-binding protein (a plasma protein), which in turn transfers it to the blood phase of CD14. The CD14–LPS complex binds to Toll-like receptor (TLR)-4 protein (see Chapter 5) on the surface of macrophages triggering cell-signaling pathways that ultimately cause release of pro-inflammatory cytokines. Recent studies have shown that TLR signaling is required for the virulence of *S. Typhimurium*. Recognition of *S. Typhimurium* is largely mediated by TLR2, TLR4, and TLR5. Mice deficient in both TLR2 and TLR4 are highly susceptible to *S. Typhimurium*, consistent with reduced innate immune function. TLR signaling enhances the rate of acidification of *Salmonella*-containing phagosome, and inhibition of this acidification prevents induction of the *Salmonella* pathogenicity island 2 (SPI-2). Hence, *S. Typhimurium* fails to induce virulence genes when deprived of innate immune signals.

Flagella. All salmonellae except *S.* Pullorum and *S.* Gallinarum are motile. Motility is mediated by peritrichous flagella. The flagella are highly immunogenic. Subspecies I, II, IIIa, and VI are biphasic, capable of producing two and sometimes three types of functional and immunologically distinct flagella. Serological distinction among the H-antigens constitutes the second part of the Kauffmann–White scheme. Phase 1 H-antigens are designated by lowercase letters, "a" through "z" and then "z1," "z2," "z3," and so on. Phase 2 antigens are designated by numerals, "1," "2," "3," and so on. As with O-antigens, the H-antigens are determined by agglutination testing using specific antiserum.

SPI, Type III secretion systems, and effector proteins. Many genes responsible for *Salmonella* virulence are located on pathogenicity islands (PAIs). PAIs are large contiguous blocks of DNA on the chromosome that contain insertion sequences and genes for virulence factors, integrases, and mobility factors. In *Salmonella*, the PAIs are called *Salmonella* pathogenicity islands (SPIs), numbered SPI-1 through SPI-22. *S. bongori* contains a different collection of SPIs than different serotypes of *S. enterica*. No strain studied has contained all 22 PAIs, and there is variation in the SPIs among *S. enterica* serotypes. This variation in SPIs reflects the variation in host range and the degree of adaptation of the pathogen for the host. *S. bongori* contains SPI-1, SPI-3A, SPI-3B, SPI-4, SPI-9, and SPI-22. *S.* Typhimurium contains SPI-1 through −6, −9, −12 through 14, and −16. *S.* Enteritidis and *S.* Gallinarum contain SPI-1 through SPI-6, SPI-9, SPI-10, SPI-12 through SPI-14, SPI-16, SPI-17, and SPI-19. *S.* Typhi contains SPI-1 through SPI-10, SPI-12, and SPI-15 through SPI-18. SPI-1 through SPI-5 each contain genes encoding type III secretion systems (T3SS), and these are named according to the SPI in which they are located; for example, T3SS-1 is on SPI-1 and T3SS-2 is on SPI-2. Each T3SS consists of an assembly of more than 20 proteins that form a hollow tube-like structure through which effector proteins are injected into host target cells.

The major function of SPI-1 is to promote invasion of the intestinal epithelium. Following bacterial contact with host cells, SPI-1 T3SS and effector genes are expressed. The effector proteins induce actin cytoskeletal rearrangement (membrane

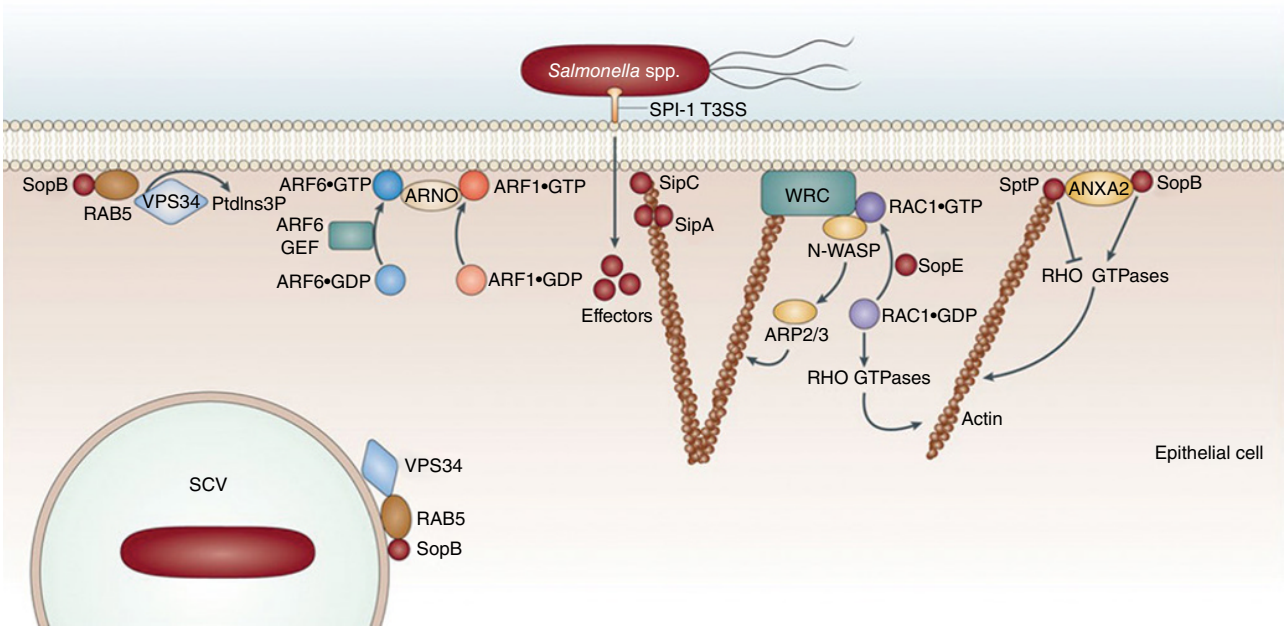


Figure 7.1 Host pathways manipulated by *Salmonella* spp. during epithelial cell invasion. Following contact with host cells, *Salmonella* spp. translocate effectors through the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system (T3SS) to mediate invasion. On the *Salmonella*-containing vacuole (SCV) and potentially at the plasma membrane, SopB recruits RAB5 and the phosphatidylinositol 3-kinase (PI3K) VPS34 to generate phosphatidylinositol-3-phosphate (PtdIns3P). SopB can also recruit annexin A2 (ANXA2) to the membrane, which functions as a platform for actin rearrangements, and SopB also activates RHO GTPase-dependent actin rearrangements. SopE and SopE2 activate host RHO GTPases to promote bacterial internalization by actin reorganization. Activation of RAC1 (conversion of RAC1•GDP to RAC1•GTP) by SopE in particular promotes the recruitment of factors to the host cell membrane (such as the Wiskott–Aldrich syndrome protein (WASP) family WAVE regulatory complex (WRC) and neural WASP (N-WASP)), which promote actin rearrangements at the host cell membrane, proximal to extracellular *Salmonella* spp. The host ADP-ribosylation factor 1 (ARF1) guanine nucleotide exchange factor (GEF), ARNO, is activated by binding to ARF6 and is recruited to the plasma membrane by PtdIns3P, which is possibly generated by SopB. Activation of ARF1 (conversion of ARF1•GDP to ARF1•GTP) by ARNO promotes WRC-dependent actin polymerization and bacterial uptake. WRC and N-WASP activate actin-related protein (ARP2/3), which results in localized actin polymerization to promote bacterial uptake into non-phagocytic cells. The effectors SipA and SipC promote bacterial invasion through their actin bundling functions. SipA may need to be cleaved into two domains (potentially by caspase 3) for activation. SptP promotes restoration of epithelial cell architecture after bacterial entry by reversing the activation of RHO GTPases. *Source:* Reproduced with permission by LaRock et al. (2015).

ruffle formation), which results in bacterial entry into the enterocyte (Figure 7.1). The SPI-1 locus is a 40-kb region containing 39 genes that encode components of the T3SS apparatus, effector proteins, transcriptional regulators of genes encoded both within SPI-1 and elsewhere in the chromosome, and chaperones required for assembly of the apparatus or secretion of effectors. The effector proteins encoded on SPI-1 include SipA-D, AvrA, SptP, SopA, SopB, SopD, SopD2, SopE, and SopE2. Transcriptional regulators, namely, HilA, HilC, HilD, and InvF, are also encoded on SPI-1, and *invF* and *hilA* expression are required for transcriptional activation of SPI-1 genes. The expression of *hilA* is under the influence of environmental changes, such as osmolarity, pH, and oxygen tension. Effectors secreted by the SPI-1 T3SS that are encoded on other PAIs include SopA, SopB (SigD), SopD, SopE (SopE1), SopE2, SspH1, and SlrP.

SopB, SopE, SipA, SipC, and SptP mediate bacterial invasion into the intestinal epithelial cells through their effects on the cell membrane and actin cytoskeleton (Figure 7.1). The effects of SopB are to (i) recruit RAB5 and the phosphatidylinositol 3 kinase (PI3K) VPS34 to generate phosphatidylinositol 3 phosphate; (ii) recruit annexin A2 (ANXA2) to the membrane, which functions as a platform for actin rearrangements; and (iii) activate RHO GTPase-dependent actin rearrangements. The effects of SopE and SopE2 are to activate host RHO GTPases to promote bacterial internalization by actin reorganization. Activation of RAC1 by SopE promotes the recruitment of factors to the host cell membrane, which stimulates actin rearrangements at the host cell membrane. The effects of SipA and SipC are to promote bacterial invasion through their actin bundling functions, and SptP promotes restoration of epithelial cell architecture after bacterial entry by reversing the activation of RHO GTPases.

Colonization of the intestine by non-typhoidal salmonellae is enhanced by the induction of a localized inflammatory

response, which provides a growth advantage. Many of the SPI-1 effectors (including SopB, SopE, SopE2, SipA, SipC and SopA) contribute to intestinal inflammation by stimulating production of the pro-inflammatory cytokine interleukin-8 (IL-8). IL-8 production occurs through the mitogen-activated protein kinase (MAPK) and NF-κB pathways. Important effects of this process are to destabilize mucosal interepithelial tight junctions and chemotactically effect neutrophil transepithelial migration into the intestinal lumen (Figure 7.2). Host TLRs are activated following the sensing of salmonellae components (for example, LPS), which promotes macrophage activation and increased microbicidal activity by the phagosomal compartment, in addition to transcriptional activation of inflammatory caspase genes.

Neutrophil emigration into the intestinal lumen results in luminal accumulation of lipocalin 2, a neutrophil gelatinase-associated protein that sequesters the bacterial siderophore enterochelin. Enterochelin is required by the microbiota for the acquisition of iron. In contrast, the siderophore salmochelin, produced by salmonellae, is not sequestered by lipocalin 2, thereby providing them with a growth advantage (Figure 7.2). Another growth advantage is provided by salmonellae invasion of host cells, which results in the release of reactive oxygen species (ROS) into the intestinal lumen. ROS react with thiosulfate, a respiratory by-product that is generated by the microbiota in the intestinal lumen, to generate tetrathionate. Tetrathionate is used by *Salmonella* spp., but not the other microbiota, as a terminal electron acceptor to support anaerobic or microaerophilic growth. In addition, *Salmonella* spp. induce SopE-dependent production of nitrate by the host, which is utilized in anaerobic nitrate respiration to enhance the growth of S*almonella* spp. and other members of the *Enterobacteriaceae* family. These and other changes enable *Salmonella* spp. to outgrow the intestinal microbiota, and provide for effective transmission owing to increased proliferation in the intestinal lumen.

The major function of SPI-2 is to promote survival within macrophages, and to elicit movement of the SCV in enterocytes to allow for release of bacteria into the intestinal lumen, thereby facilitating infection of neighboring cells. These functions depend on approximately 30 effector proteins secreted by a T3SS, with genes for both encoded on SPI-2. The T3SS translocates the effectors across the SCV membrane, and the effectors, released into the host cell cytoplasm, mediate SCV localization, vacuole membrane dynamics, host immune signaling, host cytoskeleton remodeling, and other functions.

Some of the SPI-2 effectors with identified functions include SseF, SseG, SifA, PipB2, SopD2, SseJ, SpvC, SseL, SspH1, SteC, SspH2, SrfH/SseI, and SpvB. SseF is involved in microtubule bundling and SCV perinuclear migration. SifA is involved in *Salmonella*-induced filament (Sif) formation, maintenance of vacuolar membranes, and enhanced kinesin-dependent anterograde movement along the microtubules. Sifs are long, filamentous membrane structures necessary for the proper positioning of the SCV in close proximity to the Golgi apparatus and near the perinuclear region of the host. PipB2 is involved in recruitment of kinesin-1 to the SCV and in Sif extension. Sif tubules extend from the surface of the SCV and appear to be derived from late endosomal compartments. They contain lysosomal associated membrane protein 1 (LAMP-1), vacuolar adenosine triphosphatase, lysobisphosphatidic acid, and cathepsin. SopD2 contributes to Sif formation and vacuole integrity. SseJ is involved in esterification of cholesterol in infected cells and vacuole integrity. With regard to host immune signaling: SpvC is involved in dephosphorylation of MAP kinases; SseL in macrophage delayed cytotoxicity, downmodulation of Nf-κB-dependent cytokine production, and altered lipid metabolism in infected cells; and SspH1 in inhibition of NF-κB-dependent gene transcription. With regard to host cytoskeletal effects: SteC and SspH2 are involved, respectively, in the formation and recruitment of SCV-associated F-actin meshworks; SrfH/SseI also contributes to the last-named effect; SrfH/SseI is involved in migration of infected phagocytes; and SpvB is involved in inhibition of actin polymerization, macrophage cytotoxicity, and P-body disassembly in infected cells. The means by which SPI-2 effectors mediate movement of the SCV are detailed in Figure 7.3.

The functions of the genes located on the other SPIs are less well understood. The effector proteins associated with SPI-3 include Mgts (magnesium transport system, encoded by a number of *mgt* genes, located within SPI-3). These genes (e.g. *mgtC*) are induced by low concentration of magnesium ions (as occurs within macrophages), and the encoded proteins appear to be important for survival inside macrophages. Different studies have concluded that SPI-4 genes are required for both the intestinal and systemic phases of disease. SPI-4 is a 27-kilobase (kb) region that carries six genes designated *siiABCDEF*. SiiC, SiiD, and SiiF form a type I secretion apparatus for the secretion of SiiE, which is an adhesin that contributes to the colonization of bovine intestines, and is required for efficient translocation of SPI-1 effectors in *S.* Typhimurium. All six genes (*siiABCDEF*) are required for *S.* Typhimurium virulence in a mouse model. Like SPI-1, SPI-4 and SPI-5 are under the control of the SirA/HilA global regulatory cascade.

Enterotoxin. *Salmonella* produces an enterotoxin called Stn (*Salmonella* enterotoxin). Original reports noted that Stn induced water and electrolyte secretion by host target cells. However, subsequent studies have concluded that Stn is neither enterotoxigenic, nor does it play a role in virulence; but it helps maintain the membrane integrity of the bacterium.

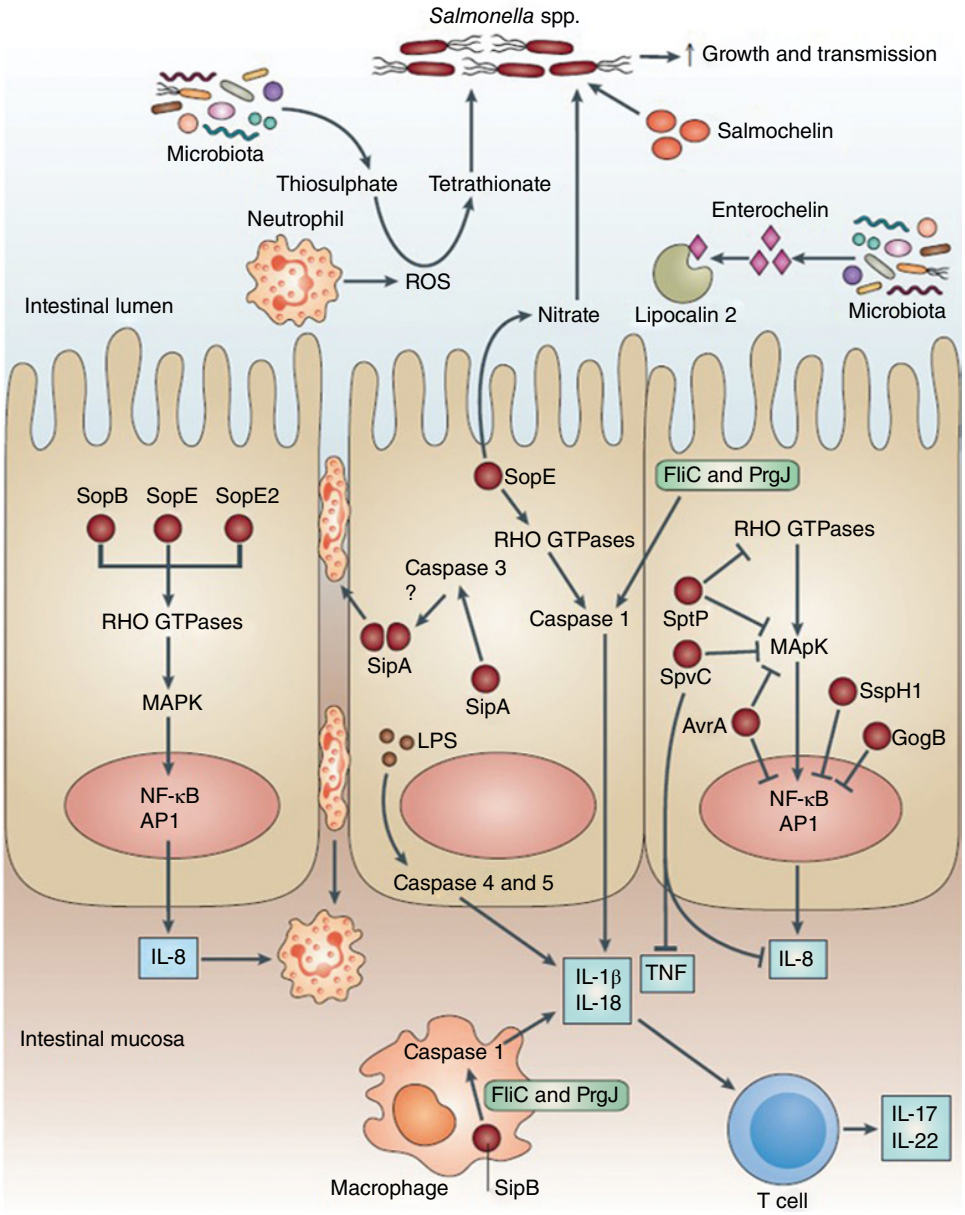


Figure 7.2 *Salmonella*-induced inflammation promotes pathogen transmission. Localized inflammation in the intestinal tract is important for promoting transmission of salmonellae. Activation of RHO GTPases by the effectors SopB, SopE, and SopE2 induces mitogen-activated protein kinase (MAPK) pathways, thereby activating nuclear factor-κB (NF-κB) and activator protein 1 (AP1), which stimulate production of the pro-inflammatory cytokine interleukin-8 (IL-8) to promote transepithelial migration of neutrophils from the intestinal lumen to the intestinal mucosa. SipA activates caspase 3 and may be subsequently cleaved by this protease; cleavage of SipA also promotes transepithelial migration of neutrophils. Caspase 1 is activated by FliC (a flagellin protein), PrgJ (a rod protein of the type III secretion system apparatus), and possibly by the effector SipB in macrophages, which may stimulate the release of IL-1β and IL-18. SopE activation of RHO GTPases can also activate caspase 1 in epithelial cells. Intracellular lipopolysaccharide (LPS) can activate caspase 4 and caspase 5, resulting in the release of mature IL-1β and IL-18, which promote the synthesis of IL-17 and IL-22 by T cells and amplifies inflammation in the intestinal mucosa. SopE also induces the production of nitrate by host cells, which can be used by luminal *Salmonella* spp. for respiration. Pathogen invasion also induces the release of reactive oxygen species (ROS) and lipocalin 2. ROS convert the respiratory by-product thiosulphate (which is generated by the microbiota) into tetrathionate, which can be used by *Salmonella* spp. (but not the microbiota) for respiration. Lipocalin 2 sequesters the iron siderophore enterochelin from the microbiota, but the *Salmonella* spp. siderophore salmochelin escapes sequestration. Together, these changes promote outgrowth and transmission of *Salmonella* spp. that reside in the intestinal lumen. The MAPK inflammatory responses are dampened by the activities of SptP, SpvC, AvrA, SspH1, and GogB, but the mechanistic basis of this is not fully understood. TNF, tumor necrosis factor. *Source:* Reproduced with permission by LaRock et al. (2015).

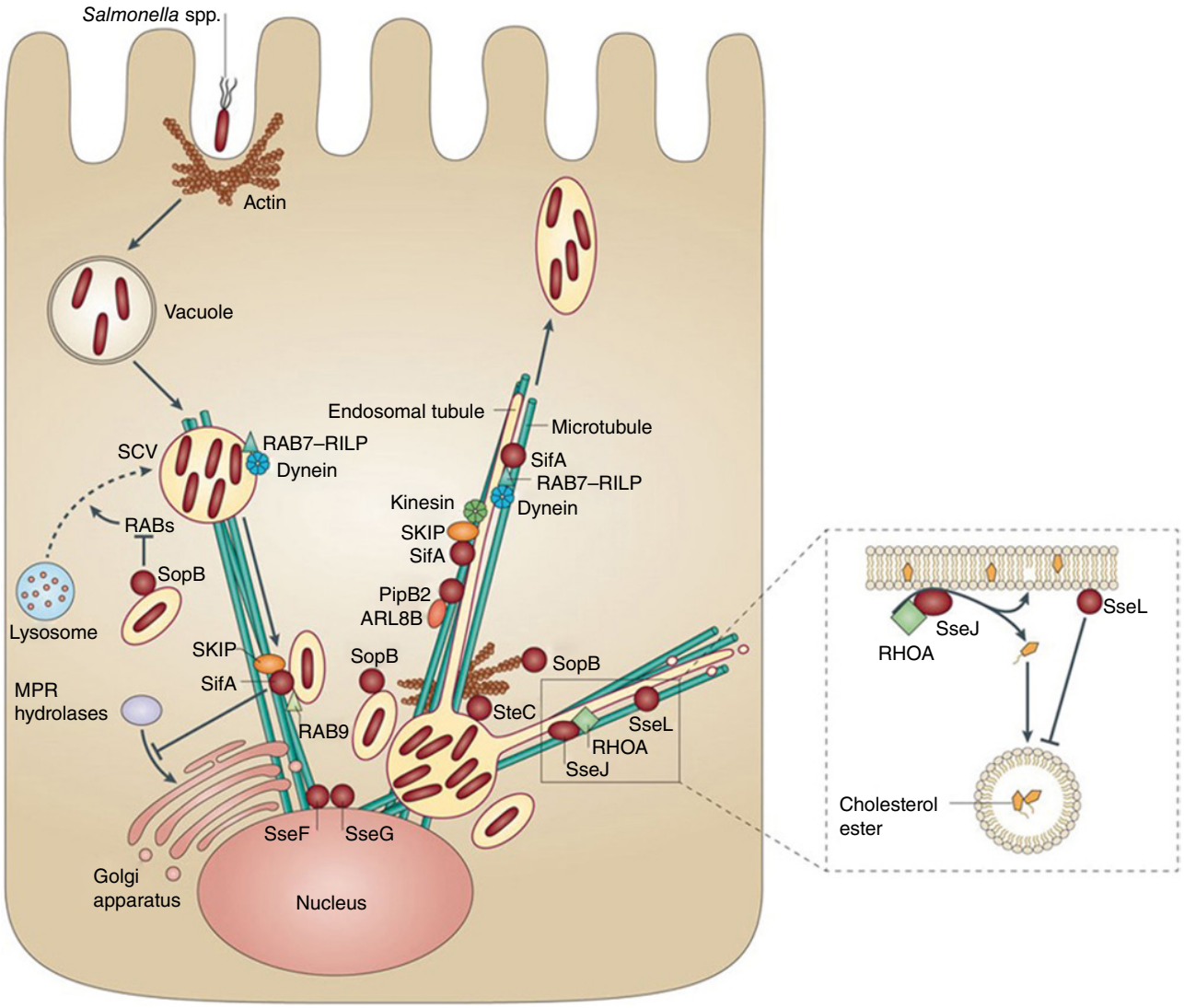


Figure 7.3 *Salmonella* spp. manipulation of host membranes. After invasion, *Salmonella* spp. reside in a vacuolar compartment that undergoes various surface modifications and alterations that distinguish it morphologically, forming the *Salmonella*-containing vacuole (SCV). In some cell types (such as epithelial cells), the SCV acquires markers of late endosomal compartments, including lysosome-associated membrane protein 1 (LAMP1), RAB7, vacuolar ATPase, and cholesterol. RAB7 interacts with AB-interacting lysosomal protein (RILP) and the microtubular motor protein dynein, and this complex is important for centripetal movement of the SCV in the cell early in infection. Salmonella pathogenicity island 2 (SPI-2) effectors (shown in dark red) are released across the SCV membrane into the host cell cytosol by the type III secretion system. These effectors distribute to different locations in the cell and are required for SCV vacuolar modifications and microtubular-based movement, including promoting the dynamic formation of endosomal tubules. Binding of SseJ to RHOA results in direct modification of the lipid content of the SCV and endosomal tubules (producing cholesterol esters), whereas SseL counteracts this activity by decreasing the accumulation of lipid droplets in cells. SseF and SseG promote microtubule bundling and the aggregation of endosomal vesicles, as well as recruiting Golgi-derived vesicles to the SCV. SteC induces actin rearrangements around the SCV and can alter the position of the SCV within the cell, and SopB induces phosphorylation and activation of myosin II indirectly. SopB phosphatase activity reduces the membrane charge of the SCV by preventing the accumulation of RABs, which are important for promoting fusion of the SCV to the lysosome (dashed arrow). SifA binds to the eukaryotic SifA and kinesin interacting protein (SKIP), which activates the microtubular motor protein kinesin to mediate the extension of endosomal tubules. PipB2 is involved in recruiting kinesin to the SCV and endosomal tubules, where it may contribute to endosomal tubulation with ARL8B. SifA–SKIP also binds PLEKHM1 (pleckstrin-homology-domain-containing protein family member 1), which results in the recruitment of the RAB7–HOPS (homotypic fusion and protein sorting) complex to mediate phagolysosomal membrane delivery to the SCV (not shown). SifA binding to RAB7 may disrupt interactions between RAB7–RILP and dynein, which would result in increased peripheral movement of endosomal tubules. SifA binding to RAB9 blocks retrograde transport of mannose-6-phosphate receptors (MPRs) and MPR hydrolyases to the Golgi apparatus, which blocks lysosomal fusion with the SCV. *Salmonella* spp. manipulate host membranes to direct the SCV toward the nucleus and to then promote movement of the SCV toward the cell periphery, where bacteria are presumably released into the intestinal lumen to infect neighboring cells.
Source: Reproduced with permission by LaRock et al. (2015).

Siderophores. In response to iron limitation, all pathogenic *Salmonella* serovars produce the catecholate siderophore enterobactin, and all salmonellae with the exception of *S. bongori* produce the glycosylated enterobactin derivative salmochelin. *Salmonella* mutants deficient in salmochelin, but not enterochelin synthesis or secretion exhibit reduced virulence during systemic infection of mice.

Stress proteins. Stress proteins are expressed when the bacterium is placed under stressful conditions (e.g. heat, cold, low pH, high pH, oxidative, hyperosmotic, and other environments). Different RNA polymerase (Rpo) sigma factors govern transcription initiation when the cell is under environmental stress; for example, RpoS is a general response factor, RpoH responds to heat shock, and RpoE responds to hyperosmotic states. In addition to Rpo sigma factors, other regulatory proteins are involved in response to these signals, and are needed for survival. Exposure to mild stress prior to more severe stress helps the bacteria adjust. *S.* Typhimurium can survive extremely low pH after growth in mildly acidic conditions (pH 4–5), a process termed the acid tolerance response (ATR). *S.* Typhimurium contains two different ATR systems, one in the log phase and the other in the stationary phase. The log phase ATR induces the expression of more than 50 proteins, termed acid shock proteins. Several regulatory proteins are required for the log phase ATR, including RpoS, Fur, and PhoPQ. A second ATR system in *S.* Typhimurium is an RpoS-independent stationary phase ATR regulated by OmpR/EnvZ. RNA polymerase containing RpoS regulates genes found on *Salmonella* virulence plasmid (Spv) (see in the following text). Many other stressful environmental conditions require similar types of responses involving different regulatory proteins. Some of these are encoded on the Spv (see the following section "Virulence plasmids").

Virulence plasmids. Salmonellae possess plasmids of various sizes, some of which have been associated with virulence. The most notable is a family of large (~50–100 kb) plasmids, termed Spv plasmids, which are found within serovars with potential to produce disseminated disease. Within subspecies I, nine serovars, namely, Abortusovis, Abortus-equi, Choleraesuis, Dublin, Enteritidis, Gallinarum, Pullorum, Sendai, and Typhimurium, are known to harbor a serovar-specific virulence plasmid that contains the *spv* operon. The *spv* operon consists of five genes, *spvRABCD*. The *spvR* gene product regulates *spvABCD*. In addition to the virulence plasmid, the *spv* operon can be found in the chromosome of subspecies I, II, IIIa, IV, and VII. The *spv* genes are essential for virulence of the different serovars in their specific hosts. Expression of *spv* genes is induced in the intracellular environment of the phagolysosome. Expression of these genes is regulated by the conditions the bacterium is exposed to, for example, those within the phagosome of the macrophage; carbon starvation; low-pH and low-iron environment in the presence of carbon; and stationary phase under the control of RpoS (see the section "Stress proteins"). Other genes on these plasmids are responsible for serum resistance and may be involved in adherence and invasion of the cellular target. The virulence plasmids modulate the immune reaction, including complement activation (serum sensitivity), of the animal host in favor of the infecting salmonellae. There are a number of loci that influence the serum sensitivity of the organism. The *rck* gene is required for serum resistance; it encodes an outer membrane protein that prevents C9 insertion into the bacterial outer cell membrane.

Miscellaneous products. The transcriptional regulator, SlyA (for salmolysin), is in part responsible for survival of salmonellae within macrophages, by activating the expression of proteins that afford protection from the toxic products generated by oxygen-dependent pathways. Several two-component signal transduction systems (TCS), such as PhoPQ, CpxAR, BaeSR, EnvZ/OpmR, and PmrAB, allow salmonellae to react to external stimuli. They consist of sensor proteins, usually histidine kinase bound to the membrane, and a cytoplasmic response regulator. The best-known TCS involved in virulence is PhoPQ, which is the main activator of the T3SS encoded on SPI-1. PhoP negatively regulates *hilA* via direct repression of *hilA* transcription, and by other effects. PhoPQ regulates genes (e.g. *pagABC* for pho-activated genes) important for survival in macrophages, resistance to cationic proteins (defensins), acidic pH, and invasion of epithelial cells. The following conditions are sensed by PhoQ and activate PhoP: carbon starvation, nitrogen starvation, low pH, and high oxygen levels. PhoQ is a kinase that phosphorylates (activates) PhoP, which is a transcriptional regulator of other target genes, thereby transmitting the signal. *pho*P mutants are avirulent. The products of Pho-repressed genes (*prg*) are mainly located in the outer membrane, and assist in secretion of proteins through the outer membrane.

The product of the *shdA* (for shedding) gene governs fecal shedding of salmonellae by an infected host. This gene is restricted to serotypes of subspecies I. Arc (for aerobic regulation control) is a two-component global regulator system involved in intracellular survival.

Ecology

Reservoir

The reservoir for members of the genus *Salmonella* is the gastrointestinal tract of warm- and cold-blooded animals. Sources of infection include contaminated soil, vegetation, water, and components of animal feeds (such as

bone, meat, and fish meal), particularly those containing milk-, meat-, or egg-derived constituents, and the feces of infected individuals. Lizards and snakes are commonly infected with several serotypes, although these infections are usually subclinical. Subspecies I is almost exclusively found in warm-blooded mammals and birds (evidence suggests that the possession of the *shdA* gene product is responsible).

Some salmonellae have become adapted to certain hosts; that is, they are not usually detected in host species other than the one to which they have adapted. Examples include Abortus-equi in horses, Abortusovis in sheep; Choleraesuis in swine (and occasionally humans); Dublin in cattle (and occasionally humans); Gallinarum (the cause of fowl typhoid) in poultry; Pullorum (the cause of pullorum disease) in poultry; Typhi (the cause of typhoid fever) in humans; and Paratyphi (also a cause of typhoid fever) in humans.

Some salmonellae are non-host-adapted, that is, capable of infecting many different host species. These include Anatum, Derby, Newport, Tennessee, and Typhimurium.

Transmission

Salmonellae are primarily transmitted by the fecal–oral route, often through ingestion of contaminated food and water. The outcome of the interaction between the host and *Salmonella* depends upon the state of the colonization resistance of the host, the infectious dose, and the particular species or serotype of *Salmonella*. Disease may or may not occur following ingestion. If it occurs, it may do so immediately, or at some later date. In the latter instance, the initial interaction may result in the colonization (without disease) of the host, but with a change in the intestinal environment, brought on, for example, by stress or antibiotics (activities that affect the normal microbiota), and disease may follow.

Pathogenesis

Mechanisms

The most common clinical manifestation of salmonellosis is diarrhea. In certain instances (defined by host factors, the strain of *Salmonella*, and dose) septicemia occurs. Host factors include age, immune status, concurrent disease, and composition of the normal flora (i.e. providing resistance to colonization).

Stationary phase salmonellae appear best suited to initiate disease, because under these conditions, RNA polymerase containing the alternative sigma factor, RpoS, initiates transcription of genes responsible for acid tolerance and subsequent survival through the stomach. Also, RNA polymerase containing RpoS is a positive regulator for the genes found on the Spv plasmids.

The target cells are the M cells in the follicle-associated epithelium overlying gut-associated lymphatic tissue in the distal small intestine and the upper large bowel. Lack of competitive microbiota caused by poor nutrition, stress, or antibiotics may potentially reduce the infectious dose. Adhesion to the M cell is the first step in the disease process, mediated by one or more of the adhesins, namely, Agf, Pef, and Lpf, or by others yet to be determined. Following adhesion, salmonellae are internalized following the induction of membrane ruffles in the target cells triggered by Ssps and Sops subsequent to their injection by the T3SS. The target cell is irreversibly damaged by this interaction, undergoing apoptosis. Salmonellae are now found within the target cells, the lymph nodule, and submucosal tissue. An inflammatory response is initiated by release of various chemokines from affected host cells, as well as release of pro-inflammatory cytokines following host interaction with cell wall LPS – activities that result in an influx of PMN leukocytes and macrophages. The influx of PMNs may be reflected in a transient peripheral neutropenia. PMNs are highly efficient in phagocytosing and killing salmonellae, whereas nonactivated macrophages are less so. If the salmonellae are unable to survive the neutrophilic infiltrate and immune response, the infectious process is arrested at this stage. Diarrhea is thought to result from prostaglandin synthesis by the recruited PMNs (and perhaps by the affected host cells), as well as activation of various inositol-signaling pathways within affected host cells. The net result is the secretion of chloride ions and water.

If the infecting strain of *Salmonella* has properties that allow dissemination (possession of SPI-2, SPI-3, SPI-4, and SPI-5 – associated gene products that allow growth within macrophages; Spv plasmid encoding ability to grow intracellularly and serum resistance; PhoQ/PhoP system allowing resistance to defensins; SlyA allowing resistance to oxygen-dependent by-products; *arcA*), septicemia may result. The likelihood of this occurring increases if the immune status of the host is diminished. Salmonellae disseminate and multiply within phagocytic cells (macrophages mainly) within phagosomes. Following systemic dissemination of the salmonellae, septicemia and endotoxic shock may develop. Strains producing this form of disease escape destruction by the host and multiply within macrophages of the liver and spleen, as well as intravascularly. During the dissemination process, salmonellae are occasionally outside of the intracellular environment and therefore at risk from the formation of complement membrane attack complexes on their surfaces. This occurrence is discouraged by at least two mechanisms: a product of the

Spv plasmid and the length of the O-repeat unit of the LPS (there is a direct correlation between O-repeat length and virulence). Invasive salmonellae are capable of secreting a siderophore, salmochelin, that removes iron from the iron-binding proteins of the host. Uncontrolled multiplication of the organism results in endotoxemia, severe vascular damage, and death.

Pathology

If the infectious process is limited to the intestinal tract, the lesions will consist of a fibrinosuppurative, necrotizing and hemorrhagic inflammation of the distal small intestine and large bowel. Necrosis of the intestine is at first erosive and frequently becomes ulcerative, resulting in the formation of a diphtheritic membrane. This lesion is especially common in swine and cattle (Figures 7.4 and 7.5). The liver is frequently affected with random, multifocal necrotizing inflammation that reflects bacterial spread via the portal vein and phagocytosis by Kupffer cells without effective bacterial killing. In the septicemic form of the disease, there may be fibrinoid change in blood vessels in many different organs as well as vasculitis, thromboembolism, hemorrhages, and infarcts. In swine with septicemic *S.* Choleraesuis infection, the spleen is usually markedly enlarged due to hyperemia, and the ears of white-skinned pigs may be dark blue from thrombosis and venous congestion.

Disease Patterns

Ruminants. Salmonellosis is a significant disease of ruminants, mainly cattle. The disease affects young (usually four to six weeks of age) as well as adult animals, although neonatal calves may also be affected, especially in dairies. Animals in feedlots and dairies are commonly affected. The disease may be a septicemia or be limited to an enteritis or enterocolitis (Figures 7.4 and 7.5). Pneumonia, hematogenously acquired, is a common presenting sign in calves with septicemia due to *S.* Dublin. Abortion may follow septicemia.



Figure 7.4 Jejunum of a 7-day-old calf naturally infected with *S. Typhimurium*. The mucosa is hyperemic and edematous, with erosions, and is overlaid by an adherent fibrinosuppurative exudate that extends into the lumen.

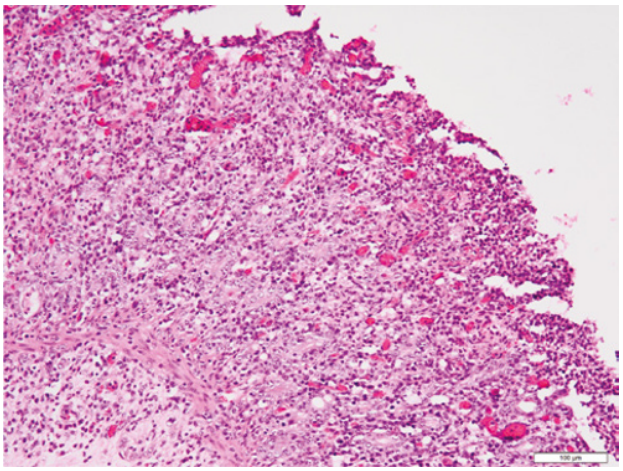


Figure 7.5 Photomicrograph of jejunum from a 2-week-old calf naturally infected with *S.* Typhimurium. There is severe diffuse villus atrophy with an attenuated, regenerative epithelial layer where intact. Neutrophils diffusely infiltrate the mucosa and submucosa, and are especially numerous at the mucosal surface, which is necrotic and eroded. Capillaries and venules in the mucosa and submucosa are hyperemic. The lesions are compatible with severe acute diffuse necrosuppurative enteritis caused by salmonellosis.

S. Typhimurium, *S.* Dublin, and *S.* Newport are the serotypes commonly isolated from cattle, and *S.* Typhimurium the main serotype affecting sheep.

Swine. Salmonellosis in swine can present as an acute, fulminating septicemia or as a chronic debilitating intestinal disease. The form depends upon the strain of *Salmonella*, the dose, and the colonization resistance of the infected animal. The disease is seen most often in pigs that have been stressed. Such conditions occur often in feeder pigs, an age group in which salmonellosis commonly occurs. *S.* Typhimurium and *S.* Choleraesuis are the predominant serotypes.

Horses. Adult horses are commonly affected with *Salmonella*. The pattern is diarrhea, though septicemia is seen occasionally. Colic, gastrointestinal surgery, and antimicrobial agents predispose the horse to the development of clinical signs. The agent is either carried normally (as in ~3% of clinically normal horses) or acquired from other sources (e.g. a veterinary hospital). *S.* Typhimurium and *S.* Anatum are the serotypes most commonly isolated from horses.

Dogs and cats. Salmonellosis is uncommon in dogs and cats, although carriage is reportedly high in clinically normal pound dogs (upward of 35%). When outbreaks occur, they are usually associated with a common source, such as contaminated dog food or "treats" (e.g. dried pig's ears). *Salmonella* should be high on the microbiological differential list for cats with signs of septicemia.

Poultry. See Section 7.3.2.

Epidemiology

Salmonella spp. are ubiquitous, geographically and zoologically. Some serotypes are relatively host specific (*S*. Dublin – cattle; *S*. Typhisuis – swine; *S*. Pullorum – fowl), while others, notably *S*. Typhimurium, *S*. Anatum, and *S*. Newport, affect a wide host range among which feral birds and rodents play important roles in interspecific dissemination of infection. Long periods of subclinical and convalescent shedding ensure widespread, unchecked distribution of the organisms.

Clinical outbreaks are correlated with depressed immune states, as in newborn animals (e.g. calves and foals) and stressed adults, parturient cows, equine surgical patients, and swine with systemic viral diseases. All animals are at increased risk of developing disease if their normal microbiota is disrupted (e.g. stress and antibiotics). These circumstances render animals susceptible to exogenous exposure or activation of silent infections.

Humans appear to be susceptible to all *Salmonella* serotypes, with typhoid fever caused by *S*. Typhi, a disease restricted to humans, and infections with other serotypes being food-borne zoonoses. Poultry and poultry products (eggs) are a major source of salmonellosis in humans. *S*. Enteritidis (e.g. phage type 4) is especially adapted for egg transmission. Whether a person develops disease following ingestion of salmonellae from the environment depends upon the dose of organisms, the serotype of *Salmonella*, and the colonization resistance of the infected individual. *S*. Typhimurium is most common, usually producing gastroenteritis. Some serotypes have greater invasion potential and greater potential for causing septicemia, for example, *S*. Choleraesuis (from swine) and *S*. Dublin (contaminated milk).

S. Typhimurium DT104 is a more virulent strain in both animals and humans than most other strains of this serotype. The case-fatality rate with DT104 is 3%, compared to 0.1% with other nontyphoid salmonellae. DT is the definitive type designation, which specifies a particular phage type. In addition to increased virulence, DT104 isolates are resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. DT104 infection in humans is usually acquired by eating contaminated beef, pork, or poultry, or by direct contact with animals, particularly those with diarrhea (e.g. cattle and cats). In the United States, the prevalence of *S*. Typhimurium isolates with the five-drug pattern of resistance increased from less than 1% in 1979–1980 to 34% in 1996, most of which were DT104 with one pulsed-field electrophoresis pattern predominating among the DT104 types. Reptiles have also become an important source of *Salmonella* in humans, with pets (e.g. turtles) in classrooms a common means of exposure.

Immunologic Aspects

Protection depends upon both innate and adaptive immunity. Competitive microbiota help reduce the numbers of pathogens by competing for nutrients, masking receptors, and producing toxic compounds. Direct-fed microbials, for example, certain strains of *Lactobacillus acidophilus* or other bacteria, may help prevent infections by providing competitive exclusion. However, complete protection can only be afforded by an adaptive immune response in the form of specific antibodies and T cells. Antibodies specific for surface structures of *Salmonella*, possibly adhesins, prevent adherence to target cells. The neonate is protected passively by ingesting specific sIgA or IgG_1 (bovine). The immunologically mature animal is protected by the active secretion of specific immunoglobulins (IgM, IgG, or IgA) into the intestinal lumen by plasma cells in the lamina propria, which prevent bacteria from adhering and invading gut epithelial cells.

Antibodies in the circulation act as opsonins and promote the phagocytosis of the organism. Destruction of the salmonellae that have been phagocytosed follows the immunologic activation of the macrophages by specifically stimulated lymphocytes (T_H1 cells) though activated macrophages are damaged or killed by Ssps. NK cells lyse *Salmonella*-infected cells.

Acquired immunity revolves around activation of macrophages, which takes place as follows. After initial interaction between salmonellae and macrophage, IL-12 is released by the affected macrophage. IL-12 activates the T_H1 subset of T helper cells. This subset secretes, among other cytokines, interferon-γ, which activates macrophages. Activated macrophages are efficient killers of intracellular salmonellae.

Artificial immunization against salmonellae is difficult. Bacterins have had limited success. Apparently, they do not stimulate strong cellular immunity, even though abundant antibody is produced. Antibodies that are produced locally or passed in colostrum or milk interfere with adsorption to the target cell and protect against disease in this location. Macrophages can be activated and antibody production stimulated in response to modified live vaccines. If given orally, these vaccines stimulate local secretory immunity and cell-mediated activation of phagocytic cells. Aromatic-dependent mutants of *Salmonella* show promise as effective modified live vaccines, especially for calves. *aroA* mutants of *Salmonella* cannot multiply within the host because vertebrate tissue does not contain the needed precursors for aromatic acid synthesis.

Laboratory Diagnosis

In cases of intestinal infection, fecal samples are collected; in systemic disease, a blood sample is collected for standard blood culture. Spleen and bone marrow are cultured

for the salmonellae when postmortem diagnosis of systemic salmonellosis is required.

Fresh fecal samples are placed onto nutrient, for example, blood agar (Figure 7.6), and one or more selective media, including MacConkey agar (Figure 7.7), xylose lysine deoxycholate (XLD) agar (Figure 7.8), Hektoen enteric medium, and brilliant green agar. For enrichment, selenite F, tetrathionate, or gram-negative broth is recommended.

Salmonellae appear as lactose-non-fermenting colonies on lactose-containing media (e.g. MacConkey agar; Figure 7.7).

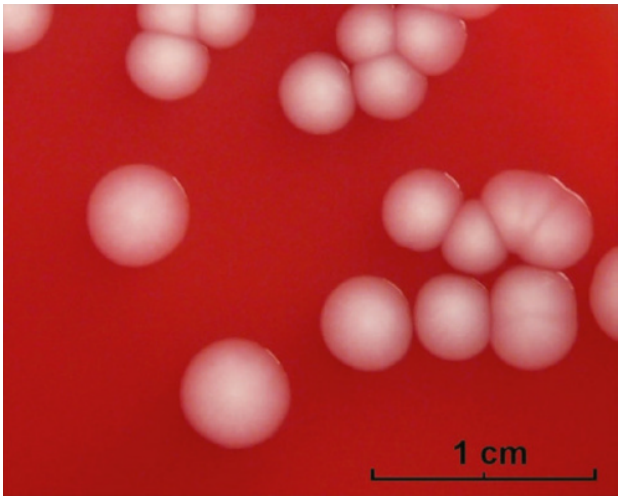


Figure 7.6 Colonies of *Salmonella enterica* ssp. *enterica* serotype Enteritidis on blood agar. Bacteria were cultivated for 24 hours in an aerobic atmosphere at 37 °C. *Source:* Bacteria in Photos. www.bacteriainphotos.com, permission from Mr. Hans Newman, accessed December 1, 2021.

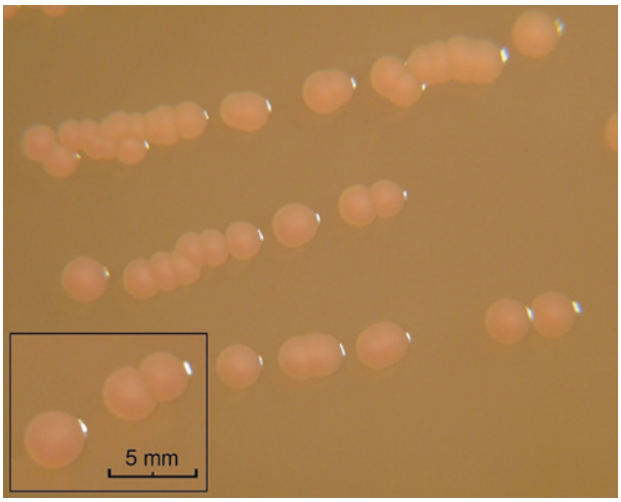


Figure 7.7 Colonies of *Salmonella enterica* ssp. *enterica* serotype Enteritidis on MacConkey agar. Bacteria were cultivated for 24 hours in an aerobic atmosphere at 37 °C. *Source:* Bacteria in Photos. www.bacteriainphotos.com, permission from Mr. Hans Newman, accessed December 1, 2021.

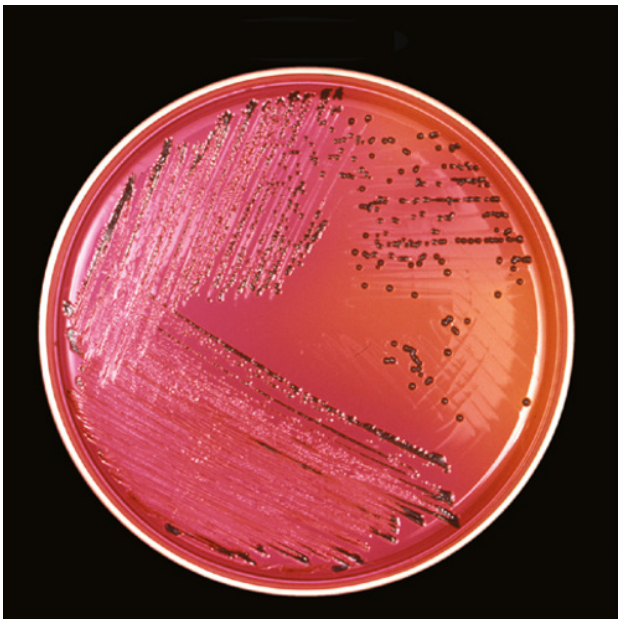


Figure 7.8 *Salmonella* colonies, XLD agar. When xylose lysine (XL) agar is supplemented with sodium thiosulfate, ferric ammonium citrate, and sodium deoxycholate, it is then XLD agar. The presence of any black-colored area indicates the deposition of hydrogen sulfide (H_2S), under alkaline conditions, and is highly suggestive of *Salmonella*. *Source:* US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID# 6619, accessed December 1, 2021.

Because most serotypes of salmonellae produce H_2S, colonies on iron-containing media (e.g. XLD agar) will have a black center (Figure 7.8). Suspicious colonies can be tested directly with polyvalent anti-*Salmonella* serum or inoculated into differential media and then tested with antisera. To cultivate salmonellae from tissue, blood agar (Figure 7.6) can be used.

Definitive identification involves determination of somatic and flagellar antigens and possibly bacteriophage type.

Various *Salmonella*-specific DNA probes and primers for the polymerase chain reaction have been developed for identification as well as detection in samples (food, feces, and water) containing other microorganisms.

A multiplex polymerase chain reaction assay using primers designed to detect the common-diarrhea-associated microorganisms of swine (*Brachyspira hyodysenteriae*, *Lawsonia intracellularis*, and *Salmonella*) has been described.

Treatment, Control, and Prevention

Nursing care is the principal treatment for the enteric form of salmonellosis. The use of antimicrobial agents is controversial. Some studies show that antibiotics do not alter the course of the disease. In addition, there is

evidence that antibiotics promote the carrier state and select for resistant strains. Treatment of the systemic form of salmonellosis includes nursing care and appropriate antimicrobial therapy as determined by retrospectively acquired susceptibility data. Because salmonellae survive in the phagocytic cell, the antimicrobial drug should be one that penetrates the cell. Examples of those that distribute in this manner include ampicillin, enrofloxacin, trimethoprim–sulfonamides, and chloramphenicol/ florfenicol. Treatment options may be compromised due to acquisition of R plasmids or integrons encoding resistance to multiple antibiotics. A serious global epidemic of *S.* Typhimurium DT104, a type of Salmonella that affects humans and other animals worldwide, contains a cluster of antibiotic-resistance-encoding genes within its chromosome. This cluster, called *Salmonella* genomic island 1 (SGI1), contains the genes for resistance to ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides, and tetracyclines bounded by two integrons. SGI1 has moved into *S.* Albany (fish in Southeast Asia) and *S.* Paratyphi B (tropical fish in Singapore).

Salmonellosis is controlled through strict attention to protocols designed to curtail the spread to susceptible animals of any contagious agent found in feces. Artificial immunization with modified live products has shown promise (e.g. *aroA* mutants). Attempts have been made to treat and prevent the endotoxemia produced by the systemic form of the disease by administering serum containing antibodies to the core LPS. Likewise, the administration of J5, a rough variant of *E. coli*, has been shown to stimulate the production of antibody to the core LPS. Both methods appear to prevent and control the signs of disease produced by systemic salmonellosis.

Salmonellosis of Poultry

Paratyphoid. "Paratyphoid" of poultry (in quotation marks because true paratyphoid is a disease of humans caused by paratyphoid serotypes of *Salmonella*) is salmonellosis produced by any of the motile strains of *Salmonella*. All salmonellae except *S.* Pullorum and *S.* Gallinarum are motile. The disease produces its highest losses in the first two weeks of life as a septicemic disease. Survivors become subclinical excretors. Infection is through ingestion. The source is usually feces or fecally contaminated materials (e.g. litter, fluff, and water).

Diagnosis is made by culturing the organism from affected tissue (e.g. spleen and joints) from birds that had been showing clinical signs of disease. It is more difficult to detect subclinical carriers because they shed the organism in feces only periodically. Some have suggested that culture of fluff and litter could be used to detect carrier flocks.

Treatment does not eliminate carriers, although it does control mortality. Treatment regimens have included avoparcin, lincomycin, furazolidone, streptomycin, and gentamicin. Exclusion of salmonellae by feeding "cocktails" of normal flora has been used with some success to reduce the number of salmonellae shed by carrier birds (competitive exclusion).

Pullorum disease. Pullorum disease, caused by *S.* Pullorum, is rare in North America but not in the rest of the world. The disease has almost been eliminated in the United States due to a breeding flock testing program.

S. Pullorum infects the ova of turkeys and chickens. Thus, the embryo is already infected when the egg is hatched. The hatchery environment is contaminated following hatching of an infected egg, leading to infection of other chicks and poults. Mortality is due to septicemia and is greatest in the second to third weeks of life. Surviving birds carry the bacterium and may pass it to their offspring. It is difficult to detect infected breeding hens by bacteriologic means. Agglutination titers, produced 3–10 days after infection, are used to detect carrier birds.

Eliminating infected breeding birds detected serologically controls this disease. Treatment with antimicrobial agents (mainly sulfonamides) reduces mortality in infected flocks.

Fowl typhoid. Fowl typhoid, caused by *S.* Gallinarum, is an acute septicemic or chronic disease of domesticated adult birds, mainly chickens. Fowl typhoid is rare now in the United States due to control programs.

The disease is diagnosed by culturing the organism from liver or spleen. It is treated with antimicrobial agents, mainly sulfonamides (sulfaquinoxaline) and nitrofurans. Fowl typhoid is controlled by managing and eliminating infected birds. A bacterin made from a rough variant of *S.* Gallinarum, 9R, has been shown to decrease mortality.

Avian arizonosis. *S. enterica* ssp. *arizonae* and *S. enterica* ssp. *diarizonae* are most often isolated from reptiles and fowl, although these species can be isolated from any animal. Turkeys are most commonly affected. There are 55 serological types affecting fowl, with types 7:1, 7, and 8 most commonly isolated in the United States.

S. enterica ssp. *arizonae* and *S. enterica* ssp. *diarizonae* are maintained in turkey flocks via hatching eggs, which become infected following ingestion by the hen. Feces also spread it.

Diagnosis is made by culturing salmonellae from the liver, spleen, blood, lungs, or kidneys of affected birds, or from dead poults and hatch debris.

Most serotypes of *S. enterica* ssp. *arizonae* and *S. enterica* subsp. *diarizonae* possess R plasmids, which sometimes makes it difficult to prevent and treat this disease. Various antimicrobial agents such as furazolidone and sulfamerazine added to feed have shown some success in lowering mortality. Injection of day-old poults with gentamicin or spectinomycin decreases mortality, but survivors still harbor (and shed) the organism.

Control measures should be aimed at prevention rather than treatment. Because of the multiplicity of serotypes, no effective vaccine is available.

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8

Family *Yersiniaceae*

Rodney A. Moxley

The genus *Yersinia* is included in the family *Yersiniaceae*, newly formed in 2016 following the division of the order *Enterobacteriales* into seven families: *Enterobacteriaceae*, *Erwiniaceae*, *Hafniaceae*, *Morganellaceae*, *Pectobacteriaceae*, *Thorselliaceae*, and *Yersiniaceae*. *Yersiniaceae* includes 8 genera (*Chania*, *Chimaeribacter*, *Ewingella*, *Rahnella*, *Rouxiella*, *Samsonia*, *Serratia*, and *Yersinia*), 67 species, and 3 subspecies, validly published and correctly named. *Yersinia* includes 26 species: *aldovae, aleksiciea*, *alsatica*, *artesiana*, *bercovieri*, *canariae*, *enterocolitica*, *entomophaga*, *frederiksenii*, *hibernica*, *intermedia*, *kristensenii*, *massiliensis*, *mollaretii*, *nurmii*, *pekkanenii*, *pestis*, *proxima*, *pseudotuberculosis*, *rochesterensis*, *rohdei*, *ruckeri*, *similis, thracica*, *vastinensis*, and *wautersii*. *Yersinia enterocolitica* includes two subspecies, *enterocolitica* and *palearctica*. The type species is *Yersinia pestis*. Some references include two subspecies for *Y. pestis*: subsp. *pestis* and subsp. *microtus*.

Disease caused by *Yersinia* infection is called yersiniosis. Yersinioses are zoonotic infections that predominantly affect rodents, pigs, and birds. The main pathogens for humans and nonhuman primates are *Y. pestis, Yersinia pseudotuberculosis*, and *Y. enterocolitica. Y. pestis* is the cause of plague, a septicemic disease of major importance in humans, rodents and occasionally domestic animals. Transmission is primarily through bite wounds of infected rodent fleas. *Y. enterocolitica* mainly causes disease in domestic animals and primates, and is the most prevalent species in humans. *Y. pseudotuberculosis* mainly affects birds and rodents, and only occasionally domestic animals and primates. *Y. enterocolitica* and *Y. pseudotuberculosis* infections are food- and water-borne illnesses that result in mesenteric lymphadenitis, terminal ileitis, acute gastroenteritis, and septicemia.

The three main pathogenic species have several features in common: tropism for lymphoid tissues; capacity to resist nonspecific immune responses; behavior as facultative intracellular pathogens; ability to resist phagocytosis by macrophages; formation of extracellular colonies in tissues; carriage of a plasmid of 70–75 kb that contains virulence genes [pCD1 in *Y. pestis*, pYV (also known as pCad and pIB1) in *Y. pseudotuberculosis*, and pYV in *Y. enterocolitica*]; carriage of a pathogenicity island known as a High Pathogenicity Island (HPI) on the chromosome; production of *Yersinia* outer proteins (Yops); and transmission to humans directly or indirectly via animal reservoirs.

Yersinia ruckeri is primarily a pathogen of salmonid fish (e.g. salmon and trout), although it infects other fish species, such as carp, catfish, sturgeon, perch, and burbot. *Y. ruckeri* causes enteric red mouth disease, a septicemic disease of economic importance to the aquaculture industry. Although primarily a fish pathogen, *Y. ruckeri* has been isolated from a wound infection in a human patient and, in addition to *Yersinia rohdei*, *Y. intermedia, Y. pseudotuberculosis*, *Yersinia mollaretii*, and *Yersinia bercovieri*, from meat and milk products.

Descriptive Features

Morphology and Staining

Members of the genus *Yersinia* are gram-negative coccobacilli (Figure 8.1) that exhibit bipolar ("closed safety pin") staining, especially when seen in smears from tissue specimens and stained with Giemsa (Figure 8.2). Most species are flagellated at ambient temperatures.

Growth Characteristics

Yersinia spp. grow on blood, chocolate, MacConkey, and other ordinary laboratory media incubated at 35 °C in ambient air, although their rate of growth is relatively slow compared to most members of the family *Enterobacteriaceae*. Hence, they may be outcompeted by other bacteria in cultures of clinical and environmental samples. *Yersinia* spp. generally grow well at a temperature range of 4 to 43 °C

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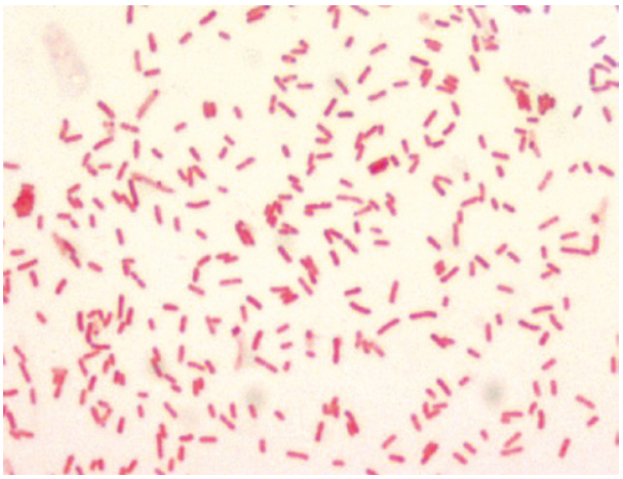


Figure 8.1 *Yersinia pestis*, Gram stain, showing gram-negative bacilli. *Source:* Courtesy of Larry Stauffer, Oregon State Public Health Laboratory, and US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#1914.

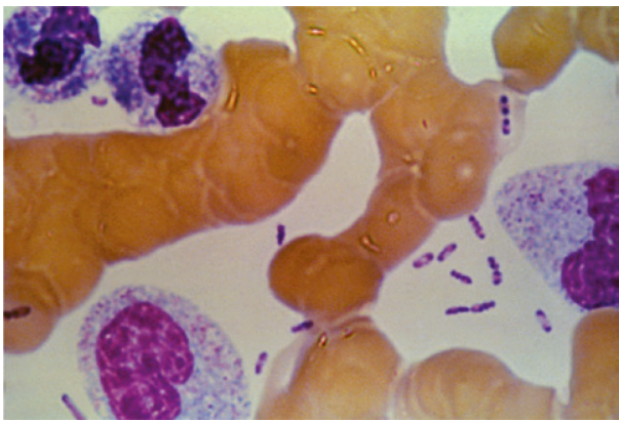


Figure 8.2 *Yersinia pestis,* Wright's stained blood smear from a plague victim showing dark stained bipolar ends of bacteria. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#2050.

(*Y. pestis* from 4 to 40 °C), with optimum temperatures ranging from 25 to 28 °C (*Y. pestis* from 28 to 30 °C), and form 1–2-mm-diameter colonies after 48 hours of incubation (Figure 8.3). The ability of *Yersinia* spp. to grow at 4 °C is a concern for blood banks, because some donors may carry species such as *Y. enterocolitica* asymptomatically.

In culture, *Yersinia* spp. yield colonies that range from <1 to 1.5 mm in diameter, with those at the low end of the spectrum being more typical of *Y. pestis*. *Yersinia* spp. are not hemolytic on blood agar (Figure 8.3); they are catalase-positive, oxidase-negative, and ferment glucose with the production of acid but no gas. *Yersinia* spp. do not typically grow well nor form turbid suspensions in liquid media. All *Yersinia* spp. except *Y. pestis* are motile at 25 °C.

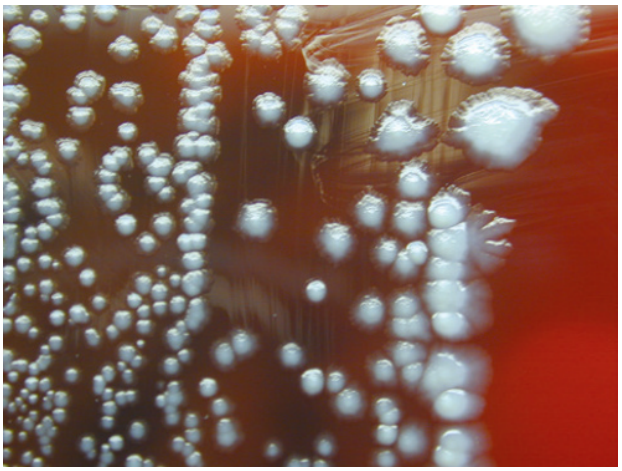


Figure 8.3 *Yersinia pestis* colonies on sheep blood agar after 72 hours of culture. *Y. pestis* grows well on most standard laboratory media. After 48–72 hours of incubation, colonies are gray-white to slightly yellow opaque and raised, and have an irregular "fried egg" appearance. Alternatively, colonies may have a "hammered copper" shiny surface. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#1921.

Yersinia pestis (Plague Bacillus)

Y. pestis is the cause of the disease called plague. Plague in human patients manifests in five principal forms: bubonic, septicemic, pneumonic, meningeal, and pharyngeal. Bubonic plague presents with a local lymphadenitis called a bubo (Figures 8.4 and 8.5), pneumonic plague with pneumonia (Figure 8.6), and septicemic plague with lesions of septicemia (Figures 8.7 and 8.8). Patients also may present with skin



Figure 8.4 An axillary bubo and edema exhibited by a patient with bubonic plague. After the incubation period of two to six days, signs and symptoms of the plague appear, including severe malaise, headache, shaking chills, fever, and pain and swelling, or adenopathy, in the affected regional lymph nodes, also known as buboes. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#2061.

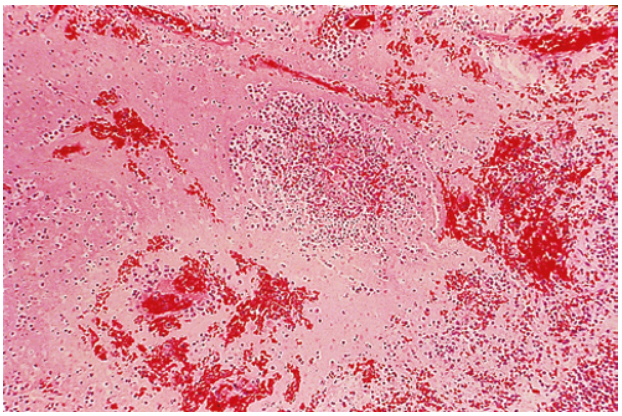


Figure 8.5 Histopathology of lymph node in fatal human plague. Medullary necrosis with fluid and *Yersinia pestis*. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#731.



Figure 8.6 Radiograph of the thorax of a patient with pneumonic plague. There is bilateral involvement, with greater consolidation of the left lung. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#4068.

lesions (ulcers, carbuncles, pustules, petechiae, bruising, and gangrene).

Historically, there have been three pandemics: the Justinian Plague (541 to 750/767 CE) the second pandemic (1346 to the 1700s CE), which included the "Black Death" period (1346 to 1353 CE), and the third pandemic beginning in approximately 1772 CE and extending to the present day. The "Black Death" pandemic was characterized by extremely high mortality, especially from about 1347 to 1351 CE, and was named such because of the characteristic lesions (infarcts) involving the hands and feet (Figures 8.7 and 8.8). An estimated 17–28 million people (approximately one-third of the population) in Europe died from the "Black Death" during this time.

Initial signs and symptoms in human plague victims appear 2 to 6 days after contact with the organism, and



Figure 8.7 Acral gangrene of the digits of the right foot of a patient with septicemic plague. Systemic infection with *Yersinia pestis* resulted in thrombosis and infarction of the terminal segments of the digits. It was because of this type of lesion that the disease became known as the "Black Death." *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#4139.



Figure 8.8 A shaved anterior thoracoabdominal region of a rock squirrel, *Spermophilus variegatus,* which is afflicted with the plague. This squirrel is displaying a petechial rash, which is similar in appearance to those found on humans also afflicted with *Yersinia pestis*. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#6720.

include fever, headache, chills, and swollen tender lymph nodes (buboes). Buboes usually involve the inguinal and femoral lymph nodes, which is reflective of lymphatic drainage of organisms originating from fleabites on the lower legs. If untreated, plague often progresses to

septicemia, characterized clinically by prostration, lethargy, high fever, and seizures.

In humans, pre-septicemic plague, if untreated has a 40–60% case-fatality rate, and if treated has a 14% case-fatality rate. Septicemic plague, if untreated, has a 100% case-fatality rate; with treatment, it has a 30–50% case-fatality rate. Pneumonic plague can be a rare secondary complication of bubonic or septicemic plague or can be the primary infection following direct inhalation of aerosolized organisms from other pneumonic cases, (human or animal), infected tissues, or cultured organisms. The case-fatality rate in untreated pneumonic cases is essentially 100%, and often exceeds 50% in treated cases.

Necropsy of animals infected with plague is a distinct risk factor for veterinarians. In 2007, a fatal case of pneumonic plague occurred in a Grand Canyon National Park wildlife biologist who had necropsied an infected mountain lion carcass seven days earlier. Face-to-face contact with infected pets and laboratory exposure are two other examples of hazards associated with veterinary practice (see below).

Descriptive Features

Cellular Composition and Products of Medical Importance

Analysis of DNA sequences of the genes encoding the 16S ribosomal RNA indicates that *Y. pestis* is a subspecies of *Y. pseudotuberculosis* that has lost a number of the enteropathogenic *Yersinia* virulence genes. The majority of *Y. pestis* strains regardless of biotype/biovar or origin contain three virulence plasmids: pCD1 (70- to 75-kb encoding the type III secretion system, Yops, and low calcium response genes); pMT1 [100- to 110-kb encoding the Caf1 (F1) capsule and *Yersinia* murine toxin (Ymt)]; and pPCP1 [9.5-kb encoding pesticin, coagulase, and plasminogen activator (Pla)]. The *ybt* genes encoding the siderophore yersiniabactin are found on the 35-kb HPI, which in turn is within the 102-kb *pgm* (pigmentation) locus on the chromosome. An additional operon within the *pgm* region and unique to *Y. pestis* is the 6-kb *hms* locus. The *hms* genes encode hemin storage proteins that enable biofilm formation and proventricular blockage in fleas. Also located on the chromosome are the genes encoding the protein Gsr (global stress requirement).

Capsule (Fra1, Caf1, or F1). The capsule plays many roles, the most important of which are interference with phagocytosis, and the protection of the outer membrane from the deposition of membrane attack complexes generated by activation of the complement system. The capsule of *Y. pestis* is called Fra1 for fraction 1 or Caf1 (for capsular antigen fraction 1) or F1 (fraction 1 antigen), and is encoded by genes that reside on the 110-kb plasmid pMT1 (which also carries the genes encoding the toxin Ymt; see below). In contrast to Ymt, F1 is expressed at temperatures >30 °C and during mammalian infection. Upon flea inoculation of *Y. pestis*, some of the bacteria are engulfed and transported to regional lymph nodes by macrophages. Here, during intracellular growth, F1 is expressed and forms a capsule on the bacterial surface. Subsequent to release of *Y. pestis* from its intracellular location, the anti-phagocytic properties of the new F1 surface enable widespread dissemination and replication, and result in host sepsis.

Cell Wall. *Y. pestis* has lost the genetic capacity to express a complete lipopolysaccharide (LPS) layer; hence, it lacks an O-antigen. This organism does, however, elicit clinical effects typical of those due to endotoxemia. This is the result of LPS binding to the LPS-binding protein and then to Toll-like receptor (TLR)-4. The mechanism of endotoxin-mediated effects is described further in Chapter 5.

High Pathogenicity Island. The HPI is so called because its presence is related to increased virulence as compared to strains that do not contain it. It is a pathogenicity island that contains genes encoding the siderophore yersiniabactin and hemin storage proteins (see sections "Hms Phenotype" and "Iron Acquisition").

Hms Phenotype. The Hms (for hemin storage) phenotype is associated with iron acquisition and colonization of fleas (see section "Iron Acquisition"). In addition to playing a role in iron acquisition, the hms locus encodes proteins that serve to block the proventriculus of the flea with aggregated bacteria. This causes regurgitation and repeated attempts at feeding. Although this enhances the possibility of transmission of the bacteria to new rodent hosts, it also eventually causes death of the flea. Hemin is Fe^{2+} heme and contains chloride. The Hms^{+} phenotype is dark greenish-brown colonies on hemin agar.

Iron Acquisition. Iron is an absolute growth requirement and must be removed from iron-binding proteins of the host. The genes encoding products involved with iron acquisition reside on the HPI. The HPI of *Y. pestis* encodes the genes for the iron-acquiring siderophore yersiniabactin and the Hms (for hemin storage) phenotype. Colonies growing on the surface of blood agar plates that display the Hms phenotype appear pigmented (dark greenish-brown) due to the storage of excess amounts of hemin or Congo Red if present, when grown at 26 °C, but not 37 °C. It is probable that stored hemin is used as a source of iron. The genes responsible are encoded by the *pgm* (for pigment) locus.

Type III Secretion System. The type III secretion system consists of an assemblage of proteins (more than 20) encoded by *ysc* (Yop secretion apparatus) genes that form a hollow tube-like structure through which effector proteins (Yops, LcrV) are injected into host target cells. The *ysc* genes encoding the proteins needed by the type III secretion system reside on plasmid pYV (along with the

genes encoding Yops and LcrV; see below). Most of the proteins of the type III secretion system make up the membrane-spanning channel that will eventually fuse with the eukaryotic cell to form the injection pore. Genes for the core of the membrane-spanning complex are expressed at 37 °C. Contact with the eukaryotic cell is not needed for this core to form [only exposure to body temperature (37 °C)].

Type VI Secretion System. The type VI secretion system includes 13 protein subunits that when assembled directly inject effector proteins into target cells in a one-step process with a bacteriophage-like cell-puncturing device. These effectors mediate pathogenic, antibacterial, and stress-resistant roles important in the pathogenesis. Effectors secreted by the type VI secretion system function include a bacteriocin, an RNAse toxin, bacterial membrane proteins, and a protein that protects against oxidative/nitrosative stress.

Toxins. *Y. pestis* produces a number of toxins and other effector proteins:

1) **Yops**. Following their injection into macrophages, Yops (for *Yersinia* outer protein) interfere with the actin cytoskeleton, thereby blocking phagocytosis, and downregulating the inflammatory responses by the inhibition of NF-kB. Injection of Yops into neutrophils results in a decrease in the expression of endothelial cell-adhesion proteins, thereby reducing effective inflammatory responses. The genes encoding Yops reside on the pCD1 or pYV plasmid. The genes residing on this plasmid encode Yops, LcrV, and the type III secretion system. They are downregulated at 26 °C, and upregulated at 37 °C and low calcium ion concentrations.

   The six Yops that are injected into the target cell, namely, YopB, YopD, YopE, YopH, YopM, and YopT, are not secreted unless contact with the cell is made. YopB and YopD are translocated early; they form the portion of the pore that transits the membrane of the eukaryotic cell. However, the low Ca^{2+} response keeps the channel closed in the absence of contact. The cytoplasm of the eukaryotic cell is low in free Ca^{2+} due to its content of calcium-binding proteins, for example, calmodulin. Exposure of the tip of the secretion apparatus to the low calcium environment of the eukaryotic cytoplasm is thought to be the signal that causes the pore to open. If Ca^{2+} concentrations are high, the protein gate remains closed, and no effector proteins are translocated. YopB and YopD form the part of the pore that transits the eukaryotic cell membrane. YopH blocks phagocytosis and inhibits the oxidative burst. It also elicits tyrosine phosphatase activity, which blocks signal transduction pathways in the phagocyte. YopE depolymerizes actin filaments in phagocytes, providing an anti-phagocytic effect. YopM prevents thrombin-platelet aggregation, and this reduces inflammatory response and clot formation. YopT disrupts actin filaments by inactivation of Rho GTPases.
2) **LcrV**. LcrV (for low calcium response virulence, also known as Factor V) is a protein located on the cell surface of *Y. pestis*. LcrV has several roles: it aids the injection of effector proteins (e.g. Yops) into target cells; after injection into phagocytic cells, it reduces the excretion of pro-inflammatory cytokines and inhibits neutrophil chemotaxis. The genes encoding LcrV reside on the plasmid pYV (also known as pCD1). As noted above, the genes residing on this plasmid encode Yops, LcrV, and the type III secretion system, and they are downregulated at 26 °C, and upregulated at 37 °C and low calcium ion concentrations.
3) **Ymt**. The genes encoding Ymt (for *Yersinia* murine toxin) reside on the plasmid pMT (which also contains the genes for the F1 capsule; see above). Ymt is a phospholipase D, and previous studies had indicated that this protein is toxic to mice and rats, causing circulatory collapse of the animal. However, it is poorly expressed at 37 °C. More recent studies have shown that it is expressed at lower temperatures (e.g. 25 °C) more typical of the flea vector. Its main function is to enhance bacterial colonization within the midgut of the flea, by helping to protect it from digestive enzymes.
4) **Pesticin**. Pesticin is a bacteriocin produced by *Y. pestis* with an uncertain role in the production of disease. Genes on plasmid pPCP1 encode pesticin (along with plasminogen activator). Pesticin inhibits growth of *Y. pseudotuberculosis* serotype IA and IB, highly invasive serovar O:8 isolates of *Y. enterocolitica*, and specific clinical strains of *Escherichia coli*. Pesticin utilizes siderophore receptors to enter into sensitive bacteria, and their antibacterial activity can be inhibited by exogenous Fe^{3+} that downregulates these receptors. Yersiniae that produce pesticin are protected by a periplasmic immunity protein encoded on pPCP1.
5) **Plasminogen Activator and Coagulase**. Plasminogen activator (Pla) is an outer membrane protein of *Y. pestis* that provides prothrombin activation (coagulase), plasminogen activation (fibrinolysis), and complement C3 proteolytic activity. Genes on plasmid pPCP1 encode Pla (along with pesticin and the periplasmic protein that confers immunity to pesticin). Pla is closely related to OmpT of *E. coli*, an outer membrane protein found in many enteric bacteria. Pla enhances the ability of *Y. pestis* to colonize the viscera and thus cause lethal infection. Adherence to host basement membranes and extracellular matrix is dependent upon Pla expression, where plasminogen activation facilitates bacterial metastasis. The

fibrinolytic and coagulase activities of Pla are temperature dependent. The fibrinolytic and proteolytic activities are higher at 37 °C than at 28 °C, whereas the coagulase activity is higher at 28 °C than at 37 °C. When Pla protease is activated, it lyses the blood clot in the proventriculus of the flea. This liquefies the blood and lets the bacteria pass through the flea's midgut and hindgut (providing it with nourishment). Bacterial replication and production of coagulase (e.g. at 28 °C) results in blockage of the proventriculus; consequently, the flea regurgitates blood and bacteria out of its mouthparts, transmits the bacteria to the mammalian host, and also starves.

6) **Gsr**. Gsr (for global stress requirement) is expressed at 37 °C while *Y. pestis* is within the macrophage phagolysosome. The Gsr protein is responsible for the survival of *Y. pestis* within this environment.
7) **Ypk**. Ypk (for *Yersinia* protein kinase) is a serine/threonine kinase that interferes with signal transduction events in the phagocyte.
8) **PsaA**. PsaA (for pH 6 antigen) is a fibrillar protein that provides entry into macrophages and mediates delivery of certain Yop proteins (e.g. YopE) into the phagocyte. It is named for the observation that it is expressed at acidic pH (5–7). It is also expressed at low calcium concentrations and at 37 °C. Acidic conditions in the phagolysosome induce its expression. It is expressed when bacteria infect the spleen and liver.

Variability. *Y. pestis* is serologically uniform. Biotypes (also known as biovars) are based on the biochemical pattern (carbohydrate fermentation, denitrification, fibrinolytic and coagulase activity, pesticin production and susceptibility, and dependence on amino acid sources). *Y. pestis* subsp. *pestis* is divided into biovars Intermedium, Antiqua, Medievalis, and Orientalis, all causing disease in humans. *Y. pestis* subsp. *microtus* is typically zoonotic, with genetic analyses suggesting that it is the closest ancestral relative of *Y. pseudotuberculosis*.

Ecology

Reservoir

Tolerant rodents in endemic areas (see below) constitute the primary plague reservoir. They rarely develop fatal disease and are called maintenance or enzootic hosts. In North America along the coastal regions of California, the meadow mouse *Microtus californicus* is such a host. In the urban cycle, the rat is the main intermediate host, and the oriental rat flea is the main vector. In the United States, the current main sources of infection are prairie dogs, squirrels, chipmunks, mountain lions, cats, dogs, coyotes, marmots, rabbits, hares, mice, voles, rats, and fleas.

Transmission

Transmission is primarily through the bite wounds of infected rodent fleas (bubonic plague; Figure 8.9), but also may occur through droplet inhalation after close contact with infected mammals (primary pneumonic plague), or rarely, consumption of contaminated raw meat (pharyngeal or gastrointestinal plague). Infected rodent fleas may transmit *Y. pestis* to more susceptible, epizootic, or amplification hosts, such as ground squirrels or rats. In sylvatic (rural) environments, squirrels, prairie dogs, deer mice, gerbils, voles, rats, and rabbits are all intermediate hosts. Some species are more resistant than others to full-blown disease and septicemia. However, like humans, prairie dogs, ground squirrels, and many other species develop bubonic or septicemia plague (Figure 8.8). When these die, still other hosts, such as humans, are attacked. Infected mammals may spread plague by the airborne route. Oral acquisition is by predation, cannibalism, and scavenging.

Pathogenesis

Fleas feed on an infected host. *Y. pestis* in the midgut of the fleas proliferate until they block (obstruct) the flea's proventriculus (function of Ymt and Hms), a process that takes about two weeks. Blocked fleas infest a new host and contaminate the feeding site with *Y. pestis* when attempting to feed. At flea temperature, the type III secretion system, Yops, LcrV, Caf1, and Gsr are not produced. The bacteria, thus introduced into a vertebrate host, lack defense against the host's innate immune system, and are killed when ingested by neutrophils (an inflammatory response is generated due to products of the flea bite along with the



Figure 8.9 *Xenopsylla cheopis,* oriental rat flea, with a proventricular plague mass. During feeding, the flea draws viable *Yersinia pestis* organisms into its esophagus, which multiply and block the proventriculus just in front of the stomach, later forcing the flea to regurgitate infected blood into the host when it tries to swallow. *Source:* Courtesy of US Health and Human Services, Centers for Disease Control and Prevention, Public Health Image Library, ID#2025.

gram-negative cell wall components of *Y. pestis*). In mononuclear phagocytes, at mammalian temperatures, and low calcium ion concentrations, and while protected by Gsr, *Y. pestis* activates the type III secretion system; produces Yops, LcrV, and F1; and is released from the phagocytic cell following initiation of apoptosis. Yersiniae acquire resistance to further phagocytosis and intracellular killing by neutrophil and mononuclear phagocytes (LcrV reduces the excretion of pro-inflammatory cytokines, and inhibits neutrophil chemotaxis; injected Yops prevent phagocytosis; Caf1 prevents phagocytosis and promotes serum resistance). Thus, early in the disease process, *Y. pestis* is an intracellular parasite, and later, an extracellular one.

Extracellular multiplication made possible by iron acquisition systems and capsule production elicits a hemorrhagic inflammatory lesion, followed by local lymph node involvement (bubo). This form is called bubonic plague.

The infection commonly becomes septicemic and, if untreated, terminates fatally (an endotoxemia aided by the function of Pla, which accelerates initiation of disseminated intravascular coagulation). Some individuals develop plague pneumonia and shed *Y. pestis* in sputum and droplet nuclei. Others contract primary pneumonic plague from this source and transmit it by the same route. Under epidemic conditions this form is nearly always fatal.

Among domestic animals, cats acquire natural clinical infection, often by ingestion of infected prey. Signs include regional (particularly mandibular) lymphadenitis, fever, depression, anorexia, sneezing, coughing, and occasionally central nervous system disturbances. Most cases end fatally. Lesions, mainly in the respiratory and alimentary tracts, include lymphadenitis, tonsillitis, cranial and cervical edema, and pneumonia. Cattle, horses, sheep, and pigs are apparently not susceptible to clinical plague. However, goats and camels are susceptible.

Human plague can be acquired directly from infected cats, ingestion of meat from infected animals (e.g. goats or camels), and necropsy of infected animals (e.g. rodents, mountain lions, etc.). In the case of domestic cats, suspected inoculation routes are via cuts, bites, scratches, and airborne and flea-borne pathways, although the latter is unlikely because the cat flea (*Ctenocephalides felis*) does not become blocked.

Epidemiology

Plague is concentrated in certain endemic areas in southern and southeastern Asia, southern and west central Africa, western North America, and north central South America. Endemicity largely parallels presence of enzootic and epizootic rodent hosts. Human plague epidemics have historically been precipitated by importation of infected rats on board ships coming from endemic regions. Today, most human cases result from infection following contact with rural wild animals (sylvatic plague). From 2000 to 2018, 26 237 plague cases in 21 countries, collectively, in the Americas, Africa, and Asia, were reported to the World Health Organization. Africa experienced the largest number of cases worldwide, whereas, in the Americas, 371 cases were reported, due mainly to sporadic cases in the United States and clustered cases in South America.

Plague is a disease of rodents. The organism is maintained in endemic hosts (certain species of *Microtus* and *Peromyscus*, i.e. voles and meadow mice). Endemic hosts are infected following the bite of infected fleas. Though endemic hosts are fairly resistant to development of serious disease, when their populations rapidly increase and spread of *Y. pestis* is rapid, die-off of endemic hosts occurs. Infected fleas, owing to the shortage of preferred hosts, feed on epidemic, highly susceptible species such as prairie dogs, rats, mice, and ground squirrels. What constitutes an endemic host and an epidemic host is somewhat clouded, because there is considerable overlap between the two.

In endemic areas, infections are clustered during the warm months. "Off-season" plague affects mostly persons handling infected rabbits, bobcats, and occasionally house cats. Carnivores such as canids (wild and domestic), bears, raccoons, and skunks, as well as raptors, seroconvert following infection (ingestion, flea bite), but rarely develop clinical disease. Cat fleas and dog fleas, *Ctenocephalides felis* and *C. canis*, respectively, do not transmit *Y. pestis* efficiently because neither flea becomes blocked. Thus, infection of humans from an infected cat (rarely dog) occurs from infections of scratches or bites with infected saliva or from inhalation of infected droplets.

Immunologic Aspects

Specific resistance to plague probably requires antibody- and cell-mediated responses. Capsular antigens (F1 antigen) evoke opsonin formation. Antibody to the LcrV antigen is protective. Disposal of intracellular organisms depends on activated macrophages. Immunity following recovery is good, but temporary.

Detection of antibodies to *Y. pestis* in resistant species (e.g. canids) is a way of determining the presence of the organism in a particular environment.

Laboratory Diagnosis

Diagnostic attempts should be supervised by qualified public health personnel (see below). Samples from affected sites (i.e. edematous tissues, lymph nodes, and nasopharynx), transtracheal aspirates, cerebrospinal fluid, and blood (for culture and serology) are collected.

Direct smears are examined following immunofluorescent, Wayson's staining, or Gram staining. Culture is done on blood or infusion agar. Identification is confirmed by immunofluorescence or bacteriophage susceptibility. Mice or guinea pigs injected subcutaneously with *Y. pestis* die within three to eight days. DNA techniques utilizing molecular probes or the amplification of specific DNA sequences by the polymerase chain reaction are available.

Serologic tests (hemagglutination, hemagglutination-inhibition, enzyme-linked immunosorbent assay [ELISA]) are useful for retrospective studies.

Treatment and Control

If plague is suspected in domestic cats, the following recommendations from the Centers for Disease Control and Prevention (CDC) apply:

1) Arrange immediately with local and state public health officials for laboratory diagnostic assistance and steps to prevent spread and contamination.
2) Place all suspect cats in strict isolation.
3) When handling such cats, wear gown, mask, and gloves.
4) Treat every suspect for fleas (5% carbaryl dust for residual effect).

Flea elimination should precede rodent control.

Aminoglycosides, chloramphenicol, fluoroquinolones, and tetracycline are effective antimicrobics. Although research continues, to date, no field-proven, efficacious vaccines for animals or humans have been developed. Protection of humans by bacterins is transient.

Yersinia pseudotuberculosis

Y. pseudotuberculosis is associated with mesenteric lymphadenitis, terminal ileitis, acute gastroenteritis, and septicemia, affecting mainly birds and rodents, and only occasionally domestic animals and primates. *Y. pseudotuberculosis* is closely related to *Y. pestis*, and many consider *Y. pestis* to be a subspecies.

Descriptive Features

Cellular Composition and Products of Medical Importance

Y. pseudotuberculosis contains the plasmid pYV (encodes the type III secretion system, Yops, and LcrV). A chromosomal locus called the HPI contains the genes for iron uptake. Also located on the chromosome are the genes encoding the proteins Ail, Inv, YadA, and Gsr.

Adhesins. *Y. pseudotuberculosis* produces three adhesins that are responsible for adherence to ß-integrins on the luminal surface of M cells and the basolateral surface of ileal epithelial cells: Ail, Inv, and Yad:

1) Ail (for attachment invasion locus) adheres to receptors on the surface of M cells. Ail also protects the outer membrane from insertion of the membrane attack complexes generated by activation of the complement system.
2) Inv (for invasin) adheres to receptors on the surface of M cells and the basolateral surface of ileal epithelial cells.
3) Yad (for *Yersinia* adhesin) adheres to receptors on the surface of M cells and the basolateral surface of ileal epithelial cells. Yad also protects the outer membrane from insertion of the membrane attack complexes generated by activation of the complement system.

Cell Wall. The cell wall of *Y. pseudotuberculosis* has O-antigens (smooth phenotype). The cell wall of the members of this genus is typical of gram-negatives. The LPS in the outer membrane is an important virulence determinant. Not only is the lipid A component toxic (endotoxin), but the length of the side chain in the O-repeat unit hinders the attachment of the membrane attack complex of the complement system to the outer membrane. LPS binds to LPS-binding protein (a serum protein), which in turn transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins (see Chapter 5) on the surface of macrophage cells, triggering the release of pro-inflammatory cytokines.

High Pathogenicity Island. See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Iron Acquisition. Iron is an absolute growth requirement, and must be removed from iron-binding proteins of the host. The genes encoding products involved with iron acquisition reside on a chromosomal pathogenicity island. The HPI of *Y. pseudotuberculosis* encodes the genes for the iron-acquiring siderophore yersiniabactin.

Type III Secretion System. See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Type VI Secretion System. See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Gsr (Global Stress Requirement). See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Toxins. *Y. pseudotuberculosis* produces toxins that are involved with pathogenicity:

1) Yops – See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."
2) LcrV – See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Variability

There are 21 serotypes based on variability of the somatic (O) antigens, and 6 biotypes (1A, 1B, and 2–5), which include several serotypes. Most strains pathogenic for humans are biotype 4, serotype O:3. Serotypes associated with disease vary in different parts of the world.

Ecology

Reservoir

Y. pseudotuberculosis is a parasite of wild rodents, lagomorphs, and birds, but it infects other mammals and reptiles and persists in the environment. The cat is the most commonly infected domestic mammal. Minor epidemics occur among sheep, pigs, nonhuman primates, fowl, and pet birds.

Transmission

Transmission is fecal–oral, with exposure primarily through ingestion of contaminated food and water.

Pathogenesis

Y. pseudotuberculosis attaches to M cells of lymphoid nodules of the distal small intestine following expression of the cell surface proteins Inv, Ail, and YadA. Attachment triggers actin cytoskeletal changes resulting in a "zippering" phenomenon that leads to the enclosure of the cell membrane around the attached yersiniae, resulting in their internalization. Internalized microorganisms pass through to the lymphoid nodule, where they are phagocytosed by macrophages within the nodule. Expression of Gsr permits intracellular survival. Within macrophages (37 °C, low calcium), the type III secretion apparatus is activated, and Yops and LcrV are produced. Ingested yersiniae are released after initiation of apoptosis of the macrophage. Now extracellular, yersiniae interfere with further phagocytosis. Invasion of the basolateral surface of ileal epithelial cells occurs following attachment of YadA and Inv, and internalization follows. Inflammation induced by extracellular yersiniae (lps), together with interferon secretion by natural killer cells and gamma delta T cell-recognition of infected epithelial cells (as well as LPS), results in an influx of polymorphonuclear neutrophil leukocytes (PMNs). Extracellular yersiniae avoid phagocytosis (Yops), and destruction by complement-mediated mechanisms by expression of Ail and YadA, both of which impart complement resistance. *Y. pseudotuberculosis* acquires iron from iron-binding proteins of the host following secretion of yersiniabactin (encoded within the HPI). Diarrhea is thought to result from prostaglandin synthesis by the recruited PMNs (and perhaps by the affected host cells), as well as activation of various inositol-signaling pathways within the affected host cells. The net result is the secretion of chloride ions and water. Septicemia results from the host's inability to "clear" yersiniae from infected sites (serum resistance, anti-phagocytic traits, and iron scavenging). The result is enteritis and septicemia.

Y. pseudotuberculosis causes intestinal infections with formation of necrotic foci in the intestinal wall, abdominal lymph nodes, and viscera, particularly the liver (Figure 8.10)

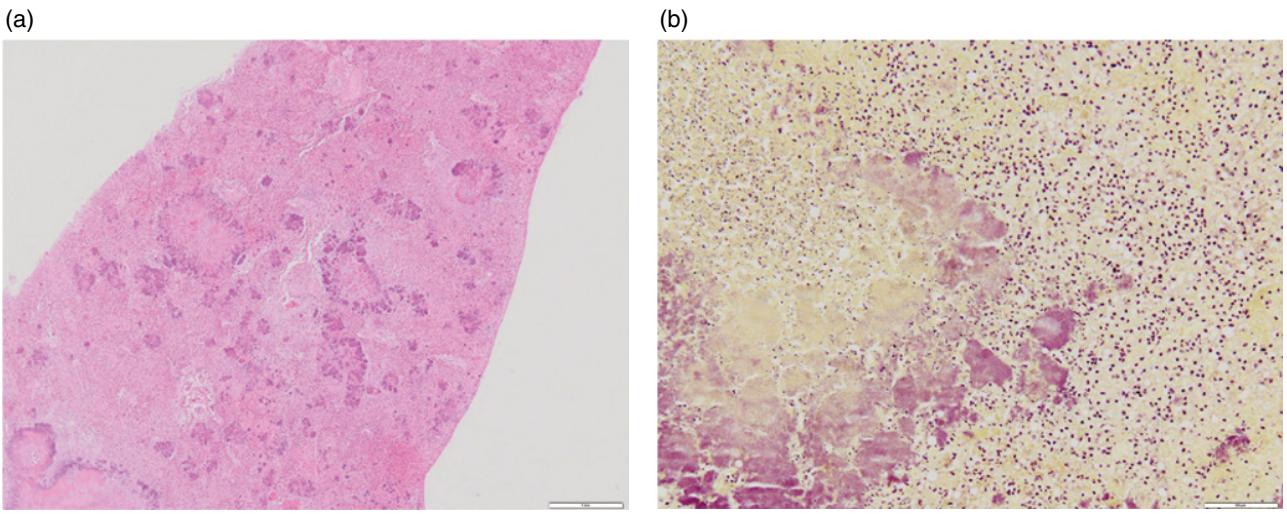


Figure 8.10 Histopathologic tissue sections from the liver of a captive *Perodicticus potto* (a "prosimian" primate belonging to the Lorisidae family) that died from yersiniosis. The animal was found dead, and at necropsy had multiple 1–2 mm white foci in all liver lobes. Histopathologic sections of liver, as shown in this figure, revealed severe multifocal coalescing massive necrosis associated with bacterial colonies. *Yersinia pseudotuberculosis* was isolated. Sections of lung were affected with diffuse pulmonary hyperemia and edema with intravascular bacterial colonies consistent with those seen in the liver. (a) Hematoxylin and eosin stain, and (b) Brown-and-Brenn Gram stain.

and spleen. There may be vomiting, diarrhea, or constipation, and weight loss, pale to subicteric mucous membranes, and depression. Fever is inconsistent. Few cases are diagnosed clinically antemortem. Mastitis is seen in cattle and abortion in ruminants and monkeys. In immunocompetent humans, the disease is generally an enteritis and abdominal lymphadenitis that is self-limiting or responsive to treatment.

Epidemiology

Pseudotuberculosis occurs worldwide. Cases tend to cluster in the cold months. In cats, prevalence is biased toward adult, rural, outdoor cats.

Immunologic Aspects

Natural infection leaves surviving individuals immune. Avirulent live vaccine protects against homologous challenge. It is not available commercially.

Laboratory Diagnosis

Diagnosis involves isolation of the agent antemortem from feces or lymph node aspirates. Isolation, particularly from mixed sources, is enhanced by cold enrichment, that is, incubation of a 10% mixture of inoculum in a minimal medium for several weeks at 4 °C. DNA techniques utilizing molecular probes or the amplification of specific DNA sequences by the polymerase chain reaction are available.

Treatment and Control

Pseudotuberculosis responds to the same antimicrobics as plague.

Yersinia enterocolitica

Y. enterocolitica is associated with mesenteric lymphadenitis, terminal ileitis, acute gastroenteritis, and septicemia in domestic animals and primates.

Descriptive Features

Cellular Composition and Products of Medical Importance

Y. enterocolitica contains the plasmid pYV (encodes the type III secretion system, Yops, and LcrV). A chromosomal locus called the HPI contains the genes for iron uptake. Also located on the chromosome are the genes encoding the proteins Ail, Inv, YadA, Gsr, and Yst.

Adhesins. See above for *Y. pseudotuberculosis*, "Cellular Composition and Products of Medical Importance."

Cell Wall. See above for *Y. pseudotuberculosis*, "Cellular Composition and Products of Medical Importance."

Gsr (Global Stress Requirement). See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

High Pathogenicity Island. See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Iron Acquisition. See above for *Y. pseudotuberculosis*, "Cellular Composition and Products of Medical Importance."

Type III Secretion System. See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."

Toxins. *Y. enterocolitica* produces a number of toxins that are involved with pathogenicity:

1) Yops – See above for *Y. pestis*, "Cellular Composition and Products of Medical Importance."
2) LcrV – See above *Y. pestis*, "Cellular Composition and Products of Medical Importance."
3) Yst (for *Yersinia* stable toxin) – Yst is a chromosomally encoded enterotoxin unique to *Y. enterocolitica*. Yst affects the guanylyl cyclase system by deregulating cGMP synthesis (increases in intracellular cGMP lead to the opening of chloride channels with the resultant flow of chloride and water into the intestinal lumen), resulting in fluid and electrolyte accumulation in the bowel lumen subsequent to blockage of sodium and chloride ion (and thus water) absorption (tip cells) and loss of chloride ions (crypt cells) (see also STa enterotoxin of *E. coli*; Chapter 6).

Variability

Y. enterocolitica includes two subspecies, *enterocolitica* and *palearctica*. The *enterocolitica* subspecies was proposed for strains with the 16S rRNA type of American origin, whereas *palearctica* was proposed for strains of European origin. *Y. enterocolitica* strains are differentiated by bio- and serotyping. Serotyping is based on reactivity to O-antigen polysaccharides. There are more than 70 serotypes, although only a small number are pathogenic. O groups 3, 5, 8, 9, and 27 are associated with classical disease of the gastrointestinal tract in human patients. There are six biogroups (biotypes), namely, BT1A, BT1B, BT2, BT3, BT4, and BT5. BT1A strains are mostly non-pathogenic; the rest (BT1B through BT5) are pathogens. BT1B predominates in the United States. BT1A and BT2 through BT5 are found in Europe. The biogroups (or biotypes) are differentiated on the basis of reactivity to esculin, indole, D-xylose, trehalose, pyrazinamidase, β-D-glucosidase, and lipase. Strains are also referred to by a combination of the serotype and biotype; for example, a strain that is serotype O:3 and BT4 is referred to as serobiotype O:3/4.

Ecology

Reservoir and Transmission

Y. enterocolitica has been isolated from a wide range of sources, for example, soil, water, food (fruits, vegetables, milk, retail meat products, cheese, eggs), and animals. The number of species of animals from which it has been isolated is also numerous and includes pets (cats and dogs), stock (chinchilla, mink, pig, rabbit, cow, goose, horse, sheep, buffalo), zoo animals (monkeys), and wild animals (raccoon, fox, snail, frog, beaver, deer, ocelot, crab, flies, fleas, birds, oysters, and many species of small rodents). Pathogenic BT2 through BT5 are mainly isolated from animals (sheep, cattle, goat, and poultry) or food. Serobiotype O:3/4 strains are frequently isolated from pigs; O:9/2 strains are often isolated from cows and goats; and O:2,3/5 strains are isolated from sheep, rabbits, and goats. Expression of certain virulence determinants at 22 °C to 25 °C suggests that mammals acquire *Y. enterocolitica* from a "cold" source (water and food, for example) rather than a warm-blooded animal. Infection follows ingestion of organisms expressing the adhesin. One of the major sources of human infection with serobiotype O:3/4 is through the handling of swine and consumption of pork. Pigs are known to carry the organism in their oropharynx, nasopharynx, and their intestine. Human-to-human transmission may also occur, although it is rarely documented.

Transmission

Transmission is by the fecal–oral route. Infection is mainly acquired through ingestion of food and water that has been contaminated with animal feces or by direct contact with animal feces (e.g. abattoir workers). Children below the age of five are much more prone to *Y. enterocolitica* infections.

Pathogenesis

The pathogenesis of disease produced by *Y. enterocolitica* is the same as for *Y. pseudotuberculosis* (see above). In addition to diarrhea associated with invasion of epithelial cells and inflammation (as with *Y. pseudotuberculosis*), *Y. enterocolitica* produces Yst.

Epidemiology

As noted above, certain subspecies, serotypes, and biotypes are geographically restricted. Subspecies *enterocolitica* is found in the United States, and subspecies *palearctica* is found in Europe. BT1B predominates in the United States. BT1A and BT2 through BT5 are found in Europe. Serotype O:8 is indigenous to the United States but not the rest of the world. Until recently, serotype O:3 was rarely isolated in the United States but is common in the rest of the world, and is becoming more so in the United States. Serotype O:9 has not been reported outside Europe.

Immunologic Aspects

Y. enterocolitica is an extracellular microorganism. Phagocytic cells readily destroy it, even though the microorganism excretes proteins (Yops) that interfere with this process. Most often, *Y. enterocolitica* disease is self-limiting due to the innate immune response: phagocytosis, lysis of infected epithelial cells, iron sequestration, and complement proteins.

A serologic relationship between O:9 serotype, common in swine, and *Brucella* spp. has complicated swine brucellosis eradication programs.

Laboratory Diagnosis

Samples of feces, lymph node biopsy, and biopsy from affected tissues are examined microbiologically. Selective media containing bile salts are somewhat inhibitory to *Y. enterocolitica*, especially at 37 °C. MacConkey agar is least inhibitory. There are special media designed for the isolation of *Y. enterocolitica* [e.g. cefsulodin-Irgasan-novobiocin (CIN) medium; see Chapter 5]. Cold enrichment of the sample at 4 °C aids attempts to isolate small numbers of *Y. enterocolitica* from a contaminated environment. Isolation from tissue necessitates the use of blood agar plates incubated at 37 °C. DNA techniques utilizing molecular probes or the amplification of specific DNA sequences by the polymerase chain reaction are available.

Treatment and Control

Antimicrobial agents useful for treating disease produced by *Y. enterocolitica* are the fluoroquinolones, tetracycline, trimethoprim-sulfonamides, and chloramphenicol. R plasmids are common in *Y. enterocolitica*, and genes encoding resistance to tetracycline and streptomycin are most commonly found.

Yersinia ruckeri

Enteric red mouth (ERM) disease is a hemorrhagic inflammation of the perioral subcutis of freshwater fish, particularly rainbow trout. Infection is systemic and causes significant mortality in hatcheries of North America, Australia, and Europe. The agent is disseminated by asymptomatic carrier fish and possibly riparian mammals (muskrats). Outbreaks appear to be related to massive exposure.

Fish with red mouth show sepsis and inflammation in most organs. A protease, Yrp (*Y. ruckeri* protease), appears to play an important role because inactivation of the encoding gene significantly reduces virulence.

Outbreaks are brought under control with antimicrobics (e.g. sulfonamides, tetracycline, trimethoprim-sulfonamide), and bacterins have been successful in lowering mortality.

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9

Enterobacteriaceae

Shigella

Rodney A. Moxley

Members of the genus *Shigella* belong to the family *Enterobacteriaceae* and are obligate pathogens, causing bacillary dysentery (shigellosis) in human and nonhuman primates. Based on phylogenetic analyses, *Shigella* spp. are strains of *Escherichia coli* that have arisen from different ancestral commensal *E. coli* by convergent evolution involving gain and loss of genes. *Shigella* should be reclassified as *E. coli*; however, the genus name *Shigella* has been retained for public health reasons because of its etiological association with shigellosis. *Shigella*, historically, and to date, includes four species: *dysenteriae*, *flexneri*, *boydii*, and *sonnei* (Table 9.1). These species are also known as serogroups (or subgroups) A, B, C, and D, respectively. The type species is *Shigella dysenteriae*. Enteroinvasive *E. coli* (EIEC) share biochemical characteristics, essential virulence factors, and clinical features with *Shigella* spp., although differences exist, and studies suggest that they evolved independently.

In nonhuman primates, shigellosis occurs almost exclusively in captive animals and appears to be related to stressful situations (e.g. transportation, crowding) or immunological dysfunctions (e.g. simian acquired immunodeficiency syndrome). Cases are most often associated with *Shigella flexneri* infection; however, nonhuman primates are susceptible to all *Shigella* spp. Rhesus macaques usually develop diarrhea with frank blood, whereas tamarins and marmosets may present with dehydration and lethargy with dried blood or wet fecal material around the anus. In humans and nonhuman primates, lesions of shigellosis are primarily seen in the large intestine (cecum, colon, and rectum), and may be focal or diffuse (Figures 9.1–9.3). Hemolytic-uremic syndrome (HUS) may occur as a sequela in cases of *S. dysenteriae* type 1 infection with Shiga toxin production.

Descriptive Features

Cellular Structure and Composition

The structure of each of the *Shigella* spp. is typical for the family *Enterobacteriaceae*. Though closely related genetically, *Shigella* spp. lack many of the traits characteristic of *E. coli*. They express neither capsules (K antigens) nor flagella (H antigens). The latter trait is the result of mutations in or complete loss of the master operon of flagellar synthesis, *flhDC*. Whereas *E. coli* are usually prototrophic, *Shigella* spp. are auxotrophic. *Shigella* isolates do not grow on synthetic media with Simmons citrate, and are negative for the deaminases for phenylalanine and tryptophan. They are also negative for arginine and lysine decarboxylase, growth in potassium cyanide (KCN) medium, liquefaction of gelatin, oxidation of gluconate, production of H_2S (on triple-sugar-iron agar), urease, utilization of Christensen's citrate or malonate, and conversion of mucic acid to mucate (mucate test). Like *E. coli*, shigellae yield both a positive methyl red and a negative Voges-Proskauer reaction. As almost all *Enterobacteriaceae*, they ferment glucose, but, with the exception of *S. flexneri* type 6, *Shigella boydii* types 13 and 14, and *S. dysenteriae* type 3, they do not produce gas. *S. dysenteriae*, *S. flexneri*, and *S. boydii* do not ferment lactose, but after several days of culture, *Shigella sonnei* and *S. boydii* serotype 9 yield lactose-positive results. Similarly, *S. sonnei* slowly ferments saccharose. *Shigella* spp. do not ferment adonitol, inositol, or salicin, or utilize acetate.

Shigella spp. have a typical gram-negative (GN) cell wall that includes lipopolysaccharide (LPS) and, like other *Enterobacteriaceae*, express an O-antigen that is useful for serogrouping and serotyping. Serogroup determination is required for the identification of the species of *Shigella*.

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Companion website: www.wiley.com/go/mcvey/microbiology4

Table 9.1 *Shigella* serogroups, species, and serotypes.

Serogroup	Species	Number of serotypes
A	*dysenteriae*	15
B	*flexneri*	18
C	*boydii*	20
D	*sonnei*	1

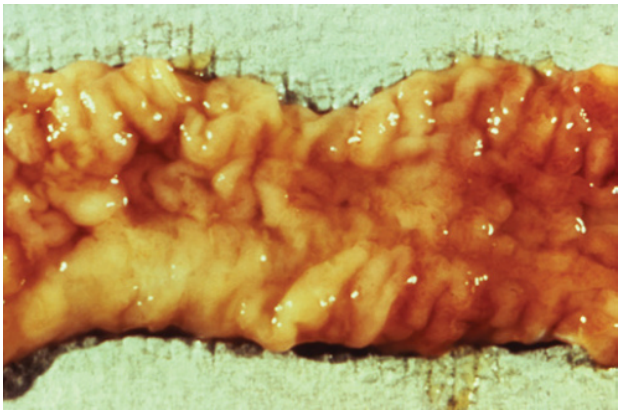


Figure 9.1 Necropsy photo of the colon of a Rhesus monkey, *Macaca mulatta*, infected with *Shigella* sp. bacteria. The mucosa is diffusely hemorrhagic and edematous. *Source:* US Department of Health and Human Services, Centers for Disease Control and Prevention, Public Health Information Library, PHIL_18695.

Traditionally, slide agglutination assays with commercially available specific antisera were used to determine the serogroup (subgroup) and serotype. However, in silico analysis of whole genome sequences with detection of serotype- and species-specific diagnostic markers is a more accurate tool. The four species of *Shigella* include >54 serotypes: *S. dysenteriae* (15); *S. flexneri* (18); *S. boydii* (20); and *S. sonnei* (1; Table 9.1). *S. boydii* serotype 13 phylogenetically clusters with and should be reclassified as *Escherichia albertii*.

Cellular Products of Medical Interest

Cell Wall

The cell wall of the members of this genus is one typical of gram negatives, containing LPS, and participates in the pathogenesis by typical means, that is, through the effects of lipid A (endotoxin) with elicitation of acute inflammation, yet also resistance to the innate immune response. In addition, glucosylation, the addition of glucose to rhamnose in the tetrasaccharide O-antigen repeats, causes a three-dimensional helical coiling of the LPS molecule, shortening it lengthwise by around half. This increases exposure of the injectisomes on the bacterial surface (see below), allowing improved contact with host cells and enhanced invasion.

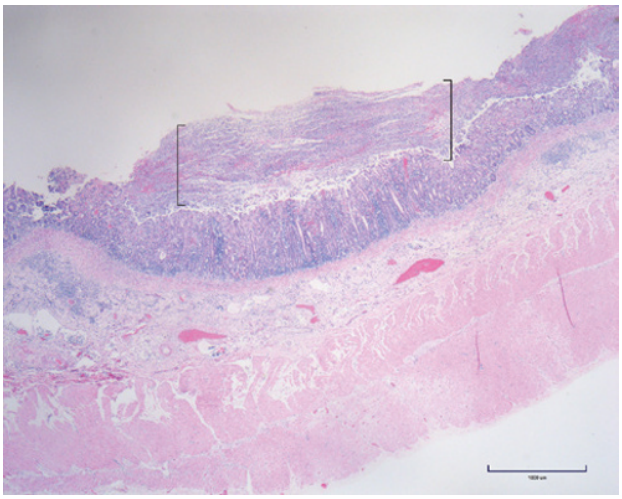


Figure 9.2 Photomicrograph of the colon of a 12-year-old, female pigtail macaque, *Macaca nemestrina*, housed in a research colony and naturally infected with *S. flexneri*. The macaque had diarrhea associated with severe diffuse subacute fibrinonecrotizing colitis and pseudomembrane formation (objective magnification, 2×). The mucosa and submucosa contain a diffuse infiltrate of inflammatory cells, and the submucosa is edematous. Overlying the mucosa is a thick fibrinonecrotic and suppurative (pseudomembrane) exudate (brackets; bar = 1000 µm). *Source:* Courtesy of Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, Department of Comparative Medicine, Hershey, PA and the Joint Pathology Center, Silver Spring, MD. Moxley, R. A. Case material, courtesy of the Wednesday Slide Conference, 2007–2008 Conference 23, Case II, Armed Forces Institute of Pathology/Joint Pathology Center, Silver Spring, MD.

Type III Secretion System (T3SS), Translocator and Effector Proteins

A requisite step in the pathogenesis of shigellosis is invasion of the large intestinal epithelium. Bacterial uptake occurs first through M cells via membrane ruffling (Figure 9.4). The bacteria transcytose the M cells being released in the M cell pockets, and then are phagocytosed by resident macrophages. Macrophages containing the bacteria undergo pyroptosis and elicit acute inflammation. Bacteria released by dying macrophages invade absorptive colonic epithelial cells via their basolateral surfaces (Figure 9.4), an event mediated by the T3SS apparatus (injectisome) and effectors secreted through it (Figure 9.5). The initial contact between bacteria and the colonic epithelial cells is at lipid microdomain rafts and is mediated by binding of the translocator protein IpaB to its receptor CD44. This binding induces early actin cytoskeletal rearrangements, but efficient and complete bacterial uptake requires T3SS effector proteins encoded on a large virulence (220-kb) plasmid. The virulence plasmid contains a mosaic of approximately 90 genes.

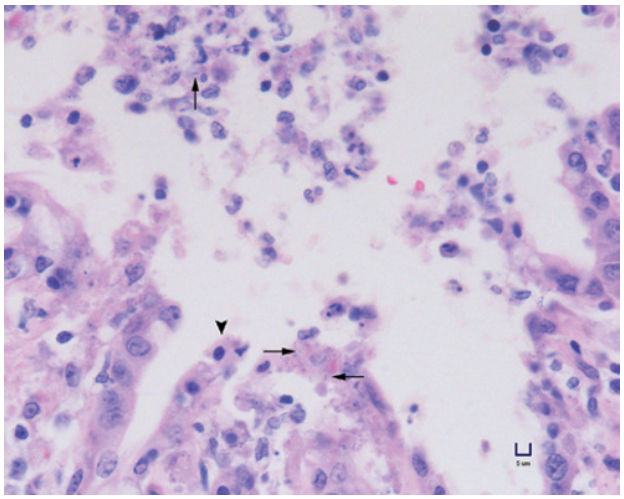


Figure 9.3 Higher detail of the interface between pseudomembrane and mucosal epithelium in the previous figure. The colonic mucosal epithelium (horizontal arrows) and neutrophils (vertical arrow) in the overlying exudate contain intracytoplasmic bacteria. Mucosal epithelial cells are necrotic (arrowhead) and sloughing into the lumen (objective magnification, 60×; bar = 5 μm). *Source:* Courtesy of Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, Department of Comparative Medicine, Hershey, PA and the Joint Pathology Center, Silver Spring, MD. Moxley, R. A. Case material, courtesy of Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, Department of Comparative Medicine, Hershey, PA and the Joint Pathology Center, Silver Spring, MD.

Invasion Plasmid Antigen (Ipa) and Invasion Plasmid Gene (Ipg) Proteins

Ipa effector proteins, that is, IpaB, IpaC, IpaD, and IpaA, are encoded by genes on an operon located within a 31-kb "entry region" on the large virulence plasmid. IpaB, IpaC, and IpaD are dominant immunogenic antigens and key virulence factors that are secreted by the T3SS. The nascent needle tip complex initially consists of a homopentamer of IpaD (Figure 9.5), and then includes the translocator IpaB. IpaB is the first of two translocators and, while positioned at the needle tip, senses the host cell membrane. IpaB and IpaC (the second translocator protein) then mediate the formation of a hydrophobic translocon (pore) in the host cell plasma membrane using IpgC as a stabilizing and partitioning chaperone. IpaD has a second role in that upon a conformational change, it acts as a signal transducer to activate bacterial effector protein secretion through the injectisome. IpaC, IpaA, IpgB1, IpgB2, and IpgD all enhance macropinocytic uptake of bacteria into the colonic epithelial cell through their collective effects on the plasma membrane and actin cytoskeleton. IpaC interacts with IpaB and inserts into the epithelial cell membrane, following which it induces actin polymerization through its interactions with Cdc42 and Rac GTPases and activation of the Src tyrosine kinase pathway. IpaA targets β1 integrins, vinculin, and Rho GTPase, resulting in depolymerization of actin to prevent uncontrolled effects of IpaC that would reduce bacterial uptake. IpgB1 and IpgB2, as guanine nucleotide exchange factors (GEF), activate GTPases (Cdc42 and Rac1 by IpgB1 and RhoA by IpgB2) that are required for actin filament remodeling to form membrane ruffles. IpgD functions as a phosphoinositide 4-phosphatase which dephosphorylates phosphatidylinositol 4,5-bisphosphate, thereby reducing the adhesion force between the plasma membrane and actin cytoskeleton of the epithelial cell. The phosphatase activity of IpgD also separately stimulates actin remodeling and membrane ruffling through Rac1 activation. The collective effects of IpaC, IpaA, IpgB1, IpgB2, and IpgD result in the extension of filopodia and membrane ruffles that mediate bacterial uptake into non-phagocytic cells.

IpaB and IpaC are also required for lysis of vacuoles in epithelial cells and macrophages (Figure 9.4, steps 3 and 11). In macrophages, release of IpaB into the cytoplasm results in it being integrated into organellar membranes in a cholesterol-dependent manner. This causes the proteolytic activation of procaspase-1 to caspase-1, and pyroptosis of the macrophage (Figure 9.4, step 6). This process also results in the cleavage, activation, and release of IL-1β and IL-18. IL-1β induces a marked inflammatory response. IL-18 activates natural killer (NK) cells and enhances production of interferon-gamma (IFN-γ), both involved in innate immunity.

Mxi-Spa T3SS

A second group of genes on the large virulence plasmid encodes for proteins needed for the molecular structure, assembly, and function of the injectisome (Figure 9.5). These include the membrane expression of *ipa* (*mxi*) and surface presentation of *ipa* antigen *(spa)* genes. The Mxi-Spa components include a basal body that spans the bacterial envelope; a hollow needle structure that is anchored in the basal body and protrudes extracellularly; and a sorting platform, a cytoplasmic ring that energizes and mediates the transport of proteins from the bacterial cytoplasm into the needle structure. A third group of genes on the large virulence plasmid encodes for two transcriptional activators, VirB and MxiE, which regulate T3SS-associated genes. A fourth group of genes encodes chaperones (*ipgA*, *ipgC*, *ipgE*, and *spa15*) needed to unfold and insert proteins into the needle complex.

Intercellular Spread (Ics) Proteins

Another group of genes on the large virulence plasmid encodes the Ics proteins. IcsA (VirG) is an outer membrane protein autotransporter secreted by the Sec pathway. IcsA

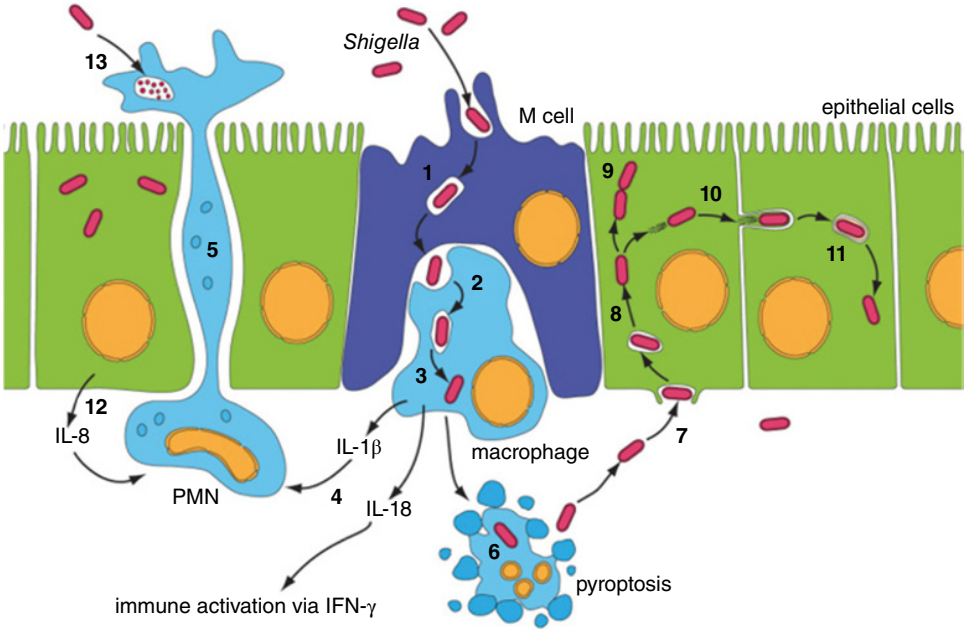


Figure 9.4 Infectious cycle of *Shigella* (Roerich-Doenitz 2013) modified from Schroeder and Hilbi (2008). Entry into the colonic epithelium is mediated in two ways: M-cell membrane ruffling and epithelial barrier destabilization. Entry via M-cells is achieved through membrane ruffling (1), and the bacillus is then transported to the M-cell pocket, where it is endocytosed by resident macrophages (2). Epithelial barrier destruction is mediated by pro-inflammatory (IL-1) and chemotaxic cytokines (IL-8). IL-8 produced by neighboring epithelial cells recruits PMN leukocytes (12), which travel from the basolateral to the apical colonic epithelium, destabilizing the junctions between the epithelial cells and allowing further invasion of *Shigella* (5). Induction of pyroptotic macrophage death occurs after *Shigella* escape from the phagocytic vacuole (3 and 6). Caspase-1, when activated, cleaves and activates IL-1β and IL-18, leading to the release of these pro-inflammatory cytokines (4) (Jennison and Verma 2004). Uptake of *Shigella* is a macropinocytic process at the basolateral membrane of epithelial cells (7). Stimulation of Rho-family GTPases triggers actin polymerization and then depolymerization, forming filopodial and lamellipodial extensions of the epithelial membrane, leading to engulfment of the bacilli. Lysis of the macropinocytic vacuole allows *Shigella* to gain access to the epithelial cytoplasm, where it rapidly multiplies, escapes autophagy, and fragments the Golgi (8 and 9). Exploitation of the epithelial actin assembly machinery allows *Shigella* to move both intra- and intercellularly (9). Protrusions mediated by bacilli are actively endocytosed by the clathrin-mediated endocytic pathways at intercellular junctions, and the double membrane vacuole is lysed to give *Shigella* access to the neighboring cells cytoplasm (11). PMN leukocytes eventually eliminate *Shigella* infection from the colonic epithelium (13). *Source:* Mattock and Blocker (2017). Licensed under CC BY.

mediates polar adhesion to epithelial cells, and once inside the host cell, the bacterium uses the Ics proteins to move within the cytoplasm and spread to adjacent epithelial cells (Figure 9.4, step 9). To mediate this function, surface-bound IcsA localizes to one pole of the bacterium binding and activating N-WASP (neural Wiskott-Aldrich syndrome protein) and the Arp2/Arp3 complex. Activation of the complex allows for nucleation of actin at that site. Once actin polymerization at one pole of the bacterium has begun and progresses, it propels the organism through the host cell cytoplasm. Polar positioning of IcsA is mediated by PhoN2, an apyrase (adenosine diphosphatase). IcsB camouflages IcsA to protect it from autophagic recognition and destruction. IcsP (SopA), a serine protease that cleaves IcsA, modulates the actin-based motility.

Virulence A (VirA) Protein

VirA, also encoded by a gene on the large virulence plasmid, is secreted by the T3SS. VirA is a cysteine protease that targets α-tubulin and assists in the mediation of intracellular motility by causing disassembly of microtubules.

Shigella Enterotoxin 1 and 2 (ShET1 and 2)

ShET1 is encoded by *set1A* and *set1B* genes on the chromosome (described below), and produced only by *S. flexneri*. ShET2 is encoded by a gene (*ospD3* or *sen*, for *Shigella* enterotoxin) on the large virulence plasmid, and is produced by all serotypes. ShET1 and 2 lack homology. ShET1 is an A1-B5 55-kDa holotoxin presumably secreted via the Sec pathway and Type II secretion, whereas ShET2 is a 63-kDa protein secreted by the T3SS. ShET1 and 2 mediate fluid secretion through their effects on the jejunum that precedes dysentery. Both increase the short circuit current in rabbit and mouse jejunal mucosa mounted in Ussing chambers, a model for ion exchange. Because watery diarrhea but not dysentery occurs in almost all patients, the enterotoxins are considered important virulence factors. Hypothetically, increased

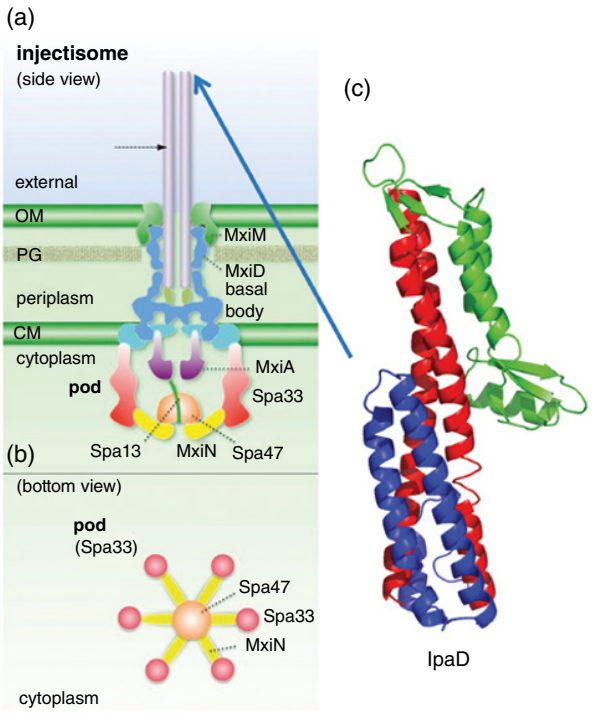


Figure 9.5 The proposed architecture of the *Shigella* injectisome. In panel A, a side view of the injectisome is shown with the major basal body components indicated (in blue hues). Beneath the bacterial inner membrane is the cytoplasmic sorting platform with the main components indicated. At the top is the extracellular needle comprising a polymer of MxiH. Panel B shows a bottom view of the sorting platform depicting the sixfold symmetry of this complex. The tip complex is not indicated here, but panel C shows the crystal structure of IpaD, which is the main component of the tip complex. IpaD resides as the most distal structure from the surface of the bacterium – presumably as a pentamer in the nascent injectisome. *Source:* Panels A and B are adapted from (Hu et al. 2015). Copyright National Academy of Sciences. Panel C is PDB ID: 2J0O, (Johnson et al. 2007). *Source:* Reproduced by permission of Picking and Barta (2020).

fluid secretion in the jejunum facilitates bacterial access to the colon, the site of invasion.

Proteins Encoded by Genes on Chromosomal Pathogenicity Islands (PAIs)

Five different chromosomal PAIs have been described in *Shigella* spp., and their prevalence varies by species, serotype, and isolate. These PAIs include four named *Shigella* PAIs (SHI; SHI-1, SHI-2, SHI-3, and SHI-O) and one called *Shigella* resistance locus (SRL). SHI-1 contains *sigA*, *pic*, and *set* genes that encode, respectively, a serine protease enterotoxin (SigA), a serine protease/mucinase (Pic), and *Shigella* enterotoxin 1 (ShEt1). SigA is a cytopathic enterotoxin, Pic causes mucus permeabilization, and ShET1 induces intestinal fluid accumulation. SHI-2 and SHI-3 contain copies of the genes for production of aerobactin (*iucA-D*) and a receptor for the iron-siderophore complex (*iutA*). SHI-2 is contained within all strains of *S. flexneri*, whereas SHI-3 is found only in *S. boydii*. In addition, SHI-2 contains novel open reading frames (ORFs) involved in attenuating inflammation. SHI-O contains genes for modification of the O antigens and serotype conversion (*gtrA*, *gtrB*, *gtrV*; glucosyltransferases and flippase). SRL contains genes encoding for iron acquisition (*fecA-E*, *fecI,* and *fecR*; ferric dicitrate uptake), aspartate metabolism (novel ORFs; aspartate racemase and transporter), and resistance to antibiotics. SRL genes encode resistance to tetracycline (*tetA-D*, and *tetR*; efflux system), chloramphenicol (*cat*; chloramphenicol acetyltransferase), ampicillin (*oxa-1*; β-lactamase), and streptomycin (*aadA1*; adenylyltransferase).

Shiga Toxin (Stx)

With rare exceptions, *S. dysenteriae* serotype 1 is the only member of the genus that contains the genes for Stx production. As in essentially all Shiga-toxin-producing *E. coli* (STEC) except edema disease strains, the Stx genes in *S. dysenteriae* are carried on the chromosome. However, unlike STEC, *S. dysenteriae* serotype 1 does not carry intact Stx-converting phages. This is thought to be the result of loss of essential phage genes caused by transposition and recombination events. A human isolate of *S. sonnei* isolate with an integrated bacteriophage carrying Stx genes has been detected. Experimentally, other *S. sonnei* strains could be lysogenized with the phage and thereby produce Stx. The *stx* DNA sequence in this phage was more closely related to stx_2 than stx_1. These findings suggest that Stx-positive *S. sonnei* and other Stx-positive species of *Shigella* may possibly emerge as important pathogens in the future.

The production of Stx is iron regulated (by way of Fur; see below), with more toxin being produced in conditions of low iron concentration. Although the other virulence factors described above are important in the pathogenesis, the virulence of a particular *S. dysenteriae* strain is also directly related to the amount of Stx produced. As described for STEC, Stx causes severe vascular lesions in the colonic mucosa, renal glomeruli, and other organs that result in hemorrhagic colitis and, in some cases, HUS. The molecular, cellular, and pathophysiologic mechanisms of Stx toxicity and the corresponding clinical effects are described in detail in Chapter 6.

Regulatory Genes

Shigellae possess a number of genes whose activation results from specific environmental cues. The primary elements of the regulatory cascade that sense and respond to environmental changes are encoded on the chromosome. A change in pH, temperature (increase to 37 °C), or change in

osmolarity would be sensed by chromosomally encoded sensor proteins (e.g. VirR) which would activate expression of *virF* on the virulence plasmid. VirF then activates transcription of *icsA* and *virB*, also located on the virulence plasmid. VirB activates T3SS assembly genes (*ipa*, *mxi*, *spa*), and "first-set" effectors. The "first-set" effectors lead to increased transcription of "second-set" T3SS effectors. In this manner, an environmental change indicating presence of the bacterium in the host would initiate a cascade of sequential gene activation steps culminating in bacterial invasion of the colonic epithelium. Some genes that are activated in this process serve as negative regulators, preventing wasted energy usage by the bacteria as they invade the host.

The genes that encode Fur (ferric uptake regulator) and an RNA polymerase containing RpoS are additional regulatory genes. The Fur protein "senses" available iron concentration, and when it is low (as it would be inside the host, because most iron is bound to host iron-binding proteins) it activates the synthesis and secretion of aerobactin, ShET1, and Shiga toxin (see above). RNA polymerase containing RpoS preferentially transcribes genes effecting acid tolerance (survival at pH 5), providing protection against the acidic pH of the gastric lumen.

Variability

Shigellae are serogrouped and serotyped solely on the basis of the O-antigen, because no H or K antigens are expressed. Serogroup determination is essential for the identification of the species of *Shigella*, and within three of the four species, there are multiple serotypes (see Table 9.1).

Ecology

Reservoir

The distribution of *Shigella* is essentially limited to the intestinal tracts of humans and large, captive primates in which shigellosis naturally occurs, and sewage. Humans are the reservoir host; there is no evidence that the disease occurs naturally in nonhuman primates in the wild without prior contact with humans. Shigellae are consistently present in sewage, and accidental contamination of water supplies by sewage effluents often causes outbreaks of shigellosis in humans. In humans, *Shigella* is endemic throughout the world, although 99% of the cases occur in the developing world.

Transmission

The disease is transmitted by the fecal-oral route, but the infective dose is so small (only 9–90 cells) and the organisms survive long enough that fomites also play a role. *S. flexneri* serotype 4 is associated with periodontal disease of nonhuman primates; the mode of transmission is unknown, but it is assumed to be feces. Antimicrobial drugs, stress, or dietary changes promote risk by reducing colonization resistance (by decreasing the numbers of the competing normal flora), which may lead to disease in subclinically infected nonhuman primates or humans, or by lowering the oral dose needed for infection and subsequent disease. Studies have demonstrated that an important vehicle for transmission of shigellosis is the hand, which points out the importance of thorough hand-washing in disease prevention. *S. dysenteriae* serotype 1 survives for up to one hour in culturable form on human skin.

Clinical Disease

The classical disease begins as watery diarrhea, then after three to four days the feces becomes scant and contains blood and mucus. There is fever, abdominal pain, cramping, and straining. There may be malaise, myalgia, and anorexia.

The severity of the clinical course usually varies with the species. *S. sonnei* mainly causes a watery diarrhea, whereas *S. flexneri* and *S. dysenteriae* produce more severe signs (e.g. more pronounced blood in the stool with either *S. flexneri* or *S. dysenteriae* and systemic complications such as HUS or brain damage in the case of *S. dysenteriae*). In all forms of shigellosis, microscopic examination of the stool will reveal large numbers of leukocytes.

Shigellosis is characterized pathologically by a mucopurulent and hemorrhagic colitis. Microscopic abscesses may be seen in the colonic mucosa, involving the crypts or intercryptal regions. Crypt abscesses often slough, producing ulcers.

HUS may be seen in human patients infected with *S. dysenteriae* 1, mainly the result of the effects of Stx production. Reiter's syndrome characterized by arthritis, sometimes appears after the intestinal infection has passed. This syndrome also may be seen with the other bacterial infections: *Yersinia enterocolitica*, *Salmonella enterica* var. Typhimurium, *Klebsiella pneumoniae*, and *Campylobacter jejuni*.

Pathogenesis

Within 12 hours after ingestion, the bacteria multiply in the small intestine to concentrations of 10^7–10^8 viable cells per milliliter of luminal contents. Abdominal pain, cramping, and fever occur while the bacteria are localized in the small intestine.

The bacteria pass to the colon and invade the colonic mucosa by a series of steps (Figure 9.4). The events at the

molecular level are described above. Shigellae adhere to the surfaces of M cells, and are taken up into phagosomes. The bacteria escape from phagosomes, multiply in the cytoplasm, and move to adjacent enterocytes by causing the induction of actin cytoskeletal rearrangement at one pole of the organism. This propels the bacteria through the cytoplasm and allows contact to be made with adjacent epithelial cells. Spread to adjacent absorptive epithelial cells occurs by induction of endocytosis through the cell membranes of both cells. The bacteria continue to spread through the epithelial layer in this manner and the cycle continues to be repeated. Phagocytosis of bacteria by neutrophils and macrophages occurs, but the bacteria cause pyroptosis of macrophages, thereby reducing the effectiveness of the host defense and causing the release of IL-1β and IL-18, which exacerbate the inflammatory response. Neutrophils effect bacterial killing, but also cause tissue damage which enhances bacterial spread. Infiltration and death of neutrophils generates the lesion, purulent colitis. Bacteria are released by dead or dying epithelial cells and macrophages, and drain via the lymphatics into gut-associated lymphoid tissue, where they are ultimately killed.

In addition to the effects of inflammation and necrosis of the colonic mucosa, diarrhea is brought about by the activation of phospholipase C, leading to increases in intracellular calcium ions, activation of protein kinase C and subsequent phosphorylation of proteins of the chloride ion channels and those of the membrane-associated ion transport proteins involved in NaCl absorption.

Based on mutagenesis and animal model studies, ShET-1 and ShET-2 are thought to play a significant role in the causation of watery diarrhea; however, the mechanism of these effects is poorly understood.

Stx produced by *S. dysenteriae* serotype 1 causes apoptosis of vascular endothelial cells and activation of leukocytes with upregulation and expression of tumor necrosis factor-α (TNF-α). TNF-α induces increased expression of the Gb3 receptor for Stx, which increases the extent of endothelial toxicity and vascular damage. The vasculature in the mucosa of the colon is affected with Stx toxicity, causing thrombosis and infarction of the colonic mucosa. These effects exacerbate those of colonic inflammation and cause hemorrhagic colitis. Stx also may affect the vasculature in extraintestinal locations, for example, the kidneys and brain, resulting in the development of the HUS. The effects of Stx are described in more detail in Chapter 6.

Epidemiology

As noted above, among animals, the disease is seen almost exclusively in large, captive primates. Humans are the reservoir host. In developed countries, shigellosis is a disease of the disadvantaged and those having close contact in group settings. In the United States, shigellosis is also mainly a disease of children one to four years old, and is caused by *S. sonnei*. Outbreaks are often seen in preschool children and toddlers in day-care centers. Outbreaks are also seen in mental institutions. Large urban outbreaks have been seen in following accidental contamination of a city water supply by sewage effluents. Worldwide in humans, *S. flexneri* and *S. sonnei* are typically responsible for endemic shigellosis, whereas *S. dysenteriae* serotype 1 is responsible for the epidemic form of the disease.

Immunologic Aspects

Protection from bacillary dysentery is by specific secretory immunoglobulin in the intestinal lumen. These antibodies prevent adherence and subsequent uptake. Shigellae are serum-sensitive (i.e. they are susceptible to complement-mediated lysis), and they are susceptible to killing by neutrophils. As a result, shigellae do not typically cause septicemia.

Whether nonhuman primates are resistant to reinfection following recovery from shigellosis is not known. Reinfection or exacerbation does occur in humans in stressful situations such as in prisoner-of-war camps. Bacterins given orally or parenterally have been ineffective. Some protection has been demonstrated following vaccination with avirulent, live oral vaccines, but these are not universally available.

Laboratory Diagnosis

Fecal samples from the live animal, or in the case of necropsy, colonic samples, should be plated onto a selective medium that is less inhibitory than media for isolation of salmonellae. There is no reliable enrichment medium for all shigellae; however, GN broth is preferred for general use. Selenite broth and tetrathionate broth, both primarily intended for enrichment of *Salmonella*, are usually too inhibitory. For the optimal isolation of *Shigella*, both a medium of low selectivity, for example, MacConkey agar, and a more selective agar medium, such as xylose-lysine-deoxycholate (XLD) agar should be used. Hektoen enteric medium is a suitable alternative to XLD (Figure 9.6), whereas *Salmonella-Shigella* (SS) agar is often too inhibitory for some strains of shigellae, for example, *S. dysenteriae* serotype 1. *Shigella* will not grow on more inhibitory media primarily intended for isolation of *Salmonella*, for example, brilliant green agar.

Shigellae appear as lactose-nonfermenting colonies on lactose-containing media, although some species (*S. sonnei*

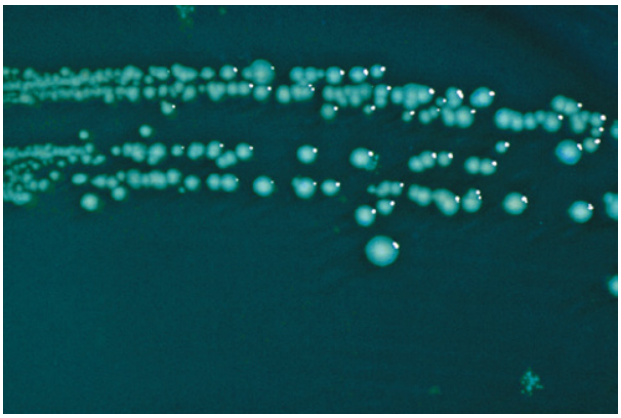


Figure 9.6 *Shigella boydii*, isolated colonies on Hektoen enteric (HE) agar. Description: This photograph depicts the colonial morphology displayed by *S. boydii* bacteria cultivated on an HE agar surface; colonies of *S. boydii* bacteria grown on HE agar display a raised, green, and moist appearance. *Source:* US Department of Health and Human Services, Centers for Disease Control and Prevention, Public Health Information Library, PHIL_6688.

and *S. boydii* serotype 9) ferment this sugar after several days of incubation. Suspicious colonies are tested directly with shigellae-specific antisera or inoculated into differential media and then tested with antisera.

Primers have been designed to amplify (by the polymerase chain reaction) DNA targets specific for shigellae. This assay has been used to detect and identify members of this genus. However, currently there are no FDA-approved nucleic acid detection methods for the clinical diagnosis of *Shigella* infections.

Treatment, Control, and Prevention

Treatment of shigellosis involves nursing and supportive care. Antimicrobial agents are indicated in serious cases but not routinely because their use in an animal facility selects for resistant strains. The first choices are ampicillin or trimethoprim-sulfonamide; organisms resistant to these have been treated with fluoroquinolones, but resistance to the latter drugs also has increased and is of especial concern, given the implications for human health.

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10

Pasteurellaceae

Avibacterium, *Bibersteinia*, *Mannheimia*, and *Pasteurella*

William B. Crosby and Amelia R. Woolums

The family Pasteurellaceae contains many organisms that contribute to animal disease. Many are normal inhabitants of the upper respiratory and gastrointestinal tracts and cause disease opportunistically when other factors enhance their ability to advance to deeper levels of these systems. Others are infrequently isolated from normal individuals and cause severe disease when animals are exposed to these agents.

There have been many changes in the taxonomy of the family Pasteurellaceae since the third edition of this textbook was written, and more changes will likely occur in the future. At the time of this writing, there are over 30 genera included in the family: *Actinobacillus*, *Aggregatibacter*, *Avibacterium*, *Basfia*, *Bibersteinia*, *Bisgaardia*, *Caviibacterium, Chelonobacter*, *Conservatibacter, Cricetibacter, Frederiksenia, Gallibacterium*, *Glaessaria, Haemophilus*, *Histophilus*, *Lonepinella*, *Mannheimia*, *Mesocricetibacter, Muribacter, Necropsobacter*, *Nicoletella*, *Otariodibacter, Pasteurella*, *Phocoenobacter*, *Rodentibacter, Seminibacterium, Spirabiliibacterium, Terrahaemophilus, Testudinibacter, Ursidibacter, Vespertiliibacter,* and *Volucribacter*. Bacteria from genera that contribute to important diseases of animals are described here and in Chapters 11 and 12. At this time bacteria in the genera not listed in Table 10.1 do not appear to be importantly associated with animals. The source of agents in the genera *Basfia*, *Chelonobacter*, *Lonepinella, Necropsobacter, and Nicoletella* are listed in Table 10.1 but these agents are not discussed further here.

All the members of the family Pasteurellaceae are gram-negative coccobacilli. They are facultative anaerobes, and typically oxidase positive, which sets them apart from members of the family Enterobacteriaceae.

Discussed in this chapter are the genera *Avibacterium*, *Bibersteinia*, *Mannheimia*, and *Pasteurella*, whose members play an important role in diseases of several animal species (Table 10.1). Briefly discussed is also *Ornithobacterium rhinotracheale*, a bacterium in the family Weeksellaceae that phenotypically and clinically resembles some strains of *Avibacterium paragallinarum*.

Descriptive Features

Morphology and Staining

Members of the genera *Avibacterium*, *Bibersteinia*, *Pasteurella*, and *Mannheimia* are gram-negative coccobacilli measuring 0.2 μm by up to 2.0 μm (Figure 10.1). Bipolarity, that is, the staining of only the tips of cells, may be demonstrable with polychrome stains (e.g. Wright's stain).

Structure and Composition

Capsule

Several species within these genera produce capsules, which contain acidic polysaccharides. The capsule plays many roles, the most important of which are interference with phagocytosis (antiphagocytic) and the protection of the outer membrane from the deposition of membrane attack complexes generated by activation of the complement system. The amount of capsule produced is proportional to the amount of available iron. *In vivo*, where the amount of available iron is low, the amount of capsule formed is less, but sufficient to protect the microorganism from phagocytosis and complement-mediated lysis. Capsules may be made of substances such as hyaluronic acid, heparin, or chondroitin, which are similar or identical to host tissue components, and are thus poorly antigenic; they also bind complement components poorly, and are therefore antiphagocytic.

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Table 10.1 Some members of the family Pasteurellaceae of importance in animal disease.

Genus and species	Associated disorder or site
Avibacterium avium (formerly *Pasteurella avium*)	Normal respiratory flora of chickens
Avibacterium endocarditidis	Endocarditis in chickens
Avibacterium gallinarum[a] (formerly *Pasteurella gallinarum*)	Respiratory disease in poultry
Avibacterium paragallinarum[a] (formerly *Haemophilus paragallinarum*)	Infectious coryza in chickens
Avibacterium volantium (formerly *Pasteurella volantium*)	Normal respiratory flora of chickens
Basfia succiniciproducens	Normal rumen flora
Bibersteinia trehalosi[a]	Respiratory disease in ruminants, bacteremia in lambs
Chelonobacter oris	Various infections in tortoises
Lonepinella koalarum	Tannin degrading bacterium in normal koala feces
Mannheimia glucosida	Mastitis in sheep, respiratory disease in ruminants
Mannheimia granulomatis	Respiratory and other infections in deer, ruminants, and hares; skin disease (lechiguana) in cattle; mastitis in sheep
Mannheimia hemolytica[a]	Respiratory disease in ruminants, mastitis in sheep
Mannheimia ruminalis	Normal rumen flora, mastitis in sheep
Mannheimia varigena	Respiratory infections and bacteremia in ruminants and swine, mastitis in cattle
Necropsobacter rosorum	Isolated from birds, significance uncertain
Nicoletella semolina	Respiratory disease in horses
Pasteurella aerogenes	Gastroenteritis in swine, abortion in swine
Pasteurella caballi	Respiratory disease in horses
Pasteurella canis[a]	Respiratory and oral infections in dogs, canine bite wound infections
Pasteurella dagmatis	Normal respiratory flora of birds, respiratory and oral cavity infections in dogs and cats, canine or feline bite wounds
Pasteurella langaaensis (formerly *Pasteurella langaa*)	Normal respiratory flora of birds
Pasteurella lymphangitidis	Bovine lymphangitis
Pasteurella mairii	Abortion in swine, bacteremia in piglets
Pasteurella multocida subspecies gallicida[a]	Fowl cholera-like disease in birds
P. multocida subspecies multocida[a]	Fowl cholera, respiratory disease in ruminants and swine, septicemia in ruminants, mastitis in ruminants, respiratory disease and other infections in rabbits, respiratory and oral cavity infections in dogs and cats, canine and feline bite wound infections
P. multocida subspecies septica[a]	Fowl cholera-like disease in birds, respiratory and oral cavity infections in dogs and cats, canine and feline bite wound infections, respiratory and other infections in bats
Pasteurella pneumotropica[a]	Respiratory disease in rodents
Pasteurella skyensis	Bacteremia in farmed Atlantic salmon
Pasteurella stomatis[a]	Respiratory and oral cavity infections in dogs and cats, canine and feline bite wound infections
Pasteurella testudinis	Infections in tortoises and turtles
Phocoenobacter uteri	Isolated from uterus of harbor porpoise, significance uncertain

[a] Organisms of particular importance, as defined by relative frequency of identification and relative severity of disease associated with infection.

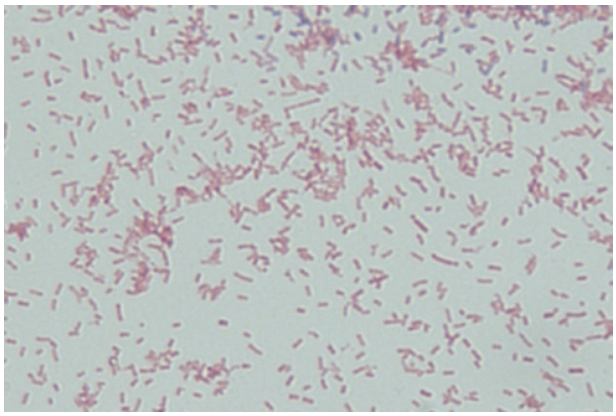


Figure 10.1 *Mannheimia haemolytica,* gram stain.

Cell Wall

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, elicits an inflammatory response following binding to LPS-binding protein (a serum protein); the complex is then transferred to CD14 on the surface of leukocytes. The CD14-LPS complex then binds to Toll-like receptor 4 (TLR4) on the surface of macrophages and other leukocytes, triggering the release of pro-inflammatory cytokines and inducing local or systemic inflammatory responses (Figure 10.2). LPS also acts synergistically with the *Mannheimia haemolytica* leukotoxin by increasing expression of the leukotoxin receptor, CD18, on the surface of macrophages and other leukocytes.

Cellular Products of Medical Interest

Adhesins

Members of the family Pasteurellaceae produce adhesins, which facilitate attachment to host cells. A type 4 fimbria that acts as an adhesin has been described for avian strains of *Pasteurella multocida*, and an adhesin termed Adh1 is expressed by *M. haemolytica*. As with other microorganisms, the expression of adhesins probably depends upon environmental cues. That is, adhesins are expressed while the microorganism inhabits an epithelial surface, but repressed when the microorganism is inside the host, where adherence to a phagocytic cell could be disastrous. The hyaluronic acid capsule of type A strains *P. multocida* serves as an adhesin.

Toxins

Bibersteinia, *Mannheimia*, and *Pasteurella* produce a number of proteins with toxic activity. At least two of these are important in the pathogenesis of disease: the leukotoxin and the *P. multocida* toxin (PMT).

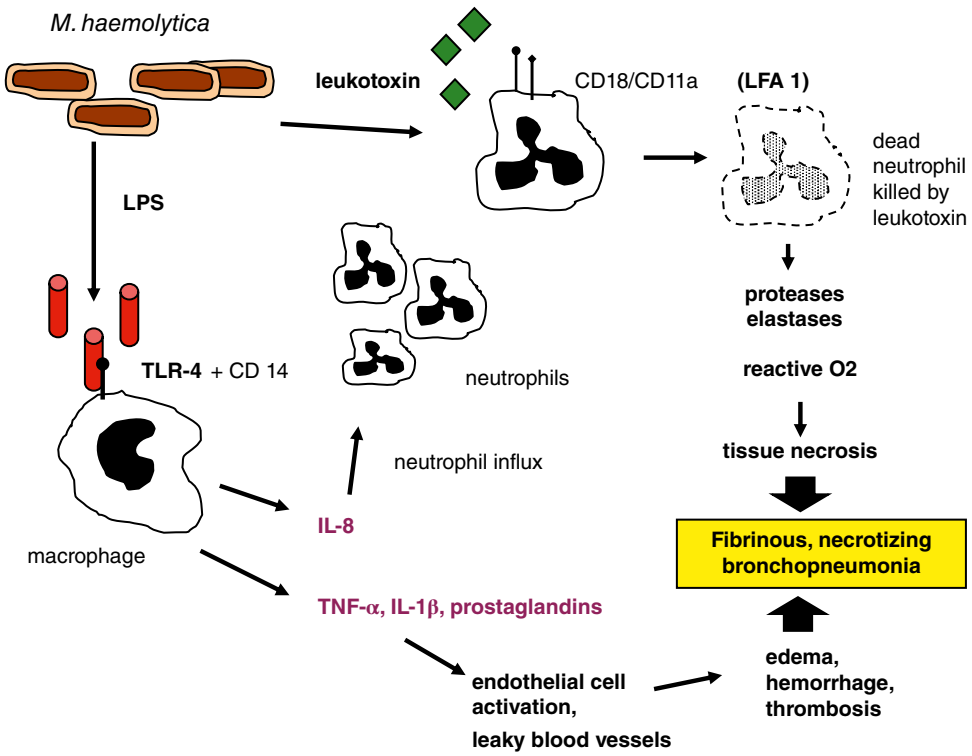


Figure 10.2 Events in the pathogenesis of fibrinous bronchopneumonia due to *Mannheimia haemolytica*. *Source:* Reprinted from Snyder and Credille (2020) Copyright (2020). With permission from Elsevier.

1) Leukotoxin. Leukotoxin is an RTX toxin (for "repeats in toxin," named after the common feature of repeats in glycine-rich sequences within the protein) produced by most species of *Mannheimia* and *Bibersteinia trehalosi*. Though similar in amino acid sequence to other members of the RTX family, leukotoxin specifically affects bovine leukocytes and erythrocytes; cells of non-ruminant species are resistant to the effects of leukotoxin. The effect on erythrocytes probably has little importance *in vivo*, but is responsible for the hemolysis observed when most *Mannheimia* and *B. trehalosi* isolates are grown on blood agar plates (Figure 10.3c). Leukotoxin binds to CD18 (a $ß_2$ integrin), which is expressed on the surface of bovine leukocytes (neutrophils, macrophages, platelets, and lymphocytes). At low concentrations, leukotoxin causes target cell activation; at higher concentrations, apoptosis is initiated; and at very high concentrations, necrosis is produced. Upon contact with leukotoxin, neutrophils degranulate, releasing potent enzymes that induce as well as aggravate inflammation (Figure 10.2); macrophages release pro-inflammatory cytokines (magnified in the presence of LPS); lymphocytes undergo apoptosis and necrosis; and platelets respond by increased adhesion. In addition, tissue mast cells degranulate, releasing vasoactive amines. In summary, leukotoxin drives an inflammatory response that can be tissue-destroying. The importance of leukotoxins in the pathogenesis of ruminant pneumonia due to *M. haemolytica* is evidenced by the fact that strains of *M. haemolytica* in which the gene for leukotoxin is disrupted produce much less severe disease than strains that can express leukotoxin normally.
2) PMT: PMT is a dermonecrotic toxin encoded by the *toxA* gene on a lysogenic bacteriophage carried by toxigenic *P. multocida* strains. PMT is produced by capsule type D or A isolates of *P. multocida* that contribute to atrophic rhinitis in swine; PMT activates signal transduction pathways in osteoclasts, leading to uncontrolled activity of these cells, and thus bone resorption leading to nasal turbinate bone loss. Recently, capsule types A and D from cases of pneumonia in sheep and goats have also been found to produce PMT. In addition to being directly cytotoxic, PMT is also a mitogen, and so can activate lymphocytes, possibly leading to dysregulated immune responses by the infected host.

Iron Acquisition

Because iron is an absolute growth requirement, microorganisms must acquire this substance if they are to exist within the host. Expression of numerous genes is upregulated in bacteria grown *in vitro* under iron-poor conditions, and many of the resulting proteins contribute to virulence. For example, *Pasteurella* and *Mannheimia* bind transferrin-iron complexes by virtue of iron-regulated outer membrane proteins (IROMPs) expressed under iron-poor conditions (such as transferrin-binding proteins, or Tbps). Iron is acquired from the transferrin-iron complexes that bind to the surface of the microorganism. *P. multocida* also binds heme and hemoglobin, which are other sources of iron.

Outer Membrane Vesicles (OMVs)

Pasteurellaceae and other gram-negative bacteria can release small lipid vesicles from their outer membranes, which contain enzymes, structural molecules, and other proteins. When isolated in laboratory experiments, OMVs have been shown to induce inflammatory and immune response by exposed cells or animals, indicating that they may contribute to pathogenesis and/or immunity in natural infection. Experimental vaccines containing OMVs are under evaluation.

Growth Characteristics

Avibacterium, Bibersteinia, Mannheimia, and *Pasteurella* grow best in the presence of serum or blood. After overnight incubation (35–37 °C), colonies are up to 2 mm in diameter, clear to grayish, and smooth or mucoid, with *P. multocida* tending to be larger and more mucoid than *M. haemolytica* (Figure 10.3a, b). *Mannheimia haemolytica*, *M. glucosida*, and some isolates of *B. trehalosi* produce hemolysis on ruminant blood agar (Figure 10.3c). All are gram-negative, nonmotile coccobacilli. They are facultative anaerobes, typically oxidase positive, reduce nitrates, and attack carbohydrates fermentatively. The pattern of carbohydrates fermented can be used to differentiate individual species.

Pasteurellaceae vary in their requirement for ß-nicotinamide adenine dinucleotide (ß-NAD, also known as V factor), with *Avibacterium avium*, *Avibacterium volantium*, and some isolates of *A. paragallinarum* requiring ß-NAD and the remaining members of these genera characterized to date as not requiring ß-NAD. *A. paragallinarum* isolates not requiring ß- NAD are designated as biovar 2 isolates, whereas isolates requiring ß-NAD are designated as biovar 1. Phenotypically, *Avibacterium gallinarum*, *Avibacterium endocarditidis*, and biovar 2 isolates of *A. paragallinarum* can be difficult to differentiate from *Gallibacterium anatis*, but differentiation is possible based on differences in the ortho-Nitrophenyl-β-galactoside (ONPG) reaction and fermentation of glycerol, D-mannitol, D-galactose, and maltose. Biovar 2 isolates of *A. paragallinarum* can also be difficult to differentiate phenotypically from *O. rhinotracheale*, another bacterial pathogen also associated with respiratory disease in poultry (see below).

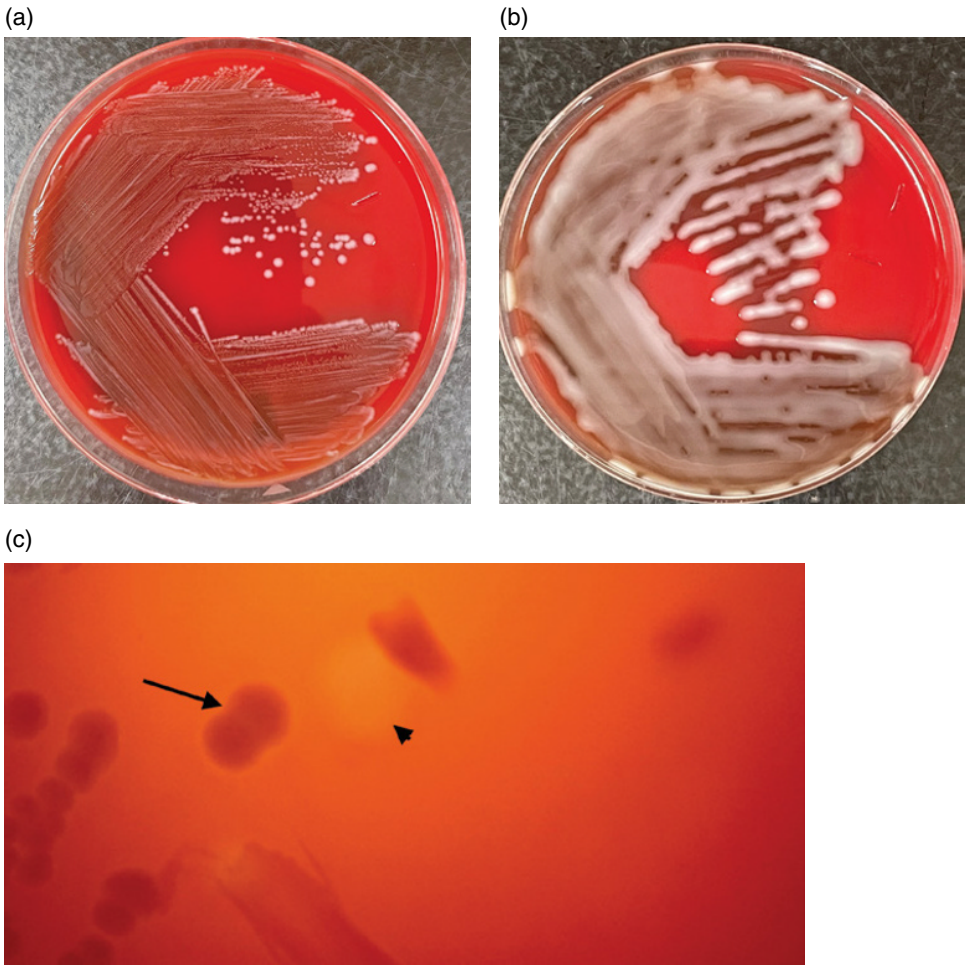


Figure 10.3 (a) *Mannheimia haemolytica* on blood agar; (b) *Pasteurella multocida* on blood agar, demonstrating more mucoid phenotype compared to *M. haemolytica*. (c) Typical hemolysis around (arrow) and under (arrowhead) *M. haemolytica* colonies on blood agar. *Source:* Photo (c) courtesy of Dr. Matthew A. Scott.

Routine diagnostic differentiation of these agents is most often accomplished by polymerase chain reaction (PCR) testing or MALDI-TOF MS) (described below).

Pasteurella aerogenes and *Actinobacillus rossii*, both of which can be associated with reproductive tract infections in sows, can be difficult to differentiate based on phenotypic and even genotypic testing. *B. trehalosi* and *P. multocida* can both overgrow *M. haemolytica* in culture (and possibly *in vivo*), a fact that may be relevant to accurate diagnosis of cases of ruminant respiratory disease.

Resistance

Cultures die within one or two weeks. Disinfectants, heat (50 °C for 30 minutes), and ultraviolet light are promptly lethal; however, *P. multocida* can survive for months in bird carcasses.

P. multocida and *M. haemolytica* have become increasingly resistant to penicillins, tetracyclines, and macrolides, with multidrug resistant isolates being found with increasing frequency in bovine pneumonia. Similarly, surveys of *P. multocida* isolates from other host species, including dogs, swine, and poultry, have in some cases identified a substantial proportion of isolates to be resistant to one or more antimicrobials. The gene encoding tetracycline resistance is unique to *Pasteurella* and *Mannheimia*. Resistance-encoding genes are frequently associated with mobile genetic elements such as R plasmids and integrative conjugative elements (ICE). ICE discovered in *P. multocida* and *M. haemolytica* have been shown *in vitro* to transfer to susceptible strains between genera both within and outside of the Pasteurellaceae, including *Escherichia coli*.

Biofilm formation can occur involving multiple members of Pasteurellaceae, leading to decreased susceptibility to antimicrobials.

Variability

P. multocida consists of five capsular serogroups (A, B, D, E, F) and 16 somatic serotypes (1–16). Recently, PCR tests to identify somatic serotypes have been developed.

M. haemolytica consists of 12 capsular types (1, 2, 5–9, 12–14, 16, 17), with types 1 and 6 most often associated with pneumonia in cattle, and type 2 most often associated with pneumonia in sheep. *B. trehalosi* consists of four capsular serotypes (3, 4, 10, 15). Recently, whole genome sequencing has facilitated the classification of *M. haemolytica* into two major genotypes. Genotype 1 is more commonly isolated from respiratory tracts of healthy cattle, whereas Genotype 2 is more commonly isolated from respiratory tracts of cattle with bronchopneumonia or pleuropneumonia. Though both specific serotypes and genotypes of *M. haemolytica* are each associated with diseased lungs in cattle, the relationship between serotype and genotype is still under investigation.

A. paragallinarum is also encapsulated, with evidence suggesting that there may be differentiation in capsule composition similar to that seen among different capsular types of *P. multocida*. There are three serotypes of *A. paragallinarum* (Page serotypes A, B, and C).

Ecology

Reservoirs

Bacteria in the genera *Avibacterium, Bibersteinia, Mannheimia*, and *Pasteurella* are carried on mucous membranes (most commonly in the oro- or nasopharyngeal region) of susceptible host species. Carriage may be widespread, as with *P. multocida* in carnivores, or exceptional, as with avian cholera-producing strains in birds or hemorrhagic septicemia-producing strains in ruminants. For some disease syndromes associated with these genera, one host species may serve as a reservoir for another. For example, domestic cattle or sheep can serve as a reservoir for *M. haemolytica* which can be transferred to highly susceptible bighorn sheep, leading to death of large numbers of this wildlife species.

Transmission

Infection is by inhalation, ingestion, or bites or scratch wounds. Many infections are probably endogenous. In bovine hemorrhagic septicemia and avian cholera, environmental contamination contributes to indirect transmission.

Pathogenesis

Mechanisms

In general, there are three manifestations of disease commonly induced by bacteria in the genera *Avibacterium*, *Bibersteinia, Mannheimia*, or *Pasteurella*: respiratory tract disease, septicemia, and trauma-associated conditions:

1) Respiratory tract involvement is either pneumonia or pleuropneumonia, or upper respiratory tract disease (e.g. atrophic rhinitis in swine, infectious coryza in chickens). Pneumonia or pleuropneumonia is seen most frequently in ruminants and is usually associated with *M. haemolytica*, *P. multocida*, or *B. trehalosi*. Environmental stress (e.g. recent shipment or weaning), virus infection, or other bacterial infections (e.g. mycoplasma) usually precede pneumonia, and are thought to decrease host defenses of the tract, allowing commensal bacteria living in the upper tract (*M. haemolytica, P. multocida*, or *B. trehalosi*) to infect the lung. *M. haemolytica* or *B. trehalosi* from the upper respiratory tract is deposited in the lung, and secrete leukotoxin and, along with LPS from their cell walls, initiate an intense inflammatory response with fibrin deposition and thrombosis (Figure 10.2). Though *P. multocida* has not been shown to produce leukotoxin, deposition of the organism in the lung initiates an inflammatory response due to its LPS. Capsule, iron-scavenging abilities, and perhaps binding of fibrinogen enhance survival of the bacteria within the lesion.
   A. paragallinarum causes outbreaks of respiratory disease in chickens (infectious coryza). *P. multocida* in combination with *Bordetella bronchiseptica* (Chapter 11) causes progressive atrophic rhinitis in swine. In this disease, *B. bronchiseptica* first adheres to the nasal mucosa and secretes a toxin called dermonecrotic toxin, which mildly damages the epithelium. Capsule type D or A strains of *P. multocida* adhere to this mildly damaged epithelium (these strains do not readily adhere to normal epithelium) and secrete PMT. It is PMT that is responsible for the destruction of the nasal turbinates. The action of *B. bronchiseptica* toxin alone, without the participation of *P. multocida*, results in a mild, nonprogressive turbinate hypoplasia.
2) Bacteremic and septicemic disease can be produced by *P. multocida* infection in ruminants and avian species. Certain *P. multocida* strains, usually of serotype B or E, cause hemorrhagic septicemia, an important disease of cattle and water buffalo in Asia, Africa, and the Middle East. Sporadic cases have occurred in other regions and in other host species. *P. multocida* and *B. trehalosi* can

cause septicemia in sheep, and *P. multocida* causes avian cholera. Why these strains and not others in the family Pasteurellaceae are able to cause bacteremia and septicemia is not completely understood. However, characteristics of their capsules and iron-scavenging mechanisms may be relevant. Outer membrane proteins on avian strains of *P. multocida* appear to play some role by decreasing phagocytosis. Signs and outcomes of these diseases are attributable to severe systemic inflammatory response and multi-organ infection.

3) Trauma-related conditions are those in which "mouth" microorganisms (*P. multocida* is the most common) are inoculated into the site of infection. Examples include canine or feline bite wounds and infections caused by dogs and cats licking compromised sites (e.g. surgical wounds).

Pathology

Lesions vary with site of infection, virulence of strains, and host resistance. In septicemias, vascular damage results in hemorrhage and fluid loss but little cellular inflammatory response. Focal necrosis in parenchymatous organs or ulcerations of mucous membranes may occur. Mammals develop generalized hemorrhagic lymphadenopathy. In chronic fowl cholera, caseopurulent inflammation occurs in joints, middle ear, ovaries, or wattles.

In ruminant pneumonias due to *Mannheimia*, *Bibersteinia*, and *Pasteurella*, gross lesions are those of bronchopneumonia or pleuropneumonia. *M. haemolytica* infection of cattle can induce a severe fibrinous necrotizing pleuropneumonia, with exudation of proteinaceous fluid in the pleural space. *B. trehalosi* can also cause necrotizing pleuropneumonia in cattle or small ruminants; infection with *B. trehalosi* or *P. multocida* can also cause bronchopneumonia without pleural reaction. Microscopically, the inflammatory response mirrors the gross lesions, with massive neutrophil infiltration and necrosis, hemorrhage, fibrin deposition, and thrombosis seen in acute *M. haemolytica* or *B. trehalosi* infection. In contrast, infiltration of neutrophils in airways and alveoli (purulent bronchopneumonia) without tissue necrosis and fibrin deposition is more commonly seen in pneumonia associated with *P. multocida* and some *B. trehalosi* cases.

Atrophic rhinitis of pigs (see also under *B. bronchiseptica*, Chapter 11) is a chronic rhinitis accompanying disturbed osteogenesis adjacent to the inflamed areas. Increased osteoclastic and diminished osteoblastic activity destroys the turbinates and bones of the snout, resulting in distortions of facial structures. Histologically, fibrous tissue replaces osseous tissue. Bone atrophy is accompanied by inflammation of varying acuteness.

The pathology of lesions that are "mouth" related is unremarkable, with neutrophils predominating.

Disease Patterns

Cattle

Pneumonia

Infection with *M. haemolytica* or *P. multocida*, contributes to the development of "shipping fever," also known as the "bovine respiratory disease complex" (BRDC). Bronchopneumonia or fibrinous pleuropneumonia (Figure 10.4) is seen when cattle, especially recently weaned calves, are transported, assembled, and handled under stressful conditions. The onset, usually one to two weeks after shipment, is marked by fever, inappetence, and listlessness. Respiratory signs (nasal discharge, cough) are inconsistently present. At more advanced stages, the fever may drop, but respiratory distress will be obvious. Abnormal lung sounds may be detected, especially over the apical lobes, which are first and most severely affected.

P. multocida is commonly isolated from young dairy calves with pneumonia, which can occur either in an endemic pattern (enzootic calf pneumonia) or in epidemics (epizootics). It is likely that other insults, such as primary viral respiratory infection, environmental factors such as poor air quality and crowding, and inadequate host immunity all enhance the ability of *P. multocida* to advance into the lung and cause disease. Serogroup A isolates of *P. multocida* are most commonly associated with ruminant respiratory disease in North America and Europe.

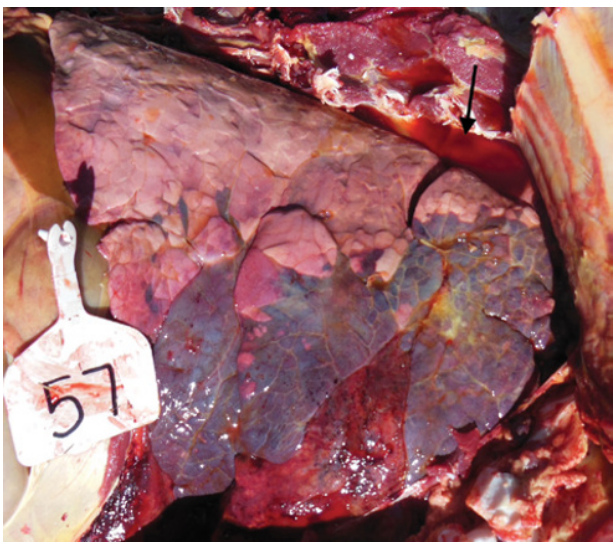


Figure 10.4 Bovine lung, fibrinous pleuropneumonia due to *Mannheimia haemolytica* infection. Note dark purple consolidated ventral lung, and serohemorrhagic fluid in pleural cavity (arrow). *Source:* Photo taken by Dr. Danielle Doyle.

Hemorrhagic Septicemia

Hemorrhagic septicemia is an acute systemic infection with *P. multocida*, most commonly serotype B (Southern and Southeast Asia) or E (Africa), most often occurring in tropical areas as seasonal epidemics with high morbidity and mortality. Signs include high fever, depression, subcutaneous edema, hypersalivation and diarrhea, or sudden death. All excretions and secretions are highly infectious.

Sheep and Goats

Septicemia

Septicemic infection, usually due to *B. trehalosi* in feeder lambs and *M. haemolytica* in nursing lambs, resembles bovine hemorrhagic septicemia, although intestinal involvement is often absent and the morbidity rate is much lower.

Pneumonia

As is the case in cattle, pneumonia in sheep and goats can occur following shipment, or in groups of animals in either enzootic or epizootic patterns. *M. haemolytica, B. trehalosi*, and *P. multocida* are most commonly involved. As described above for calves, other factors, including coinfection with viral pathogens, environmental stressors, and inadequate host immunity, enhance the ability of these bacteria to cause bronchopneumonia in small ruminants.

Mastitis

Mastitis in small ruminants can be caused by various *Mannheimia* species or *B. trehalosi*. Disease often occurs late in lactation, when large lambs bruise the udder and provide the inoculum from their oropharyngeal flora. Acute systemic reactions accompany disease of the udder, parts of which undergo necrosis ("blue bag") and may slough.

Swine

Atrophic Rhinitis

Atrophic rhinitis of young pigs (three weeks to seven months of age) leading to turbinate destruction and secondary complications results from synergistic nasal infection by *P. multocida* (usually serogroup D or A) and *B. bronchiseptica* (Chapter 11). Signs include sneezing, epistaxis, and staining of the face due to tear duct obstruction. Nasal skeletal abnormalities produce lateral deviation of the snout or wrinkling due to rostrocaudal compression. Secondary pneumonia is due in part to the elimination of the turbinates as defenses of the respiratory tract.

Pneumonia

As is the case for ruminants, bronchopneumonia can occur in swine following shipment, or in groups of swine in enzootic or epizootic patterns. *P. multocida* is commonly isolated from such cases. As for ruminants, it is likely that other insults, such as primary viral respiratory infection, environmental factors such as crowding and poor air quality, and inadequate host immunity, enhance the ability of *P. multocida* to cause bronchopneumonia.

Rabbits

Respiratory Tract Conditions

"Snuffles," a mucopurulent rhinosinusitis of rabbits due to *P. multocida*, develops when bacteria normally residing in the nasopharynx cause disease following stress of pregnancy, lactation, or mismanagement. Coinfection with other pathogens, particularly *B. bronchiseptica* (Chapter 11), may exacerbate disease. Complications include bronchopneumonia, middle and inner ear infection, conjunctivitis, and septicemia.

Genital Tract Disease

In the genital tract of ruminants, *P. multocida* may cause orchitis, balanoposthitis, and pyometra.

Avian Species

Infectious Coryza

Infectious coryza (caused by *A. paragallinarum*) is an acute contagious infection of chickens that is usually confined to the upper respiratory tract. Birds of all ages can be infected, and disease can become chronic. Signs include nasal discharge, swelling of sinuses, facial edema, and conjunctivitis. Air sac and lung involvement may occur in severe cases. When disease is uncomplicated, mortality is low, and loss of production is the most important consequence. Superimposed infections with viruses, other bacteria, or helminth parasites, or management-related factors such as poor ventilation, exacerbate and prolong outbreaks. Complicated outbreaks can cause significant economic losses to poultry producers. Of other species, only Japanese quail are highly susceptible.

Avian Cholera

Avian cholera, a systemic infection due to *P. multocida* (most commonly serogroup A), is acquired by ingestion or inhalation and affects mainly turkeys, waterfowl, and chickens. The peracute form kills a high percentage of infected birds without preceding signs of illness. The acute type, marked by listlessness, anorexia, diarrhea, and nasal and ocular discharges, may last several days, with lower total mortality than the peracute form. The subacute form is mostly respiratory and is manifested by mucopurulent nasal discharges. In chronic fowl cholera, caseous lesions are found. Inapparently infected carrier birds appear to be important in the epidemiology of fowl cholera. *A. gallinarum* is sometimes co-isolated from chronic cases.

Dogs and Cats

Mouth-Related Conditions

P. multocida (cats) and *Pasteurella canis* (dogs) are found alongside predominantly anaerobic flora in oral cavity infections, bite wound infections, serositides (e.g. feline pyothorax), and oral cavity-related foreign body lesions.

Equine

Respiratory Tract Conditions

Pasteurella caballi occurs in equine respiratory disease, usually in association with *Streptococcus equi* subspecies *zooepidemicus*.

Laboratory Rodents

Pasteurella pneumotropica, a common commensal, may contribute to opportunistic infections such as pneumonia. Phenotypically similar organisms in other hosts probably belong to different species (e.g. *Pasteurella dagmatis*).

Immunity

Basis of Immunity

Circulating antibody is significant in protection against hemorrhagic septicemia and fowl cholera. The type-specific capsular antigens are essential immunogens in hemorrhagic septicemia. In birds recovering from infection with *A. paragallinarum*, immunity provides heterologous protection across various capsular types. With other forms of disease associated with *Pasteurella* and *Mannheimia*, the picture is less clear. Both antitoxic and antibacterial antibodies are important in protection; for example, anti-leukotoxin antibodies are an important requirement for immunity to severe disease induced by *M. haemolytica*; immunity to PMT can limit severity of atrophic rhinitis in swine.

Vaccination

Vaccines against *A. paragallinarum* are commercially available and can protect chickens from disease following experimental exposure to the bacteria, but vaccines must contain the serotype causing disease to be most effective.

Vaccines to prevent bronchopneumonia associated with *P. multocida* in swine and cattle, and bronchopneumonia or pleuropneumonia associated with *M. haemolytica* in cattle, are commercially available. These vaccines can prevent disease associated with experimental challenge, but their efficacy in the field is less consistent. Vaccines manufactured for use in cattle are sometimes administered to sheep and goats, but in general these are unlikely to provide much benefit because different serotypes of *Mannheimia* and *Pasteurella* not included in bovine vaccines are most commonly associated with disease in small ruminants.

In countries where bovine hemorrhagic septicemia occurs, vaccines are used to prevent disease and can be effective for up to two years. Antiserum is useful for short-term protection.

Vaccines are used to aid in the control of fowl cholera. *P. multocida* and *B. bronchiseptica* bacterins with toxoid are commercially available and are useful in the control of atrophic rhinitis.

Laboratory Diagnosis

Isolation and Identification

Bacteria in the genera *Avibacterium, Bibersteinia, Mannheimia*, and *Pasteurella* grow overnight (35–37 °C) on media containing blood or serum and can be identified using differentiating biochemical tests. Capsule and somatic typing can be done, but these tests are more often used in research applications and are not likely available at regional veterinary diagnostic laboratories. DNA probes and primers designed to amplify specific regions of the bacterial chromosome by PCR can be used to identify specific species, and such DNA testing, sometimes in multiplex assays that can identify multiple organisms simultaneously, is commonly used in diagnostic laboratories.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS, often simply called "MALDI") has become increasingly used for the identification of bacterial species, including the Pasteurellaceae. MALDI-TOF MS can also be used to rapidly differentiate between Genotype 1 and Genotype 2 *M. haemolytica*.

Definitive identification of members of the family Pasteurellaceae can be completed through sequencing 16S ribosomal RNA gene sequences. Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) has been shown to usefully differentiate *A. paragallinarum* isolates, and multi-locus sequence typing (MLST) is often used to differentiate *P. multocida* isolates. However, these molecular tests are beyond the scope of most diagnostic laboratories and are generally used only in research or epidemiologic investigations.

Treatment and Control

Bacterial isolates in the genera *Avibacterium, Bibersteinia, Mannheimia*, and *Pasteurella* can be susceptible to a variety of antimicrobials effective against gram-negative bacteria. Although historically antimicrobial resistance was

uncommon in these genera, resistant and multidrug resistant isolates are being increasingly reported. Metaphylactic administration of antimicrobials, especially macrolides, to all individuals immediately after shipping is sometimes used to control BRDC in groups of cattle at high risk for the disease. However, this practice may be contributing to increased prevalence of antimicrobial resistance in *M. haemolytica* and *P. multocida* in cattle.

Vaccination is used to control disease associated with infection by *M. haemolytica* in cattle; *P. multocida* in swine, cattle, and avian species; and *A. paragallinarum* in chickens. Additionally, vaccines for *M. haemolytica* may provide partial protection against severe disease associated with *B. trehalosi* in cattle. Although vaccines against these agents are effective in preventing disease following experimental infection, efficacy in the field is more variable and likely depends on a variety of factors related to relative severity of challenge, ability of vaccinated animals to respond appropriately to vaccination, and the severity of environmental stressors that exacerbate host susceptibility to disease.

Ornithobacterium rhinotracheale

O. rhinotracheale is a gram-negative rod in the family Weeksellaceae that resembles some members of the family Pasteurellaceae. It is a facultative anaerobe whose growth is enhanced by carbon dioxide, it is oxidase positive, and attacks carbohydrates fermentatively. At this time 18 serotypes (A–R) have been reported, although no clear relationship between serotype and virulence has yet been determined.

Infection by *O. rhinotracheale* is associated with respiratory tract disease (sinusitis, airsacculitis, pneumonia) in birds (domestic and wild). In turkeys and chickens, respiratory tract disease may be relatively mild, resulting in poor performance, or severe with increased mortality. Transmission is horizontal (aerosols, contaminated fomites) or vertical; it is unclear whether vertical transmission is transovarian or through cecal contamination of eggs. Coinfection with other viral or bacterial respiratory pathogens is common.

With the appearance of NAD-independent strains of *A. paragallinarum* (see above), differentiation of *A. paragallinarum* from *O. rhinotracheale* can be difficult by classical culture techniques. A PCR-based test quickly and easily differentiates the two organisms.

Although historically *O. rhinotracheale* was susceptible to a variety of antimicrobials, antimicrobial resistant isolates are increasingly being identified. Autogenous vaccines have been used in an effort to reduce morbidity.

Acknowledgment

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11

Pasteurellaceae

Actinobacillus

Bradley W. Fenwick and Andrew N. Rycroft

The family *Pasteurellaceae* contains the genera *Actinobacillus, Gallibacterium, Haemophilus, Histophilus, Lonepinella, Mannheimia, Pasteurella, Aggregatibacter, Phocoenobacter*, and several others. As is the case with the *Actinobacillus* species, these genera contain species of primary medical importance as well as a range of opportunistic pathogens causing disease characterized by septicemia and associated sequelae under certain and generally infrequent to rare conditions.

Although they are genetically distinct, the genera *Actinobacillus, Gallibacterium*, and *Pasteurella* overlap phenotypically. As is the case with the genus *Pasteurella,* the genus *Actinobacillus* has and continues to undergo taxonomic reclassification. This is due to our understanding of relational characteristics shifting from phenotype to metabolomics to genetic means (including whole genome sequencing). Adding to the uncertainty is the host-specific nature of most infections and the differences in the nature of the clinical disease that *Actinobacillus* species are known to cause as primary pathogens, most notably in animals. There are numerous case reports and reports of isolated and limited disease outbreaks, with true disease outbreaks limited the involvement of mostly *Actinobacillus pleuropneumoniae*.

Currently there are 16 recognized species and 2 subspecies in the genus *Actinobacillus*, several of which are associated with specific diseases in animals (Table 11.1). Of the greatest clinical and economic significance are *A. pleuropneumoniae*, *Actinobacillus lignieresii, Actinobacillus suis, Actinobacillus equuli, Actinobacillus seminis*, and *Actinobacillus capsulatus*. The type strain is *A. lignieresii*, which is not a strong reference because of its unique characteristics among the other members of the genus.

Although phenotype-based and molecular methods have been developed to identify and differentiate some of the most common taxa, the restricted host range and characteristic disease conditions associated with each species allow for a reliable presumptive identification of *Actinobacillus spp*. in animal disease.

All the members of the family *Pasteurellaceae*, including *Actinobacillus* spp., are gram-negative coccobacilli. They are facultative anaerobes, and typically oxidase positive (setting them apart from members of the family *Enterobacteriaceae*). Most are commensal parasites of the respiratory tract and oral cavity of animals that sporadically cause disease in individuals or small groups. The most notable exception is *A. pleuropneumoniae*, which like other *Actinobacillus sp.,* can be asymptomatically carried by pigs but is viewed as a primary pathogen. This is because of its ability to cause significant naturally occurring disease outbreaks characterized by high morbidity and mortality in healthy pigs.

Discussed in this chapter are *A. lignieresii* (the agent of pyogranulomatous inflammatory processes, primarily of ruminants), *A. pleuropneumoniae* (the agent of contagious porcine pleuropneumonia), *A. equuli* (causing neonatal septicemia in equids), *A. suis* (associated with respiratory disease, septicemia, and localized infections in swine and horses), and *A. seminis* (causing periorchitis, and particularly epididymitis, in rams).

Properties of Genus

The organisms of the genus *Actinobacillus* are small (0.3–0.5 by 0.6–1.4 μm) facultative anaerobic/microaerophilic, gram-negative, non-acid-fast, non-motile, non-spore-forming rods. They are often interspersed with coccobacillus forms providing a "Morse code" (random dots and dashes) appearance.

After 24 hours incubation, colonies on agar are translucent to gray, small (1–2 mm in diameter), often described as

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Table 11.1 Some members of the genera *Actinobacillus*, *Gallibacterium*, and *Volucribacter* that are of importance in animal disease.

Genus and species	Associated disorder or site
Actinobacillus arthritidis	Bacteremia and septicemia in foals
Actinobacillus capsulatus	Arthritis in rabbits
Actinobacillus delphinicola	Bacteremia and septicemia in sea mammals
Actinobacillus equuli ssp. *equuli*[a]	Bacteremia/septicemia in foals, respiratory disease in horses, pericarditis in horses, abortions in mares (mare reproductive loss syndrome), bacteremia/septicemia in swine
A. equuli ssp. haemolyticus (formerly A. suis–like)[a]	Respiratory disease in horses, pericarditis in horses, abortions in mares (mare reproductive loss syndrome)
Actinobacillus indolicus	Respiratory disease in swine
Actinobacillus lignieresii[a]	Chronic pyogranulomatous glossitis in cattle ("wooden tongue"), pyogranulomas of the proximal gastrointestinal tract in cattle
Actinobacillus minor	Respiratory disease in swine
Actinobacillus muris	Respiratory disease in rodents
Actinobacillus pleuropneumoniae[a]	Respiratory disease in swine
Actinobacillus porcinus	Respiratory disease in swine
Actinobacillus rossi	Reproductive tract infections in sows
Actinobacillus scotiae	Bacteremia and septicemia in harbor porpoises
Actinobacillus seminis	Epididymitis in rams
Actinobacillus suis	Respiratory disease and bacteremia/septicemia in swine

[a] Organisms that cause disease of particular importance, as defined by relatively frequent identification of infection and relative severity of disease associated with infection.

sticky, waxy, or adherent (especially on primary isolation). Growth is usually improved by enriching the medium with serum or other complex nutrients, and is sometimes enhanced by inclusion of 5% CO_2 in the atmosphere at 37 °C. Carbohydrates are fermented without gas production. Urease, β-galactosidase, and the ability to reduce nitrates are typically present; indole is not produced from tryptophan. Some species grow on MacConkey agar (sometimes as very small colonies). Selective and enriched media have been developed to increase the chances of isolation, including from highly contaminated locations. Notably, cultures of *Actinobacillus* species often die within a week (at room temperature or at 6 °C) and may be difficult to recover after as few as three days from the initial isolation on agar medium. It has been proposed that this is due to oxygen toxicity: at the end of exponential phase growth, the enzymes necessary for controlling the effect of oxygen free radicals may decline rapidly. They therefore often require regular subculture or storage at −70 °C to maintain the viability of cultures in the laboratory.

Actinobacillus colonies are variably hemolytic on blood agar, depending upon expression and secretion of cytolytic RTX (repeats in the structural toxin) group toxins with hemolytic activity (ApxI, ApxII, and variants of these). Erythrocytes from some species (especially sheep) are notably more sensitive to this hemolytic action than others. Most *Actinobacillus* species are non-hemolytic on blood agar. However, colonies of *A. suis* and *A. equuli* subsp. *haemolyticus* are surrounded by a wide zone of complete hemolysis on sheep blood agar.

Some *Actinobacillus* species (*Actinobacillus indolicus*, *Actinobacillus minor*, *A. pleuropneumoniae* biotype 1, and *A. porcinus*) require V factor β-nicotinamide adenine dinucleotide (NAD) for growth, but not X factor (heme). *A. pleuropneumoniae* biotype 2 and other species such as *A. lignieresi* do not require an exogenous supply of NAD. To detect NAD-dependence, a culture on blood agar may be co-inoculated with a single streak of a staphylococcus: a so-called nurse strain. Staphylococci produce excessive NAD and "feed" those colonies that cannot grow without the NAD. There is therefore a gradient of NAD concentration away from the staphylococcus such that the colony size of the *Actinobacillus* gradually decreases with distance from the staphylococcus. Only very small colonies form at a distance of more than 8–10 mm from the staphylococcus. This old technique detects the phenomenon of satellitism. While the blood in blood agar may naturally contain a small amount of NAD, an enzyme present in the erythrocytes destroys that NAD. As a result, although a concentration of 0.5 μg/ml of NAD is adequate for the growth of

NAD-dependent organisms, supplementation of blood agar must be of the order of 20–50 µg/ml to allow growth.

A second characteristic, used to recognize *A. pleuropneumoniae* in particular, is the CAMP effect (named after the authors, Christie, Atkins, and Munch-Peterson, who described the phenomenon in 1944). If the staphylococcus nurse strain chosen is one that produces the sphingomyelinase known as β-toxin, this enzyme alters the membrane of the sheep erythrocytes in sheep blood agar, and they become sensitive to the effect of weak hemolysins. In this way, ApxI, ApxII, and ApxIII are seen as causing hemolysis around the *Actinobacillus* colonies growing within the well-demarcated zone of β-toxin. ApxIII is not itself haemolytic but contributes as a co-hemolysin in the CAMP effect. Colonies growing from lesions in the pig respiratory tract that show both satellitism and the CAMP phenomenon are presumptively identified as *A. pleuropneumoniae* (Figure 11.1).

Overall, the variable and dynamic nature of the phenotypic characteristics of *Actinobacillus* as well as the overlap with related genera can make conclusive culture-based identification of some species difficult. Molecular genetic methods have helped address this challenge.

Apart from *A. pleuropneumoniae*, the *Actinobacillus spp*. are considered commensal organisms of the mucosal surface of the respiratory, alimentary, and genital tracts of animals and become pathogenic if introduced into deeper tissues. This may result in characteristic, often diagnostic, localized or systemic clinical disease characteristics and lesions. Subclinically infected carrier animals are common and provide a reservoir and the primary means of transmission. Penetration that results in disease is sporadic and when involving an "outbreak" is often associated with a specific environmental or husbandry factor. *Actinobacillus* spp. do not appear to survive in the environment except for some species where survival is prolonged by organic matter. *A. pleuropneumoniae* has been said to survive up to 30 days in water at 4 °C, apparently because of its ability to produce biofilm. However, experimental data show survival of non-biofilm suspensions in aqueous suspension was for less than 24 hours, but this could be prolonged by both NaCl and mucin (mimicking nasal secretion). Under dry conditions, viable organisms persisted for three to four days.

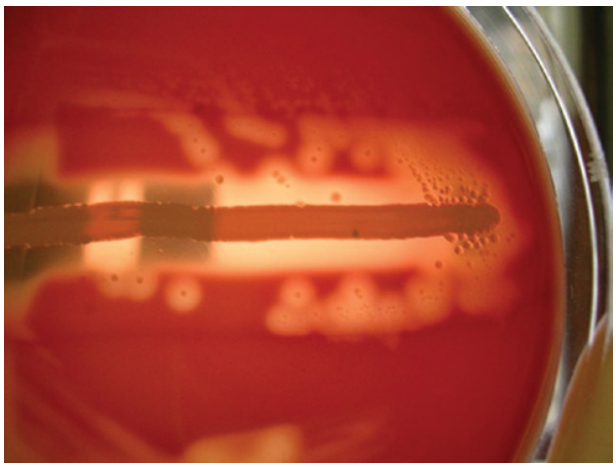


Figure 11.1 A primary culture of *Actinobacillus pleuropneumoniae* from a lung lesion shows both satellitism (NAD-dependence) and CAMP effect (co-hemolysin) visible around a central streak of **β**-toxin producing *Staphylococcus*, on sheep blood agar after 24 hours at 37 °C.

Under certain conditions, *A. equuli*, *A. lignieresii*, *A. pleuropneumoniae*, and *A. suis* can cause primary disease in domestic animals. *A. lignieresii* is commonly found in the oropharynx of cattle and sheep, and can also become pathogenic, causing "wooden tongue" in cattle. The same is true for *A. suis* in pigs and horses, causing septicemia and localized infections. *A. equuli* is pathogenic for horses and pigs, causing purulent nephritis and arthritis, especially in foals and piglets. *A. pleuropneumoniae* can be found in the nasal cavities and tonsillar crypts of healthy pigs but can also cause significant acute and ongoing disease outbreaks, especially when introduced into fully susceptible populations or when immunity declines in certain age ranges.

Descriptive Features

Structure and Composition

Lipopolysaccharide, Capsule, and Biofilm

The cell envelope is typical of gram-negative microorganisms consisting mainly of lipopolysaccharide (LPS), proteins, and lipoproteins. However, the outer membrane of *Actinobacillus* spp. and other *Pasteurellaceae* appears more fragile and less able to exclude small molecules than that surrounding enterobacteria such as *Escherichia coli*. This is shown by their natural sensitivity to benzyl penicillin (penicillin G), which is normally excluded by the outer membrane of gram-negatives such as enterobacteria and pseudomonads.

The detailed chemical structures of the LPS and capsular polysaccharide (CP) have been known for many years, particularly for the different serotypes of *A. pleuropneumoniae*. Variations in the structures of the LPS and to a greater degree the capsule are responsible for differentiation of serotypes and are the basis of serotype-specific serological tests. Serological differentiation between strains has always been problematic. Using slide agglutination, results were often unclear when sticky, adherent colonies were mixed with antiserum. Serotyping results were also complicated

by the closeness of some of the antigens causing cross-reactions and by the carriage of identical LPS antigens among different capsular serotypes. As a consequence, many isolates of *A. pleuropneumoniae* were mistyped. For example, in the United Kingdom, it was apparent that the predominant serovar causing pleuropneumonia in the pig population was serotype 3. Later work, using accurate polymerase chain reaction (PCR)-based detection of specific capsular genes, has shown serotype 3 to be rare, and over 90% of clinical isolates are actually serotype 8.

The genes that encode the synthesis and export of *A. pleuropneumoniae* CP have been sequenced from all known serotypes. These are organized in a manner similar to that of corresponding genes of other bacteria that produce type III capsules, including *Haemophilus influenzae* type b, *Pasteurella multocida* A:1, *Mannheimia haemolytica* A1, and *Neisseria meningitidis* group b. Based on analysis of CP gene structure, 19 serotypes of *A. pleuropneumoniae* are currently recognized. PCR-based typing of all the capsular types is now possible and has largely replaced the inconsistent and inaccurate serological typing of strains using agglutination or antigen precipitation with specific antiserum.

Serological cross-reaction between species of *Actinobacillus* is also known. *A. suis* LPS includes a core LPS that is structurally similar to that in *A. pleuropneumoniae*, which may account for some serological cross-reactivity between the two species.

The CP is required for protection of *A. pleuropneumoniae* from host defenses, such as phagocytosis and possibly complement-mediated killing. Removal of the capsule has, for 30 years, been known to decrease the ability of *A. pleuropneumoniae* to cause disease and at least one licensed live vaccine is based on a capsule-deficient mutant of serotype 5.

There is evidence that some *Actinobacillus spp*. produce biofilms under certain environmental growth conditions. The role of biofilms is not fully understood but, like biofilms produced by other pathogenic bacteria, these are likely to enhance environmental and *in vivo* persistence and transmission, especially in the case of *A. pleuropneumoniae*.

Cell Products of Medical Interest

Adhesins

Colonization is often the first step toward the occurrence of disease. The role of adhesins, as in other microorganisms, is to allow the bacterium expressing them to adhere to cells lining a particular niche, as well as to the surface of target cells prior to the initiation of disease. In some cases, niche and target cells may be the same. The expression of adhesins depends upon various environmental cues and dictates both the location of colonization (carrier vs. disease) and transmission potential. Probably all members of the *Pasteurellaceae* family that colonize animals express several adhesins. This is likely to be particularly true for *Actinobacillus* given that their natural habitat is the oral cavity and respiratory tract (particularly the tonsils).

Except for *A. pleuropneumoniae*, little is known about the adhesins of the *Actinobacillus spp*. associated with animal disease. *A. pleuropneumoniae* adheres to different cell types, the epithelium of the nasal cavity and tonsillar crypts (as in the case with carrier animals), and to a greater degree the cells of the terminal bronchiole and alveoli.

A. pleuropneumoniae produces at least two structures that clearly function as adhesins. The first is an adhesin in the classic sense: that is, a structure composed of protein subunits whose main function is to bind to the surface of host cells. These adhesins are type 4 fimbriae. In the case of *A. pleuropneumoniae* these adhere to cultured primary lung epithelial cells but are only expressed under NAD-restricted growth conditions in some but not all strains. A 55 kDa and a 60 kDa outer membrane protein as well as an autotransporter protease have also been associated with culture condition-dependent adhesion. In addition, *A. pleuropneumoniae* is reported to bind to or interact with respiratory tract mucus, collagen, fibronectin, and double-stranded DNA.

The second adhesin is LPS. This is not usually considered an adhesin. Nevertheless, the LPS (in particular the core portion) of *A. pleuropneumoniae* is likely to be involved in the adherence of this microorganism to cells of the lower respiratory tract of swine by interacting with specific surface glycoproteins. Gene expression following contact with porcine lung cells and involving the *tad* locus-specific genes suggest a mechanism for the occurrence of waxy phenotypes that are common on primary culture but lost after laboratory passage (soft or mucoid). Differences in the core LPS has been demonstrated between waxy and non-waxy forms of the same clinical isolate using silver-stain in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracted LPS.

Capsule

The virulence of *A. pleuropneumoniae* in pigs is influenced by the amount and possibly the type of CP synthesized. Isolates with thick CP appear to be more virulent. The CP plays many roles, the most important of which are interference with phagocytosis (antiphagocytic) and protection of the outer membrane from the deposition of membrane

attack complexes generated by activation of the complement system (serum resistance). In addition, capsules (perhaps in association with LPS) provide sufficient space between surface-activated membrane attack complexes induced by antipolysaccharide antibodies and the cell wall such that the microorganism survives. The genes that encode export and synthesis of *A. pleuropneumoniae* CP have been cloned and sequenced from several serotypes.

Biofilm

Biofilms are structured bacterial communities surrounded by a polymer matrix that they produce to support attachment and provide protection from hostile environments, including host immune responses and antibiotics. It is likely that most, if not all, *Actinobacillus sp*. can, under the right conditions, form biofilms. The conditions necessary to induce the expression of biofilms is not well understood, and the phenotype can be easily repressed under standard laboratory growth conditions. In the case of *A. pleuropneumoniae*, and probably other species, biofilm production is enhanced under anaerobic conditions. The biofilm matrix produced by *A. pleuropneumoniae* when grown on polystyrene is highly hydrated polyglucosamine.

Cell Envelope

The Lipid A component of LPS is recognized as a signal of bacterial infection, and is sometimes referred to as a modulin. This elicits an inflammatory response following association with LPS-binding protein and CD14. The CD14–LPS complex binds to Toll-like receptor proteins (see Chapter 2) on the surface of macrophage cells, triggering the release of proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α). The outer LPS core can interact with RTX-type toxins in a Ca^{2+}-dependent fashion that enhances hemolytic and cytotoxic potential. In addition, the LPS plays a role in antiphagocytosis, serum resistance, and serotyping.

Toxins

Members of the genus *Actinobacillus* produce a least two products in addition to LPS endotoxin, that have toxic activity. The most important are the RTX-type toxins, and the other is urease.

RTX-Type Toxins

A. pleuropneumoniae was long known to produce haemolytic effects in culture. Using monoclonal antibody and genetic analysis, it was found that different serotypes of *A. pleuropneumoniae* produced and secreted three cytolytic and sometimes haemolytic proteins. These were found to be related at the genetic level and were all proteins of a similar size (100–120 kDa) but with different cell tropisms. All were shown to be members of the RTX group, of which the most widely recognized was the alpha-hemolysin produced by many strains of *E. coli* and the leukotoxin of *M. haemolytica*. The RTX group of toxins are so named because they have the common feature of repeating glycine-rich motifs "repeats in toxin." The toxins of *A. pleuropneumoniae* were given different names by different laboratories, but these were rationalized into three distinct toxins designated Apx: ApxI, ApxII, and ApxIII. ApxI is considered the most potent. It is both hemolytic and cytotoxic for leukocytes. ApxII is considered less potent and is also both hemolytic and cytotoxic. ApxIII is a potent cytotoxin but is not hemolytic. Most serotypes secrete two of the toxins: either ApxI and ApxII or else ApxII and ApxIII. A few serotypes secrete only a single Apx type: ApxI or ApxII. The pattern of Apx toxin secretion is linked to the serotype. Serotypes 1, 5, 9, 10, 11, and 14 produce ApxI, and all the serotypes (except serotypes 10 and 14) produce ApxII; serotypes 2, 3, 4, 6, 8, and 15 produce ApxIII. Closely related toxins were subsequently shown to be produced by *A. equuli* subsp. *haemolyticus* (Aqx) and by *A. suis* (variants of ApxI and ApxII). Similarly, *A. rossi* produces ApxII and ApxIII. *A. lignieresii* strains carry the genes encoding RTX-like proteins, but they do not appear to express them (at least *in vitro*) due to lack of a functional promoter.

All members of the RTX toxin family have a similar gene organization and distinctive structural features. Although variations do exist, the generic RTX toxin operon consists of four genes that are designated *rtxCABD* in transcriptional order. RTX toxins require post-translational modification to become biologically active. This is the addition of three acyl groups to the RtxA protein by the product of the *rtxC* gene.

The Apx toxins produced by *A. pleuropneumoniae* are thought to be the primary factors responsible for the hemorrhagic lesions characteristic of swine pleuropneumonia caused by *A. pleuropneumoniae*. The central pathogenic importance of Apx toxins in the development of clinical disease is well established in that they are necessary for full virulence, and purified, recombinant Apx toxin alone was shown to induce pleuropneumonia-like lesions when instilled into the lungs of pigs.

A. pleuropneumoniae mutants that lack the production of any Apx toxins lose their pathogenic potential to induce characteristic lung lesions. As with other RTX-type toxins, there is a dose effect. At low concentrations these toxins

interfere with macrophage and neutrophil function by triggering degranulation, and they are cytolytic for macrophages and neutrophils. This is key to the pathogenicity of *A. pleuropneumoniae*: because the toxin(s) are destroying the primary defense cells of the lung while causing the release from those neutrophils of potent inflammatory mediators that cause the edema, hemorrhage, and necrosis. In addition, some RTX toxins lyse erythrocytes (explaining the hemolytic phenotype of *Actinobacillus* spp. that express the toxins when grown on blood agar). They are responsible for the diagnostically useful CAMP reaction (the co-haemolytic effect) when cross-streaked with a β-toxin-producing strain of *Staphylococcus* (above). The effect of the RTX on erythrocytes probably plays little if any role in disease production, except perhaps increasing the availability of iron.

There is a fourth Apx protein, ApxIV. This was inferred from the detection of genes in the RTX arrangement and the presence of antibody to the molecule in pigs recovered from the disease. ApxIV has no known toxic function and does not appear to play a crucial role in virulence. It is normally produced by all strains and serotypes of *A. pleuropneumoniae* but from only that pathogen. Comparison of mutants lacking any of the three Apx toxins with one that had a further deletion in the ApxIV genes showed no detectable differences. Although ApxIV is said to be produced only *in vivo*, in fact small quantities can be detected during *in vitro* growth. The value of ApxIV is in diagnostics: antibody to ApxIV is detected by enzyme-linked immunosorbent assay (ELISA) in the serum of pigs that have recovered from or are infected with *A. pleuropneumoniae*; the *apxIV* gene is used to recognize and identify *A. pleuropneumoniae* in PCR-based tests.

Urease

Urease produced by *A. pleuropneumoniae* has been shown to be a virulence factor. It is unknown whether this enzyme plays a role in the diseases produced by the other urease-positive species of *Actinobacillus*. Urease is responsible for the liberation of ammonia from urea. The association constant of *A. pleuropneumoniae* urease is extremely high, allowing effective association with the low concentration of urea found in blood and tissue fluids. In addition to attracting and activating neutrophils and macrophages, ammonia inhibits phagolysosome fusion and increases the pH within phagolysosomes, thereby reducing the effectiveness of acid hydrolases. These effects not only decrease the ability of the host animal to clear the microorganisms from the lung but also support colonization of the upper respiratory tract, thereby promoting the carrier state. Mutants unable to produce urease are cleared more rapidly from the respiratory tract.

Iron Acquisition

Because iron is an absolute growth requirement, microorganisms have developed multiple high-affinity systems to obtain it from their environment or hosts. For several reasons, free iron is maintained at extremely low concentrations (10^{-18} M) by the host via high-affinity iron-binding proteins such as transferrin. *Actinobacillus* spp. acquire iron via several mechanisms including a complex involving iron-regulated outer membrane proteins expressed under iron-poor conditions (as a complex including transferrin-binding proteins TbpA and TbpB). Iron is acquired from the transferrin–iron complexes expressed on the surface of the microorganism. Mutation causing loss of either TbpA or TbpB weakens virulence considerably because the organism is unable to metabolize and grow *in vivo*. Interestingly, in the case of *A. pleuropneumoniae* only porcine transferrin acts as a source of iron. This may well contribute to, or be responsible for, the narrow host range: only the pig is a host for natural infection with *A. pleuropneumoniae*, and this pathogen is not known to infect other animals or humans. In addition to acquiring iron using Tbps, *A. pleuropneumoniae* binds to hemin and hemoglobin and utilizes siderophores (hydroxamates, catechols, and possibly others) produced by other species of bacteria even though it does not produce them itself.

Miscellaneous Products

Other products produced by *Actinobacillus* (specifically *A. pleuropneumoniae*) that may play a role in disease include IgA and IgG proteases. These interfere with adherence and opsonization, respectively. Another is a periplasmic superoxide dismutase that has been suggested as a strategy to prevent killing within the phagolysosome by inactivating superoxide molecules. In addition, high-molecular-weight surface carbohydrates associated with the capsule and LPS may play a role in scavenging free toxic oxygen radicals. Like iron, nickel is held at low concentrations by the host but is required for urease activity. To account for this, *A. pleuropneumoniae* has a high-affinity nickel uptake system. Finally, although the most common locations of the *Actinobacillus* spp. are oxygen rich, oxygen concentrations can be low within lesions such as abscesses. In the case of *A. pleuropneumoniae*, alternate terminal electron acceptors have been identified, and it appears that oxygen-poor environments induce the expression of some virulence factors such as biofilm.

Antigenic Variability

Apart from the serotypes and biotypes of *A. pleuropneumoniae* discussed above, *A. lignieresii* and *A. equuli* are also antigenically diverse. The six somatic types of *A. lignieresii* have some relation to geographic and host species predilection, but autoagglutinating strains are common. There are observations of differences between strains of *A. lignieresii* in their ability to cause disease. There are 28 known antigenic groups of *A. equuli*, likely based on LPS antigens. Differences in disease potential among these has not been recognized. *A. suis* has at least two somatic serotypes (1 and 2) with strains infecting pigs (*A. suis*) or horses (*A. suis*–like). There are no significant biochemical differences between *A. suis* strains as they are homogeneous, whereas *A. suis*–like strains are antigenically diverse.

Ecology

Reservoir

Actinobacillus spp. (except possibly *A. capsulatus*) are commensal parasites on mucous membranes, perhaps aided by the formation of biofilm, toxins, metabolic adjustment, and unknown factors that interfere with host defenses. *A. pleuropneumoniae* colonizes the tonsils of healthy pigs but is more closely associated with the respiratory tract of sick or recovered animals and is viewed as an obligate parasite of pigs as no other host has been identified and its survival outside the host animal is very limited. *A. equuli* and *A. suis*–like strains are largely restricted to horses.

Transmission

Except in neonates where infections likely originate from their mothers, most infections with *Actinobacillus* arise from endogenous sources. Neonatal foals acquire *A. equuli* before, at, or shortly after birth from their dams, commonly via the umbilicus. For *A. suis*, *A. pleuropneumoniae*, and other respiratory pathogens, transmission is both via direct contact with respiratory secretions and aerosol exposure; clinical disease is then dependent on the immunity of the recipient and exposure dosage. Subclinical transmission between the sow and piglets under the passive protection of colostral immunity, followed by colonization of the piglets, is viewed as being a common form of transmission in swine and the basis of persistent enzootic (often subclinical) infection within a herd.

Pathogenesis

Mechanisms

Most diseases produced by members of the genus *Actinobacillus* result from infection of a normally sterile site by microorganisms that have colonized a contiguous site (niche). Viral infections, trauma, or stress all predispose animals to disease. At times it is difficult to determine the nature of the antecedent event. In neonates with septicemia, failure of passive transfer is suspected as maternal immunity is often protective. Deposition of actinobacilli into a normal sterile site results in initiation of an inflammatory response due to innate recognition of bacterial components such as LPS as signals of bacterial infection (the so-called pathogen-associated molecular patterns (PAMPs) interacting with Toll-like receptors). Polysaccharide capsule interferes with phagocytosis (antiphagocytic) by reducing or preventing opsonization with complement C3b. Transferrin-binding proteins participate in iron acquisition. For those that produce RTX-type toxins (Apx and Aqx), the directly cytotoxic and inflammatory response is intensified by activating and destroying neutrophils and macrophages. The release of the contents of the massive quantity of neutrophils is damaging to many tissues. If the infectious process involves the lungs, alveolar epithelial cells are also damaged. Urease-producing actinobacilli that are phagocytosed resist destruction by generating ammonia, and perhaps superoxide dismutase, inside the phagocytic cell. Opsonization is also reduced by the production of IgG protease. In the case of neonatal septicemia (*A. equuli*, in particular), actinobacilli gain access to the systemic circulation. Capsules and transferrin-binding proteins promote multiplication within the bloodstream, resulting in endotoxemia and septic shock.

Pathology

Lung lesions (usually produced by *A. equuli*, *A. pleuropneumoniae*, and *A. suis*) are suppurative. Characteristically, *A. pleuropneumoniae* in its most virulent form produces a necrotizing, fibrinohemorrhagic pneumonia with pleuritis. Microscopically, inflammatory cells dominate, with erythrocytes, degenerative neutrophils, and mononuclear cells being a prominent feature. Visually, the affected tissue is reddish black to red, pink, and gray with yellow fibrin deposited on the surface (see Figure 11.2). Localized to more generalized pleuritis is common, especially in pigs. The lung lesions may nearly fully resolve over several months so that, depending on the age of the pig, prior disease may not be apparent at the time of slaughter.

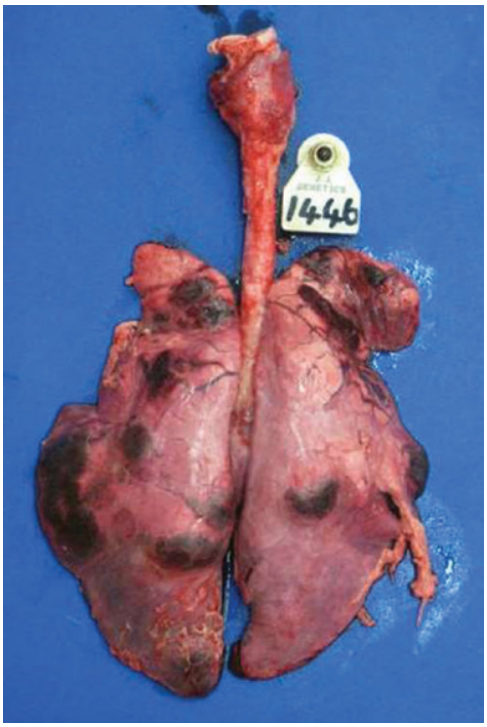


Figure 11.2 Multiple lesions in the lungs of a three-month-old pig infected with *Actinobacillus pleuropneumoniae*. There are multiple lung abscesses in apical, cardiac, and diaphragmatic lobes and deposition of fibrin on the surface of the left diaphragmatic lobe.

Inspection of the respiratory pluck in the abattoir can be an unreliable means of determining the health status of a herd.

A soft tissue process, mainly produced by *A. lignieresii*, is a chronic granuloma in the ruminant facial soft tissues and tongue. At its center are colonies of *A. lignieresii*, ringed by eosinophilic cellular material producing club-shaped processes forming a "rosette". The complex is surrounded by neutrophils and granulation tissue containing macrophages, plasma cells, lymphocytes, giant cells, and fibroblasts. Plant fibers, indicating external penetration, are often present. Through coalescence, larger granulomas (1 cm or more in diameter) may be found. Infection spreads to lymph nodes, producing granulomas along the way. If the tongue is involved, proliferative tissue reaction causes the tongue to protrude from the mouth. Superficial lesions often ulcerate. In sheep, suppurative infectious occur around the head and neck and in the skin and mammary gland. Although the condition is often called "wooden tongue," tongue involvement is not typical.

Epidemiology

Except for *A. pleuropneumoniae*, generally the actinobacilli of animal disease interest are opportunistic pathogens producing disease when their host's integrity is compromised, as by trauma, immaturity, or other stress. Trauma to mucous membranes of ruminants by rough feed may cause herd outbreaks of infection with *A. lignieresii*, falsely suggesting disease transmission rather than an overall husbandry or environmental risk factor.

In the case of porcine pleuropneumonia caused by *A. pleuropneumoniae*, chronic asymptomatic carriers are the reservoir and the basis for unexpected disease "outbreaks" and transmission between herds. Disease occurs when non-immune animals are exposed to clinically infected individuals that sporadically shed at sufficient levels and via inhaled aerosol to cause infection and clinically apparent disease. Prevalence of disease outbreaks are highest in the colder months due to management practices associated with increased animal density, reduced airflow, and mixing of individuals with different exposure histories.

Laboratory Diagnosis

The generally restricted host range and the characteristic clinical disease conditions caused by the various *Actinobacillus* species are of considerable aid in arriving at reliable presumptive etiological diagnosis. Bacterial diagnosis is generally carried out using bacteriological isolation and identification. Most *Actinobacillus* spp. will grow well on blood agar, but *A. pleuropneumoniae* requires heated blood agar (chocolate agar) because heating destroys the NAD-degrading enzyme in erythrocytes. Alternatively, a blood-free medium containing 5% horse serum and 5 μg/ml β-NAD may be used. Colonies of *Actinobacillus* are often waxy or sticky in primary culture. In most cases, speciation is accomplished by cultural and biochemical tests, which are continually being refined. A positive CAMP reaction and satellitism is particularly valuable in the identification of *A. pleuropneumoniae*. Because of a high degree of variability, biochemical methods alone may not be adequate to fully identify the species. Increasingly, as a result of the great range of species whose genome sequence has now been published (or made available in databases), PCR is now used to confirm species identity. This is particularly useful with isolates from unusual tissues and hosts. Identification is sometimes followed by PCR-based typing that provides insight concerning the epidemiology and clinical course of the disease.

Serological testing has become the primary tool in control of disease and in preventing transmission of *A. pleuropneumoniae* within and between herds. Enzyme immunoassay-based methods focused on a number of species and serotype-specific antigens are more reliable than complement fixation tests. Long chain LPS ELISA is now used in N. America to monitor the presence of

infection serologically, including subclinical infection, due to *A. pleuropneumoniae* of all serotypes.

Treatment and Control

Good management and environmental practices are recognized as the key to controlling the transmission and significant disease caused by the *Actinobacillus* species in animals. Porcine pleuropneumonia is controlled and treated by a combination of management practices, immunoprophylaxis, and antimicrobial therapy.

Management practices including minimizing contact of piglets with mature (carrier) animals (e.g. "all in, all out," "age segregation," and "early weaning" rearing practices; and the identification and elimination of carriers by serological testing, culture, and/or by polymerase chain reaction) are the most useful.

Antimicrobial use in pigs, particularly for enteric and respiratory disease, has been widespread. Both prophylactic (prevention) and metaphylactic use (control) have been used, sometimes with a rolling program of in-feed antibiotic through much of the nursery, growing, and finishing stages. This has become unacceptable with the realization that overuse in animals contributes to the environmental load of antimicrobial and the selection for resistance elements (genes, transmissible DNA, and mutations) among bacterial populations. Excessive use of antimicrobials has indeed been associated with unusual resistance among pathogens such as *A. pleuropneumoniae* and led to selection of resistance in bacterial flora that may be transmitted into the human population. Although the use of antimicrobials has been greatly reduced by the coordinated introduction of targets and incentives (as in much of Europe), and careful stewardship by veterinarians and producers, there is still a place for antimicrobial therapy, alongside other measures, in the face of uncontrolled disease. Potentially useful antimicrobial agents include penicillin G, tetracycline, gentamicin, kanamycin, cephalosporins, tilmicosin, tiamulin, florfenicol, ceftiofur, enrofloxacin, and trimethoprim–sulfonamide.

Vaccination against contagious pleuropneumonia has been attempted for more than four decades. It was demonstrated by Ragnhild Nielsen in the 1980s that recovery of a pig from infection with one serotype confers strong immunity to disease caused by some other serotypes. Conversely, any protection provided by vaccines was generally serotype specific. Immune protection by vaccination is therefore theoretically possible. Various different strategies have been used and huge research effort has been expended on understanding the disease, the pathogenicity, and the immune response. There have been some successes, yet there is no widespread, reliable, commercially available vaccine that would offer protection against clinical disease and prevent carriage and transmission of the pathogen in vaccinated animals.

Killed whole bacteria (bacterins) have been tried, sometimes enriched with addition of iron-limited proteins or Apx toxin. Unfortunately, simple bacterin vaccines have frequently shown poor efficacy and, in some cases, are known to exacerbate the disease severity.

Subunit vaccines consisting of a combination of Apx toxins or other antigens have been tried, but with mixed results. Apx toxins can be used directly, without inactivation, in pigs because they spontaneously aggregate to form large complexes and so lose toxicity within a few hours. Thus, they form a natural toxoid. The aim of some of the recent vaccine programs has been to invoke a toxin-neutralizing response in order to confer protection. However, Apx proteins appear to bind LPS and so there is a strong anti-LPS response to injection of Apx vaccine antigen, rather than of Apx-neutralizing antibody. A further problem with this is the endotoxic property that LPS contaminating Apx in vaccines causes hyperthermia in piglets following vaccination. This has limited the use of Apx-based vaccines because the adverse reaction to the vaccine has sometimes prevented commercial registration.

Live-attenuated vaccines have also been tried: again, these have shown mixed success and failure and less than reliable protection against the disease. A commercial capsule-deficient vaccine has been available in the United States. Commercial vaccines are available, but efforts continue to test new ideas and develop a successful vaccine against porcine pleuropneumonia. This has been a frustratingly difficult problem to solve.

In "wooden tongue" caused by *A. lignieresii*, iodides given orally or intravenously promptly reduce the inflammatory swelling, which is the main clinical problem. Avoidance of harsh, dry feed reduces the likelihood of developing this condition. Good foaling hygiene, including umbilical disinfection, reduces the likelihood of foal septicemia caused by *A. equuli* and *A. suis*–like strains. Potentially effective antimicrobial treatment of septicemia produced by *A. equuli* include penicillin G, ceftiofur, and gentamicin. However, treatment of foal septicemia once clinical signs are present is usually too late and ineffective. There is reason to believe, because of the very early/rapid onset of neonatal septicemia in foals, that infection sometimes takes place within the reproductive tract during parturition. If so, antimicrobial cover of the mare may be more effective and antimicrobials are occasionally warranted. The low incidence of this disease and expense of working with experimental horses inhibit effective research.

Disease Patterns

Ruminants

Wooden Tongue

Actinobacillosis or "wooden tongue" caused by *A. lignieresii* occurs in ruminants (especially cattle and sheep) worldwide. Rare individual cases have been reported in dogs, horses, ducks, and rats. In ruminants, *A. lignieresii* (normally an inhabitant of the nasopharynx but can be recovered from the rumen) is inoculated by trauma (e.g. plant fibers) into the oral mucosa, initiating the disease process. The key differential diagnosis is actinomycosis caused by *Actinomyces bovis* which causes "lumpy jaw" related to the occurrence of osteomyelitis and reactive new bone formation. In both diseases, poor-quality hay has been implicated as the basis for increased disease frequency in some herds creating the false impression of a disease outbreak associated with animal-to-animal transmission.

Multiple firm granulomatous lesions most frequently develop in the neck, cheeks, and in the classic case the tongue. Regional lymph node involvement is common. Systemic spread may occur but is infrequent and may provide a degree of diagnostic confusion. The lesions are firm, odor free, well-defined granulomatous lesions. Although the diagnosis is often straightforward from clinical findings, treatment is often protracted, and healing is slow. Most significantly, lesions may interference with feed intake and cause weight loss and dehydration.

Epididymitis

Although there are a number of bacteria that cause epididymitis in rams, *A. seminis* and *Brucella ovis* are particularly common, important, and share disease characteristics. *A. seminis* resident in the prepuce and as an opportunistic pathogen may ascend to cause infection and associated disease. *A. seminis* appears to infect all parts of the genital tract, and the epididymis is most frequently and severely involved. In some cases, this may result in severe and diffuse periorchitis. Epididymitis caused by *A. seminis* is fibrinosuppurative and necrotic involving one or both epididymides. These may fistulate through the scrotal wall. In prolonged infections the epididymides are large and fibrotic.

Swine

Pneumonia

A. pleuropneumoniae occurs worldwide (serotypes 1 and 5 are more common in North America; serotypes 2, 8, and 9 are more common in Europe). Under the right conditions, all 19 serotypes have the ability to cause primary pneumonia of swine. Clinical disease, in its most aggressive form, is remarkable among bacterial diseases in its speed, severity, and host specificity. Characteristically, in a dose-dependent fashion, and influenced by immune status of the animals, the classic disease is a necrotizing fibrinohemorrhagic pneumonia with pleuritis and serosanguinous thoracic effusion (Figure 11.2). The late stage of the disease is often accompanied by pulmonary hemorrhage which is seen as bloody froth at the nostrils. Death is most commonly the result of respiratory failure causing hypoxia. Although bacteria may be detected in blood in the terminal stages, septicemia is not a general feature of the disease.

Clinical pleuropneumonia can occur at any age in susceptible animals, even as young as three weeks, but significant "outbreaks" are particularly common in two- to four-month-old pigs. During this period the view is that passive immunity derived from the sow is no longer sufficient to protect some of the individuals. Concurrently, as the population density increases, ventilation is reduced. As the pathogen harbored in the palatine tonsil invades the lower respiratory tract of one individual, the inflammation induces coughing in that individual initiating the aerosol transmission of bacteria to many others in the herd. Coughing then accelerates further respiratory to respiratory spread throughout the herd. Under experimental conditions, infection of the respiratory tract of naïve animals with 10^4 *A. pleuropneumoniae* is sufficient to induce clinical disease in 24 hours. Higher doses can act to produce clinical signs in as little as four hours. Disease occurrence and severity are strongly influenced by production practices. Other triggers can include a rapid change in environmental temperature, ventilation system failure, and introduction of new (subclinically infected) carrier animals into an immunologically susceptible herd. Conversely, the introduction of disease-free pigs into a subclinically infected herd can initiate an outbreak.

Early clinical signs include depression, fever, and reduced appetite. This is followed by coughing and, in acute disease, by acute respiratory distress. In the peracute form of the disease, animals may die within 24 hours or sooner. Morbidity, over a period of weeks, may reach >90% with mortality up to 40%. Survivors show intermittent non-productive cough and unsatisfactory weight gain, regardless of antimicrobial treatment. Chronic infections, often without preceding acute episodes, are the source of persistent herd problems and transmission between herds. Lesions consist of fibrinous pneumonia and pleuritis. Pneumonia in older animals may be caused by *A. suis* that is difficult to differentiate from acute *A. pleuropneumoniae* infection.

Septicemia

Septicemia of young pigs, as well as arthritis, petechial hemorrhage, and endocarditis, is sometimes associated with *A. suis* infection. This is particularly associated with

high health status herds in which other forms of pneumonia have been eliminated. Septicemia caused by *A. suis* is clinically characterized by sudden death in piglets and a more prolonged disease course in other pigs. The infection produces a vasculitis that results in cutaneous petechial hemorrhages (particularly in older animals) that can also involve the kidneys, heart, and lungs. There can also be exudate in the thorax and pericardium. The primary differential diagnosis is swine erysipelas.

Horses

Septicemia

Neonatal septicemia in foals due to *A. equuli* ("sleepy foal disease") occurs worldwide. It is relatively uncommon, sporadic disease that is frequently fatal despite treatment. *A. equuli* is naturally present in the oral cavity of the mare, and infection of the neonate takes place during or immediately following birth. Disease then occurs within a few hours or days after birth and is marked by fever, inappetence, prostration, and diarrhea. The organism is found in the circulation and in major organs of the foal. Disease in the newborn foal is strongly associated with colostrum deprivation, thus allowing infection that has transferred from the mare to foal via the umbilicus or the respiratory tract to become established. Antibody to *A. equuli* is plentiful in the serum of the mare but very sparse in foals that have not ingested colostrum. The components of the colostrum associated with protective immunity were shown to be specific for the cytolytic toxin Aqx. Neutralizing immunoglobulins against Aqx may therefore confer protection. Animals that survive beyond the first few days typically develop lameness due to (poly)arthritis and/or purulent nephritis. Some foals develop umbilical infections ("navel ill"). It can also be isolated from verminous aneurysms associated with the parasite *Strongylus vulgaris*. Sporadically, *A. equuli* is associated with metritis, abortion, endocarditis, meningitis, and other sequelae of septicemia.

Pneumonia

Bacterial pneumonia in horses (of any age) is usually associated with a mixture of beta hemolytic streptococci (usually *Streptococcus equi* subsp. *zooepidemicus*) and gram-negative microorganisms (commonly *A. equuli*). In young thoroughbreds, a specific condition of poor performance in training is sometimes referred to as "the virus." Although some cases may be due to viruses, investigations have shown that a complex of infection, sometimes involving mycoplasmas, is strongly associated with the presence of *Actinobacillus* sp. in the lower airway. While these organisms have not been identified to species level, *A. equuli* subsp. *haemolyticus* and *A. suis* are the likely candidates as pathogens.

Other Species

Various *Actinobacillus spp*. have caused rare infections in other species, including humans; these are often associated with contact with horses and pigs.

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12

Pasteurellaceae

Glaesserella, Haemophilus, and *Histophilus*

Amelia R. Woolums

The family Pasteurellaceae contains many organisms that contribute to animal disease. Many are normal inhabitants of the outer respiratory, gastrointestinal, or urogenital mucosae, causing disease opportunistically when other factors enhance their ability to advance to deeper levels of these systems. Others are infrequently isolated from normal individuals and cause severe disease when animals are exposed to these agents.

There have been many changes in the taxonomy of the family Pasteurellaceae in recent years, and more changes will likely occur in the future. Many new genera are presented in Chapter 10. Discussed in this chapter are the genera *Glaesserella, Haemophilus*, and *Histophilus*, whose members include several species that contribute to animal disease (Table 12.1). Also listed in Table 12.1 but not discussed further is *Canicola* (formerly *Haemophilus*) *haemoglobinophilus*.

All the members of the family Pasteurellaceae are gram-negative coccobacilli. They are facultative anaerobes, and typically oxidase positive, which sets them apart from members of the family Enterobacteriaceae. Members of the genera *Glaesserella* and *Haemophilus*, beyond sharing the traits of the family Pasteurellaceae, require for propagation one or both of two growth factors: porphyrin (hemin) or β-nicotinamide adenine dinucleotide β-NAD, also known as X (heat-stable) or V (heat-labile) factor, respectively.

Discussed in detail in this chapter are *Glaesserella* (formerly *Haemophilus*) *parasuis*, the cause of a septicemic disease of swine characterized by polyserositis, polyarthritis, and sometimes meningitis (also known as Glässer's disease), and *Histophilus somni*, a cause of respiratory, cardiac, septicemic, and genital disease in cattle and sheep and, less commonly, goats. The bacteria formerly known as *Haemophilus somnus*, *Haemophilus agni*, and *Histophilus ovis* are all now classified as *H. somni*.

Descriptive Features

Morphology and Staining

Members of the genera *Glaesserella, Haemophilus*, and *Histophilus* are gram-negative rods, less than a micrometer wide and usually 1–3 μm long, but sometimes forming longer filaments.

Structure and Cellular Products of Medical Interest

Lipooligosaccharide (LOS)

The cell wall of *G. parasuis, Haemophilus* spp., and *H. somni* contains lipooligosaccharide (LOS), a molecule similar in structure and function to lipopolysaccharide (LPS) of other gram-negative bacteria. Lipooligosaccharide has a relatively complex core oligosaccharide, but lacks the long polymeric O-antigen chain seen in LPS. Like LPS, LOS can elicit a host inflammatory response following its binding to lipopolysaccharide-binding protein (a serum protein), which in turn transfers it to CD14 on the surface of leukocytes. The CD14-LPS complex then binds to Toll-like receptor 4 (TLR4) on the surface of macrophages and other leukocytes, triggering the release of pro-inflammatory cytokines and inducing local or systemic inflammatory responses.

In *H. somni*, LOS has been shown to undergo phase variation resulting in periodic changes in epitope expression due to changes in the carbohydrate portions of the LOS. This phase variation of LOS helps *H. somni* evade the adaptive arm of the host immune response; it also renders the bacteria resistant to complement-mediated lysis, a major component of the innate immune response. As a result, isolates that undergo phase variation are more virulent. In addition, some strains of *H. somni* have the ability to scavenge host sialic acid and transfer it to the

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Table 12.1 Some members of the genera Haemophilus and Histophilus of importance in animal disease.

Genus and species	Associated disorder or site
Canicola (formerly Haemophilus) *haemoglobinophilus*	Cystitis, vaginitis, and balanoposthitis in dogs; neonatal infection of puppies
Glaesserella (formerly Haemophilus) parasuis[a]	Fibrinous polyserositis, polyarthritis, and meningitis (Glässer's disease) in swine; respiratory disease in swine
Haemophilus aegyptius	Meningoencephalitis in sheep
Haemophilus paracuniculus	Mucoid enteritis in rabbits
Haemophilus parahaemolyticus	Respiratory disease in swine
Haemophilus parainfluenzae	Respiratory disease in rabbits and guinea pigs
Histophilus somni[a]	In ruminants: respiratory disease, bacteremia/septicemia, myocarditis and pericarditis, reproductive infection and abortion, septic polyarthritis, thrombotic meningoencephalitis (TME), suppurative meningitis

[a] Organisms that cause disease of particular importance, as defined by relatively frequent identification of infection and relative severity of disease associated with infection.

terminus of their LOS, making the bacterial cell even more resistant to complement fixation and antibody attachment.

Immunoglobulin Binding Protein A (IbpA)

H. somni expresses a large surface protein that can range in size from 120 to 350 kDA and which has many functions that contribute to virulence. Because early research showed that the protein bound host immunoglobulin (antibody), it was called "immunoglobulin binding protein A" (IbpA). IbpA binds to immunoglobulin molecules at their Fc region, rendering them ineffective for opsonization or triggering complement activation, and thus helping *H. somni* evade the host immune response. IbpA includes a filamentous hemagglutinin that helps the bacterium attach to host cells. It also contains a "filamentation induced by cAMP" (Fic) domain that adenylylates Rho GTPases of mammalian cells, blocking the ability of these GTPases to bind to downstream effector molecules, leading to disruption of the actin cytoskeleton. Interaction of the Fic domain of IbpA with bovine type 2 alveolar epithelial cells or endothelial cells causes the cells to become round and separate from neighboring cells; *in vivo* this likely facilitates escape of *H. somni* from the respiratory tract into the bloodstream, where it can travel to other organs and induce infection. Experimental vaccines containing IbpA have been shown to induce protective immunity in calves.

Biofilm Formation and Exopolysaccharide (EPS)

Members of the genera *Glaesserella, Haemophilus,* and *Histophilus* form biofilms; biofilm formation has been studied most thoroughly in *H. somni*. Generally speaking, a biofilm includes bacterial cells of one or more species along with extracellular proteins, carbohydrates, and DNA produced by the bacterial cells. Bacteria in a biofilm are resistant to environmental or host factors that could otherwise kill individual bacterial cells. Although bacterial cells in a biofilm are tightly adherent to the surface where the biofilm has been established, individual planktonic bacterial cells can be shed from the biofilm, allowing them to spread to other regions in the host, where they may establish infection, and more biofilms. In laboratory experiments, the biofilm formed by *H. somni* alone has been characterized in detail, but *H. somni* can also readily form a biofilm with *Pasteurella multocida*. It is likely that *in vivo* biofilms typically contain more than one bacterial species, enhancing their persistence in infected organs.

H. somni secretes an exopolysaccharide (EPS) that is a D-mannan polymer with occasional galactose residues on side chains. Large amounts of EPS are found in the biofilm produced by *H. somni*, with disease-causing isolates of *H. somni* producing thicker biofilm than commensal isolates not associated with disease. It is possible for *H. somni* to add sialic acid of host origin to the EPS, which makes the biofilm appear more like host material, thus increasing resistance to complement or antibody, and thus mediating

immune evasion. Biofilm formation has been demonstrated in myocardial and lung tissue of calves infected with *H. somni*.

Capsule

Some strains of *Haemophilus* spp. and *G. parasuis* can produce capsules; *H. somni* has not been shown to produce a capsule. Bacterial capsules have many functions, the most important of which are interference with phagocytosis and the protection of the bacterial outer membrane from the deposition of membrane attack complexes generated by activation of the complement system.

Pili and Other Adhesins

The role of pili and other adhesins is to allow the bacteria expressing them to adhere to cells lining a particular niche, as well as to the surface of so-called "target" cells prior to the initiation of infection. The expression of adhesins depends upon various environmental cues.

Iron Acquisition

Because iron is an absolute growth requirement, microorganisms must acquire it if they are to survive within the host. Members of the genera *Haemophilus* and *Histophilus*, and likely *Glaesserella,* bind transferrin-iron complexes by virtue of iron-regulated outer membrane proteins expressed under iron-poor conditions (so-called transferrin binding proteins, or Tbps). Iron is acquired from the transferrin-iron complexes that bind to the surface of the microorganism.

Growth Characteristics

Members of the genera *Glaesserella*, *Haemophilus*, and *Histophilus* are facultative anaerobes, typically oxidase positive, and attack carbohydrates fermentatively. *H. somni* may produce a yellowish pigment. Carbon dioxide at 5–10% enhances growth of some species and is essential for isolation of Histophilus. Within 24 to 48 hours at 35–37 °C and on adequate media, members of these genera produce turbidity in broth media or colonies on solid medium that are 1 mm in diameter. Whereas members of the genus *Haemophilus* generally require hemin (X factor) or β-NAD (V factor), *H. somni* requires neither. *Glaesserella parasuis* requires β-NAD but not hemin. A medium that provides both hemin and β-NAD is "chocolate agar," a blood agar prepared by addition of blood when the melted agar is at 75–80 °C (rather than 50 °C as is the case when making regular blood agar). This procedure liberates β-NAD from cells and inactivates enzymes destructive to β-NAD.

An alternative way of providing hemin and β-NAD when attempting to identify bacteria that require these factors is to inoculate "feeder" bacteria (e.g. *Staphylococcus*) across plates where the bacteria that require hemin or β-NAD have been streaked. The feeder bacteria will produce the required hemin and β-NAD through metabolism. On otherwise inadequate media, growth of small *Haemophilus* colonies can be seen only near the feeder streak, a phenomenon called "satellitism" or "satellite growth." It may be duplicated by commercially prepared X- and V factor-impregnated filter papers placed on the inoculated area.

Resistance

Glaesserella, Haemophilus, and *Histophilus* are readily killed by heat and die rapidly in culture and storage unless freeze dried or stored at −70 °C.

Variability

There are at least 15 serovars of *G. parasuis*, with different serovars demonstrating different levels of virulence. Isolates of *H. somni* have been shown to be variable based on assessment of genetic or antigenic characteristics.

Ecology

Reservoirs

Members of the genera H*aemophilus* and *Histophilus* are normal inhabitants of the upper respiratory tract; in addition, they may also be found among the normal flora of the distal urogenital tract. *Glaesserella parasuis* can be found in the upper respiratory tract of healthy pigs. From these sites they may extend inward to the normally sterile regions of these organ systems and cause disease. Alternatively, the bacteria may cross the epithelium of these sites and move through the host via the vascular system. *H. somni* is an obligate inhabitant of ruminant mucous membranes; it does not appear to persist in any meaningful way outside the host.

Transmission

Mechanisms of transmission of *G. parasuis* and *H. somni* have not been well characterized. It may be that infection occurs when endogenous bacteria on the upper respiratory or distal urogenital mucosa convert by some mechanism to an invasive phenotype, cross the epithelium of the mucosal surface of these sites, and move through the bloodstream to other sites in the body. Alternatively, these agents may extend from the mucosal surface to the deeper and

normally sterile sites in these organ systems when other insults disrupt the physical and functional barriers provided by the normal mucosa. Transmission may also occur through close contact or short distance aerosol from other infected animals. Indirect transmission (through fomites) may be important during outbreaks of *G. parasuis* infection in swine.

Pathogenesis

Mechanisms

Disease is induced when *G. parasuis* or *H. somni* invade normally sterile sites. Whether an invasive phenotype is triggered by some as yet undefined event, or whether it is happenstance "contamination" of a previously sterile site due to some primary insult such as trauma, primary viral infection, or immunocompromise associated with the stress of transport or other environmental factor, is currently not entirely clear.

In septicemic disease produced by *H. somni*, the microorganism is resistant to complement-mediated lysis and the adaptive immune response due to LOS phase variation and sialylation of LOS and biofilm EPS. Disease-associated strains of *G. parasuis* and *H. somni* can resist killing inside phagocytic cells by impairing phagosomal-lysosomal fusion. Iron is acquired by removal of iron from transferrins in host plasma. In addition, antibody (especially that belonging to the IgG2 isotype) binds to IbpA on *H. somni* by the Fc region of the antibody molecule. Antibody bound in such fashion does not trigger complement activation, nor does it serve as an opsonin. Through all of these mechanisms, these bacteria are able to proliferate within the bloodstream and spread throughout the body, particularly affecting joints and the central nervous system. *H. somni* also adheres to endothelial cells by way of IbpA, and triggers apoptosis of these cells. Endothelial cell death through apoptosis induces thrombosis, which likely contributes to tissue necrosis and inflammation through local hypoxia due to interrupted blood flow through small blood vessels. Intravascular multiplication of bacteria and cellular injury induces a local or systemic inflammatory response. As noted above, biofilm formation *in vivo* protects the bacteria from clearance by host immune factors, supporting persistent infection. Extension of *H. somni* or *G. parasuis* into the lower respiratory tract stimulates an inflammatory response activated by their LOS.

Disease caused by members of the genera *Glaesserella*, *Haemophilus*, and *Histophilus* are generally species specific. Cattle, sheep, and bison are susceptible to disease due to *H. somni*, whereas goats are less commonly affected.

Pathology

All infections have suppurative components brought about by the chemistry of the gram-negative cell wall, which triggers the release of pro-inflammatory cytokines from macrophages with the resultant influx of neutrophils. Infection of lungs, body cavities, and joints tends to be serofibrinous to fibrinopurulent. Because of the ability of *H. somni* to induce apoptotic death of endothelial cells, which leads to platelet aggregation and thrombus formation, thrombosis is a characteristic microscopic finding in lesions caused by it. For example, colonization of *H. somni* in the microvasculature of the central nervous system produces thrombotic vasculitis leading to encephalitis and meningitis. Thrombosis can also be seen in the lung or myocardium when these organs are infected. Thrombosis also leads to vascular leakage and decreased blood flow to tissues downstream of the thrombus; thus, hemorrhagic, necrotizing lesions are often a feature of pathology caused by *H. somni*.

Disease Patterns

Ruminants

Pneumonia and Pleuropneumonia

H. somni can cause suppurative bronchopneumonia or fibrinous pleuropneumonia in ruminants, most commonly in cattle. The agent commonly contributes to the development of "shipping fever" in recently transported cattle, along with viral agents, mycoplasmas, and *Mannheimia haemolytica*, *P. multocida,* and/or *Mycoplasma bovis*. Experimental infection of cattle with bovine respiratory syncytial virus (BRSV) prior to *H. somni* infection has been shown to lead to pneumonia of increased severity, as compared to that due to infection by either agent alone. Cattle infected with BRSV prior to *H. somni* have been shown to produce high titers of immunoglobulin E (IgE) directed against *H. somni*, which likely contributes to disease exacerbation through IgE-mediated mechanisms such as mast cell degranulation.

Cattle with pneumonia due to *H. somni* occasionally develop concurrent septic arthritis in one or more joints, and/or otitis. Bacteria are presumed to initiate infection in the respiratory tract and then spread from the nasopharynx to the auditory canal to induce otitis, and to transfer in the bloodstream from the lung to the joints.

Myocarditis and Pericarditis

H. somni can cause myocarditis, myocardial abscesses, and/or pericarditis in cattle; these conditions can be common in feedlot cattle, particularly in the Northwestern United States and Western Canada. In cattle with myocarditis, a grossly visible dark-colored region of myocardium may be evident (Figure 12.1). More chronic lesions



Figure 12.1 Gross necropsy photo of heart of feedlot steer with myocarditis due to *Histophilus somni*. Note affected myocardium, which is darkly discolored (arrow). *Source:* Photo courtesy of Dr. Eugene Janzen.

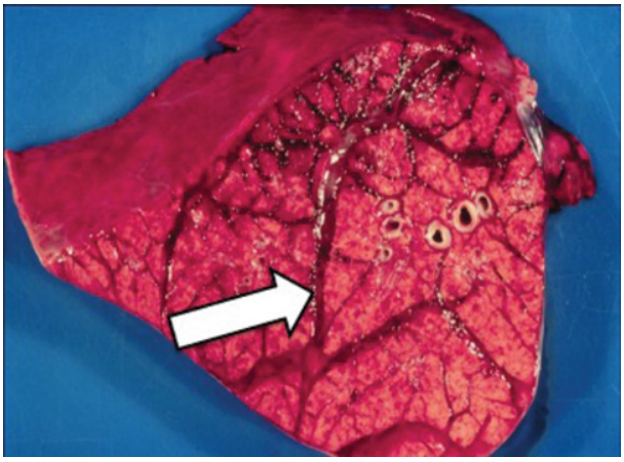


Figure 12.2 Gross necropsy photo of lung from feedlot steer with acute pulmonary edema due to left-sided heart failure caused by myocarditis due to *Histophilus somni*. Note separation of pulmonary lobules by edema (arrow). *Source:* Photo courtesy of Dr. Eugene Janzen.

may be pale or abscessed. The papillary muscles of the left ventricle are most commonly affected, although larger regions of the myocardium may also be affected. Sudden onset of heart failure may result from these lesions, with severe pulmonary edema occurring (Figure 12.2). Affected cattle may die acutely with no previous signs of disease.

Bacteremia/Septicemia

H. somni can cause bacteremia and septicemia in ruminants, resulting in signs of a systemic inflammatory response and sometimes leading to arthritis, myocarditis, meningoencephalitis, or abortion.

Bovine Thrombotic Meningoencephalitis

Through the 1950s to 1980s, thrombotic meningoencephalitis (TME) was the most common manifestation of infection by *H. somni* in cattle. However, since the 1990s, respiratory and myocardial disease have been more commonly identified; this situation has been attributed to a presumption that commercial vaccines for *H. somni* are relatively more effective in preventing neurologic disease than pulmonary or cardiac disease. This situation may alternatively be due to the evolution of strains affecting cattle in response to other forces. When TME occurs it is due to transfer of bacteria in the blood from the respiratory mucosal surface to the central nervous system, where the Fic domain of the surface IbpA induces cytotoxicity to and apoptosis of capillary endothelial cells, leading to thrombus formation, interrupted blood flow, and tissue necrosis, followed by inflammation and thus meningoencephalitis. Affected animals show clinical abnormalities referable to the inflammatory process in the affected regions of the central nervous system, with depression and stupor being common. The preencephalitic stage is marked by high fever. Infection of the ruminant central nervous system by *H. somni* can also lead to leptomeningitis and inflammation of the ventricles, without obvious thrombus formation.

Abortion, Reproductive Infections, and Mastitis

Abortion due to *H. somni* occasionally occurs in ruminants. Whether this is most often secondary to bacteremia or to ascending infection from the urogenital tract has not been determined. Reproductive infections (such as metritis or epididymitis) or mastitis in ruminants can also be caused by *H. somni*. Epididymitis in rams caused by *H. somni* can be clinically indistinguishable from epididymitis due to *Actinobacillus seminis* or *Brucella ovis*.

Swine

Bacteremia/Septicemia

In young weaned pigs, *G. parasuis* causes polyserositis, polyarthritis, and sometimes meningitis (also known as Glässer's disease). Infection leads to an acute inflammatory response with fibrinous polyserositis that can affect the pleura, peritoneum, mediastinum, pericardium, joints, and meninges. Weaning, transport, and management stress are predisposing causes. The disease strikes sporadically within days of the predisposing event. Morbidity and mortality are often low because of widespread acquired resistance, but they may be high in previously unexposed herds. Disease manifestations include fever and general malaise, respiratory distress and abdominal discomfort, lameness, and neurologic signs including seizures. Disease can be fatal or recovery can follow, typically in one to two weeks.

Although some strains of *G. parasuis* are obviously virulent, the agent can also be isolated from healthy pigs. Thus, not all strains of *G. parasuis* cause disease. However, it has been difficult to determine the specific factors that differentiate virulent from nonvirulent strains, because the bacteria are resistant to *in vitro* manipulations (such as transfection or mutagenesis) necessary to identify virulence factors. Most research has to date focused on characterizing responses of stains isolated from diseased pigs, and comparing them to isolates from normal pigs; this research indicates that LOS, capsule formation, porin proteins, and trimeric autotransporters (VtaA) contribute to virulence.

Respiratory Disease

Glaesserella parasuis can cause bronchopneumonia in swine; this may be secondary to virus infections (e.g. due to swine influenza). Other bacteria (e.g. *Pasteurella* spp. and *Mycoplasma* spp.) may also participate.

Immunity

Basis of Immunity

Circulating antibody develops in infected individuals and has a protective function. Immunity to *G. parasuis* occurs after experimental or natural infection; however, immunity to the infecting serotype is more consistent than is cross-protective immunity to other serotypes. Because *G. parasuis* and *H. somni* can persist inside macrophages, cell-mediated immune mechanisms are likely required for clearance, but the exact nature of effective cell-mediated immunity has not been determined.

Infection or vaccination of cattle with *H. somni* can induce production of IgE directed against the agent, which has been associated with disease of increased severity. This is probably due to IgE-mediated degranulation of mast cells and eosinophils and the resultant release of inflammatory mediators. Infection of cattle by BRSV prior to *H. somni* can increase production of IgE directed against the bacteria.

Vaccination

Vaccines are commercially available for use to prevent and control respiratory disease due to *H. somni* in cattle. Although vaccination can decrease the incidence of respiratory disease in at-risk animals (such as feedlot cattle), protection is not inevitable. The efficacy of these vaccines in preventing meningoencephalitis, polyarthritis, and reproductive or cardiac disease in cattle is not well characterized. In the United States, there are no *H. somni* vaccines labeled for use in sheep or goats.

In the United States, a modified live vaccine is commercially available for control of disease due to *G. parasuis* in swine; however, protection is not reliably induced for strains that not closely related to the vaccine strain. Autogenous vaccines have also been used to prevent disease due to *G. parasuis*. It is important for isolates used in autogenous vaccines to represent the virulent serotype causing disease; nonvirulent serotypes of *G. parasuis*, which may not induce protective immunity, can be concurrently isolated from diseased animals.

Laboratory Diagnosis

Identification of members of the genera *Glaesserella, Haemophilus*, or *Histophilus* from infected tissues or fluids is required to establish a diagnosis. Isolation of these agents by bacterial culture can be difficult because they are fastidious relative to other commensal or contaminating organisms that may also be present in samples. Because special media and conditions are required to isolate these agents, it is important for veterinarians submitting samples to diagnostic laboratories to specifically request that the lab attempt to isolate these agents when they are suspected.

Identification of genus- and species-specific DNA by polymerase chain reaction (PCR) may be easier than culture of these agents if the laboratory offers this test; however, diagnosis made in this manner will not result in an isolate that can be used to identify serotype (in the case of *G. parasuis*) or antimicrobial susceptibility profile. Also, some studies have shown PCR to be less sensitive than culture for isolation of *G. parasuis*.

Treatment and Control

Bacteria in the genera *Glaesserella*, *Haemophilus*, and *Histophilus* have historically been generally susceptible to antimicrobials effective against gram-negative organisms; they may also be susceptible to antimicrobials not usually chosen for treatment of gram-negative organisms, such as penicillin. However, prevalence of resistance to antimicrobials appears to be increasing, particularly for *H. somni*. *H. somni* isolates carrying integrative conjugative elements (ICEs) harboring multiple antimicrobial resistance genes have been isolated from cattle in the United States and Canada. Isolates of *G. parasuis* resistant to historically effective antimicrobials have also been identified. Thus, it is prudent to submit samples for culture and antimicrobial susceptibility testing, particularly if multiple animals are

involved, or if treated animals have failed to respond to treatment as expected, so that effective antimicrobials can be confirmed. Several antimicrobials are specifically labeled for treatment of respiratory disease due to *H. somni* in cattle in the United States and Canada. However, effective treatment of some conditions may be difficult if the nature of the pathologic lesion is such that a long course of therapy is required (e.g. septic arthritis in a feedlot steer). In such cases, it may not be practically feasible to treat the animal effectively due to management or financial constraints.

Control of disease due to these agents can be aided in some cases by vaccination (see above). Management to prevent or limit primary insults that may increase susceptibility to infection, such as primary viral respiratory infection, may also help to limit disease due to these agents.

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13

Bordetella

Bradley W. Fenwick

Bordetella are members of the *Betaproteobacteria*, which in turn are part of the Proteobacteria. As gram-negative bacteria, they have a broad diversity of morphologies and occupy a wide range of environments. *Burkholderiales* and *Neisseriales* are among the four orders within the *Betaproteobacteria* that can cause disease in animals and plants. They include pathogenic members within *Bordetella*, *Burkholderia*, and *Ralstonia*. Modern diagnostic methods as well as molecular and genomic enabled studies have expanded the number of known *Bordetella* (currently 16) and provide clarity concerning their genomic relatedness and virulence factors. Over time, additional *Bordetella* are likely to be identified in various environmental niches as well as in animals that they subclinically colonize and are involved to some degree in causing respiratory disease.

Bordetella relatively recently evolved from *Bordetella*-like environmental sources (mostly soil) to infect and cause disease in animals. *Bordetella bronchiseptica* can infect amoeba, specifically *Dictyostelium discoideum*. As with other bacteria that have an endosymbiotic relationship with amoeba, this provides an environmental reservoir and is likely the original basis for adaptation to intercellular survival, a characteristic of most *Bordetella*. As is the case with mammal intercellular adaptation, *B. bronchiseptica* gene expression is influenced by its interactions with amoeba and is highly influenced by environmental conditions.

While adapting to more host-restricted or host-specific life cycles, disease-causing species of *Bordetella* lost hundreds of genes, which significantly reduced their genome size, and concurrently developed a set of characteristic shared virulence factors. *Bordetella* gene expression is environmentally dependent and modulated via various mechanisms. For example, only *Bordetella pertussis* expresses pertussis toxin; although the gene is present in *B. parpapertussis* and *B. bronchiseptica*, it is generally viewed as being transcriptionally silenced, raising the question of its value and the reason for its persistence in these species. On the other hand, one of the hallmarks of *B. bronchiseptica* as well as the other *Bordetella* is the degree of phenotypic modulation, most notably the expression of virulence factors associated with various environmental conditions.

The 9 (perhaps 10) phenotypically and genotypically characterized species of *Bordetella* are traditionally divided among three groupings; classical, non-classical, and environmental. Several more (6+) have been recognized but not confirmed. The classical group include *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*. The non-classical group includes (in order of genome size) *B. hinzii*, *B. pseudohinzii*, *B. trematum*, *B. avium*, and *B. holmesii*. Finally, *B. petrii* is the only characterized member of the environmental group, with *B. ansorpii* being the most closely related. *B. petrii* is unique as being the only member known to being capable of anaerobic growth. Genetic analysis indicates that *B. pertussis* and *B. parapertussis* independently evolved from an ancestral strain(s) of *B. bronchiseptica* and as a group (including *B. holmesii*) share a high percentage of their genome as well as an array of virulence factors that are often not present or expressed in the other *Bordetella*. *B. pertussis*, *B. parapertussis* (human lineage), and *B. holmesii* are strict human pathogens causing respiratory tract infections that can result in whooping cough or whooping-cough-like disease. *B. trematum*, *B. petrii, B. ansorpii*, and *B. hinzii* on rare occasions can cause ear, wound, skin, and other diseases in immunocompromised humans. *B. bronchiseptica, B. hinzii*, and other known *Bordetella* spp., as well as *B. bronchialis, B. spurigena, B. flabilis* and a number of unidentified *Bordetella*, have been isolated from humans with cystic fibrosis but have not been characterized. *B. bronchiseptica* is of the greatest concern in veterinary medicine.

Although generally viewed as a commensal or opportunistic pathogen of the respiratory tract in a number of

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Table 13.1 Members of the genus *Bordetella*, usual source or associated condition.

Species	Usual source or associated condition
Bordetella pertussis	Whooping cough in humans and chimpanzees
Bordetella parapertussis	Whooping-cough-like disease in humans; chronic nonprogressive pneumonia in sheep (human and sheep strains are different)
Bordetella bronchiseptica	Atrophic rhinitis in swine and respiratory tract disease in many animals
Bordetella avium	Rhinotracheitis in poultry (especially turkeys and coryza) and wild birds
Bordetella hinzii	Commensal in poultry, rare respiratory disease in turkeys, septicemia in humans
Bordetella holmesii	Whooping-cough-like disease and septicemia in humans
Bordetella trematum	Opportunistic infections in humans
Bordetella petrii	Opportunistic infections in humans
Bordetella ansorpii	Opportunistic infections in humans

domestic and wild animals, *B. bronchiseptica* has a tropism for ciliated epithelial cells and the capability of intracellular survival. It causes or is viewed as being involved in a variety of respiratory diseases, including, in rare cases, respiratory tract infections in humans (Table 13.1). *B. avium* and *B. hinzii* are pathogens of birds, causing coryza or rhinotracheitis. A sheep-specific lineage of *B. parpapertussis* is involved in pneumonia, and *B. pseudohinzii* is known to infect laboratory mice.

Descriptive Features

Morphology and Staining

Bordetellae are small highly pleomorphic (rod, coccoid, diplococcus, ovoid) gram-negative bacteria, ranging from about 0.4–0.7 μm to 2 μm.

Structure and Composition

Bordetella spp. are typical of gram-negative bacteria in that their cell wall is composed of lipopolysaccharide (LPS) and protein. It is known that *B. bronchiseptica* and *B. pertussis* can produce a capsule under certain conditions. All members of the genus that have been evaluated produce fimbrial adhesins (pili) and biofilms. At least *B. bronchiseptica*, *B. avium*, and the most recently discovered *B. pertussis* are motile with flagella or flagella-like structures, the expression of which is variably regulated by environmental conditions.

Cellular Products of Medical Interest

Surface structures, such as flagella, fimbriae, polysaccharides, as well as various toxins, are involved with environmental survival, transmission, establishment of infection, and modulation of the host response. The ability to regulate the expression of these products is influenced by the dynamics of the interaction between *Bordetella* and the environment, and can be a source of confusion and uncertainty regarding their role in causing or enhancing clinically evident disease as well as successful treatment and control strategies.

With few – what appear to be strain-specific – exceptions, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. avium* produce (or at least have the genes to produce) many of the same products involved in the pathogenesis of clinically apparent disease (Table 13.2). Of these, *B. pertussis*, has the most limited host range and reduced genomic size but expresses essentially the same array of products, including most notably and uniquely, pertussis toxin.

Adhesins

The role of adhesins, as in other disease-associated microorganisms, is to allow *Bordetella* spp. to adhere to cells lining a particular niche, as well as to the surface of so-called target cells (e.g. respiratory ciliated epithelium) prior to the initiation of infection and perhaps disease. The expression of adhesins depends upon various key environmental cues. Binding followed by adherence appears to be at least a two-step process involving sequential involvement of different adhesins.

Bordetellae produce several structures that are responsible for adherence to host cells. These adhesins include fimbriae (or pili), filamentous hemagglutinin (FHA), pertactin, cell wall LPS, tracheal colonization factor (Tcf), and pertussis toxin (Ptx). To the degree known, the expression of all is regulated by the BvgAS two-component regulation system.

Fimbriae

Fimbriae are filamentous protein structures composed, in part, of subunits (pilins) that are likely responsible for adherence to receptors on host cells. They are known to mediate attachment of *Bordetella* to ciliated respiratory epithelium and are involved in the host antibody responses. They are in the same family as the type 1 fimbriae produced by enterobacteria and encoded by four structural genes. *B. bronchiseptica* has four unlinked fimbrial genes: fim2, fim3, fimX, and

Table 13.2 Cellular products of medical interest within members of the genus *Bordetella*.

Product	*Bordetella pertussis*	*Bordetella parapertussis*	*Bordetella bronchiseptica*	*Bordetella avium*
Adhesins and others				
Fimbria	+	+	+	+
Filamentous hemagglutinin	+	+	+	+
Pertactin	+(69 kDa)	+(70 kDa)	+(68 kDa)	−
Tracheal colonization factor	+	−	−	ND
Pertussis toxin	+	−[a]	−[a]	−
Biofilm	+	+	+	ND
Toxins				
Tracheal cytotoxin	+	+	+	+
Dermonecrotic toxin	+	+	+	+
Adenylate cyclase toxin	+	+	+	−
Pertussis toxin	+	−[a]	−[a]	−
Osteotoxin	+	−[a]	+	+
Other				
Capsule	−	−	+	−
Iron Scavenging	+	+	+	+
Type III secretion system	+	+	+	−
Brk	+	+	+	−
BatB	−	+	+	−

ND, not determined.
[a] Genes encoded but protein is not produced.

fimA. The expression products are assembled and secreted by a single apparatus encoded by the fimBCD locus which is within the fha operon. Expression of fimbrial proteins is regulated by the BvgAS system; phase variation due to slipped strand mispairing during replication can occur. The independent location of fimbrial genes allows for protein-specific phase variants, serotype switching, and perhaps survival in the face of a host immune response. Binding is via sulfated glycoconjugates on epithelial cells that can increase the expression of CR3 integrin to which FHA binds. Fimbrial proteins are strongly antigenic and are viewed as a key inducer of a T-cell-mediated immune response (perhaps in an adjuvant-like fashion). They are responsible for agglutination by immune sera and the basis of at least four different *B. bronchiseptica* serotypes.

Filamentous Hemagglutinin (FHA)

FHA is an important *Bordetella* adhesin that belongs to the larger group of fimbrial adhesins which are involved in mediating the association with the surface of respiratory epithelium. It thus plays a role in colonization and also influences the host immune response. FHA is required but not sufficient for adherence and binding to macrophages. It is also secreted and involved in biofilm formation. FHA is a highly conserved large complex protein with several well-characterized activities associated with various domains within the protein. Variability in the key binding regions appear to contribute to *Bordetella* species differences in host range and disease-causing ability. The FHA of *B. holmesii* is distinct. FHA undergoes significant posttranslational modification that results in a 220 kDa mature protein from a 370 kDa precursor forming a 50-nm-long filamentous molecule that includes a hairpin fold. SphB1 (a serine protease) is required for maturation of FHA. Similar to other gram-negative bacteria, FHA has a two-part secretion system. A heparin-binding domain provides for hemagglutinin properties, except in *B. avium*. It induces a strong clinical disease-protective antibody response. FHA stimulates TLR2, but not TLR4 or TLR5. Interaction of FHA with TLR2 suggests its involvement in induction of the innate immune system, which may contribute to immunoprotection against infection.

Pertactin (Prn)

Prn (so named because it is a protective vaccine antigen) is an outer membrane protein that functions as an adhesin and is a member of a family of proteins known as

autotransporters, which are virulence factors in many gram-negative bacteria (e.g. *Neisseria* IgA protease). Prn is conserved in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* under the same *Bordetella* virulence gene regulatory system, thus suggesting a general role in the pathogenesis of clinical disease across various hosts. The benefits of functional expression of Prn in pathogenesis are consistent with its conservation in the pathogenic *Bordetella*. Prn is involved in providing resistance to neutrophil-mediated clearance and functions as an immunomodulator by reducing the production of proinflammatory cytokines. However, Prn-deficient strains of *B. pertussis* have been isolated and are increasing in frequency among vaccinated populations, adding to the uncertainty of the function and overall importance of Prn.

There are 16 other autotransporter genes identified in the genome of *B. pertussis*, 5 of which are disrupted by frameshift mutations. Numerous other autotransporter proteins have been identified among the bordetellae (including BatB), but their role in the ecology of *Bordetella* infection and pathogenesis of disease is not fully understood and is evolving rapidly. Like FHA, Prn contains an "Arg-Gly-Asp" (RGD) sequence involved in the binding to host cell integrins. It also has two areas of repetitive amino acids (GGXXP and PQP) that vary in number and are responsible for the difference in size of the mature protein (68–70 kDa) between different species and strains and are protective epitopes. The three-dimensional structure of Prn shows 16 right-handed parallel β-helixes, among which is the largest known β-helix structure.

Prn is highly immunogenic. Convalescent and vaccinate serum contain bactericidal antibodies. Vag8 and BrkA are involved in complement (serum) resistance. The emergency of Prn-deficient strains is likely driven by acellular vaccination and the generation of bactericidal antibodies targeting Prn. These Prn-deficient *B. pertussis* strains have been reported in countries using Prn-containing vaccines. The frequency of Prn-deficient strains is variable but has risen to dominance in some countries. Interestingly, in countries where Prn is not part of the pertussis vaccine, reports of Prn-deficient isolates are rare and associated with migration from countries with Prn in their vaccines. As such, the prevailing view is that vaccine-provided selective pressure provides the basis for a strong correlation between the use of vaccines containing Prn and the appearance and rise to prominence of Prn-deficient strains. The variety of genomic lesions has been identified as being responsible for the loss of Prn, indicating independent selection events.

Prn-deficient *B. pertussis* infection shows a reduced colonization in unvaccinated mice, but advantages in both colonization and competition in mice vaccinated with Prn. Vaccine disease prevention effectiveness of against *B. pertussis* strains that produce or do not produce Prn does not appear to differ significantly. To date there has been no detectable loss of *in vivo* colonization or disease-causing characteristics of strains lacking Prn production in humans. This raises the possibility that Prn is redundant or has a complementary but not essential function to other virulence factors. Overall, the data suggest that loss of Prn may confer a selective advantage, while decreasing clinical severity.

Tracheal Colonization Factor (Tcf)

Tcf is a protein adhesin that is similar to pertactin (e.g. autotransporter, contains Arg-Gly-Asp sequences, and proline-rich regions) and binds to ciliated epithelial cells of the respiratory tract. It appears to target the trachea and is produced by *B. pertussis* and not by either *B. parapertussis* or *B. bronchiseptica*.

Tracheal Cytotoxin (Tct)

Tct is a peptidoglycan (PGN) fragment. PGN recycling is utilized by bacteria during cell division. In *Bordetella*, a transporter defect results in accumulation of extracellular fragments of PGN. The released PGN constitutes the virulence factor Tct as a 921 Da disaccharide tetrapeptide fragment. All pathogenic *Bordetella* produce an identical Tct molecule. Tct is the only factor capable of replicating ciliated cell extrusion that is the hallmark of the bordetellosis. Following Tct release, non-ciliated cells of the airway epithelium internalize Tct. This leads to IL-1α production by non-ciliated cells. Neither Tct nor *Bordetella* liposaccharides induce IL-1α production, but in combination they are a significant inducer. This is followed by the production of nitric oxide (NO), which can be toxic to ciliated cells. Tct also inhibits neutrophil chemotaxis. It has been speculated that the loss of Tct in acellular vaccine formulations contributed to their waning immunogenicity. However, these vaccines contain the *Bordetella* PGN and therefore the have a potential adjuvant effect. Loss of PT, TCT, and dermonecrotic toxin (DNT) resulted in vaccine strains that can colonize hosts and elicited a protective immune response.

Adenylate Cyclase Toxin-Hemolysin (CyaA)

CyaA is a member of the repeat-in-toxin (RTX) family of cytotoxin that is known to be produced by *B. pertussis, B. parapertussis*, and *B. bronchiseptica*. Typical of similar RTX toxins, CyaA has a hydrophobic pore-forming region, an active domain containing two posttranslational acylated lysine residues, a receptor binding domain with characteristic calcium-binding glycine- and aspartate-rich nonapeptide repeats, and a C-terminal secretion for a type I secretion

system. CyaA plays a role in establishing infection. On the other hand, antibodies to CyaA enhance phagocytosis by neutrophils. CyaA antibody-mediated neutrophil clearance is important. The RTX domain of CyaA is sufficient to generate neutralizing antibodies. CyaA also displays an adjuvant effect, with adenylate cyclase (AC) generating a significant immune response. CyaA generates of antigen-specific Th17 cells by a pore-forming-dependent mechanism. Th17 cells mediate protective immunity in animals and humans.

Cellar destruction is dependent on secreted CyaA. But unlike pertussis toxin, which is soluble, CyaA quickly aggregates and in this form fails to provide a disease-causing phenotype. As such, CyaA is thought to be a short-lived toxin acting in close proximity to the bacteria and functioning at the point of close association with the host cell surface via CD11b/CD18 integrin. It is not viewed as contributing to systemic disease. CyaA is required for colonization and lethality of *B. pertussis* in infant mice. Both AC activity and the hemolysin have been shown to be required for colonization and virulence by *B. pertussis*. CyaA uses both its AC domain and hemolysin to induce cellular damage that reduces phagocyte-mediated bacterial clearance, thus enabling colonization and bacterial survival. CyaA inhibited phagocytosis and oxidative bursts by neutrophils. CyaA induced apoptotic cell death of bronchopulmonary cells. Although AC is required for bacterial colonization and persistence, the hemolysin does not appear to be involved in colonization. The hemolysin contributes to cell death, penetration across the epithelial lining, and recruitment of myeloid phagocytic cells into *B. pertussis*-infected tissue. The pore-forming ability is not required for bacterial persistence but contributes to disease-causing potential.

Pertussis Toxin (Ptx)

Ptx is composed of five multi-subunits (AB_5) which function as both an ADP-ribosylating toxin and an adhesin. Ptx is an important virulence factor for *B. pertussis* and is central to the pathogenesis of pertussis infection and disease. Detoxified Ptx is an important component of acellular pertussis vaccines. Functionally, Ptx inhibits host G-protein-coupled receptor signaling that cause several downstream effects. Generally thought to only be produced by *B. pertussis*, a portion of Ptx is excreted from the bacterial cell and a portion remains attached to the surface. It is the surface-associated portion that serves as the adhesin. The host cell types to which Ptx adheres are ciliated epithelial cells of the respiratory tract and phagocytic cells. Although the genes for Ptx are present in both *B. parapertussis* and *B. bronchiseptica*, they are not expressed because of point mutations, particularly in the promoter. However, there appears to be an alternative expression system where under certain environmental condition Ptx can be produced by *B. bronchiseptica*.

Ptx binds to mammalian cell surface glycosylated molecules (not receptor mediated), where it is endocytosed and transported to the endoplasmic reticulum. In the cytosol, it inhibits activation of G proteins by ligand-bound G-protein-coupled receptors. This modification has multiple effects on host cell functions. Ptx has a number of systemic biological effects; perhaps the most important of these is leukocytosis, which is a characteristic of *B. pertussis* infection in infants and correlates with fatal outcome. In addition, Ptx is known to affect heart rate and cardiac function, vasoactive sensitization to histamine, and induction of insulinemia/hypoglycemia, and because of its adjuvant abilities may contribute to the occurrence or severity of autoimmune encephalitis and exacerbations of multiple sclerosis.

Ptx promotes lung inflammation through a variety of mechanisms. However, it unclear whether lung inflammation correlates with cough severity. Baboons cough paroxysmally when infected with a clinical isolate of *B. pertussis* although they do not develop cough when infected with an isogenic Ptx-deficient strain. On the other hand, there is a strong association between Ptx production and cough responses, which may be due to the greater inflammatory response induced by Ptx. This may be because Ptx is necessary to induce robust respiratory inflammation. The paroxysmal nature of pertussis cough may also be an effect of Ptx activity, since Ptx can inhibit desensitization of receptors stimulated by tussive agents, thereby preventing the cessation of the coughing response. Ptx inhibits early innate immune defenses, including recruitment of neutrophils and antibacterial activity of macrophages. Ptx interferes with adaptive immunity, including migration of dendritic cells in response to lymphatic chemokines and generation of serum antibody responses. Detoxified Ptx is a component of many vaccines because it is recognized as a protective antigen. A monocomponent detoxified Ptx vaccine indicates an immune response that is protective. Intravenous administered pertussis immunoglobulin (P-IGIV) containing anti-Ptx antibodies results in significant reduction in leukocytosis in infants with pertussis, reduced paroxysmal coughing, and bradycardic episodes.

Biofilm

Biofilms are produced by most pathogenic bacteria, providing a degree of protection against environmental and host factors, as well as providing resistance to a number of antimicrobial agents. The *B. pertussis* bps locus that encodes proteins involved in biofilm formation is distinct from the capsule locus. Impairment in biofilm production in both *B. bronchiseptica* and *B. pertussis* resulted in a reduced bacterial adherence to respiratory epithelial cells. Although

poly-glucosamine (PGA) is not required for the formation of biofilms by *Bordetella*, it contributes to biofilm stability. A xylose polymer is also produced as part of the biofilm. As with other virulence factors, biofilm production is regulated by the BvgAS system, and there is evidence that fimbria, FHA, and pertactin could be involved in the formation and/or stabilization of biofilms.

Capsule

Since the first report that bordetellae produce a polysaccharide capsule, the finding has been controversial. Genomic analysis demonstrates an incomplete capsular locus. Like other virulence and metabolic functions, the variable expression of capsule biosynthesis production is sensitive to environmental growth conditions and is regulated by the RisA and the BvgAS two-component systems. As such, under the right conditions, at least some of the *Bordetella* produce a surface-associated type II microcapsule. The role of the capsule in the transmission, colonization, and persistence of the various bordetellae is not understood. There is evidence that in the case of *B. pertussis* the capsule does not play a role in defense against complement-dependent killing or phagocytosis. On the other hand, the capsular locus is expressed during infection, and encapsulated mutants undergo variable reductions in their ability to colonize. Elements of the capsule assembly and transport system appear to contribute to the stability of the plasma membrane and, as such, the structure and function of various membrane proteins.

Cell Wall

The cell wall of the members of this genus is typical of gram-negative bacteria, being composed of carbohydrates, lipids, and proteins.

Lipopolysaccharide (LPS)

The biosynthesis of a full-length LPS molecule by *Bordetella* is essential for the expression of full virulence. The LPS structures of *Bordetella* are modified in a BvgAS-dependent manner, indicating involvement in some aspects of transmission, colonization, and persistence. Different distal structures modifying the LPS are related to subspecies and appear to serve different purposes in respiratory tract infection. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* LPS are not the same, being composed of a complex trisaccharide (*B. pertussis*), a trisaccharide plus an O-antigen-like repeat (*B. bronchiseptica*), or a modified trisaccharide plus an O-antigen-like repeat (*B. parapertussis*). Cell wall LPS of *B. avium*, and by inference *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, serves as an adhesin by binding to ciliated epithelium of the respiratory tract, and as a "shield" to cover the outer membrane protecting it from the action of membrane attack complexes generated by the complement system.

The LPS in the outer membrane is an important virulence determinant, interacting with the innate and adaptive immune responses, and likely acting in association with other toxins. Not only is the lipid A component toxic (endotoxin), but the length of the side chain in the O-repeat unit hinders the attachment of the membrane attack complex of the complement system to the outer membrane and imparts resistance to antimicrobial proteins (defensins) found in respiratory tract secretions and within phagocytic cell granules. LPS binds to LPS-binding protein, which transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells, triggering the release of pro-inflammatory cytokines including tumor necrosis factor.

The composition of the LPS (presence or absence of the O-repeat portion, its length, charge, and fatty acid substitutions) influences the extent of protection against the complement system and antimicrobial peptides, and may be involved in differences in virulence between strains. *B. pertussis* has two types of LPS, LPSI and LPSII. LPS composition is regulated by the BvgAS system. In comparison to their parent strain, the LPS-surface-exposed mutants are less able to colonize locations in the respiratory tract and the time points differed significantly. Such mutants are also more sensitive to complement-mediated killing.

Outer Membrane Protein

Brk (for *Bordetella* resistance to killing) is an outer membrane protein and provides resistance against serum-mediated killing. Brk imparts resistance to damage by the complement system and may also function as an adhesin (it contains two Arg-Gly-Asp regions as well as binding locations for sulfated glycoconjugates). Brk is also regulated by the BvgAS system. The outer membrane protein profile of *B. avium* is distinctly different from those of the other *Bordetella* species, and as predicted the growth medium influences the expression of outer membrane proteins.

Exotoxins

The presence of the genes encoding protein toxins, which include AC toxin, pertussis toxin, and DNT, are characteristic of the classical *Bordetella* ssp. Pertussis toxin is a hallmark of *B. pertussis*, and DNT is produced by *B. avium*.

There are five known exotoxins produced by *Bordetella* species that play roles in clinical diseases: tracheal cytotoxin, DNT, adenylyl cyclase toxin, pertussis toxin, and

osteotoxin. All, except osteotoxin and tracheal cytotoxin (a portion of the cell wall), are regulated by the BvgAS regulon (see section titled "Regulation of the Cellular Products of Medical Interest" below).

Tracheal Cytotoxin

Produced by the "classic" *Bordetella* species plus *B. avium*, tracheal cytotoxin is a fragment of the *Bordetella* PGN cell wall that is identical to *Neisseria gonorrhoeae* ciliostatic anhydropeptidoglycan that in other gram-negative bacteria. Acting in association with LPS, tracheal cytotoxin damages ciliated epithelial cells by interfering with DNA synthesis. Tracheal cytotoxin also induces the production of interleukin-1 (IL-1) and excessive amounts of nitric oxide from macrophages.

Dermonecrotic Toxin (DNT)

DNT is a member of a family of toxins with similar structures and biological activity. The other "dermonecrotic" toxins include PMT produced by *Pasteurella multocida* (see Chapter 10), and CNF1 and CNF2 produced by *E. coli* (see Chapter 6). These toxins are so named because dermonecrosis follows their injection into the skin, an occurrence that does not occur naturally (DNT is not secreted). DNT deaminates and transglutamates (preferred) the small GTP-binding protein Rho blocking its GTPase activity (i.e. GTPase activating proteins are unable to correctly bind to modified Rho). These modifications result in changes in the actin cytoskeleton of affected cells (actin stress fibers) and inhibition of differentiation of osteoblasts in bone tissue. DNT expression is required for *B. bronchiseptica* to cause turbinate atrophy in pigs and pneumonia in mice, and for *B. avium* to be pathogenic.

Adenylyl Cyclase Toxin

Adenylyl cyclase toxin is a bifunctional protein with independent adenylyl cyclase as well as hemolytic activity that is produced by classic *Bordetella* species but not *B. avium*. Adenylyl cyclase toxin is the only known *Bordetella* exotoxin that is secreted without proteolytic cleavage. It enters the host cell, and following "activation" by calmodulin, increases the intracellular concentration of cAMP. Affected cells lose control of intracellular levels of cAMP, resulting in an inability to regulate ion and fluid flow into and out of the "target" cell (respiratory tract epithelium). Loss of control of cAMP levels in phagocytic cells results in reduction of this cell's ability to phagocytose and kill. The hemolytic activity is associated with an RTX motif (repeats in toxin; named after the common feature of repeats in glycine-rich sequences within the protein; see also *E. coli* hemolysin, *Pasteurella/Mannheimia* leukotoxin, *Actinobacillus* hemolysin, and *Moraxella* cytotoxin). By virtue of the RTX-type toxin activity, adenylyl cyclase toxin is also a pore-forming protein that targets neutrophils, macrophages, and lymphocytes. Taken together, adenylyl cyclase production is necessary for infection and plays a central role in virulence.

Pertussis Toxin

Pertussis toxin (Ptx) is an ADP-ribosylating toxin that ribosylates "activated" heterotrimeric G proteins (GTP-bound), rendering them incapable of returning to the inactive state (GDP-bound). "Activated" G proteins stimulate adenylyl cyclase, leading to increased levels of intracellular cAMP. Abnormally high concentration of cAMP results in loss of fluid and ion flow into and out of the cell, and if the cell has phagocytic function, interference with uptake and intracellular killing. Although Ptx disrupts a number of metabolic pathways, the host cell typically is not killed. The role of Ptx as an adhesin was discussed earlier. Ptx induces a significant and fully protective immune response.

Osteotoxin

Osteotoxin is produced by *B. avium, B. pertussis*, and *B. bronchiseptica* but not by *B. parapertussis*. It is lethal to tracheal and bone cells via the reactive products of cleaved extracellular cysteine.

Iron Acquisition

Because iron is an absolute growth requirement, microorganisms must acquire it if they are to survive within the environment and their host. *Bordetella* species have multiple iron acquisition systems that in combination ensure the availability of iron regardless of the environmental conditions and stage of infection. Over the course of infection, changes in growth conditions (including intercellular) alter the source and abundance of iron. The *Bordetella* iron acquisition genetic systems are known include alcaligin, enterobactin, and heme. Under iron-replete growth conditions, the genes of these three iron systems are repressed by Fur. Each is under positive transcriptional regulation that involves induction by the cognate iron source under derepressing conditions.

Under conditions of iron starvation (as during infection), bordetellae secrete a hydroxamate-type siderophore called alcaligin. Growth of *B. bronchiseptica* in iron-depleted and serum-supplemented medium is enhanced by norepinephrine. Growth promotion is siderophore

independent. *B. bronchiseptica* alcA alcaligin biosynthesis mutants (that do not produce siderophores) can obtain transferrin-bound iron via norepinephrine.

Bordetellae acquire iron by secreting a siderophore and utilizing siderophores made by other species of microorganism. *Bordetella* can utilize enterobactin, a siderophore produced by members of the family Enterobacteriaceae, as well as xenosiderophores. These siderophores (alcaligin and enterobactin) remove iron from the iron-binding proteins of the host (transferrin and lactoferrin) and make it available to the bacterium. In addition, they utilize the iron found in heme and hemoproteins.

B. pertussis, *B. parapertussis* and *B. bronchiseptica* have 14–19 genes encoding TonB-dependent outer membrane receptors that are known or predicted to be involved in iron uptake. To obtain iron, the siderophore alcaligin (*B. pertussis* and *B. bronchiseptica* are also capable of using the xenosiderophores enterobactin ferrichrome and desferrioxamine B). *B. bronchiseptica* also can use ferrichrysin, ferrirubin, aerobactin, protochelin, schizokinen, ferricrocin, vicibactin, and pyoverdin. In *Bordetella*, norepinephrine-mediated transferrin-iron uptake requires neither the BfeA enterobactin receptor nor the alcaligin siderophore or its FauA receptor. As such, the TonB system in norepinephrine-mediated iron acquisition is likely responsible.

Utilizing the TonB system, *B. pertussis* and *B. bronchiseptica* can use hemin and hemoglobin as sole sources of iron. The outer membrane protein that binds these, BhuR (for *Bordetella* heme uptake receptor), is regulated by levels of available iron by means of the extracytoplasmic sigma factor Rhu and the Fur regulon. When the bacterium's iron demands are satisfied, iron activates the DNA-binding activity of the Fur repressor, and the iron transport system expression is repressed.

Type III Secretion System (T3SS)

The T3SS injectosome is a structurally and functionally complex protein-export apparatus that enables the delivery of bacterial effector proteins into the cytosol of the host cells through a conduit spanning the bacterial membrane and the plasma membrane of target cells. It is an assemblage of proteins (more than 20) that form a hollow tube-like extracellular needle-like appendage with a central channel of ~2 nm in diameter that protrudes from the bacterial surface through which effector proteins are "injected" into host "target" cells. T3SS injectosomes are widespread among gram-negative bacteria, including animal, plant, and insect pathogens. The genes for the components of the T3SS injectosome are generally clustered on a mobile genetic element and are acquired as an intact genetic block by horizontal gene transfer.

Although the genes for T3SS are present in *B. pertussis* and human strains of *B. parapertussis*, evidence of production is lacking. *B. avium* appears to lack the required genes. *Bordetella* effector proteins have not been identified, but host "target" cells (tracheal epithelial cells, phagocytic cells) undergo apoptosis as a result of effector protein "injection," resulting in loss of epithelial integrity and evasion of the immune system of the host (decreased phagocytosis, and decreased antigen processing that leads to reduced immune responses).

T3SS has been identified in the *B. bronchiseptica* and *B. parapertussis* (under the control of the BvgAS regulon; see section titled "Regulation of the Cellular Products of Medical Interest" below). The *bsc*-encoded genes of the *Bordetella* injectosome are induced during infection, and are responsive to blood or serum exposure and increased CO_2 concentrations. Other stimuli that can activate T3SS expression and secretion in *Bordetella* are response triggered iron limitation and/or starvation for carbon sources. Upon the internalization of *Bordetella* into macrophages, the transcription of injectosome genes is downregulated as an adaptation to intercellular persistence.

Regulation of the Cellular Products of Medical Interest

Expression of *Bordetellae* virulence factors is regulated by two independent two-component systems, the BvgAS and the RisA regulons. These regulatory systems have an environment-sensing component that detects key signals such as temperature, which in turn promotes the second component of the system that via phosphorylation directly or indirectly controls expression or repression of a very large number of cellular products, including key virulence factors that enhance survival and transmission. Individually and especially in combination, these gene regulatory systems reflect the degree of complexity associated with understanding the natural history of the *Bordetella* as a group as well as individual species. The BvgAS modulates the virulent $BvgAS^+$, intermediate $BvgAS^i$, and avirulent $BvgAS^-$ phases of virulence factor production. RisA controls gene expression that is necessary for intracellular survival.

The $BvgAS^-$ phase of *B. bronchiseptica* is characterized by the expression of flagellin and the repression of adhesins and toxins necessary for the colonization of the respiratory tract. In contrast, $BvgAS^+$ phase involves the repression of flagellin, adhesins, and chemotaxis genes but the enhanced production of virulence factors important in both colonization, host response, and disease

occurrence. Intercellular survival is likely BvgAS independent and is characterized by the expression of cellular surface components such as capsule and O-antigens but the repression of virulence genes. Intercellular survival mimics the expression of genes associated with environmental growth that avoids triggering a host response associated with disease, thus allowing for clinical disease-free persistence and an enhanced potential for transition.

Growth Characteristics

Bordetellae are fastidious and can be difficult to culture, particularly on primary isolation, where depending on the stage of infection, 50% of attempted initial isolations fail. Mature colonies appear in one to three days (Figure 13.1). All but one of the species of *Bordetella* are strict aerobes, deriving energy from oxidation of amino acids. *B. petrii* (the exception) is a facultative anaerobe. Growth can be inhibited by fatty acids, particularly in the case of *B. pertussis*, which requires the use of specialized media. *B. bronchiseptica* and *B. avium* grow on ordinary laboratory media (35–37 °C), including MacConkey agar, under atmospheric conditions; the former is inconsistently hemolytic on blood agar. *B. bronchiseptica*, *B. avium*, and *B. hinzii* are motile under laboratory conditions. *B. pertussis* does not grow on Columbia agar.

Biochemical Activities

B. avium and *B. bronchiseptica* are catalase and oxidase positive, ferment no carbohydrates, and utilize citrate as an organic carbon source. *B. bronchiseptica*, *B. avium*, and *B. hinzii* are oxidase positive. Only *B. bronchiseptica* reduces nitrate and produces urease, *B. parapertussis* is weakly urease positive.

Resistance

Bordetellae are killed by heat or disinfectants. They are susceptible to broad-spectrum antibiotics (tetracyclines and fluoroquinolones) and polymyxin, but not to penicillin. Their environmental and intercellular survival characteristics have epidemiological significance.

Variability

The variability between and among the various bordetellae is suggested by both the observed genotypic and phenotypic differences. However, because of the influence of the BvgAS and other regulon systems on gene expression, including during *in vitro* culture, the true variability is likely to be significantly underestimated.

An assortment of typing schemes has been developed, mainly for epidemiological purposes. At least four serotypes have been described for *Bordetella* based upon the Fim protein (see section titled "Adhesins" above). Except for *fimA,* the fimbrial genes of *B. avium* are not shared with the bordetellae. Related to differences in the number of proline-rich repeats, pertactin varies both in size and in antigenic character between *Bordetella* species and within strains of the same species.

B. bronchiseptica dissociates into four phases varying in colonial characteristics, hemolytic activity, suspension stability in saline, ease of colonization, and toxicity. Some strains appear to be host specific. At least 20 heat-labile (K) and heat-stable (O; 120 °C/60 minutes) antigens exist. Many are common to several species. Others

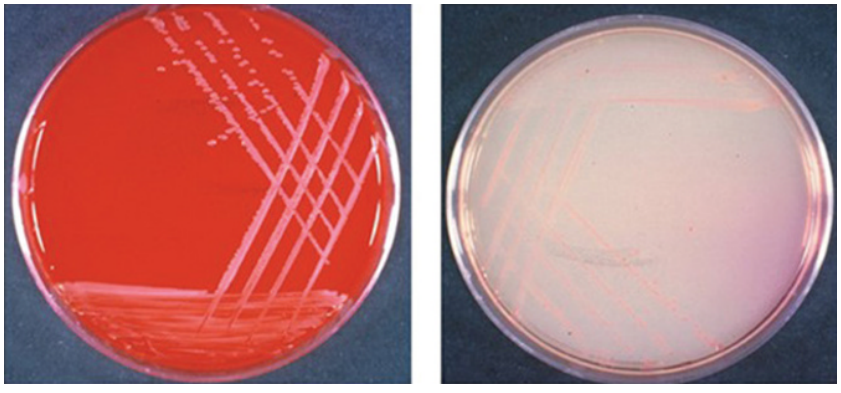


Figure 13.1 Growth of *Bordetella bronchiseptica* on (a) blood agar and (b) MacConkey agar plates.

are species and type specific. *B. bronchiseptica* from pigs appear different from the strains from dogs and horses (which are also different from each other). Other strain-specific disease, phenotype, and molecular differences have been identified and hypothesized to be the basis for vaccine failures.

Three serotypes, based on surface agglutinins, are recognized in *B. avium*. *B. parapertussis* strains that infect and cause disease in humans are distinct from those strains that cause chronic nonprogressive pneumonia in sheep.

Ecology

Reservoir

Bordetellae colonize the surface of ciliated respiratory tract epithelium and intercellularly to phagocytic cells. They are routinely found in the nasopharynx of healthy animals and in at least the case of *B. bronchiseptica* have an environmental reservoir within amoeba. Both are the likely sources of exposure that could produce disease. Although some bordetellae have or appear to have a limited host range related to the occurrence of clinically evident respiratory disease, *B. bronchiseptica* associated disease occurs in a wide range of wild and domestic animals, laboratory rodents, swine, rabbits, and occasionally horses, koalas, seals, other herbivores, primates, and turkeys. It is safe to say that under the right circumstances, as an opportunistic pathogen or co-pathogen, it has the potential to be involved in respiratory disease nearly all animals. *B. avium* inhabits the respiratory tract of infected wild and domestic fowl, resulting in disease principally in turkeys (coryza).

Transmission

Infection is primarily airborne, but environmental contamination can be a significant factor in some cases, especially where animal density is high. In turkeys, *B. avium* is also indirectly spread via contaminated water and litter. Transmission is most likely during the early catarrhal phase, when clinical symptoms are relatively mild and non-characteristic. Subclinical cases may have similar epidemiologic significance.

Experimentally, as few as 10 organisms can establish an infection, but the experimental creation of typical respiratory disease in the natural host is not reliable and can take very large *Bordetella* inoculation numbers. Disease outbreaks can occur and increase rapidly. Environmental conditions (temperature, humidity) and host density influence the frequency and severity of clinical disease. The hypothesis is that phenotype shifts (loss of adhesins) associated with the BvgAS system promote transmission and that once deposited in a new host the appropriate environment is recognized, thus reactivating the BvgAS system and resulting in the production of disease-associated virulence factors, most notably adhesins and toxins.

The natural habitat of *B. bronchiseptica* is the respiratory tract of animals such as rabbits, cats, and dogs. *B. bronchiseptica* infection in humans is rare, occur most often after close contact with carrier animals and association with immune compromise.

Pathogenesis

Mechanisms

Studies of numerous virulence factors and their corresponding biological activities have yielded plausible explanations for many of the clinical disease characteristic of *Bordetella* infection. On the other hand, the occurrence of culture-positive subclinical infections and the frequency of seropositive antibody levels indicate that *Bordetella* spp. are perhaps often an opportunistic pathogen in animals. Complicating this is the fact that bordetellae are masters at "sensing" various environmental cues and via various gene expression regulatory systems adapting to survive. Most notably, the BvgAS regulon that influences the expression of various genes encoding metabolic functions and virulence factor production was discussed earlier (see section titled "Cellular Products of Medical Interest" above). The challenge to understanding the importance of this adaptive ability is the limited capability to fully replicate these environmental conditions *in vitro* (including conditions associated with intracellular survival and the dynamics of infection). Vaccine escape variants, specific gene knockouts, and *in vitro* expression measurements in laboratory animal disease models have been helpful but do not provide a complete picture.

The attachment of bordetellae to ciliated epithelial cells follows expression of adhesins, some of which also have toxic and pro-inflammatory properties (fimbriae, FHA, pertactin, cell wall LPS, Tcf, and biofilms – see Table 2 for a listing of which adhesins are produced by which *Bordetella*). *B. bronchiseptica* produces ciliostasis within three hours in canine tracheal explants, which may be explained by the action of AC toxin and tracheal cytotoxin that can also inhibit the phagocytic activities. In addition, *B. bronchiseptica* flagellin is a potent pro-inflammatory factor that induces chemokine, cytokine, and host defense gene expression through Toll-like receptor 5.

Bordetellae adhere to inflammatory cells (by way of FHA, pertactin, and pertussis toxin), and release adenylyl cyclase (interferes with phagocytosis and killing), pertussis toxin

(interferes with phagocytosis and killing), and "injection" of an effector protein (interferes with phagocytosis). If phagocytosis occurs, by repression of the expression of some virulence factors and enhanced production of others, the bordetellae survive within phagolysosomes by blocking lysosomal fusion and persist by escaping into the endocytic compartment. Bacteremia does not occur.

Multiplication occurs (iron is scavenged from host iron-binding proteins – lactoferrin and transferrin – and from heme and hemoproteins; the *Bordetella* outer membrane is protected from complement-generated membrane attack complexes by capsule, LPS, Brk) and inflammation is initiated (LPS; death of epithelial cells due to "injection" of effector proteins; tracheal cytotoxin). Inflammation and loss of the ability to control fluid and ion flow into and out of tracheal epithelial cells (increased production of cAMP from effects of increased activity of adenylyl cyclase) leads to increased mucus and fluid accumulation in the upper respiratory tract. Ciliated epithelial cells become ineffectual in clearing secretions (cilial paralysis due to increased levels of cAMP and cytoskeletal changes brought about by the action of DNT; death of ciliated cell due to tracheal cytotoxin; and the unidentified effector protein "injected" by way of the type III secretion system).

The consequences of *Bordetella*-induced changes in the ciliated portions of the respiratory tract vary but generally include depression of respiratory clearance mechanisms, facilitating secondary complications (e.g. pneumonia); in pigs, *B. bronchiseptica* causes nasal irritation, rendering the turbinates susceptible to the action of the DNT (Pmt) of *P. multocida*, which has emerged as the primary agent of atrophic rhinitis (for "progressive atrophic rhinitis," see Chapter 10). If *P. multocida* does not become involved, DNT, as well as all of the products described above, produces a mild, reversible turbinate hypoplasia ("nonprogressive atrophic rhinitis"). Pneumonia, if it occurs, is due to inflammatory responses and the inability of host phagocytic cells and the complement system to easily clear *Bordetella* as well as other microorganisms that might colonize the respiratory system. Interestingly, there is currently no mechanistic explanation for the paroxysmal coughing that is the hallmark of pertussis. Purified pertussis toxin itself is not sufficient, and paroxysmal coughing occurs with *B. parapertussis*, which does not appear to produce pertussis toxin.

Pathology

The attachment, functional impairment, and destruction of ciliated respiratory epithelium is the hallmark of *Bordetella*-associated clinical disease. Depending on the animal species involved and their immune status, the typical process begins with a catarrhal phase characterized by sneezing, coughing, and rhinorrhoea. Fever is not common. This develops into a suppurative phase affecting various portions of the tract (rhinitis, sinusitis, tracheitis). Compromise of the clearance mechanisms of the upper respiratory tract may lead to pneumonia and airsacculitis with *Bordetella* itself and secondary bacterial superinfections that can significantly enhance disease severity. On the other hand, prior or co-infection with other respiratory microorganisms (e.g. *canine influenza virus*) may enhance the potential of *Bordetella*-associated disease that is antibiotic responsive.

Immunologic Aspects

Pathogenic Factors

Many of the virulence factors produced by the bordetellae function, either directly or indirectly, to overcome the host's immune response, resulting in disease, transmission, and persistence. As noted previously, bordetellae can also downregulate these factors to avoid triggering an immune response, especially during intracellular colonization. Clearly, the host-specific nature of some of the bordetellae (most notably, *B. pertussis* infects only humans whereas *B. bronchiseptica* has a broad host range, except for humans) provide clear evidence of innate host-specific immunity. Depressed cell-mediated responses have been observed in experimental *B. avium* infections. *B. bronchiseptica* can alter the response of dendritic cells, which decreases production of IL-10 and interferon. Not infrequently, injectable *B. bronchiseptica* bacterin vaccines may induce a local type-III-like hypersensitivity reaction in dogs.

Protective Role

The defense mechanisms preventing clinical respiratory disease cause by *Bordetella* spp. are both nonspecific (local inflammation, increase in macrophage activity, and production of interferon) and specific (proliferation of B- and T-cells). Overall, the basis of immunity is incompletely understood. Mucosal antibody is believed to prevent *B. bronchiseptica* colonization in dogs, but direct evidence is lacking including the unreliable protection provide by intranasal vaccines. The significant degree of common antigens as well as *Bordetella* species and strain-specific differences make it difficult to assess the degree of immunity to infection or disease. Localized reactions typical of a type III hypersensitivity-like reaction occasionally occur

with parenteral vaccines, especially in dogs. *B. avium* antiserum is ineffective in protecting turkeys, but maternal immunization reduces losses in challenged progeny. Antibody to various adhesins prevents adherence to tracheal epithelia under *in vitro* conditions.

Immunization

Various vaccine formulations are the cornerstone of providing protection against whooping cough caused by *B. pertussis* in humans. Over time the effectiveness of some of these vaccines has fallen to unacceptable levels because of the selection of *B. pertussis* antigen variants. The result has been the development and usage of new vaccines. A role for circulating antibody in immunity is indicated by the correlation between protection of human *B. pertussis* vaccinees and serum agglutinin titers. However, effective immunity does not necessarily depend on the presence of serum agglutinins, and immunity may be mediated in part by cellular immune mechanisms.

In animals, vaccines appear to afford a degree of protection against clinically apparent disease, but as is the case with *B. pertussis*, do not appear to prevent colonization, persistence, and transmission. As such, *Bordetella* vaccines in animals are not viewed as being a necessary part of a routine vaccine plan but rather to be used only when the risk of exposure and clinical disease is exceptionally high. The overuse of kennel cough vaccines with very little evidence of their general clinical disease prevention value is a concern. In contrast to current vaccines that at require yearly boosters to provide any measure of protection (in the case of *B. bronchiseptica* in dogs and cats), infection provides substantial immunity that appears to last for several years.

Parenteral administered killed and antigen extracts as well as internasal/intratracheal live attenuated vaccine have been beneficial but provide unreliable protection against kennel cough in dogs and cats. As in the case with vaccine failures against *B. pertussis* in humans, this is likely related to critical antigenic differences, particularly with pertactin, between vaccine and wild-type strains of *B. bronchiseptica*. Localized reactions typical of a type III hypersensitivity occasionally occur with parenteral vaccines and has motivated the development of internasal vaccines, which are claimed to provide protection in just a day or two. Such immediate protection is unlikely to be specific but rather a benefit of a nonspecific increase in general mucosal immunity.

Bacterins used on pregnant sows provide some colostral immunity to piglets, especially when including toxigenic *P. multocida* strains. Bacterin-toxoid preparations protect piglets. Of the *B. avium* vaccines, those using attenuated live organisms intratracheally have been most effective.

Laboratory Diagnosis

Laboratory testing methods include culture, cytology with direct observation and fluorescent antibody, polymerase chain reaction (PCR), and serology. The "gold standard" for the diagnosis to *Bordetella* infection is a positive culture. However, *Bordetella* is not easy to isolate on primary culture, with estimates that over 60% of culture attempts fail. Nevertheless, the attempt should be made as it allows for antibiotic sensitivity determination and strain-specific genetic evaluations that contribute to epidemiologic assessments and better transmission control. Culture success strongly depends on the technique used to collect the sample and the initial culture. *Bordetella* can be cultured/detected from nasopharyngeal swabs, nasopharyngeal secretions, bronchoalveolar lavage fluid, and transtracheal washes. It is underappreciated that swabs should be made of dacryon or calcium alginate (which also allows for PCR testing) and transported in charcoal-based agar (e.g. Ames medium that includes charcoal transport-medium, charcoal blood agar, Regan-Lowe medium) at room temperature.

Nasal swabs, sediment of transtracheal washes, and tracheal swabs are cultured on blood and MacConkey agars. The use of Bordet-Gengou and charcoal blood agar can be helpful in the initial recovery of *B. bronchiseptica*. Selective media are helpful in limiting overgrowth by faster-growing bacteria. Colonies of *B. bronchiseptica* and *B. avium* can easily be overlooked on primary isolation, as they are often less than 1 mm in size after 48 hours incubation and are variably hemolytic. Routine laboratory tests (oxidase, urase, nitrate-reductase, carbohydrate use) are used for identification. Differential agglutination of sheep and guinea pig red blood cells are helpful for differentiating *B. avium* and *B. bronchiseptica*. *B. avium* reacts like *Alcaligenes faecalis* in routine laboratory tests and can be differentiated by cellular fatty acid analysis. In addition, *B. avium* must be differentiated from *B. hinzii*.

Cytologic examination of transtracheal or endotracheal washings, or bronchoalveolar lavage samples with the direct observation of *Bordetella*-consistent microorganisms clustered on the villi of epithelial cells is diagnostic. These same samples are more valuable than nasal, oral, or nasopharyngeal samples in increasing the chances of a positive culture. Various PCR-utilizing primers designed to amplify various species-specific segments of DNA are available for determining the presence of *Bordetella* as well as for species-specific identification purposes. PCR detects roughly twice as many cases as culture.

Serology (agglutination, complement fixation, enzyme-linked immunosorbent assay or ELISA) can be useful in confirming a diagnosis, especially when clinical signs are

mild and culture has not been successful. Paired serum samples are needed with the optical timing of the second sample being several weeks following the onset of clinically evident disease. A serologic response is typically mounted by two weeks following infection. Vaccination will result in a *Bordetella* IgG response and may interfere with result interpretation.

Treatment and Control

The basis of *Bordetella* disease control in animals is good infectious disease control that is focused on preventing transmission between healthy carriers and fully susceptible new hosts, either directly by aerosol or indirectly by mechanical transfer. Significant disease outbreaks, in contrast to subclinical infection, are often associated with less than optimal environmental conditions. Vaccination, when available, reduces the likelihood of significant clinical disease but appears to have marginal impact on the potential of infection and perhaps the development of carriers.

Canine tracheobronchitis responds inconsistently to antibiotics. Vaccination, fumigation of kennels, adequate ventilation, and isolation of affected dogs and cats are useful preventive practices. Prior infection, with or without clinical disease, does not provide durable protection against reinfection. Tetracycline, or equivalents, remains the drug of choice. There is widespread resistance to cephalosporins.

The progressive form of atrophic rhinitis is not treatable. Preventive measures include maintenance of an aged sow herd with a low carrier rate; preventing transmission from sow to piglet via mediated and/or segregated early weaning methods; thorough disinfection and cleanup of farrowing houses and nurseries after each use (all-in/all-out production); vaccination; prophylactic use of sulfonamides in feed or water; and elimination of carrier sows based on nasal swabs.

B. avium is susceptible to tetracycline, erythromycin, and nitrofurantoin, but resistant to penicillin G, streptomycin, and sulfonamides. Medication during the early stages of a disease outbreak can be economically beneficial. Mass medication and vaccination may prevent outbreaks without eliminating the infection.

Animal Disease Patterns

Swine

Atrophic Rhinitis

The progressive form of atrophic rhinitis is due to combined infection with *P. multocida* and *B. bronchiseptica* (Figure 13.2). The nonprogressive form of this disease follows infection with *B. bronchiseptica* alone and is transient and self-limiting, most frequently causing disease (sneezing and nasal discharge) in pigs three to four weeks old.

Pneumonia

Compromise of the host defenses of the upper respiratory tract sometimes leads to secondary pneumonia associated with *B. bronchiseptica* and/or some other microorganism. Primary pneumonia caused by *B. bronchiseptica* occurs in neonatal pigs (three to four days old) and is characterized by coughing, dyspnea, and at times high morbidity and mortality. Disease outbreaks are likely associated with failure of passive immune transfer or low material immunity.

Dogs

Canine Infectious Tracheobronchitis (Kennel Cough)

Kennel cough is directly associated with *B. bronchiseptica*, with clinical disease being most common in young dogs as well as in mature dogs who are susceptible under the right circumstances, whether vaccinated or not. The natural disease may be accompanied by canine influenza virus, canine

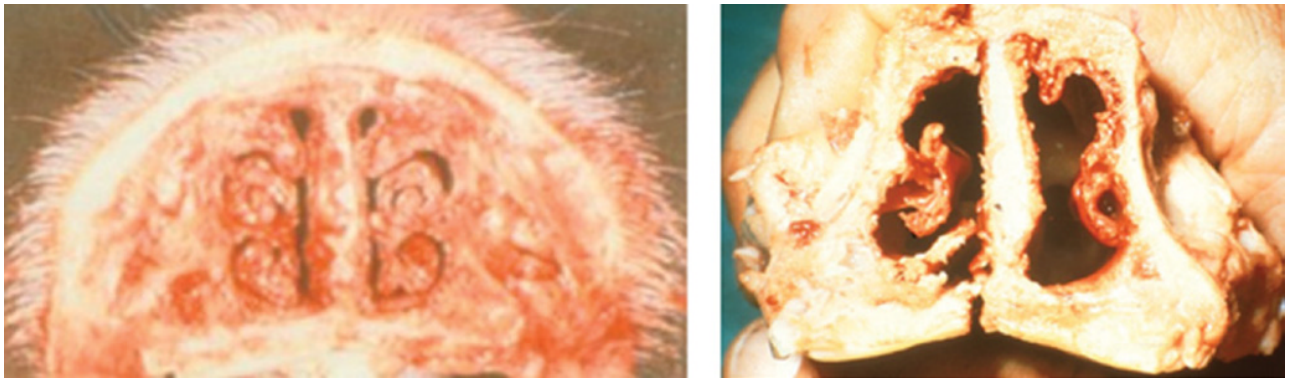


Figure 13.2 Cross-section of nasal turbinitis of (left) normal and (right) atrophic rhinitis pigs.

parainfluenza virus, canine adenoviruses 1 and 2, and canine herpesvirus. Rapidly progressing disease outbreaks occur in kennels and animal hospitals. The incubation period is around a week followed by acute coughing, in the most severe cases gagging and retching. Although most dogs recover within a few weeks without treatment, the bacterium can persist for months and relapses are possible. Transfer of the same strain to susceptible cats has been demonstrated.

Seropositive rates in healthy unvaccinated adult dogs can be 22%, recognizing that titers decline over time after infection. Isolation and PCR detection rates in healthy dogs can be as high as 45% and 52–78% in dogs with clinical respiratory disease. By modulating the immune response and locating intracellularly, *B. bronchiseptica* in dogs is adapted to establish long-term asymptomatic infection. As such, infections are typically chronic, often asymptomatic, and notoriously difficult to clear even with antibiotic treatment and vaccination. There is increasing evidence of some resistance to certain antibiotics including tetracyclines and ampicillin.

Pneumonia

B. bronchiseptica is sometimes isolated from samples collected from pneumonic lungs, most often from dogs with canine distemper and can be complicated by co-infections with *P. multicide*.

Cats

Upper Respiratory Disease

B. bronchiseptica has long been recognized as an important agent in canine kennel cough and is now increasingly understood to play a role in upper respiratory track disease (URTD) and subclinical infection in cats. *B. bronchiseptica* infections in cats are more widespread than previous recognized. This is because the clinical disease signs of *B. bronchiseptica* infection in cats (mild tracheobronchitis, conjunctivitis) are similar to common respiratory viruses and other infections caused by herpesvirus, calicivirus, *Mycoplasma*, and *Chlamydophila*. The challenges associated with isolating *B. bronchiseptica* contribute to the uncertainty.

The seroprevalence rate provide an estimate of the frequency with which cats are infected with *B. bronchiseptica*. Prevalence studies suggest that *B. bronchiseptica* infection is widespread with 24–87% being seropositive. Highest rates are seen in multi-cat households and rescue catteries. As noted previously, given the broad host range of *B. bronchiseptica*, there is strong molecular evidence that strains can be transmitted between dogs and cats.

B. bronchiseptica is capable, under certain circumstances, of causing clinically evident URTD in the absence of other identified pathogens. Experimental infections in kittens result in signs (nasal discharge, sneezing, spontaneous or induced coughing, and dry or wet rales) within five days, which spontaneously resolve in 10 days. Cats can carry the organism asymptomatically (up to 19 weeks) following recovery. Less frequently, severe bronchopneumonia associated with *B. bronchiseptica* may occur, particularly in kittens and immunocompromised cats.

Rabbits

Snuffles

B. bronchiseptica infections in rabbits are common and usually asymptomatic. The pathogenicity is uncertain. The organism has been noted to prefer the cilia of the respiratory epithelium in rabbits, and so infection with *B. bronchiseptica* may impair mucociliary clearance and allow for the entry of more pathogenic organisms. It is thought to contribute to "snuffles" (rabbit upper respiratory tract infections) and is often found as a co-infection with *P. multocida*. *B. hinzii* has been described in rabbits.

As a carrier, rabbits can shed *Bordetella* into the environment, potentially putting other animals at risk for infection. This means that even healthy rabbits with no signs of illness can spread *Bordetella* to a guinea pigs (who suffer more significant disease) that may be in the same environment.

Guinea Pigs

Guinea pigs are perhaps one of the most susceptible animals to clinically serious *B. bronchiseptica* infections. Unlikely rabbits, guinea pigs may develop illness from *B. bronchiseptica* infection alone.

In guinea pigs, *B. bronchiseptica* outbreaks can be clinically significant, resulting in acute development of respiratory disease associated with high mortality. Morbidity and mortality are most commonly seen in young animals. Clinical signs are much less common in modern colonies even when *B. bronchiseptica* is detected, suggesting that past disease outbreaks may have been due to combined infections of *B. bronchiseptica* and some other infection(s). Most reports of *B. bronchiseptica* pneumonia in guinea pigs were before guinea pig adenovirus pneumonia was recognized.

Affected guinea pigs appear ill, with ruffled fur, labored breathing, sneezing, and anorexia. They may also have a mucopurulent or catarrhal exudate at the nares. Without quick appropriate treatment, it is not uncommon for the disease to quickly progress (24–48 hours) to potentially fatal pneumonia. Guinea pigs that survive an infection with *B. bronchiseptica* may become chronic asymptomatic

carriers. At necropsy, cranioventral areas of the lungs are consolidated, and there may be purulent exudate in the airways.

Poultry and Birds

B. avium and *B. hinzii* are pathogens of birds, causing coryza and rhinotracheitis. *B. avium* was isolated from young turkeys and has also been isolated in chickens, finches, noble macaws, Japanese quails, and ostriches. *B. hinzii* is found in the respiratory tract of poultry. Although it was initially described as a commensal bacterium in birds, some isolates appear to have pathogenic properties.

Chickens and other birds may be infected with *B. avium*. *B. hinzii* is generally viewed as a commensal but under the right environmental conditions can cause mild respiratory disease in turkeys. Wild birds commonly asymptomatically carry *B. avium* and *B. hinzii*. There have been a number of cases of *B. hinzii* in immunocompromised humans causing whooping-cough-like disease and septicemia.

Turkey poults infected with *B. avium* may develop tracheobronchitis, sinusitis, and airsacculitis (turkey coryza). A contaminated environment is important in perpetuating and transferring infection. Signs include nasal exudate, conjunctivitis, sneezing, tracheal rales, and dyspnea. Morbidity can be very high, but mortality, except by secondary infection, is generally low (<5%). Recovery may begin after two weeks, although some illnesses may last six weeks and are associated with stunted growth and tracheal collapse.

Horses

B. bronchiseptica is not infrequently isolated from tracheal cultures of healthy horses. The association of *B. bronchiseptica* with clinical disease in horses is much less common, and it is considered an opportunistic pathogen in horses. Disease associations that have been reported include respiratory track disease including pneumonia, conjunctivitis, and infertility. Depending on the severity, signs of *B. bronchiseptica*–associated respiratory disease include mucopurulent nasal discharge, cough, lethargy, and weight loss.

Sheep and Goats

Bordetella-related respiratory disease is sheep and goats is rarely reported. A sheep-specific lineage of *B. parpapertussis*, which can infect and cause disease in humans, is involved in pneumonia in sheep.

Laboratory and Wild Animals

B. bronchiseptica has been associated with mild respiratory disease, in most cases viewed as a secondary infection, in laboratory animals as well as in a very wide range wild animals, mostly as individual cases associated with respiratory disease, especially pneumonia.

Rats are frequently used as an animal model of *B. pertussis* infection and in understanding the role of individual virulence factors and immunity. Infectious are generally subclinical, but mice and rats with impaired immune system are susceptible to clinical disease caused by *B. bronchiseptica*. *B. hinzii* has been reported to cause pulmonary disease in laboratory mice and bacteremia in rats. *B. pseudohinzii* colonization of wild rats has been reported and is known infect laboratory mice. The few surveys of *B. bronchiseptica* infections of wild rats show a low prevalence.

Acknowledgment

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Further Reading

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14

Brucella

S.C. Olsen and P. Boggiatto

The genus *Brucella* encompasses a group of gram-negative bacteria that survive almost exclusively in infected hosts with a tropism for intracellular compartments of phagocytic, reticuloendothelial, and specialized epithelial cells. The genus has traditionally been divided into species based on microbial characteristics and host preference, but most *Brucella* species are capable of infecting many hosts. Clinical disease caused by infection with bacteria in this genus, brucellosis, is generally associated with chronic infections and pathologic effects in reproductive tissues.

One of the most important characteristics of brucellae is the number of species in the genus that can cause zoonotic infections. Human brucellosis is a significant, resurgent, worldwide zoonosis with particularly high prevalence in Middle Eastern, Central Asian, and Mediterranean countries. Because clinical symptoms are not pathognomonic, human brucellosis is frequently misdiagnosed. Addressing the disease in animal reservoirs is the most economical approach to preventing human brucellosis.

Descriptive Features

The *Brucella* genus has traditionally been classically characterized as having six species: *Brucella abortus, Brucella melitensis, Brucella suis, Brucella canis, Brucella ovis*, and *Brucella neotomae*. The high degree of homology at the genomic level has led to interpretation that the genus is composed of only one species, *B. melitensis,* with the other classical species proposed as strains of *B. melitensis*. However, nomenclature still utilizes the classical designations based on the preferred host species. Recent isolations of new *Brucella* species from sea mammals (cetaceans and pinnipeds), common voles, amphibians, baboons, a prosthetic breast implant, and Austrian foxes have expanded the number of species included in the *Brucella* genus (Table 14.1). Some of the classical species (*B. melitensis, B. abortus, and B. suis*) are divided into biovars based on biochemical (Table 14.2), phenotype, and antigenic properties. Although division into biovars has been used for epidemiologic purposes, biotyping can be somewhat subjective as it is based on subtle differences including requirements for higher CO_2 tensions for growth, production of hydrogen sulfide, growth on media containing dyes (thionin or basic fuchsin), and agglutination with monospecific A and M antisera. However, biovar classifications have been supported by whole genome sequencing analysis.

Morphology and Staining

Brucella are gram-negative, coccobacilli, that are usually single but can be in pairs or small groups. Coccobacilli are usually 0.6–1.5 μm in length and 0.5–0.7 μm in width. Pleomorphic forms are rare except in old cultures. *Brucella* are nonmotile and do not have capsules or form spores. True bipolar staining is not observed. Although not truly acid fast, they are resistant to decolorization with weak acids and therefore stain red with a modified Ziehl-Nielsen stain.

B. abortus, *B. suis*, *B. melitensis*, and *B. neotomae* are considered to have a smooth colony morphology (Table 14.1), a characteristic associated with expression of the lipopolysaccharide (LPS) O side chain. In comparison, *B. ovis* and *B. canis* have rough colony morphologies and do not express the LPS O side chain. Smooth colonies appear round, glistening, and blue-green in color and do not take up crystal violet stain. In comparison, rough colonies have a dry, granular appearance, are yellowish-white color, and stain with crystal violet.

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Table 14.1 Preferred hosts, colony phenotype and zoonotic characteristics of known *Brucella* species.

Species	Biovar	Preferred Host(s)	Smooth or Rough Phenotype	Zoonotic Potential
Brucella abortus	1	Cattle	Smooth	+
	2	Cattle	Smooth	+
	3	Cattle	Smooth	+
	4	Cattle	Smooth	+
	5	Cattle	Smooth	+
	6	Cattle	Smooth	+
	9	Cattle	Smooth	+
Brucella canis		Canine	Rough	+
Brucella ceti		Porpoises/dolphins	Smooth	−/+[a]
Brucella melitensis	1	Sheep/goats	Smooth	+
	2	Sheep/goats	Smooth	+
	3	Sheep/goats	Smooth	+
Brucella neotomae		Wood rat	Smooth	−
Brucella ovis		Sheep	Rough	−
Brucella inopinata		Frogs/toads	Smooth	+[b]
Brucella microti[c]		Soil/voles/foxes/frogs	Smooth or rough[c]	−[d]
Brucella papionis		Baboon	Smooth	−[d]
Brucella pinnipedialis		Seals	Smooth	−/+[a]
Brucella suis	1	Swine	Smooth	+
	2	European hare	Smooth	−[e]
	3	Swine	Smooth	+
	4	Reindeer/caribou	Smooth	−
	5	Rodents	Smooth	−
Brucella vulpis		Foxes	Smooth	−[d]

[a] Zoonotic infections associated with ST27 genotype.
[b] Isolated from infected human breast implant but not associated with typical symptoms of brucellosis.
[c] Some isolates from soil found to have rough phenotype.
[d] Not known to be zoonotic at this time.
[e] Potential for infection in immunocompromised individuals.

Cellular Structure and Composition

The cell wall of *Brucella* is typical for gram-negative bacteria. The outer membrane, approximately 4–5 nm in thickness, is composed of asymmetric layers of LPS and phospholipids and is supported by an underlying 3–5 nm layer of peptidoglycan. Some proteins, such as outer membrane protein A (OmpA), are covalently bound to the peptidoglycan layer and stabilize the outer membrane. The hydrophobic region of the membrane provides an anchor for proteins and forms a functional and structural barrier between the periplasm and the exterior of the cell. The periplasmic space varies from 3 to 30 nm. Porins in the outer membrane function as channels to the interior of the cell. Other proteins, such as lipoproteins, are also embedded in the outer membrane.

The lipopolysaccharide (LPS of *Brucella*) is composed of lipid A, core oligosaccharides, and O-poly-saccharides. The structure of LPS in *Brucella* differs from that in gram-negative enteric bacteria in that the backbone sugar of the lipid A is different and the LPS has a low phosphate content. The LPS protects the bacteria from cationic peptides, oxygen metabolites, and complement-mediated lysis. The O-polysaccharide on the LPS, expressed by smooth strains, is highly antigenic and can express A and/or M antigens dependent upon the species of *Brucella*. The *Brucella* cell envelope, LPS, lipoproteins, and flagellin display a reduced pathogen-associated molecular pattern (PAMP) for recognition by innate immunity, most likely due to hydrophobic moieties of the outer membrane including ornithine lipids. The altered PAMP of *Brucella* fails to induce a robust innate immune response, which contributes to the pathogen's *in vivo* stealth.

Table 14.2 Growth requirements for six classical Brucella species.

Species	Biovar	CO_2 Requirement	H_2S Production	Growth on Thionin[a]	Agglutination[b]	
					A	M
Brucella melitensis	1	−	−	+	−	+
	2	−	−	+	+	−
	3	−	−	+	+	+
Brucella abortus	1	+[c]	+	−	+	−
	2	+[c]	+	−	+	−
	3	+[c]	+	+	+	−
	4	+[c]	+	−	−	+
	5	−	+	+	−	+
	6	−	−	+	+	−
	9	+/−	+	+	+	+
Brucella suis	1	−	+	+	+	−
	2	−	−	+	+	−
	3	−	−	+	+	−
	4	−	−	+	+	+
	5	−	−	+	−	+
Brucella neotomae		−	+	−	+	−
Brucella ovis		+	−	+	−	−
Brucella canis		−	−	−	−	−

[a] On media containing a concentration of 10 μg/ml (1:100 000) thionine.
[b] Agglutination in monospecific sera for A (abortus) or M (melitensis).
[c] Usually on primary isolation.

Cellular Products of Medical Interest

The lipid A of *B. abortus* LPS has been demonstrated to have immunogenic properties of reduced toxicity as compared to LPS from other gram-negative bacteria. A 14-kDA protein, present in all traditional *Brucella* species, can function as a lectin by binding IgG's from several animal species and has hemagglutination properties. *Brucella* bacteria and LPS are also very poor activators of complement. However, heat-killed preparations of *B. abortus*, its LPS, and/or lipoproteins strongly elicit β-chemokines, T helper 1 (Th1) cytokines, and stimulate expression of stimulatory and adhesion molecules on antigen-presenting cells. This has led to their proposed use as adjuvants for human HIV vaccines

In phagocytic cells, virulent *Brucella* spp. inhibit phagolysosome fusion in phagocytic cells and may express proteins that bind to human Rab GTPase molecules and influence intracellular trafficking and/or maturation of phagosome-derived vacuoles into replicative vacuoles. *Brucella* can also produce guanosine monophosphate, adenine, and other compounds that interact with neutrophils to reduce myeloperoxidase activity, prevent primary granule release, and inhibit production of reactive oxygen compounds.

Growth Characteristics

Brucellae are aerobic and many strains require carbon dioxide for growth (Table 14.2). Thiamine, nicotinamide, and biotin are also required for growth. Addition of 5–10% serum to media may stimulate growth, but bile salts, tellurite, and selenite inhibit growth. Growth in liquid media is usually slow unless vigorous aeration is provided. Colonies generally become visible on solid media in three to five days, but samples should be incubated for at least 7–10 days before considered negative. Typical colonies of virulent strains are round with smooth margins, convex, translucent, and appear pearly white in color.

Resistance

Although brucellae can survive freezing and thawing, freeze/thaw cycles are associated with reductions in viability. Long-term survival under environmental conditions is highest under cool, moist conditions with minimal exposure to ultraviolet light. *Brucella* are susceptible to most

disinfectants for gram-negative bacteria. Lysis by bacteriophages is frequently used under laboratory conditions to type *Brucella*. Pasteurization of milk is considered effective for killing *Brucella*.

Diversity

Most classical species of *Brucella* have two circular chromosomes encoding approximately 2.1 and 1.2 megabase pairs (Mbp). The genome of *B. suis* biovar 3 is the exception in that it has a single chromosome of 3.1 Mbp. The origin of replication of the large chromosome is typical of bacterial chromosomes, whereas that of the small chromosome is plasmid-like. The guanine-cytosine (GC)-content of the two chromosomes is nearly identical with an average GC-content of approximately 58–59%. Classical species in the genus are highly homogeneous, with DNA–DNA pairing studies demonstrating greater than 90% homology across all species. Only 7000 single nucleotide polymorphisms have been identified in the 3.1 Mbp of genomic sequences shared across *Brucella* spp. Differences in genomic structure are primarily due to the occurrence of genomic islands, and deletions related to these regions were found in *B. abortus* and *B. ovis*. Though many bacterial genetic islands encode pathogenicity factors, most of the *Brucella* genomic islands encode hypothetical proteins and enzymes commonly found associated with horizontally acquired DNA such as transposases and integrases. The genomes of the classical *Brucella* species display an average of >94% identity at the nucleotide levels, with *B. abortus* and *B. melitensis* being the most closely related. A close relationship has also been detected between *B. canis* and *B. suis*, whereas *B. neotomae* and *B. ovis* demonstrate greater divergence levels from other *Brucella* species. With the exception of biovar 5, all other *B. suis* isolates cluster together.

Although many atypical species are genetically close to the *Brucella* genus, they are phenotypically similar to *Ochrobactrum* spp. Most atypical strains carry additional genetic material commonly found in soil-associated Alphaproteobacteria but not found in classical *Brucella* strains.

Brucellae have multiple mechanisms to protect against DNA mutations, and the genome is very stable. This trait prevents the use of restriction fragment length polymorphism (RFLP) techniques to characterize strain lineages within biovars. No mechanisms of genetic exchange, including phages, have been found in the genus.

Ecology

Zoologic and Geographic Reservoirs

Members of the genus *Brucella* exist as intracellular pathogens in mammalian hosts. Most species of *Brucella* are obligate pathogens in that they do not exist as commensals nor are they found free-living in the environment. However, more recently identified species, that is, *Brucella microti*, may have genetic differences that support a free-living as compared to a host-associated lifestyle. Although traditional species of *Brucella* may temporarily be recovered from environmental samples associated with infected animals, environmental persistence is generally not believed to be of epidemiologic importance. Because direct or close contact is generally believed to be required for transmission, maintenance of brucellosis in an animal or human population generally requires continual infection of susceptible hosts.

The preferred host of *B. abortus* is cattle, but it can also naturally infect numerous other species including bison (*Bison bison*), elk (*Cervus elaphus* and *Cervus canadensis*), camels (*Camelus dromedaries* and *Camelus bactrianus*), yaks (*Bos grunniens*), African buffalo (*Syncerus caffer*), and swine. Biovars 1 and 2 have widespread worldwide distribution, although biovar 3 is predominantly found in India, Egypt, and Africa. Several countries in Northern and Central Europe, Canada, Australia, Japan, and New Zealand are considered to be free of this pathogen.

Sheep and goats are the preferred hosts of *B. melitensis*, but this species of *Brucella* is also known to infect cattle, camels, free-ranging Alpine ibex (*Capra ibex*), and other species. *B. melitensis* is endemic in parts of Central and South America, Africa, Asia, the Middle East, and countries in the Mediterranean region. Areas considered to be free of *B. melitensis* include Canada, the United States, South-East Asia, Northern and Central Europe, Australia, and New Zealand.

Domestic and feral swine are the preferred hosts for biovars 1, 2, and 3 of *B. suis*. Biovars 1 and 3 of *B. suis* can also infect cattle and horses. Biovar 2 can establish infection in hares (*Lepus capensis*) and has a geographical distribution in a broad range between Scandinavia and the Balkans. Biovar 4 is predominantly associated with infection in caribou (*Rangifer tarandus*) but has also been found in moose (*Alces alces*), Arctic foxes (*Vulpes lagopus*), and wolves (*Canis lupus*) in subarctic areas. Biovar 5 has been exclusively isolated from wild rodents in the former USSR. Porcine brucellosis (biovars 1, 2, and 3) has widespread distribution in domestic and feral swine globally, but prevalence is higher in some areas such as South-East Asia and South America. It should be noted that unlike biovars 1, 3, and 4, biovar 2 of

B. suis is not considered to be important for causing human brucellosis.

Dogs and sheep are the preferred hosts of *B. canis* and *B. ovis*, respectively and both species of *Brucella* have widespread geographic distribution. *B. neotomae* was isolated from desert wood rats in the Great Salt Lake Desert of Utah and appears to have a restricted host range and limited geographic distribution.

Marine strains of *Brucella* have been identified in both the Atlantic and Pacific oceans, although current knowledge is not sufficient to characterize geographic and host population distributions. Distribution of *B. microti* is not fully understood, but it has been recovered from environmental samples, and natural infection has been demonstrated in a wide range of species including frogs, common voles, wild boar, foxes, and soil. The amphibian *Brucella* has a worldwide distribution and has also been demonstrated to naturally infected fish (*Taeniura lymma*). In general, distribution and natural hosts of atypical species of *Brucella* are not fully characterized.

Transmission

Transmission of classical brucellae in preferred hosts primarily occurs through aerosol, oral, and/or venereal routes across mucosal surfaces. Transmission can be best described by dividing the species into high-virulence (*B. abortus* and *B. melitensis*) and low-virulence (*B. ovis* and *B. canis*) groups, with *B. suis* differing by sharing characteristics of both groups. For *B. abortus* and *B. melitensis,* transmission primarily occurs laterally through fluids or tissues associated with the birth or abortion of infected fetuses, or vertically to offspring through the milk. Abortion events are the most important route for transmission of *B. abortus* and *B. melitensis* with fetal fluids and placenta tissues having colonization as high as 10^9–10^{10} CFU/g. Venereal transmission is not considered to be important for *B. abortus* or *B. melitensis*.

Venereal transmission is an important route for infection by *B. suis*, *B. ovis*, and *B. canis* in preferred hosts. *B. suis and B. canis* can also be shed by excretion in urine, milk, or from mucosal surfaces, and *B. canis* has also been recovered from feces. Bitches can shed *B. canis* for four to six weeks after abortions, and vaginal discharges may contain high bacterial numbers (10^6–10^{10} CFU/ml). In general, animals infected with *B. suis* and *B. canis* effectively transmit *Brucella* for longer periods of time than animals infected with *B. abortus* or *B. melitensis* through shedding from mucosal surfaces or within urine.

The mechanisms for transmission of *Brucella* in marine mammals, amphibians, foxes, voles, and baboons are unknown but may differ from classical brucellae. The demonstration that marine *Brucella* are almost exclusively localized within gut and uterine tissues of lungworms (*Parafilaroides* and *Phocoena*) in an infected Pacific harbor seal and harbor porpoise, suggests an intriguing possibility that parasites may play a role in transmission of this atypical species.

Pathogenesis

Brucellae generally enter across mucosal membranes, and initially localize in lymphatic tissues draining the site of entry. If bacteria do not become localized and are not killed in regional lymph nodes draining the site of infection, bacteria may replicate and spread to other lymphoreticular tissues and organs via lymph and blood. Distribution to reproductive and mammary tissues occurs during the bacteremic phase, and localization in these tissues can be associated with high bacterial numbers (Figures 14.1 and 14.2). Once localized in the immunoprivileged environment of the pregnant uterus, or lumens of ducts within the mammary gland, immunologic mechanisms to eliminate brucellae are severely impaired.

The internalization of *Brucella* into phagocytic cells differs between rough and smooth organisms. The LPS O side chain of non-opsonized smooth *Brucella* interact with lipid rafts, which contain glycosphingolipids, cholesterol, and glycosyl-phosphatidylinositol anchored proteins, on the surface of phagocytes. Internalized *Brucella* initially localize within phagocytes in an acidified membrane-bound compartment (phagosome), where they are exposed to free oxygen radicals generated by the respiratory burst. Cyclic beta-1,2-glucans may help target *Brucella* to their replicative niche in the endoplasmic reticulum. Exposure to an acidified environment induces a type IV secretion system that interferes with phagosomal maturation and interacts with the endoplasmic reticulum to neutralize the

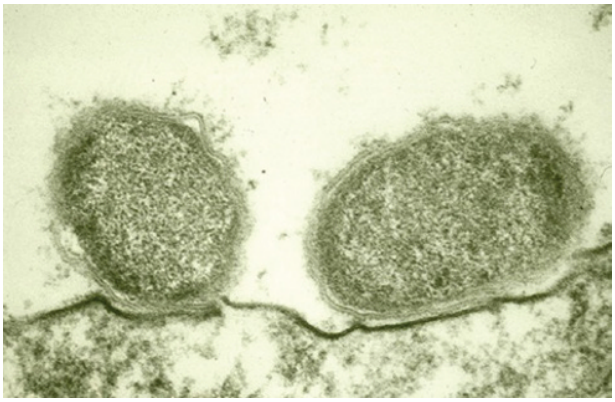


Figure 14.1 Electron micrograph of *Brucella.*

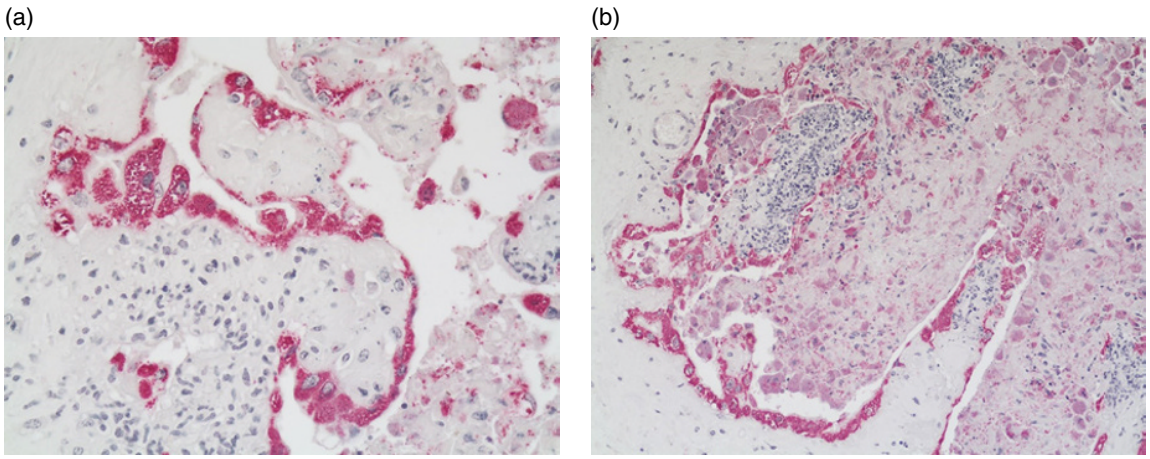


Figure 14.2 (a) Low (10×, upper) and (b) high (40×, lower) magnification of sections of bovine cotyledon/placentome infected with *Brucella abortus*. Bacteria were identified by immunostaining using a polyclonal antibody against *Brucella* lipopolysaccharide.

pH of the phagosome. The modified phagosome ("brucellosome") resists phagosome maturation and fusion with lysosomes. Although most brucellae (approximately 70–85%) are eliminated by phagolysosome fusion, creation of the brucellosome allows intracellular survival of some. *Brucella* use stationary-phase physiology and siderophores to scavenge iron as a mechanism for long-term survival within the nutrient-poor environment of the modified phagosome. Brucellae have multiple molecular mechanisms to detoxify free radicals because oxidative killing is the primary mechanism employed by host phagocytes to control replication of intracellular pathogens. The O side chain on the LPS appears to be a key molecule for invasion, protection from oxidative killing, cationic peptides, and complemented-mediated lysis. The capacity of *Brucella* to maintain long-term residence within macrophages serves as the basis for their ability to establish and maintain chronic infection.

In contrast to entry via lipid rafts, opsonization of *Brucella* increases internalization 10-fold and targets the bacteria to phagosomes that mature and fuse with lysosomes to form phagolysosomes. Localization within phagolysosome compartments increases intracellular killing of brucellae by phagocytic cells.

During persistent infection, some brucellae reside in a dormant, non-replicative state in phagocytic cells. Calves infected with *B. abortus* at young ages often do not demonstrate seroconversion until after reaching puberty. Recrudescence of clinical brucellosis is a significant problem in human patients. Latency is also an issue in that *Brucella* RNA can be recovered from culture-negative animals, and human infection has been documented several decades after the last known exposure. Molecular mechanisms controlling recrudescence, or host physiology influencing *in vivo* replication of brucellae have not been identified at this time.

The erythritol genes are highly conserved in *B. abortus*, *B. suis*, and *B. melitensis*. Erythritol is preferentially metabolized and can promote the growth of some *Brucella* strains. Although it has been hypothesized that the preferential localization of *B. abortus* within the reproductive tract is related to the high concentration of erythritol in fetal placenta, data suggests that erythritol is not an essential carbon source for *B. suis* in intracellular environments.

Epidemiology

Humans are most likely aberrant hosts as clinical disease is more severe in humans than in natural reservoirs, and humans tend to be dead-end hosts. With the exception of abortions, it should also be noted that brucellosis is rarely associated with mortality in natural or aberrant hosts.

Human brucellosis, caused by *B. melitensis*, *B. suis*, and *B. abortus* infection, occurs worldwide, but prevalence tends to be higher in parts of the Mediterranean basin, Central and South America, the Middle East, sub-Saharan Africa, and Central Asia. Although less prevalent, human brucellosis caused by *B. canis* or marine *Brucella* strains have been reported globally. Brucellosis is estimated to infect over 500 000 people yearly in non-industrialized countries of the world, with estimates of disease prevalence ranging from <1 per 100 000 people in the United Kingdom, United States, and Australia, to >70 per 100 000 people in some countries in the Middle East and Central Asia. Of concern is the observation that children appear to represent a high proportion of human brucellosis cases in endemic areas. This is likely influenced by consumption of unpasteurized milk products in these regions. The high risk of fetal loss after infection of pregnant women is

effectively prevented by antibiotic treatment. Economic instability, socioeconomic factors, and insufficient regulatory programs in domestic livestock over the last several decades are likely contributing factors to the re-emergence of brucellosis in both livestock and humans in many countries.

Brucellosis in humans is most frequently a disease of farm workers, veterinarians, and laboratory or abattoir workers but can also occur through consumption of non-pasteurized dairy products. Direct contact with infected animals, or materials associated with abortion, can lead to human infection through aerosolization into respiratory tissues, oral consumption, or opportunistic penetration through breaks in the skin. Veterinarians can also develop brucellosis from infection with vaccine strains of *B. abortus* or *B. melitensis* with inadvertent needle sticks being a frequent cause of infection. As prevention of human brucellosis is primarily related to the control of the disease in animals, human brucellosis has a low incidence in countries in which livestock brucellosis has been controlled.

It should be noted that *B. suis* has unique zoonotic features due to the long bacteremia and prolonged shedding from infected swine. Processing of infected swine in abattoirs has frequently resulted in large numbers of workers becoming infected with brucellosis. In addition to the prolonged bacteremia in pigs, the observed infections in abattoirs may also be related to the fact that *B. suis* can be readily isolated from urine within the bladder of infected swine. Processing of infected swine can result in aerosolization of *B. suis* from urine or other body fluids. Some data suggests high rates of human infection after consumption of *B. suis*-infected milk.

The incubation period associated with human infection is variable and can range from less than a week to several months. The pathophysiology of brucellosis in humans differs from the characteristics of brucellosis in animal models. Clinical symptoms in humans are not pathognomonic, and the disease has been described as having protean manifestations. Symptoms can include recurrent pyrexia (undulant fever), cephalalgia, malaise, joint and muscle pain, night sweats, and even neurological manifestations. *Brucella* can distribute to almost any tissue or *in vivo* site, with clinical symptoms related to inflammatory lesions associated with bacterial localization. Osteoarticular disease is the most common complication and can include peripheral arthritis, sacroiliitis, and spondylitis. Although human brucellosis is generally associated with low mortality, endocarditis associated primarily with *B. melitensis* infection is the principal cause of human mortality. Although reports are rare, human to human transmission of brucellosis through venereal transmission or through breast milk have been reported.

Characterization of Infection in Animal Populations

As mentioned previously, most *Brucella* spp. have widespread worldwide distribution in domestic livestock populations. Susceptibility to infection, clinical effects, and disease prevalence are influenced by vaccination status, sexual maturity, and pregnancy status. Localization in the reproductive tract or mammary gland is associated with the most severe pathology and capability to transmit infection. In general, preferred hosts are more susceptible to infection with brucellae during pregnancy, although host mechanisms responsible for the increased susceptibility are not characterized at this time. In the absence of vaccination, infected herds frequently demonstrate seroprevalence rates of 40–60%. Vertical transmission of brucellosis to offspring can occur through infected milk. With *B. abortus*, it has been shown that infection in young animals may remain latent until the initiation of puberty.

The most common clinical manifestation of *B. melitensis*, *B. suis*, and *B. abortus* in natural hosts is reproductive losses. With the exception of third trimester abortions, birth of weak offspring, and infertility in males and females, clinical symptoms of brucellosis in natural hosts are relatively rare. In uniparous species, abortions are generally fresh with minimal autolysis. In multiparous animals, abortion may not be as common due to physiological mechanisms that maintain pregnancy while viable fetuses remain in utero. Brucellae also frequently infect mammary glands, causing mastitis and reduced milk production. Occasionally, *Brucella* can localize in joints, bones, or other aberrant locations in natural hosts, leading to inflammation and associated pathology. Osteomyelitis/spinal meningitis in swine infected with *B. suis* can lead to hind limb paralysis.

Transmission of *B. ovis* infection in sheep generally occurs during the breeding seasons. The ewe acts as a mechanical vector for transmitting infection between infected and non-infected rams. Homosexual activity of rams may be another mechanism for transmitting infection. The most common clinical manifestation of *B. ovis* infection is infertility in rams, with scrotal palpation being a mechanism for identification of infected rams.

Because of the capability for venereal transmission, husbandry practices and management practices can contribute to the introduction of *B. suis* and *B. canis* into non-infected herds or kennels. Entry of infection can occur by the purchase of infected animals, male or female, or by the communal use of a male, which may transmit the disease mechanically or which may be excreting *Brucella* in semen. Use of *B. suis*-infected semen for artificial insemination is also a potential infection route in swine herds.

Immunologic Aspects

Immune Mechanisms in Pathogenesis

Cellular immune responses are generally felt to play a critical role in protection against intracellular pathogens such as *Brucella*. Development of adaptive immunity involving presentation of by antigen-presenting cells (APCs), production of Th1-type cytokines (interferon-γ, tumor necrosis factor, interleukins-2, −12, and −18), and clonal expansion of antigen-specific CD4+ and CD8+ T-cells are key to the development of protective immunity. In general, it is felt that antigens derived from outside (exogenous) and which enter the antigen processing cell through phagocytosis for degradation in the phagolysosome are presented to CD4+ cells in association with Class II MHC. In contrast, antigens synthesized within the antigen-presenting cell cytoplasm and transported to the endoplasmic reticulum are processed through endogenous pathways and presented to CD8+ cells in association with Class I MHC. Most endogenous proteins are processed through ubiquitin-proteasome degradation pathways; these autologous sequences generally do not provoke responses by CD8+ cells. The intracellular location or entry of antigen into the cell is important, as processing via the endogenous pathway tends to evoke protective cell-mediated immune responses. In contrast, peptides expressed through exogenous antigen processing are typically not associated with strong protective immune responses. However, antigens processed via the exogenous pathway are typically associated with a non-protective Th2 response. This may explain why efficacious brucellosis vaccines are composed of live bacteria, whereas vaccination with killed bacteria generally fails to induce adequate protection.

In general, antibodies are considered to play a minor role in long-term protection against brucellosis, although they may opsonize bacteria and facilitate phagocytosis and may lead to processing/presentation of *Brucella* epitopes on APCs. Cytotoxic T lymphocytes may play an important role in *in vivo* protection by killing infected cells and releasing intracellular bacteria within the cells for uptake and destruction by activated macrophages. As some smooth *Brucella* strains downregulate genes associated with apoptosis, prevention of host cell apoptosis may also be a mechanism used by *Brucella* to facilitate intracellular survival and persistence.

Pathogen recognition receptors (PRRs), such as Toll-like receptors, play an important role in recognizing PAMPs and initiating innate immune responses. APCs, including dendritic cells (DCs) and macrophages, express various PRRs. Triggering of these receptors results in activation of these cells. In particular, activation of dendritic cells (DCs) results in their migration to secondary lymphoid organs, where they present antigen and stimulate naïve T-cells to initiate adaptive immune responses. The observation that *Brucella* display reduced PAMP is likely a mechanism the bacteria use to elude innate immunity. In addition, brucellae have been shown to directly interfere with APC function by preventing DC maturation, modulating cell signaling pathways, and interfering with antigen processing and presentation, to name a few.

Mechanisms of Resistance and Recovery

Long-term protection against brucellosis is associated with stimulation of cellular immunity, whereas antibodies are considered to play a minor role in protection. Although specific correlates of protective immunity are currently not known, it is believed that protection is mediated by the Th1 subset of CD4+ lymphocytes and is associated with production of IFN-γ and other cytokines associated with cellular immunity. The ability of preferred hosts to clear infection, with or without treatment, remains controversial due to the intracellular location of the pathogen, and observations of recrudescence of infection under field conditions. Like other intracellular bacteria and as mentioned previously, latency is an issue in natural hosts and aberrant hosts (humans).

Vaccination

Vaccination is a critical tool for control or eradication of brucellosis that primarily prevents clinical effects of disease (i.e. abortions or infected offspring), which lead to transmission. Approved vaccines are available to prevent *B. abortus* infection in cattle and *B. melitensis* infection in sheep and goats. Approved vaccines are not available for other hosts of brucellae, although the *B. melitensis* Rev1 vaccine has been recommended to prevent *B. ovis* infections in sheep. For *B. melitensis* and *B. abortus* infections in preferred hosts, vaccination programs have not included males as they are not considered to play a significant role in disease transmission. The most efficacious vaccines are live attenuated strains that are frequently administered to prepubescent animals. Rough vaccine strains of *B. abortus* (i.e. RB51 or killed 45/20) that do not express the O side chain on the LPS that function as differential (DIVA) vaccines for diagnostic purposes have been utilized. Conjunctival administration of smooth vaccine strains has also been used, particularly in adult animals, in an effort to reduce positive responses on brucellosis serologic tests. Live attenuated brucellosis vaccines have disadvantages in that they may induce abortions in pregnant animals, be shed in milk of lactating animals, and are themselves potential zoonoses. Although isolated, recently in the United States,

several instances of RB51-vaccinated cattle shedding bacteria in milk have been reported. Consumption of unpasteurized milk and milk products from these animals has resulted in at least one human infection.

Laboratory Diagnosis

Specimens

As brucellosis is one of the most common laboratory-acquired infections, manipulations of live cultures of zoonotic *Brucella* spp. should be handled under Biosafety level 3 conditions using appropriate safety procedures. All work should be conducted in biological safety cabinets and avoid procedures that produce aerosols. Biosafety level 2 conditions are recommended for processing routine clinical specimens of human or animal origin. In the United States, *B. abortus*, *B. melitensis*, and *B. suis* (vaccine strains are exempt) are classified as potential bioweapons and must be handled in accordance with Select Agent regulations.

Optimal microbiologic samples for diagnosis would include milk or vaginal swabs from live animals, lung and/or gastric contents from aborted fetuses, or tissues such as uterus, mammary gland, male reproductive tissues, lymphatic tissues associated with the mammary gland, or reproductive tract obtained at necropsy, or bursitis/hygroma fluid. Although blood collected in anticoagulant may be beneficial for isolation of *B. canis* or *B. suis*, it is generally a poor sample for bacteriologic evaluation for other brucellae as bacteremias associated with infection are generally of very short duration.

Direct Examination

Gross lesions caused by *Brucella* spp. are not pathognomonic but can include variable necrosis of cotyledons across the placenta (Figure 14.3a, b), thickening of intercotyledonary areas, and retained placenta. Common histologic lesions of maternal tissues include necrotic placentitis and lymphosuppurative or lymphohistiocytic interstitial mastitis. In males, orchitis (Figure 14.4) with multiple abscess, epididymitis, vesicular adenitis, and testicular degeneration have been reported. The most frequent fetal

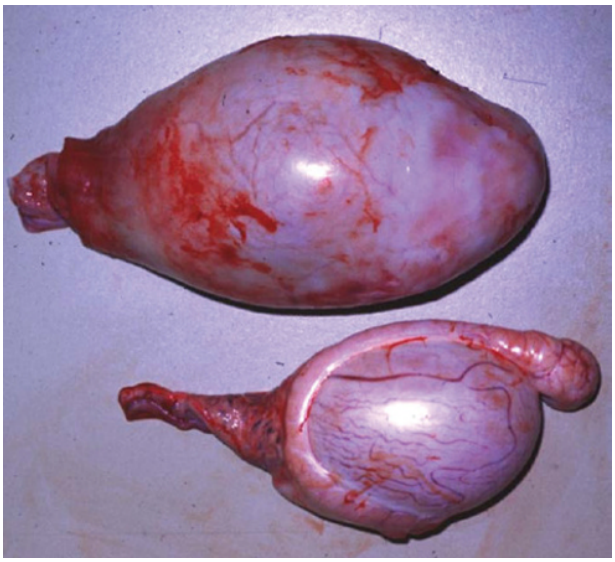
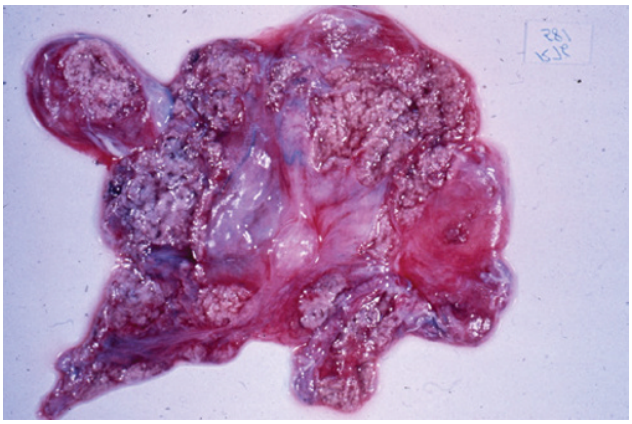


Figure 14.4 Ovine testicle demonstrating hypertrophy associated with *Brucella* infection. *Source:* Courtesy of Noah's Archive, https://davisthompsonfoundation.org/noahs-arkive. Original contribution of Dr. Anderson, University of Idaho.

(a)



(b)

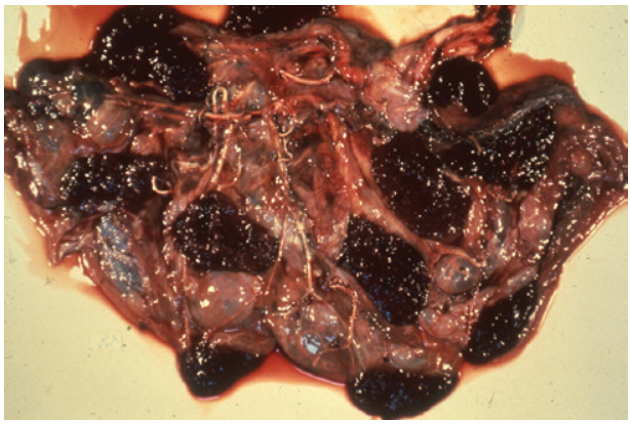


Figure 14.3 (a) Pyometra associated with brucellosis. *Source:* Courtesy of Noah's Archive, https://davisthompsonfoundation.org/noahs-arkive. Original contribution of Dr. Richard Jakowski, Tufts University. (b) Aborted bovine placenta demonstrating necrosis, hyperemia, and thickening between attachments to cotyledons. *Source:* Courtesy of Noah's Archive, https://davisthompsonfoundation.org/noahs-arkive. Original contribution of Dr. Richard Jakowski, Tufts University.

lesion is a multifocal, histiocytic bronchopneumonia with, or without, suppurative infiltrates. Fetal lesions may also include variable foci of necrotizing arteritis, necrosis, and granulomas in the lung and other tissues.

Additional lesions caused by *B. suis* infection in pigs include fibrinopurulent arthritis in compound or large joints, osteomyelitis in lumbar vertebral epiphysis, and military uterine nodules. *B. abortus* is known to cause bursitis and hygromas in cattle. Horses are an aberrant host of *B. abortus* and can develop suppurative and granulomatous bursitis lesions at the supraspinous (fistulous withers) or atlantal (poll evil) bursae.

Isolation

Because brucellae are slow growing in relation to other bacteria, contaminants may overgrow and severely impair the ability to isolate *Brucella* spp. For that reason, samples obtained for culture should preferentially be obtained aseptically. Alternatively, selective media (i.e. Farrell's media or Kuzdas and Morse media) have been developed to inhibit the growth of contaminants and facilitate the isolation of *Brucella* spp. Regardless, at minimum, it will take 72 hours for *Brucella* spp. colonies to be visible on media, and samples should be incubated for a minimum of 7–10 days before arriving at a negative determination.

Identification

Traditionally, after isolation of gram-negative coccobacilli with culture and biochemical characteristics consistent with identification as *Brucella*, species differentiation has been based on phage and dye sensitivity, CO_2 dependence for growth, production of H_2S, and serum agglutination tests. However, as microbial isolation can be slow and tedious, other more rapid methods have been developed for identification, such as polymerase chain reaction (PCR) using species-specific primers. Classifying *Brucella* isolates into biovars has been used for epidemiologic purposes but is somewhat subjective because it is based on subtle differences including requirements for higher CO_2 tensions for growth, production of H_2S, growth on media containing dyes (thionin or basic fuchsin), and agglutination with monospecific A and M antisera.

Serologic Tests

For serologic detection of *B. suis*, *B. abortus*, and *B. melitensis* in preferred hosts, the immunodominant antigen involved in the serologic responses is the O side chain of the LPS. Most standard brucellosis serologic tests (agglutination, complement fixation, fluorescence polarization assay, and ELISA) use the polysaccharide O side chain from *B. abortus* as antigen and were initially developed for detection of *B. abortus* infections in cattle. Although considered adequate for use in detection of *B. melitensis* infection in small ruminants and cattle, they are generally felt to have reduced sensitivity and specificity in detection of *B. suis* infections in swine. Sensitivity and specificity of standard brucellosis serologic tests may differ by host species and the *Brucella* spp. causing infection. The structural component of the O side chain (repeated residues of 4-formamindo-4, 6-dideoxymanose linked in α-1,2 or α-1,3 conformation) is also similar to epitopes on the LPS of other bacteria such as *Yersinia enterocolitica* O:9, *Escherichia coli* O:157, and others, and can lead to reductions in specificity under some conditions.

For detection of *B. ovis* in sheep, complement fixation, ELISA, and agar gel immunodiffusion (AGID) tests have been utilized. For detection of *B. canis* infection in kennels, tube agglutination, AGID, indirect fluorescence, rapid slide agglutination (with/without 2-mercaptoethanol), and ELISA tests (all using *B. canis* antigens) have been recommended.

The delayed-type hypersensitivity reaction (allergic skin test) based on the use of S-LPS free *Brucella* cytoplasmic proteins (widely known as Brucellin) extracted from rough *B. melitensis* strain B115 has been used as a diagnostic in many countries. The Brucellin test has been proposed to be beneficial in differentiating seropositive responses caused by infection with *Y. enterocolitica* O:9.

Molecular Tests

Multiple PCR techniques are currently available for differentiating *Brucella* species. However, current PCR techniques have not been able to fully differentiate all biovars within all *Brucella* spp.

Because of the stability of *Brucella* genomes, molecular assays designed to assess genetic relationships between isolates, such as RFLP, are ineffective for epidemiological investigations or strain comparisons. Molecular techniques, including comparison of variable nucleotide repeats in non-coding areas of the chromosome, the Bruce ladder, a multiplex PCR technique, and whole genomic sequencing, have been used to compare genetic and epidemiologic relationships between strains. However, molecular biotyping is more complicated than standard PCR methods and requires special equipment, which may limit their use by diagnostic laboratories.

Treatment

In general, no treatment strategies are recommended for preferred hosts for *B. abortus*, *B. suis*, and *B. melitensis* due to the requirement for long-term administration and the

inability to adequately ensure total clearance of *Brucella* from its intracellular niche and prevent re-emergence at later times. In some countries, antibiotic treatment (oxytetracycline with/without streptomycin), alone or in combination with test-and-slaughter, has been used to minimize disease in infected swine or sheep farms.

Treatment of humans usually involves a prolonged course of antibiotic therapy, and relapse of infection of treatment is not uncommon. Relapses are usually not associated with the emergence of antibiotic resistant strains.

Control and Prevention

Control Methods for *B. abortus, B. melitensis,* and *B. suis*

Many countries have programs to control or eradicate zoonotic brucellae (*B. abortus, B. melitensis*, and *B. suis*) from domestic livestock. Although brucellosis can cause severe economic losses to livestock producers, most regulatory programs for brucellosis were developed to reduce brucellosis in animal reservoirs as a mechanism to prevent human infections. Multiple studies have demonstrated that that the most cost-efficient mechanism for controlling human brucellosis is to control brucellosis in animal reservoirs.

As *Brucella* are intracellular pathogens, most test and removal programs are based on removal of animals that are positive on serologic tests that detect antibodies against the immunodominant O side chain of the *Brucella* LPS. Although antibodies indicate that the individual's immune response has responded to infection with *Brucella*, it should be emphasized that seropositive responses do not necessarily indicate the animal is currently infected or capable of transmitting brucellosis.

The full depopulation of all animals on infected premises is the strategy of choice for controlling brucellosis. However, because this strategy may be too expensive or not acceptable to the owner, other approaches may need to be utilized

Regulatory programs for cattle and small ruminants can include sanitation programs to prevent disease transmission, vaccination programs to reduce herd susceptibility, and test and removal programs to eliminate animals demonstrating humoral evidence of prior infection with brucellosis. Efficacious vaccines are effective tools in both control and eradication programs. Although vaccination is highly effective in reducing production losses (i.e. abortions or weak offspring) caused by brucellosis, it is less effective at preventing infection after exposure to field strains or seroconversion after exposure. Although vaccination is an effective tool in controlling brucellosis, vaccination alone has never been effective in eradicating brucellosis. Apart from the preferred depopulation resolution, the most effective brucellosis programs incorporate combinations of sanitation, vaccination, and test and removal procedures.

For control of *B. suis* in swine, regulatory efforts, including serologic surveillance, to control or eradicate *B. suis* in swine should be directed toward herds, rather than individual animals. Owing to the present lack of effective tools to manage brucellosis in swine, whole-herd depopulation appears to be the only viable option to eradicate *B. suis* from domestic swine herds. If feral swine or other wildlife reservoirs were the source of infection, management/biosafety practices may need to be modified to prevent reintroduction of brucellosis.

Control Methods for *Brucella ovis*

Use of a test-and-slaughter approach based on scrotal palpation and/or serologic testing (complement fixation, ELISA, or gel diffusion test) has been successfully used to eradicate *B. ovis*. As shedding in semen can be intermittent, a single negative sample is not predictive of non-infected status. Antibody responses are also unreliable as only 35% of infected rams develop detectable titers. Vaccination with *B. melitensis* Rev1 is considered the most viable method of control against *B. ovis* in sheep, but seroconversion on diagnostic tests makes this approach unattractive. Prolonged antibiotic therapy results in prevention of shedding in semen and improvement in sperm motility in 80–100% of treated rams. In some instances, culling of the entire ram flock may be the most economical approach for eradication of *B. ovis*.

Control Methods for *Brucella canis*

Best control in infected kennels is removal of seropositive dogs and de-sexing of infected animals. Disinfection of the kennel after removal of infected dogs is recommended. Treatment of infected dogs is not encouraging due to frequent failure of antibiotic regimens or relapse of infection. Resolution of infection has been reported using long-term treatment (4–96 weeks) with a combination of antibiotics (doxycycline, enrofloxacin, and streptomycin or gentamycin) combined with monitoring for reversion to negative status on serologic tests (AGID). Repeat testing 3–6 months after negative tests is required to check for relapse of infection. Relapse of infection is possible. Owners should be warned of possible zoonotic infections with *B. canis*. Prevention of re-entry of brucellosis into kennels is prevented by isolation of new additions, appropriate biosecurity measures, and serologic testing.

15

Burkholderia mallei and *Burkholderia pseudomallei*

Sanjeev Narayanan

Members of the genus Burkholderia are gram-negative, aerobic rods belonging to the phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, and family Burkholderiaceae. As of this writing, there are more than 100 species belonging to this genus, most of which live in acidic soil; some are closely associated with soil fungi. Although some are plant pathogens, others are used for beneficial effects in plants, for producing beneficial bioactive products commercially, and for degrading environmental pollutantsA well developed secretory system enhances its pathogenicity. Members of the *Burkholderia cepacia* complex produce disease in compromised human patients (e.g. those with cystic fibrosis; chronic granulomatous disease; immunosuppression; nosocomial infections). Two members of *Burkholderia pseudomallei* species group, *Burkholderia mallei* (the cause of "glanders," primarily in equids) and *B. pseudomallei* (the cause of "melioidosis" in a variety of species), are important in veterinary medicine and are the topics of discussion in this chapter. Both produce pyogranulomatous disease and have gained notoriety by being designated as category B bioterrorism agents by the Centers for Disease Control and Prevention, United States, and is considered an emerging and re-emerging infectious agent of humans by the National Institutes of Health, United States.

Burkholderia mallei

B. mallei is a facultative intracellular bacterium that causes a systemic pyogranulomatous disease called "glanders," once a worldwide disease of Equidae that remains endemic in parts of Asia, Africa, and South America. Focal outbreaks were also reported in the former USSR in 2007. No naturally occurring cases of glanders have been reported in North America since the 1940s. Natural infections have been reported in dogs, camels, horses, goats, and sheep, but felids are particularly susceptible. Guinea pigs and hamsters are highly susceptible to glanders and are therefore used in diagnostic testing or experimental studies. Glanders varies in severity and can be an acute, chronic, or latent disease.

At one time or another, this bacterium was called *Pseudomonas mallei*, *Bacillus mallei*, *Actinobacillus mallei*, *Acinetobacter mallei*, *Malleomyces mallei*, *Pfeiferella mallei*, and *Loefferella mallei*.

Descriptive Features

Morphology and Staining

B. mallei are gram-negative, 0.5-μm-wide rods of variable length. The bacilli may be arranged singly, in pairs end to end, in parallel bundles or palisades.

Structure and Composition

B. mallei produces a carbohydrate capsule. The cell wall is typical of gram-negative bacteria composed of lipopolysaccharide (LPS) and protein. Being nonmotile, flagella are not produced (differentiating it from *B. pseudomallei*, which is motile).

Cellular Products of Medical Interest

Capsule. The only demonstrable role the capsule plays in disease produced by *B. mallei* is to protect the outer membrane from membrane attack complexes generated

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following activation of the complement system (which is manifested as a serum-resistant phenotype). Capsule-negative mutants show a significant reduction in virulence. Also, capsular glycoconjugates have been demonstrated to be viable vaccine candidates in experimental animal models.

Cell Wall. The cell wall of *B. mallei* is typical of gram-negative bacteria. The LPS in the outer membrane is an important virulence determinant. LPS consists of Lipid A, a core region, and cell wall (O) antigens. Not only is the lipid A component toxic (endotoxin), but the length of the side chain in the O-repeat unit hinders the attachment of the membrane attack complex of the host complement system to the outer membrane. LPS binds to LPS-binding protein in serum, which transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor protein-4 (see Chapter 2) on the surface of antigen-presenting cells, triggering the release of pro-inflammatory cytokines and chemokines.

Miscellaneous Products. *B. mallei* possess type IV pili, type III (animal and plant types), and type VI secretion systems. Although many effector proteins injected into the host cells by the Type III secretion apparatus have been identified, their exact function is not known. Many effectors have been hypothesized to play an integral role in bacterial survival in intracellular (including cytosolic) environments in the host cells (predominantly macrophages).

Proteases, lipases, and phospholipase C have been demonstrated in the culture fluids of *B. mallei*. None of these products have been shown to play a significant role in disease. Surface protein autotransporter BpaB has been shown to mediate adherence, biofilm formation (*in vitro*), and pathogenesis, including *in vivo* and intracellular survival. BoaA outer membrane proteins have been demonstrated to play a role in binding to host epithelial cell surfaces.

Growth Characteristics

The organism grows in routine bacteriological media, but media containing glycerol or blood are best. Nonhemolytic colonies develop in 48 hours or more at 20–41 °C (optimal 37 °C). They range from mucoid to rough forms (see Figure 15.1). Confluent growth is common. *B. mallei* does not grow on MacConkey agar or at 42 °C. *B. mallei* grows on MacConkey agar.

Biochemical Characteristics

B. mallei is aerobic, nonmotile and oxidase variable, catalase-positive, indole-negative, and resistant to colistin and polymyxin B; it reduces nitrates (without gas) and hydrolyzes urea. Glucose is catabolized oxidatively.



Figure 15.1 Rough colony morphology of *Burkholderia mallei* on blood agar with no hemolysis. *Source:* Courtesy of Gavin Koh, https://commons.wikimedia.org/w/index.php?curid=4975784.

Resistance

Resistance is unremarkable, although in dark, damp, and cool environments, the agent can survive for months. Aminoglycosides, chloramphenicol, fluoroquinolones, macrolides, sulfonamides, trimethoprim, imipenem, ceftazidime, piperacillin, and tetracyclines inhibit *B. mallei in vitro*. The incidence of resistance to antibiotics is low compared to other species but has been demonstrated by mechanisms such as reduced penetration, efflux pumps, enzymatic degradation, and target modification.

Ecology

Reservoir

Infected Equidae is the reservoir. Restricting animal movement is the primary method of disease control.

Transmission

Exposure occurs via contaminated feed, water, fomites, and sometimes through inhalation and wounds. Skin exudates and respiratory secretions are the most common source of infection in equids, especially in crowded and unsanitary conditions. Carnivores can be infected by ingesting meat from infected animals.

Pathogenesis

Pathology. Lesions develop primarily in the liver, spleen, lungs, and lymph nodes but can also be found in any tissue and organ. *B. mallei* produces typical pyogranulomas characterized by a central core of necrotic debris admixed with fibrin and intact and degenerate neutrophils

(Figure 15.1). This core is surrounded by a layer of histiocytes, epithelioid macrophages, and multinucleated giant cells. The entire nodule is surrounded by an outer rim of collagenous capsules embedded with small to large infiltrates of lymphocytes and few plasma cells. A multifocal and coalescing distribution is common in systemic infections and lesions near epithelial surfaces; ulceration is common.

Strain variations determine the suppurative versus granulomatous predominance in lesions.

Mechanisms. Although toxins are suspected in pathogenesis, the mechanisms are uncertain. Primary lesions form at the point of entry – the pharynx, for example. The infection spreads along lymphatics, producing nodular lesions on the way to lymph nodes and the bloodstream, which disseminates the agent. Monocytes and macrophages are suspected to carry the bacterium to draining lymph nodes because *B. mallei* are facultative intracellular bacterium capable of surviving and replicating in the cytosol of macrophages. Metastatic lesions form in the lungs or other organs, such as the spleen, liver, and skin, producing cutaneous glanders ("farcy"). Lesions in the nasal septum may be primary, hematogenous, or secondary to a pulmonary focus.

Disease Patterns

Glanders. The disease predominates in the upper and lower respiratory tracts and in the skin. Multiple nodules and ulcers develop in the upper airways and nasal cavity, which rupture to release thick mucopurulent nasal discharge (Figures 15.2 and 15.3). Lung lesions are multifocal coalescing pyogranulomas with caseous or calcified centers. Cutaneous glanders ("farcy") is characterized by subcutaneous abscesses. Local, regional, or generalized lymphadenopathy is typical in all types of glanders.

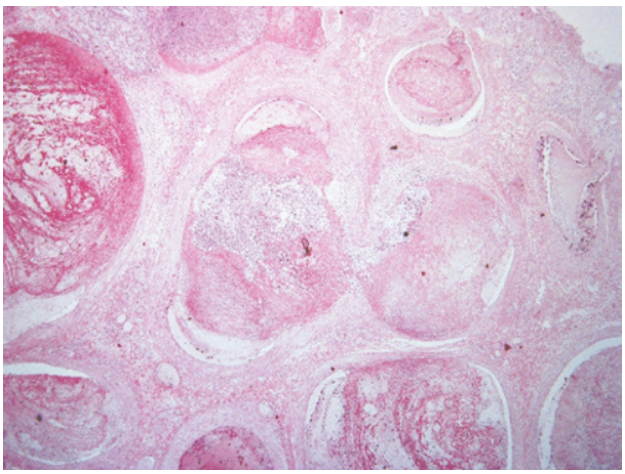


Figure 15.2 Section from the nasal cavity of a horse with *Burkholderia mallei* infection (H&E). There is leukocytoclastic vasculitis in many vessels, and their lumen contain thrombi.



Figure 15.3 Gross appearance of multifocal to coalescing mucosal ulcers and nodules on the nasal septum of a horse. *Source:* Courtesy of Noah's Arkive, https://davisthompsonfoundation.org/noahs-arkive. Original contribution of Dr. D.D. Harrington or Purdue University.

Acute infections are characterized by fever, nasal discharge, and lymphadenitis of the head and neck, with swelling along the upper respiratory tract. They tend to end fatally in about two weeks and predominate in donkeys and felids, less so in mules.

In horses, protracted chronic and subclinical infections are typical; signs, if present, include occasional fever, persistent respiratory problems, skin abscesses ("farcy buds"), and nodular induration of cranial lymph nodes.

Natural infections in humans are traced to acutely ill horses and may lead to acute or chronic infections.

Epidemiology

The persistence of glanders depends on an infected horse population. The movement of animals contributes to the spread of disease in facilities and geographic regions. Susceptible non-equids acquire glanders from infected horses or horsemeat and appear to be dead-end hosts.

Immunologic Aspects

Humoral and cell-mediated responses occur.

Apparent recovery from glanders, including loss of dermal hypersensitivity, has been observed under natural conditions but without increased resistance to reinfection.

Killed whole-cell preparations, polysaccharide-based preparations, and live-attenuated bacteria have all been tested as vaccine candidates and have shown to provide some protection. However, no vaccines are commercially available.

Laboratory Diagnosis

Nodular contents are cultured on blood or glycerol agar. They may be examined for gram-negative rods and by immunofluorescence. Guinea pigs and hamsters are highly susceptible to fatal infection with virulent strains.

Any suspect isolates should be submitted to a qualified reference laboratory. Differentiation from *B. pseudomallei* is important.

Serologically, glanders is diagnosed by complement fixation tests employing aqueous bacterial extracts as antigen. Enzyme-linked immunosorbent assays (ELISAs), indirecthemagglutination,counterimmunoelectrophoresis, immunoblotting, and indirect fluorescent testing are used for diagnostic purposes in some areas. However, complement fixation tests and ELISAs are approved for international trade. The intradermal-palpebral mallein test detects cell-mediated hypersensitivity, which indicates infection and has served as a basis for glanders eradication. Mallein is a heat extract of old *B. mallei* broth cultures.

Polymerase chain reaction-based assays have high sensitivities of detection; however, due to the high genetic variability of *B. pseudomallei*, differentiation between *B. mallei* and *B. pseudomallei* using DNA-based techniques is complicated.

Differential diagnoses for equine glanders include strangles (*Streptococcus equi*), epizootic lymphangitis (*Histoplasma farciminosum*), sporotrichosis (*Sporothrix schenckii*), ulcerative lymphangitis (*Corynebacterium pseudotuberculosis*), and melioidosis (*B. pseudomallei*).

Treatment and Control

Although glanders is treatable by many antimicrobials (see above), treatment is inappropriate in countries committed to glanders eradication. Equine imports from endemic areas are mallein-tested, and reactors are euthanized.

Burkholderia pseudomallei

B. pseudomallei are aerobic gram-negative, facultative intracellular rods that cause a pyogranulomatous disease called "melioidosis," a disease superficially resembling glanders. Important distinctions are that (i) melioidosis affects a wide host range and (ii) the agent is a saprophyte (an endosymbiont of amoebae living in the environment), whose prevalence is unaffected by the elimination of infected animals.

Previous synonyms for this bacterium include *Pseudomonas pseudomallei*, *Bacillus pseudomallei*, *Bacterium whitmori*, *Malleomyces pseudomallei*, and *Loefferella mallei*.

Descriptive Features

Morphology and Staining

B. pseudomallei are gram-negative rods 0.5 μm wide and variable in length. They frequently appear as long bundles of densely packed organisms. The bacterium in clinical samples may occasionally appear bipolar (safety-pin shaped).

Structure and Composition

B. pseudomallei produces a carbohydrate capsule. The cell wall is typical of gram-negative bacteria composed of LPS and protein. Being motile, flagella are produced (differentiating it from *B. mallei*, which is nonmotile).

Cellular Products of Medical Interest

Adhesin. *B. pseudomallei* adheres to amoebic trophozoites of *Acanthamoeba* species prior to uptake (and by inference adherence to phagocytic cells). Adherence is by means of the flagellar protein Fli (for flagellin). Surface proteins of B pseudomallei (BoaA and BoaB) that affect its adherence to respiratory epithelium have been characterized.

Capsule. A function the capsule has been shown to play in *B. pseudomallei* is to protect the outer membrane from membrane attack complexes generated following activation of the complement system (which is manifested as a serum-resistant phenotype). Capsules contain repeating units of [3)-2-O-acetyl-6-deoxy-beta-D-manno-heptopyran osyl-(1]and may also play an integral role in the intracellular survival of this bacterium, as mutants that lack functional genes encoding the capsule are avirulent.

Cell Wall. The cell wall of *B. pseudomallei* is typical of gram-negative bacteria. The LPS in the outer membrane is an important virulence determinant. Not only is the lipid A component toxic (endotoxin), but the length of the side chain in the O-repeat unit hinders the attachment of the membrane attack complex of the complement system to the outer membrane. LPS binds to LPS-binding protein (a serum protein), which transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells, triggering the release of pro-inflammatory cytokines.

Miscellaneous Products. The chromosome of *B. pseudomallei* possesses at least one pathogenicity island (a cluster of genes encoding virulence determinant(s), an integrase protein, a specific insertion site, and mobility) encoding a Type III secretion system. Studies have shown that the type III secretory system of B pseudomallei is extensive, and the genome contains at least three separate gene clusters. A Bsa type III secretion system has been

shown to be vital for the escape of this bacterium from the primary endosomes into the cytoplasm of host cells, thereby playing a critical role in replication and persistence in the host cell. A type VI secretory system is also present.

Proteases, lipases, and a phospholipase C have been demonstrated in the culture fluids of *B. pseudomallei* grown *in vitro*. None of these products have been shown to play a significant role in disease production.

Growth Characteristics

B. pseudomallei can grow in routine bacteriological media, especially those containing blood. In sheep blood agar, the organism reveals small, smooth colonies, which after a few days, will appear dry and wrinkled. Unlike *B. mallei*, *B. pseudomallei* grows on MacConkey agar, in the presence of 2% sodium chloride, and at 42 °C.

Biochemical Characteristics

B. pseudomallei is aerobic, motile and oxidase-positive, catalase-positive, indole-negative, and resistant to colistin and polymyxin B; it reduces nitrate to nitrogen gas (often produces only one small bubble of gas) and hydrolyzes urea.

Resistance

B. pseudomallei is killed by disinfectants and does not survive chilling and freezing in biological specimens. It is generally susceptible to imipenem, doxycycline, and minocycline. Most clinical strains have been shown to be resistant to amoxicillin, ticarcillin, cefoxitin, cefoperazone, cefsulodin, sulfonamides, and aztreonam. Resistance to ceftazidime (drug of choice) is on the rise. The majority of clinical isolates are also resistant to fluoroquinolones (except finafloxacin), aminoglycosides, and macrolides.

Ecology

Reservoir

B. pseudomallei is considered a soil and water dweller (most likely an endosymbiont of amoebae). Although most prevalent between 20° northern and southern latitudes, extratropical foci do exist, for example, in France, Iran, China, and North America.

Transmission

Direct contact with contaminated soil and surface waters is the most likely source of initial infection. Inhalation, ingestion, wound infection, transplacental (in goats), and possibly arthropod bites introduce infection. Animal to animal and sexual transmission are rare. In humans, inhalation and consumption of infected animal products may be significant.

Pathogenesis

Pathology. The lesions are primarily pyogranulomatous. Single to multiple suppurative or caseous nodules may be a predominant feature in affected organs. Lungs, spleen, liver, and associated lymph nodes are commonly affected. Small abscesses tend to coalesce, developing into larger suppurative foci or granulomas.

Mechanisms. Being an endosymbiont of amoebae, *B. pseudomallei* is adapted to survive within phagocytic cells of the host. Microorganisms are taken up by a process of "coiling" phagocytosis and survive within the cell by being resistant to lysosomal contents (e.g. defensins) and by escaping phagosomes and phagolysosomes. Actin-based motility has been observed within phagocytic cells, and actin-associated "budding" occurs from affected to unaffected cells. Host cells infected with *B. pseudomallei* release pro-inflammatory cytokines and undergo apoptosis.

Disease Patterns

Melioidosis. Also called Whitmore's disease, it is typically systemic, although it can be localized to specific systems (as in pulmonary melioidosis). They can be acute, chronic, subclinical, latent, or fulminant diseases. Manifestations depend on the extent and distribution of lesions. Strain differences and the overall innate and adaptive immune status of the host have been demonstrated to play a role in disease presentation and manifestation. The equine disease may mimic glanders. In cattle, acute and chronic infections can localize in the lung, joints, and uterus. Arthritis and lymphadenitis occur in sheep. Goats suffer a loss of condition, respiratory and central nervous system disturbances, arthritis, and mastitis. Similar signs are seen in swine, along with abortions and diarrhea. Dogs develop a febrile disease with localizing suppurative foci.

Epidemiology

Clinical disease is usually sporadic but is endemic in humans in southeast Asia and northern Australia. The host range is virtually unlimited. Melioidosis has been reported in sheep, goats, pigs, cattle, horses, deer, camels, alpaca, dogs, cats, dolphins, wallabies, kangaroo, koala, and primates. Non-mammal cases have been documented in birds, tropical fish, and reptiles. Humans, especially veterinarians, may acquire infections from a wide range of

animals, and the infections range from the rapidly fatal to the subclinical. A wet environment, such as swampy terrain or rice paddies, is related to exposure. Many recent studies have shown that persons with uncontrolled diabetes are highly prone to melioidosis.

Immunologic Aspects

Complement-fixing and indirect hemagglutinating antibodies are produced during infections. Cell-mediated hypersensitivity has been demonstrated in infected goats. Successful vaccination of horses and zoo animals is reported.

Laboratory Diagnosis

The methods for isolating and identifying *B. mallei* apply to *B. pseudomallei*. Chilling and freezing of specimens should be avoided. Motility, growth on citrate, growth at 42 °C, and reduction of nitrates to gaseous nitrogen distinguish *B. pseudomallei* from *B. mallei*. Polymerase chain reaction–based assays are available for detection and identification. They are, however, not as effective in differentiating *B. pseudomallei* from *B. mallei*.

Treatment and Control

Treatment in animals is expensive, prolonged, and often unsuccessful. Companion and zoo animals may be treated after antimicrobial susceptibilities are verified by laboratory tests. Vaccines are not commercially available.

Further Reading

Bacterial taxonomy browser at http://www.ncbi.nlm.nih.gov/Taxonomy

Center for Food Security and Public Health. Glanders. Fact Sheet. https://www.cfsph.iastate.edu/Factsheets/pdfs/glanders.pdf

Center for Food Security and Public Health. Melioidosis. Fact Sheet. https://www.cfsph.iastate.edu/Factsheets/pdfs/melioidosis.pdf

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16

Francisella tularensis

Marilynn A. Larson and Peter C. Iwen

Introduction

Francisella tularensis is a facultative intracellular bacterium that causes an acute zoonotic disease known as tularemia, which is also referred to as rabbit fever, deerfly fever, or lemming fever. This disease can be transmitted via multiple routes, including tick bite, deer fly bite, inhalation, ingestion, or skin contact with an infected animal or fomite. Tularemia is generally characterized by systemic symptoms, frequently accompanied by the formation of a localized cutaneous ulcer. Although the disease has been reported throughout the United States, most cases occur in the southern and south-central states, which include Missouri, Kansas, Arkansas, Oklahoma, and Texas. Between 2015 and 2018, a mean of 253 human cases (ranging from 229 to 314) of *F. tularensis* infections has been reported in the United Sates, in which the majority of these tularemia cases occurred during the months of April through September (https://www.cdc.gov/tularemia/statistics/index.html, accessed May 18, 2021). Tularemia is a nationally notifiable disease through the National Public Health Surveillance System. A case definition for reporting is considered a clinically compatible case with confirmatory laboratory results.

Classification

F. tularensis is a species that belongs to a large group of intracellular bacteria that includes the mycobacteria, *Listeria, Legionella, Brucella, Coxiella*, and *Rickettsia*. The genus *Francisella* is named after Edward Francis, an American bacteriologist who extensively studied the etiology of this gram-negative coccobacillus and pathogenesis of tularemia. The genus *Francisella* comprises 14 valid species (https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/Francisella, accessed May 18, 2021), one of which includes the reclassification of *Wolbachia persica* to *Francisella persica* (Larson et al. 2016). Of these species, only *F. tularensis* is considered a true pathogen to humans and animals. The species epithet *tularensis* pertains to Tulare County, California, where the disease was first described in rodents. *F. tularensis* has three widely accepted subspecies known to cause infections. These include *F. tularensis* subsp. *tularensis* (also referred to as Type A), *F. tularensis* subsp. *holarctica* (also referred to as Type B), and *F. tularensis* subsp. *mediasiatica* (see Table 16.1). A fourth subspecies, *F. tularensis* subsp. *novicida*, has also been proposed, but has not been widely acknowledged and is generally considered a separate species (*Francisella novicida*). In addition, *F. novicida* is not classified as a Tier 1 select agent by the Centers for Disease Control and Prevention (CDC), unlike the other three pathogenic subspecies. Most cases of tularemia are caused by the Type A and Type B strains, with the Type A strain being further divided into two distinct genotypes, more specifically Subtype A.I and Subtype A.II, due to differences in genomic organization and virulence. Importantly, *F. tularensis* A.I strains are considered some of the most infectious bacteria known. *F. tularensis* strains are typed using pulsed-field gel electrophoresis, genomic content and sequences, and now more commonly by targeted polymerase chain reaction (PCR) assays. These strains and subtypes appear to have distinct pathological characteristics and geographical distributions.

Epidemiology

F. tularensis is endemic in Europe, Asia, and North America. The Type A strains have been found mostly in North America, whereas the Type B strains are found more widespread in Europe and Asia as well as in North America. *F. tularensis* subsp. *mediasiatica* on the other hand has been isolated only in Kazakhstan and Turkmenistan. For the Type

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Table 16.1 Description of *Francisella tularensis* subspecies and subtypes.[a]

Subspecies	Type/Subtype	Strain Designation	Type Strains[b]	Isolate Information
tularensis	A.I	SCHU S4	FSC 237	Human ulcer, 1941, Ohio
tularensis	A.II	B-38	ATCC 6223 FSC 230	Human lymph node, 1920, Utah
holarctica	B	LVS	ATCC 29684 FSC 155	Live vaccine strain, Russia, 1936
mediasiatica	NA	NA	FSC 147 GIEM 543	Midday gerbil, 1965, Kazakhstan

Abbreviations: FSC, *Francisella* strain collection, Swedish Defense Research Agency, Umea, Sweden; ATCC, American Type Culture Collection, Manassas, Virginia, USA; GIEM, Gamaleya Scientific Research Institute of Epidemiology and Microbiology, Moscow, Russia; NA, not applicable.

[a] All classified as Tier 1 select agents by the CDC.

[b] Prepared from the List of Procaryotic Names with Standing in Nomenclature, J.P. Euzeby curator, Societe de Bacteriologie Systamatique et Veterinaire, France.

A subtypes, the A.I strains appear to be more common in the eastern and midwestern part of the United States, whereas the A.II strains are more commonly observed in the western region of the United States. Both the Type A and Type B strains are associated with sporadic outbreaks of tularemia in the United States. *F. tularensis* has been seen in a variety of environments that includes over 250 species of wild mammals, domestic animals, and blood sucking arthropods, as well as water and soil. Animals associated with acute disease after exposure include rodents such as the muskrat, water vole, domestic mouse, prairie dog, rat, and beaver; lagomorphs such as the cotton tail rabbit, hare, and jackrabbit; and insectivores such as the red-toothed shrew. Domestic animals include sheep and cat. Due to the predatory nature of cats, tularemia cases are often due to bites and scratches from an infected cat. Outbreaks of human tularemia frequently follow an outbreak of tularemia in animals. Due to this widespread association with animals and the environment, there are numerous diverse modes of transmission for *F. tularensis*. These include direct contact with infected mammals (e.g., skinning of rabbits), bites of infectious arthropods (e.g., ticks and deer flies), ingestion of contaminated water or food (e.g., raw milk and undercooked meat), inhalation of contaminated aerosols or dusts, and bites from infected animals (e.g., cats). However, there is no evidence for person-to-person transmission. Transmission from infected animals to humans can occur in a variety of ways. Arthropods are frequent vectors for disease transmission, with ticks considered a common mode of transmission east of the Rocky Mountains and deer flies common vectors in Utah, Nevada, and California. Cases of tularemia from occupational and recreational exposure to a *F. tularensis*–contaminated ticks tend to be seasonal and occur in late spring and throughout the summer. Overall, tularemia is considered one of the major tick-borne illnesses in the United States. The tick vectors most commonly associated with transmission are *Amblyomma americanum* (lone star tick: South Central and Eastern United States), *Dermacentor andersoni* (Rocky Mountain wood tick: Western North America), and *Dermacentor variabilis* (American dog tick: Eastern, Central, and Southern North America). A differential diagnosis of tularemia from other tick-borne diseases is necessary following initial presentation of a patient. Tularemia is characterized by a high fever, headache, normal WBC count, and elevated liver transaminases without a rash (the rash is typical of Lyme disease and Rocky Mountain spotted fever) or anemia (this sign is typical of ehrlichiosis and babesiosis).

Clinical Manifestations

F. tularensis subsp. *tularensis* is one of the most infectious pathogens to humans. An infectious dose of as few as 10 organisms can cause an acute infection. Type A–caused tularemia is generally associated with the more severe disease, with the Type A.I subtype causing more serious disease than Type A.II serotype. Type B–caused tularemia is generally associated with milder symptoms and frequently occurs after exposure to a *F. tularensis*–contaminated stream, pond, lake, or river, or to an infected semi-aquatic animal such as a muskrat or beaver. Therefore, this disease is sometimes considered to be a waterborne disease. However, severe infection does periodically occur in patients infected with *F. tularensis* Type B strains, which should be appropriately treated. *F. tularensis* subsp. *mediasiatica* has rarely been reported as a cause of human disease, and *F. novicida* is considered of low virulence and

only occasionally causes disease in immunocompromised patients. The incubation period following exposure to the Type A and B strains is usually 3 to 5 days, ranging from 1 to 21 days. Tularemia is a potential lethal infectious disease characterized by seven different clinical syndromes. *Ulceroglandular* is the most common condition recognized, which occurs after exposure to infected animal tissue (direct contact) or by the bite of an insect vector that has fed on an infected animal. This condition is characterized by the formation of a primary ulcer (painful maculopapular lesion) that develops at the site of bacterial exposure with the presence of acutely inflamed regional lymph nodes. *Glandular* disease is characterized by a regional lymphadenopathy with no ulcer present. This disease may have originated with a primary ulcer, but the ulcer may not be detected. The *oropharyngeal* form occurs in individuals who ingest *F. tularensis*–contaminated water or food. The patient with this form presents with an ulcerative-exudative stomatitis and pharyngitis with or without tonsillar involvement. The disease is also associated with an excessive regional neck lymphadenitis (cervical lymphadenopathy), which may mimic streptococcal disease. This condition may also occur in patients who may have been exposed to an infected insect or tick bite to the head and neck region without the detection of an ulceroglandular lesion. The *pneumonic* or respiratory form of tularemia is contacted by inhalation of aerosolized *F. tularensis*. This form occurs after exposure to the carcasses of rodents or lagomorphs found in the environment after death from tularemia. Respiratory tularemia may present with symptoms of pneumonia, including cough, chest pain, and an increased respiratory rate. An intentional aerosol release of *F. tularensis* Type A would be expected to result in clinical manifestations similar to those recognized in natural respiratory tularemia (fever, dry cough, chest pain, and hilar adenopathy). Other rare forms of tularemia include *oculoglandular* disease, which is characterized by severe conjunctivitis and preauricular lymphadenopathy; *typhoidal* tularemia, which is characterized by high fever, hepatomegaly, and splenomegaly; and *intestinal* disease, characterized by intestinal pain, vomiting, and diarrhea.

Pathogenesis

F. tularensis is a facultative intracellular pathogen, and the pathogenesis of this organism is its ability to replicate and survive within different cell types. However, macrophages are the primary replication niche. Although the course of infection involves the spread of the organism to multiple systems, including the lungs, liver, spleen, and lymphatic system, the ultimate course of disease differs depending on the route of infection. The cell surface of *F. tularensis* contains a carbohydrate that is a unique version of lipopolysaccharide (LPS), which leads to phagocytosis via the formation of asymmetrical pseudopod loops. Once inside the cell, *F. tularensis* exits the phagosome, avoiding phagolysosomal fusion, and then replicates within the cytosol of the infected cell. Following a period of replication, the bacteria induce apoptosis in the infected host cell, and are then released from this cell to quickly infect another host cell. The ability to cause disease is related to a large set of virulence genes, including *mgl*A and *mgl*B (macrophage growth locus), *igl* (intracellular growth locus), and other genes in the *Francisella* Pathogenicity Island (FPI). The FPI contains 19 genes that encode for essential qualities of intracellular growth and virulence. Both the Type A and Type B strains are noted for causing disease; however, differences in virulence have been noted among the subtypes of Type A and between Type A and Type B strains. Type A.I tends to produce a severe form of tularemia with a higher mortality rate, whereas Type A.II and Type B strains produce less severe and rarely fatal infections in humans. Evidence suggests that this difference in pathogenicity is related to genomic rearrangements that alters the regulation of virulence factors in the strains, potentially leading to a broader range of immunosuppression and increased virulence.

Immunology

The bulk of data on immune responses to *F. tularensis* infection has been generated using mammalian animal models of infection and by studying responses in humans following vaccination. Additional information has been compiled following studies on individuals who have had natural infection. Studies following the administration of the attenuated vaccine strain derived from Type B *F. tularensis*, designated LVS for "live vaccine strain," have been a model for intracellular pathogens although this information should not be assumed to apply directly to fully virulent *F. tularensis* strains. This vaccine has not been licensed by the United States Food and Drug Administration (FDA) because of a lack of understanding of the basis of attenuation and the possibility of this strain reverting back to a pathogen. This attenuated vaccine was derived by repeated passage of a Russian vaccine strain on peptone cysteine agar and subsequent manipulations including lyophilization and serial passage through mice. The LVS strain is administered by the dermal route using a scarification method to produce a ulceroglandular lesion at the site of inoculation. Following immunization, a robust specific anti-*Francisella* antibody response occurs; however, the

contribution of B cells toward protective immunity has been found to offer little advantage in intracellular infections. On the other hand, activation of specific T cells involving memory cells is known to have a protective response following both vaccination and natural disease. Similar to other intracellular pathogens, long-term protective immunity to a *F. tularensis* infection relies primarily upon T cells. T cells use a variety of both soluble and contact-dependent mechanism to ultimately control and eliminate an infection by *F. tularensis*. Multiple research groups are studying the essential role of the various T cell subsets to characterize the mechanisms that confer protection against tularemia.

Vaccination

Numerous approaches have been employed to develop a tularemia vaccine, ranging from crude culture extracts to subunit vaccines. So far, these approaches have failed to achieve good levels of protection against virulent strains. Bacterial LPS appears to be a key component that is recognized by the human immune system, and the search for other protective antigens continues. Part of the hope in the development of a subunit vaccine lies in the availability of new adjuvants, such as immune stimulating complexes. However, use of this adjuvant system has not been evaluated in humans. Therefore, the attenuated strain of *F. tularensis* Type B (LVS) continues to be the only vaccine available for protection in humans. Although this vaccine has been utilized successfully for decades to immunize large groups of people, the vaccine has not been licensed by the regulatory agencies. There are many reasons for this, but the lack of understanding of the basis of attenuation and the inability to conduct clinical studies due to the infectious nature of the organism are some key reasons why a universal vaccine is not available. The FDA issued a regulation in 2002 as an alternative licensing pathway for pharmaceutical products that targets highly lethal pathogens. This regulation, called by some the "animal rule," has been used in the development of biodefense vaccines. This allows for approval based on animal efficacy data conducted under Good Laboratory Practice controls and human safety, along with immunogenicity data conducted under Good Clinical Practice controls, which support the correlation of protection defined in the animal model. The animal rule is intended to be used as a pathway for regulatory review only after exhausting other ways to license a vaccine. However, utilization of this regulation still requires an understanding of the mechanism of pathogenesis, which is not available for *F. tularensis*. Further, the immunologic response in animals differs significantly from that in humans. This makes it unlikely that animal studies will completely predict what happens in humans. Currently, the LVS strain continues to be the lead candidate to protect individuals in the event of a deliberate release, based on its long history of use in humans.

Laboratory Diagnosis

The laboratory diagnosis of tularemia is based on recovery of the causative agent or detection of specific antigen or DNA in a clinical specimen or recognition of a serological response to *F. tularensis* in serum. The Council on State and Territorial Epidemiologists has described, based on laboratory criteria, a supportive case of tularemia as an elevated serum antibody titer to *F. tularensis* antigen (without documented fourfold or greater change) in a patient with no history of a tularemia vaccination or detection of *F. tularensis* in a clinical or autopsy specimen by a fluorescent assay or detection of *F. tularensis* in a clinical or autopsy specimen by a PCR-based test (https://www.cdc.gov/nndss, accessed May 28, 2021). A confirmatory case of tularemia requires the isolation of *F. tularensis* in a clinical specimen or a fourfold or greater change in serum antibody titer to *F. tularensis*–specific antigen when testing acute and convalescent sera.

Laboratory Response Network (LRN)

Special precautions are necessary when working with cultures or specimens suspected to contain *F. tularensis*, because this organism is considered a risk group 3 agent by the United States National Institutes of Health and is classified as a Tier 1 select agent by the federal government. Therefore, the handling and testing of this risk group 3 agent in the laboratory requires biosafety level 3 containment. As a part of the bioterrorism preparedness program in the United States for the evaluation of specimens and isolates potentially containing *F. tularensis*, the CDC has developed a network of Sentinel Level Clinical Microbiology Laboratories that comprise part of the United States LRN. The CDC and the Association of Public Health Laboratories in coordination with the American Society for Microbiology has developed protocols designed to assist these sentinel laboratories with information and techniques to rule out microorganisms that might be suspected as agents of bioterrorism (such as *F. tularensis*), or to refer suspect isolates to LRN Reference Laboratories (e.g., public health laboratories) for confirmation testing.

Serological Methods

Serology is the most common method used for the confirmation of tularemia. Antibody responses against *F. tularemia* are detected in patients about 10–20 days after infection. A negative serological test soon after the onset of illness (acute serum) does not exclude a diagnosis of tularemia, and therefore, a second serum (convalescent serum) should be obtained several weeks later to confirm or exclude the diagnosis. Agglutination protocols using antigens such as *Francisella*-specific LPS, an outer membrane carbohydrate-protein fraction, or whole killed cells are used as antigens to measure the immune response. Testing of both sera are necessary to demonstrate a fourfold rise in specific antibody titer for confirmatory diagnosis of tularemia.

Morphology and Staining

F. tularensis is a tiny, pleomorphic, nonmotile, gram-negative, facultative intracellular coccobacillus (0.2–0.5 µm by 0.7–1.0 µm) (see Figure 16.1). From culture, the organism stains poorly. Therefore, the small size of this microbe and poor staining may make interpretation difficult (Figure 16.1).

Culture Isolation

F. tularensis can be isolated from specimens of blood, skin, ulcers, lymph node drainage, gastric washings, or respiratory tract in cases of tularemia. Isolation of *F. tularensis* in culture provides a confirmed diagnosis of tularemia and provides an isolate for molecular, epidemiological, and susceptibility testing. As a fastidious aerobe, this organism prefers medium supplemented with cysteine (e.g., cysteine hemoglobin agar), but it will also grow on other enriched media such as chocolate agar and Modified Thayer-Martin agar. However, growth on sheep blood agar is generally poor. Adequate growth generally takes 48 hours or more at 35–37 °C in 5% CO_2 after which time the colonies are about 2–4 mm in diameter, white to gray opaque, flat with an entire edge, smooth, and a shiny surface (see Figure 16.2). *F. tularensis* will not grow on gram-negative selective/differential media such as MacConkey or Eosin methylene blue, but will grow in commercial blood culture media (Figure 16.2).

Preliminary Biochemical Reactions

F. tularensis is oxidase negative, weakly catalase positive (3% hydrogen peroxide), beta-lactamase positive (e.g., Cefinase test reagent), urease negative (e.g., Christensen agar), negative for satelliting, and shows no requirement for factor X (hemin) and factor V (NAD) for growth. Sentinel laboratories that cannot rule out a potential *F. tularensis* at this point are requested to consult with their LRN reference laboratory to determine the next step for submission of confirmation testing.

Limitations of Culture

Commercial identification systems should not be used to identify *F. tularensis* because they have a high probability of misidentification and the potential for generating aerosols. *F. tularensis* is most often misidentified as *Haemophilus*

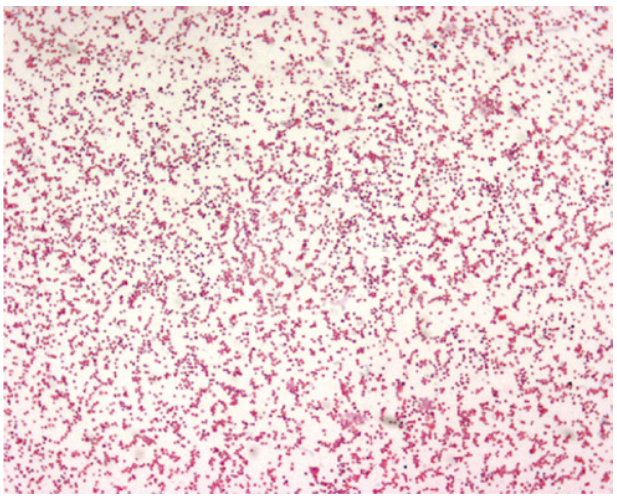


Figure 16.1 Gram stain of *Francisella tularensis* showing poor staining of tiny gram-negative coccobacillus mostly seen as single cells, magnification ×100. *Source:* CDC Public Health Image Library/Larry Stauffer.

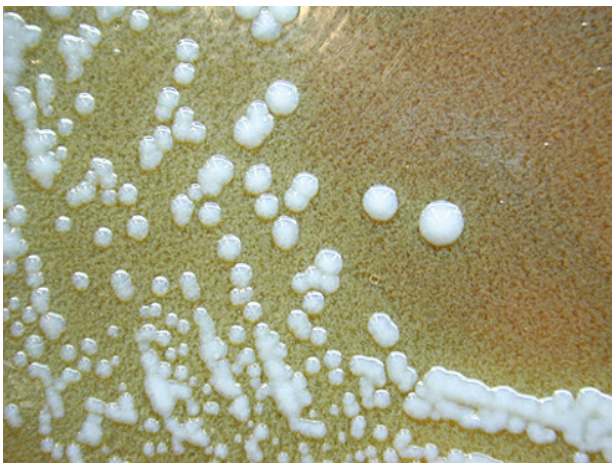


Figure 16.2 Culture of *Francisella tularensis* on cysteine heart agar after 72 hours of incubation showing colonies that are 2–4 mm, smooth, entire, green-white color with an opalescent sheen. *Source:* CDC Public Health Image Library/Larry Stauffer.

influenzae, which forms satellite colonies on sheep blood agar and has a growth requirement for X and V factors, or as *Actinobacillus actinomycetemcomitans*, which are generally beta-lactamase negative.

Antigen Detection Methods

Antigen detection methods are most useful for the direct detection of *F. tularensis* in clinical specimens. Direct fluorescent antibody staining, using an FITC-labeled rabbit antibody directed toward whole-cell killed *F. tularensis* are commonly used. In addition, immunohistochemical staining using a monoclonal antibody directed against the LPS can be used to visualize *F. tularensis* in formalin-fixed tissues.

Molecular Methods

Numerous PCR-based amplification methods have been described for the molecular detection of *F. tularensis* from clinical specimens and for confirmation identification from culture isolates. The increased sensitivity and specificity along with the rapidity compared with other diagnostic methods makes molecular testing attractive for future diagnostics. However, the lack of standardization of assays and validation testing protocols limit the use of molecular methods for laboratory confirmation at the present time. Genomic methods using sequence analysis comparison have also been used for the accurate identification of isolates recovered in culture. Targets within the rDNA complex (e.g., 16S ribosomal DNA) have been shown to be useful for identification purposes. Moreover, the complete assembly of genomic sequences and omics data associated with strains from the different *F. tularensis* subspecies and subtypes that are publicly available in various databases contribute to the further characterization and development of diagnostic tools for this pathogen.

Treatment

Parenteral administration of an aminoglycoside is the first choice for the treatment of tularemia in adults and children, and the aminoglycoside drug of choice is gentamicin at 5 mg per kg, which is divided into two or three daily doses and is monitored by assaying for serum concentrations of the drug. When available, streptomycin is a viable alternative drug treatment that can be given by intramuscular injection for a daily dose of 2 g, which is divided into two doses, for up to 10 days. Streptomycin is not widely used due to its potential to cause vestibular toxicity and the frequent appearance of hypersensitivity reactions among personnel involved in its administration. For post-exposure prophylaxis where laboratory personnel have been accidentally exposed, the recommended therapy is either ciprofloxacin (1000 mg daily divided in two doses) or doxycycline (200 mg daily, divided in two doses) for up to 14 days. In cases where exposure may have occurred without verification, the exposed person should be instructed to be alert to the development of fever within 14 days of exposure and a readiness to be treated is warranted. Antimicrobial susceptibility testing is not routinely done for *F. tularensis* because no natural resistance to antibiotics used for clinical therapy has been documented. However, the potential for *F. tularensis* to be used for bioterrorism makes resistance an area of concern. Therefore, standardized methods for antimicrobial susceptibility testing are available to test *F. tularensis* susceptibility to various antibiotics that include aminoglycosides (gentamicin and streptomycin), tetracyclines (doxycycline and tetracycline), quinolones (ciprofloxacin or levofloxacin), and chloramphenicol.

Safety Measures

Both the general public and laboratorians are at high risk for infection when exposed to infected arthropods, infected animals, or when handling *F. tularensis* cultures in the laboratory. To prevent exposure in the environment, individuals should protect themselves against arthropod bites by wearing protective clothing and by using insect repellents with frequent inspection for and removal of ticks from the skin and scalp. Children should be instructed not to handle dead or sick animals. Landscape workers should be aware of the potential for inhalation of infected aerosols (e.g., lawn mowing) where an infected carcass might be present. Hunters, trappers, and food preparers should be instructed to wear rubber gloves or other protection when handling the carcasses of wild rabbits and other potentially infected animals. Game meats should also be cooked thoroughly. Veterinarians and physicians who suspect tularemia should alert the laboratorians, technicians, and other appropriate personnel to the need for special diagnostic and safety procedures. Tularemia has been reported as the fourth most common reported laboratory-associated infection worldwide caused by bacteria, after brucellosis, Q fever, and typhoid fever. The United States Department of Health and Human Services provides a document for guidance in the laboratory when handling *F. tularensis*. The CDC has also published a guideline for managing potential laboratory exposures to *F. tularensis* (http://www.cdc.gov/tularemia, accessed May 18, 2021). This guideline provides assistance on how to determine if the exposed individual

should be managed by a fever watch and who would benefit from immediate prophylaxis.

F. tularensis as a Biological Weapon

The high virulence of the pathogen in humans, the low infectious dose (10–50 organisms), and the ease of dissemination by aerosolization have led to the concerns of *F. tularensis* for utilization as a bioweapon. Therefore, the United States government has classified *F. tularensis* as a Tier 1 select agent, which requires the handling and possession of this pathogen to be highly regulated. Any laboratory (human or veterinary) where diagnostic testing is performed must document destruction of confirmed cultures of *F. tularensis* or be registered by the national Federal Select Agent Program to have viable cultures in their possession.

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17

Moraxella

John Dustin Loy and Gabriele Maier

Members of the genus *Moraxella* are gram-negative rods or cocci belonging to the family Moraxellaceae. The genus *Moraxella* had been previously subdivided into two subgenera: *Moraxella* (containing the rod-shaped members of the genus) and subgenus *Branhamella* (the coccoid members). Some of the coccoid species were also formerly classified as *Neisseria*. There are 19 valid species currently contained within the genus, many of which are associated or have been found in animals (Table 17.1). From a veterinary perspective, *Moraxella bovis* (*M. bovis*) is the most clinically significant member of the group. *M. bovis* is the cause of infectious bovine keratoconjunctivitis (IBK), the most common and costly ocular disease of cattle. Other significant veterinary species include *Moraxella bovoculi* (*M. bovoculi*) and *Moraxella ovis* (*M. ovis),* both hemolytic and coccoid organisms associated with IBK in cattle and infectious keratoconjunctivitis (IKC) in sheep, respectively. *M. bovoculi* is newly described, and thus there may be some taxonomic overlap with *M. ovis* before the recognition of a separate species.

Descriptive Features

Morphology and Staining

Members of the genus *Moraxella* are short, thick gram-negative rods or cocci, 1–1.5 μm × 1.5–2.5 μm, and are often arranged in pairs or short chains. Variation in morphology is commonly observed in culture (Figure 17.1).

Structure and Composition

Cell Wall and Outer Membrane

The outer membrane of *Moraxella* spp. is composed primarily of lipooligosaccharides (LOS), which are similar to lipopolysaccharide (LPS), but lack O-antigen. These molecules are likely involved in virulence and pathogenesis, including immune evasion. *M. bovis* has an outer membrane composed of three distinct types of LOS, which like other *Moraxella* spp. are uniquely devoid of heptose, and have been shown to confer serum resistance, enhanced adhesion properties, and resistance to some detergents and antibiotics. Capsules may be present in fresh isolates of certain strains of *M. bovis*.

Cellular Products of Medical Interest

Adhesins. The role of adhesins, as in other microorganisms, is to allow the bacterium to attach to host cells. *M. bovis* produces a type IV pilus (plural: pili) or fimbria that adheres to bovine conjunctival and corneal epithelial cells. This pilus is similar to those of *Pseudomonas aeruginosa* and *Dichelobacter nodosus* among other related organisms. *M. bovis* pili are necessary virulence determinants that can be lost during subculture (also see section below titled "Variability"). Pilus strain variation confers serotype specificity. *M. bovoculi* produces a similar pilus, which has approximately 38% similarity on a sequence level to the *M. bovis* pilus. Although additional characterization has not been described, these pili likely perform a similar function in both species.

Exotoxins

The most significant *M. bovis* exotoxin is an RTX type of excreted cytotoxin and is similar in structure and function to this type of exotoxin found in other bacterial pathogens (see also *Escherichia coli* hemolysin, Chapter 6; Pasteurella/Mannheimia leukotoxin, Chapter 10; Actinobacillus hemolysin, Chapter 11; adenylyl cyclase toxin of Bordetella, Chapter 13). RTX stands for repeats in toxin, named after the common feature of repeats in glycine-rich sequences within the protein. The RTX cytotoxin, sometimes referred to as hemolysin or hemolytic cytotoxin due to hemolytic activity observed on blood agar plates, has been termed Mbx (for *M. bovis* toxin). Mbx is a pore-forming toxin with

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Table 17.1 Moraxella species of veterinary interest.

Moraxella species	Animal host	Location/tissue	Significance
A. Commensals			
Moraxella pluranimalium	Pig	Nasal flora	Commensal
Moraxella porci	Pig	Nasal flora	Commensal
Moraxella osloensis	Cat	Ocular conjunctiva	Commensal
Moraxella oblonga	Sheep	Oral cavity	Commensal
Moraxella boevrei	Goat	Nasal / ocular flora	Commensal
Moraxella caprae	Goat	Nasal flora	Commensal
Moraxella caviae	Guinea pig	Pharynx	Commensal
Moraxella cuniculi	Rabbit	Nasopharynx	Commensal
Moraxella canis	Dog, Cat	Muzzle	Commensal
Moraxella ovis, Moraxella bovis	Spanish ibex (*Capra pyrenaica hispanica*)	Ocular and nasal mucosa	Commensal
Moraxella sp.	Tuatara (*Sphenodon punctatus*)	Cloaca	Commensal
Moraxella sp.	Black cobra *(Naja naja karachiensis)*	Distal gut	Commensal
Moraxella-like sp.	California sea lion	Vaginal and preputial mucosa	Commensal
Moraxella atlantae	Black rat *(Rattus rattus)*	Skin	Commensal
B. Isolated from lesions in pure culture			
M. bovis	Cattle	Ocular surface	Infectious bovine keratoconjunctivitis
M. bovoculi	Cattle	Ocular surface	Infectious bovine keratoconjunctivitis
	Horses		Conjunctivitis
M. ovis	Sheep	Ocular surface	Conjunctivitis
M. ovis[a], *M. bovis*	Mule deer, moose	Ocular conjunctiva	Keratoconjunctivitis*
M. caprae	Goat	Ocular surface	Infectious keratoconjunctivitis
Moraxella lacunata	Elk, eland	Lung	Pulmonary abscess, considered rare, also a human pathogen
Moraxella catarrhalis	Cynomolgus macaque (*Macaca fascicularis*)	Nasal cavity	"Bloody nose syndrome," sneezing, epistaxis and mucohemorrhagic nasal discharge
M. osloensis	Cat	Pericardium	Fibrinous pericarditis, suspected secondary to respiratory infection
C. Isolated from lesions as part of a mixed flora			
Moraxella equi	Horse	Ocular surface	Conjunctivitis
M. osloensis	Bowhead whale (*Balaena mysticetus*)	Skin lesions	Not found on normal skin
M. caprae	Sheep	Gingival pockets	Broken mouth periodontitis in sheep grazed on rough pasture
M. bovis	Sheep and goats	Lung	Pneumonia

[a] May overlap with *M. bovoculi*.

specificity for conjunctival and corneal epithelial cells and neutrophils. Mutants unable to produce Mbx are avirulent. *M. bovoculi*, and *M. ovis* secrete similar RTX toxins. *M. bovoculi* RTX toxins are encoded by an operon called Mbv, similar in structure and function to Mbx, which is necessary for hemolytic activity. Some genes within the Mbv operon appear to have undergone recombination with *M. bovis* sequences found in some strains. Mbv presence is detectable using MALDI-TOF MS protocols and specialized media formulations.

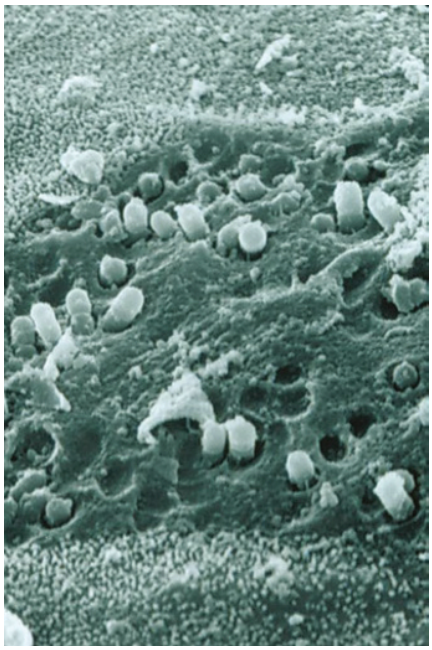


Figure 17.1 *Moraxella bovis* in the cornea of an experimentally infected calf. There is evidence of digestion of corneal substance around the bacterial cells. Scanning electron micrograph, 22 000×. *Source:* Courtesy of Dr. Doug Rogers, University of Nebraska Veterinary Diagnostic Center.

Iron Acquisition

Because iron is an absolute growth requirement, microorganisms must acquire this substance if they are to exist within the host. *Moraxella* spp. acquire iron from the iron-binding proteins transferrin and lactoferrin of the host by expressing Tbp and Lbp (for transferrin- and lactoferrin-binding proteins, respectively) on their surface.

Miscellaneous Products

M. bovis produces a number of proteins that are toxic or potentially virulence related such as complement-degrading proteases, lipases, phosphoamidases, esterases, hyaluronidase, peptidases, and proteases. Some strains secrete phospholipase, which, in addition to a potential virulence factor, may be a vaccine antigen target. Similar to genes found in members of the genus *Bordetella,* filamentous hemagglutinin-like proteins are carried by some strains on plasmids and may facilitate colonization.

Growth Characteristics

M. bovis grows best at 35 °C in the presence of serum and blood (Figure 17.2). Bovine blood and calcium can enhance hemolytic activity in hemolytic strains. No growth occurs on MacConkey agar or anaerobically. In 48 hours, fresh isolates produce flat, hemolytic, friable colonies, about 1 mm in size that corrode the agar and autoagglutinate when suspended in saline. *M. bovoculi* vary in hemolytic activity, are more rounded, and can vary in color from white to cream. Some species also appear as small domed colonies that slide across the agar surface when touched with a loop, which has been described as the "hockey puck sign." The addition of cloxacillin to bovine blood agar has been used as a semi-selective media to isolate *M. bovis* from lacrimal secretions.

Biochemical Activities

M. bovis is oxidase positive, asaccharolytic, nonfermenting, and catalase variable. *M. ovis* as well as most *M. bovoculi* reduce nitrate, whereas *M. bovis* does not. *M. bovoculi* can usually be distinguished from *M. ovis* and *M. bovis* by the presence of phenylalanine deaminase activity.

Resistance

Resistance to physical and chemical agents is not remarkable. *Moraxella* spp. are typically susceptible to commonly used antibiotics, including penicillin. Some isolates of *M. bovis* and *M. bovoculi* are resistant to tetracyclines and macrolides. *Moraxella catarrhalis* is the only species that commonly produces a betalactamase. However, resistance, or the potential for resistance, in veterinary *Moraxella* is increasing, as a genomic island with 10 or more antimicrobial resistance genes has been found in some strains of *M. bovoculi*. This island confers *in vitro* resistance to macrolides and tetracyclines, which are frequently used for treatment among other important antimicrobials. Many members of the genus have been shown to produce biofilms on abiotic surfaces, including *M. bovis* and *M. bovoculi*. The capacity for biofilm formation varies between strains. Biofilm formation in *M. bovis* involves pili, but this has not been determined in *M. bovoculi*. Biofilm formation increases resistance to chemicals and antimicrobials, including lysozyme, which is important in the innate defense of ocular and other mucous membranes.

Variability

In culture upon continued passage, *M. bovis* undergoes colonial dissociation (phase variation), producing smooth colonies composed of cells which lack pili due to inversion of the pilin encoding gene, which are less infective and less autoagglutinable. Pili are immunogenically diverse, a trait that has led to a classification scheme based on serological similarities. At least seven different serotypes of *M. bovis*

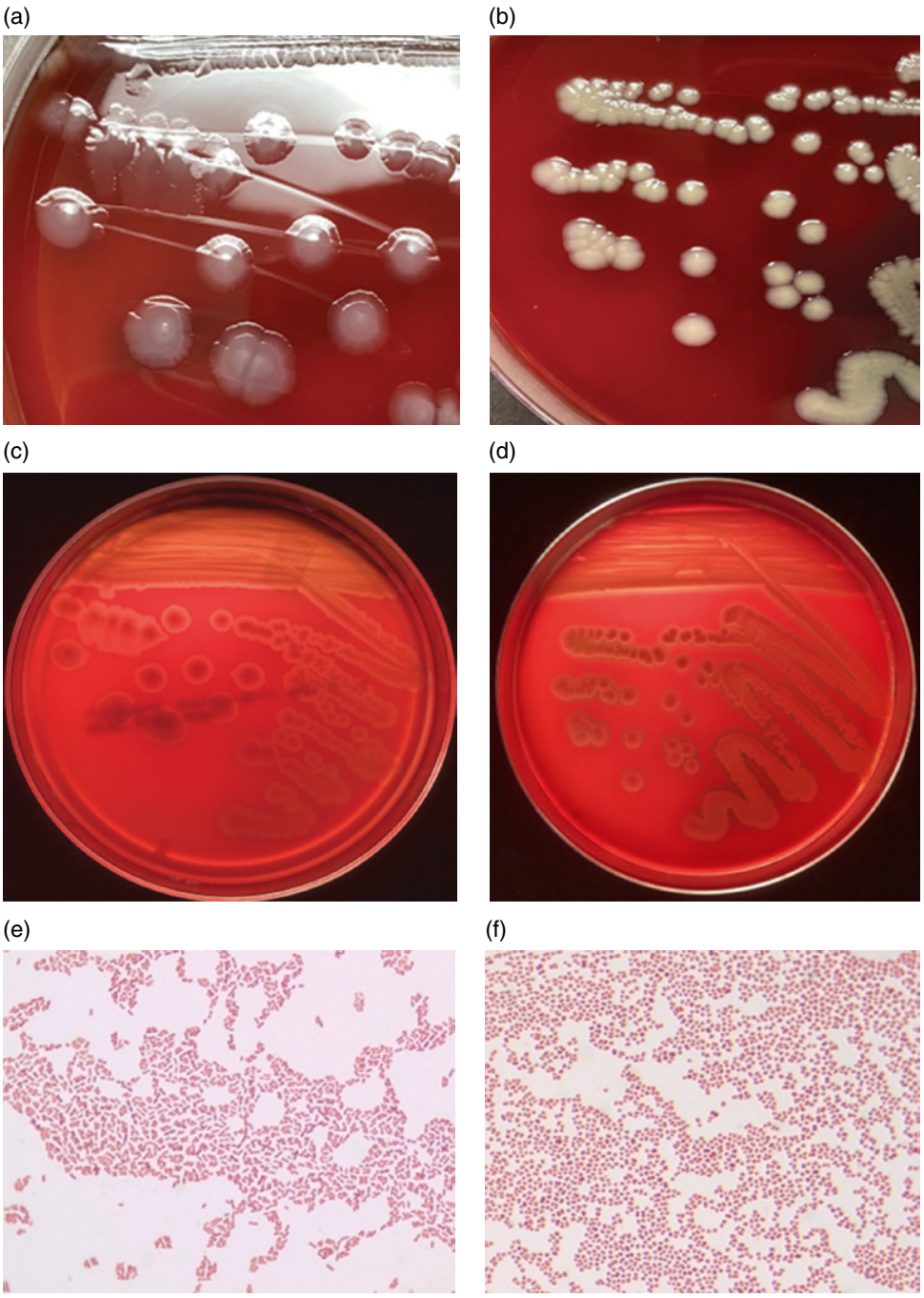


Figure 17.2 (a) and (c) *Moraxella bovis* and (b) and (d) *Moraxella bovoculi* growth on 5% sheep's blood agar after 48 hour incubation. (e) *Moraxella bovis* and (f) *Moraxella bovoculi* Gram's stain of culture visualized using light microscopy at 1000X oil immersion. *Source:* Courtesy of Matt Hille and J.D. Loy, University of Nebraska-Lincoln Veterinary Diagnostic Center.

have been described. Pili are expressed as two mutually exclusive forms called Q (quick) and I (intermediate) forms apart from the serotype level variation. The Q form has been shown to have enhanced binding properties to host cells. Nonhemolytic variants of *M. bovis* exist and are considered nonpathogenic.

Ecology

The *Moraxellaceae* are parasites or commensals (normal flora) primarily of the upper respiratory and ocular mucosal surfaces of mammals. Most of these bacteria are not strict pathogens (see Table 17.1.)

Reservoir

Moraxella bovis occurs worldwide on the bovine conjunctiva and upper respiratory mucosa, often without clinical manifestations. Age and seasonality have been shown to affect the relative abundance of *Moraxella* in hosts, with higher numbers found in younger animals. *M. bovoculi* is also found in these same niches; however, a genotypic difference has been found between strains isolated in association with IBK and those found in the upper respiratory tract of normal cattle.

Transmission

Transmission is by direct and indirect contact, including flying insects and possibly other airborne transmission.

Pathogenesis

Mechanisms. Disease produced by *M. bovis* is closely linked to cytotoxin (Mbx) and pili. Attachment of pili to conjunctival epithelium is followed by destruction of conjunctival and corneal cells. Growth of *M. bovis* in the conjunctival and corneal lesions leads to inflammation (gram-negative cell wall). Mbx-mediated lysis of neutrophils amplifies inflammation and tissue destruction due to release of degradative enzymes from neutrophilic granules (Figures 17.1 and 17.3).

Microinjuries of target tissues caused by flies, dust, pasture plants, or ultraviolet (UV) radiation may enhance the ability of organisms to colonize and invade corneal epithelium. Concurrent infections with viruses, such as bovine herpesvirus 1 (infectious bovine rhinotracheitis virus) and adenovirus, mycoplasma (*Mycoplasma bovoculi*), bacteria (*Listeria monocytogenes*), and nematodes (*Thelasia*), may complicate the disease. *My. bovoculi* has been shown to potentially increase colonization with *Moraxella* spp., possibly due to an increase in corneal "dark cells," which *Moraxella* pili appear to bind more robustly.

Disease Pattern and Pathology

IBK

IBK begins with invasion of conjunctiva and cornea by *M. bovis*, resulting in edema and a predominantly neutrophilic inflammatory response. This invasion of the cornea has been shown to occur within hours of inoculation into gnotobiotic calves. It may progress from mild epiphora and corneal clouding to production of severe edema, corneal opacities, vascularization, ulceration, and rupture leading to uveal prolapse and panophthalmitis (Figure 17.3). Ulcer healing proceeds from the periphery and requires several weeks. Frequently, cattle owners indicate observation of refractory cases of IBK that do not respond to treatment, but this is mostly just indicative of the extended ocular healing process. Central scarring may persist for months. Though not all lesions require treatment for full recovery, losses occur because painful, vision-impaired animals do

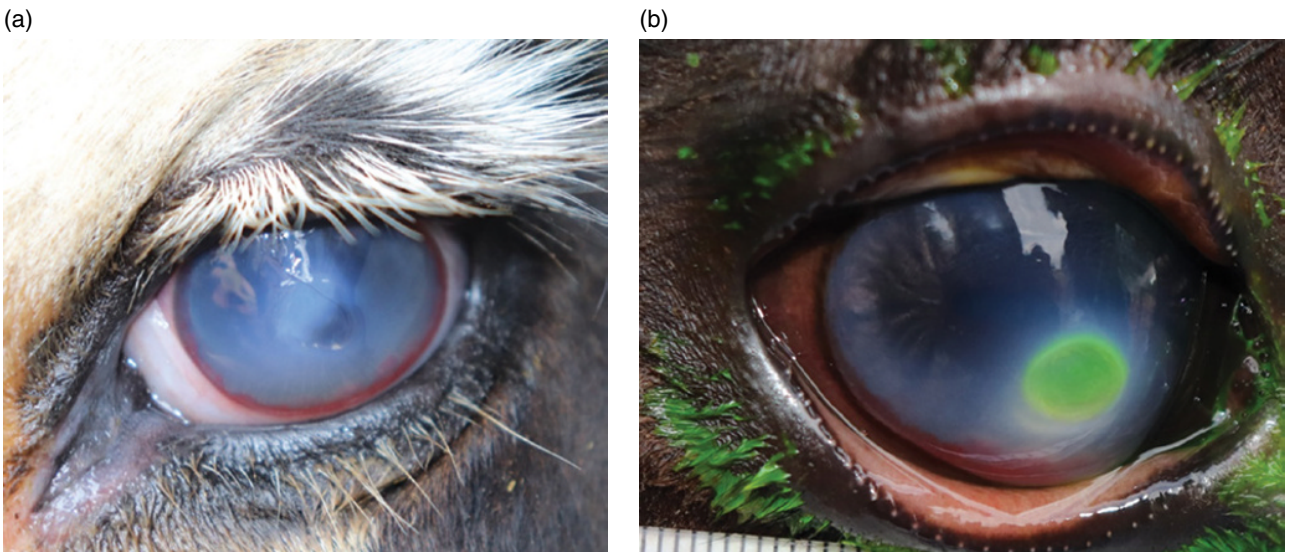


Figure 17.3 (a) Calf with infectious bovine keratoconjunctivitis. (b) Corneal ulcer is visualized via fluorescein sodium stain. *Source:* Courtesy of Gabriele Maier, University of California-Davis.

not forage and lose condition. Additionally, scarring may be present that reduces economic value. Long-term impairment to vision and blindness can occur.

Immunologic Aspects

Antibodies of all isotypes are produced during natural infection, with secretory IgA predominating locally. Temporary resistance to reinfection follows recovery. The relative roles in immunity and recovery of general versus local responses and humoral versus cell-mediated responses are poorly understood.

Experimental bacterins and fimbrial antigens stimulate resistance, optimally to homologous challenge. In addition, Mbx and proteolytic enzymes have shown variable levels of protection-inducing activity. Bacterins are commercially available; however, field trials for commercially available vaccines have not produced consistent results.

Laboratory Diagnosis

For sample collection, the authors recommend the use of a flocked swab in liquid transport media (Amies or similar) to enhance recovery at the laboratory. Bacteria may be also demonstrated in smears of ocular exudate, most convincingly by immunofluorescence using antibody specific for *M. bovis* antigens. Exudate or ocular secretion is cultured on blood agar, and *Moraxella* spp. are identified by colony characteristics, oxidase activity, hemolysis, proteolysis, and failure to ferment carbohydrates, reduce nitrate, or reduce phenylalanine. Specific fluorescent antibody conjugates can be applied directly to suspect colonies on plates for identification even of dissociant colonies (epifluorescence) although these antibodies may not be routinely available. Polymerase chain reaction (PCR)-based assays followed by restriction fragment length polymorphism tests have been shown to distinguish between *M. bovis*, *M. ovis*, and *M. bovoculi*. However, some genotype 2 strains of *M. bovoculi* may lack the differential restriction site and may be misidentified. MALDI-TOF mass spectrometry has been shown to readily distinguish between the different *Moraxella* species, and it is preferred to other methods, except potentially whole genome sequencing, if a robust database is available. MALDI MS–based assays to distinguish genotypes and toxin phenotypes are available for *M. bovoculi*. Several multiplexed real-time PCR-based panels that distinguish *M. bovis* and *M. bovoculi* based on detection of RTX genes that include other potential causes of IBK or IBK-like disease (*Mycoplasma* spp. and Bovine Herpesvirus-1) that can be used to detect agent nucleic acids directly from clinical samples such as swabs are also available.

Epidemiology

Even though *M. bovis* has been recognized as the most important etiologic agent of IBK, its causal role in the disease is not entirely understood as it is frequently isolated from healthy eyes and is often absent from cultures taken from IBK lesions. Under laboratory settings, experimental infections of cattle eyes with *M. bovis* have resulted in corneal lesions consistent with IBK in gnotobiotic calves. The development of IBK in *M. bovis*–infected eyes, while uninfected control eyes stay normal, is evidence for causality. Reproduction of IBK lesions in experimental infections is often achieved by accompanying inoculations with corneal insults through UV light or corneal scarification but have also occurred without these additional measures. The requirement for corneal insults to establish IBK lesions during experimental infections may be dependent upon the virulence of the *M. bovis* strain used. Under field conditions, the evidence for *M. bovis* as a causative agent of IBK is often less clear. Results of field studies may be confounded by the sampling timing relative to the host's immune response or sampling technique resulting in contamination of swabs with commensal microbial species of the eye or adnexa that outperform *M. bovis* during culture. Exposure to UV light and to the face fly *Musca autumnalis* de Geer have been associated with the occurrence of IBK and may be part of a component cause model for the disease. UV light leads to aging of corneal epithelial cells from light to dark cells. *M. bovis* has been observed to preferentially adhere to aged corneal dark cells, which are devoid of the surface projections microvilli and microplicae. While feeding on eye secretions, the face fly's sharp prestomal teeth cause microscopic conjunctival and corneal damage, which may facilitate bacterial entry into corneal stroma. Weak correlations between face fly numbers and occurrence of IBK have been observed under field conditions. There is also evidence that reducing fly numbers with insecticides reduces the incidence of IBK. The face fly is also often cited as a mechanical vector for transmission between cattle eyes and has successfully transmitted IBK under laboratory conditions. However, the evidence from field studies, where higher isolation rates of *M. bovis* from flies are expected to result in higher IBK rates in cattle, is missing. Mechanical irritation of cattle corneas from plant awns while grazing is also often implicated as a contributing factor to IBK, but scientific evidence is likewise missing from the literature. A breed predilection to developing IBK

seems to exist for Hereford cattle, which is mainly attributed to a lack of periocular pigmentation. Compared to breeds belonging to the *Bos taurus* subspecies, breeds in the *Bos indicus* subspecies are less frequently affected by IBK. Age of the animal is another risk factor that has been consistently associated with IBK, with younger calves being more susceptible to develop IBK than older animals; this has been attributed to their immature immune system.

M. bovoculi is isolated regularly from IBK lesions in the absence of *M. bovis* or potential other pathogens during field outbreaks. Nevertheless, *M. bovoculi* has not yet shown to produce IBK-like lesions following experimental infections of cattle eyes following controlled infections as has been shown for *M. bovis*. However, only a limited number of studies have attempted to reproduce IBK via experimental *M. bovoculi* inoculations. Temporality – exposure prior to disease – must be established to definitively establish *M. bovoculi* as a causative agent of IBK. *M. bovoculi* has been classified into two genotypes, 1 and 2, and only genotype 1 appears to be isolated from IBK-affected eyes. In addition, there is evidence of genomic recombination between *M. bovis* and *M. bovoculi* in the form of recombinant 16S rDNA found in both *M. bovis* and *M. bovoculi* genomes, further complicating the role these bacterial species play in causing IBK.

Treatment

Antimicrobials are considered the treatment of choice for IBK. In the United States, oxytetracycline and tulathromycin are labeled for the treatment of IBK caused by *M. bovis*. In clinical trials, other antimicrobials, including subcutaneous (SC) and intramuscular (IM) florfenicol, SC ceftiofur, subconjunctival (S-con) penicillin, SC tilmicosin, topical cloxacillin, and S-con clindamycin have shown efficacy in the treatment of IBK versus placebo. Nonsteroidal anti-inflammatories such as flunixin meglumine are recommended as supportive care. Addition of dexamethasone into S-con penicillin injections has not been shown to affect outcomes.

Non-antimicrobial treatment options, for example, for organic production, include a commercially available dilute hypochlorous acid spray, and other topical disinfectants such as iodine solution, and autologous patient serum harvested from whole blood, which contains corneal growth factors and anticollagenases. However, infections typically occur in field settings, where frequent topical applications are often not practical. Cloth patches to cover affected eyes are thought to protect from UV light and mechanical insults. Overall, published data on efficacy of non-antimicrobial treatments are sparse.

Prevention

Killed vaccines targeting *M. bovis* or *M. bovoculi* are commercially available or can be custom-made as autogenous vaccines from inactivated bacterial isolates from cases. Vaccine efficacy in clinical trials has often been shown during challenge trials with homologous strains, but protection has for the most part not been achieved during field trials with natural infections.

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18

Pseudomonas

Deepti Pillai

Members of the genus *Pseudomonas* are gram-negative aerobic rods. Although there are more than 200 characterized and uncharacterized species and subspecies of *Pseudomonas*, most veterinary diseases are caused by *Pseudomonas aeruginosa*. Previously named pseudomonads of veterinary importance, *Pseudomonas mallei* and *Pseudomonas pseudomallei* have been moved to the genus *Burkholderia*. This chapter will focus on *P. aeruginosa* and its significance as a pathogen in veterinary medicine.

Most members of the genus *Pseudomonas* live in soil, water, plants, and sewage. It can be found on the skin, mucous membrane, and feces of normal animals, but not as a member of the normal flora (i.e. they are transients). *P. aeruginosa* is very rarely involved with primary disease. However, it is highly problematic in clinical medicine and is frequently found in hospital settings, including medical devices such as catheters, endoscopic devices, and respiratory equipment. Its ability to thrive on and in the indwelling medical devices makes it is an emerging nosocomial pathogen in veterinary clinics. In animals, *P. aeruginosa* is mainly involved in secondary bacterial infections associated with compromised host defenses, and hence is considered as an opportunistic pathogen.

They are resistant to the commonly used antimicrobial agents. Their different resistance mechanisms complicate the treatment regimen when they contaminate a compromised site.

Microbiology

P. aeruginosa is a gram-negative rod, 0.3–0.5 µm by 1.2–2.0 µm, and causes disease in animals, humans, and plants. It is motile through polar flagella and produces diffusible pigments: pyoverdin (fluorescent green), pyorubin (red brown), pyomelanin (brown/black) and pyocyanin (blue-green) (Figure 18.1 and Table 18.1). The pigments produced in the lesions give a characteristic greenish tinge to the exudates. Pigment production is also evident in the colonies grown on agar media (see Figures 18.2 and 18.3). The bacterium also produces an aromatic compound, 2-aminoacetophenone, giving a characteristic grape-like odor to the bacterial colonies.

Growth Characteristics

P. aeruginosa is an obligate aerobe, deriving energy from the oxidation of organic materials using oxygen as a terminal electron acceptor. It grows on all standard media over a wide range of temperatures, 4–41 °C.

Virulence Factors and Their Role in Different Stages of Infection

P. aeruginosa produces several virulence factors that help in establishing infection in the host.

1) ***Attachment***
   a) Flagella and Type IV Pilli: These two factors act as adhesins and play an essential role in the attachment to the host epithelial cells. Flagella and Type IV pili initiate an inflammatory response by inducing pro-inflammatory cytokines.
2) ***Multiplication and Colonization***
   a) Quorum sensing (QS): The four QS circuits – *Las* system, *Rhl* system, Pseudomonas quinolone signal (PQS), and integrated QS system (IQS) – are interconnected and coordinate the production of virulence factors (toxins and enzymes), biofilm maturation, and virulence phenotypes (motility).
   b) Biofilm: Biofilm formation promotes bacterial colonization of *P. aeruginosa* during the infection,

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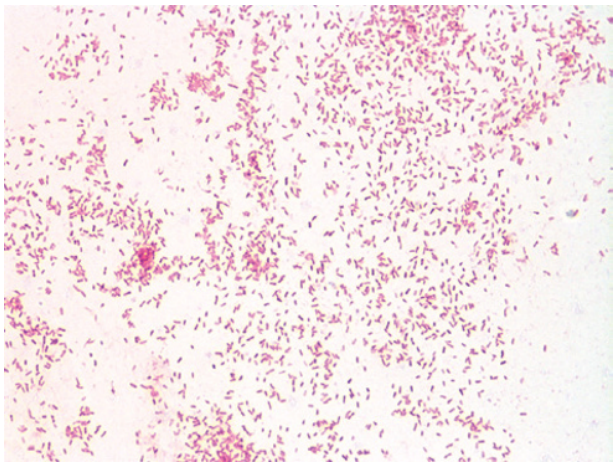


Figure 18.1 A Gram stain of *Pseudomonas aeruginosa* from a blood agar plate (1000×).

Table 18.1 Morphological and biochemical features of *Pseudomonas aeruginosa*.

Features	*Pseudomonas aeruginosa*
Colonial morphology on blood agar	>1 mm, gray (gunmetal), and flat with serrated edges
Diffusible pigment production	Positive
Colony odor	Grape-like
Growth at 42 °C	Positive
Motility	Positive
Catalase	Positive
Oxidase production	Positive
Indole production	Negative
Methyl red test	Negative
Voges–Proskauer test	Negative
Citrate test	Positive
Oxidation of:	
Glucose	Positive
Lactose	Negative
Sucrose	Negative

Adapted and modified from *Veterinary Microbiology and Microbial Disease* (second edition).

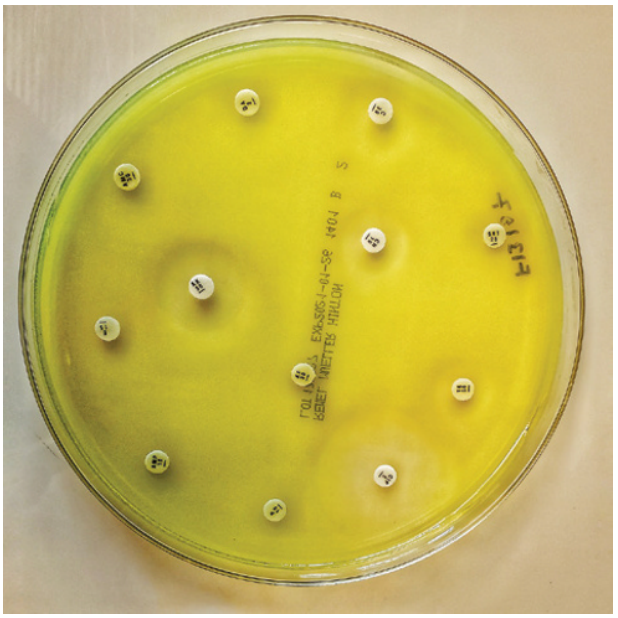


Figure 18.2 Pyoverdin pigment (fluorescent green) produced by *Pseudomonas aeruginosa* on a Muller Hinton Agar.

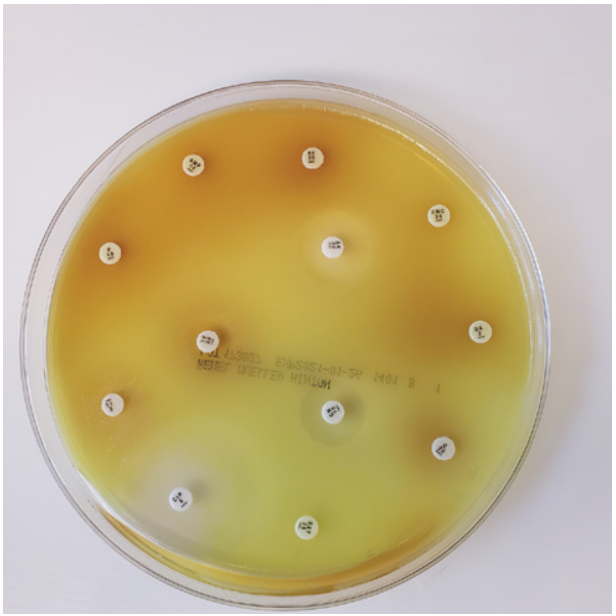


Figure 18.3 Pyorubin pigment (reddish brown) produced by *Pseudomonas aeruginosa* on a Muller Hinton Agar.

especially in biomedical devices such as indwelling catheters.

3) ***Invasion and tissue damage***
   The toxins and enzymes secreted by *P. aeruginosa* that cause host tissue damage are the following:
   a) Exotoxin A: This toxin binds to host cells and becomes internalized. It inhibits protein synthesis by ribosylation of elongation factor-2, resulting in cell death.
   b) Phospholipase C: *P. aeruginosa* produces two types of phospholipase C, contributing to tissue necrosis and cell death.
   c) Elastase and alkaline proteases: These are involved in tissue destruction and invasion of *P. aeruginosa* during the infection.

d) Exotoxins S, T, U, and Y: These are type II secreted toxins that facilitate tissue destruction and invasion of *P. aeruginosa*.
e) Pyocyanin: It is an iron-acquiring siderophore and is associated with toxic activity on the host cell.

4) **Dissemination**
a) Alginate: The gel-like material secreted around the bacteria during the infection helps to evade the host immune system by preventing phagocytosis and complement system activation.
b) Lipopolysaccharide (LPS): The LPS in the outer membrane (OM), along with flagella and Type IV pili, induces an inflammatory response and protects the bacteria from phagocytosis and the serum-killing effect.
c) Capsule: The capsule protects the OM from the membrane attack complex of the complement cascade. The capsule also prevents opsonization and ingestion by phagocytic host cells.
d) Type Secretion Systems (TSS): *P. aeruginosa* uses several secretion systems to evade, escape, and survive in the host body. T3SS helps evade phagocytosis and spread infection, whereas T6SS has an antibacterial function and is utilized as a defense mechanism in the environment.

Epidemiology

The organism is adaptable to diverse conditions. Most often, *P. aeruginosa* favors moist environments and can survive in low-nutrient conditions. These pathogens are found in wet and poorly aerated environments of the veterinary hospital, such as inadequately dried hoses on anesthetic machines or contaminated invasive devices. These situations increase the number of pseudomonads in the environment of the compromised animal (site), thereby increasing the risk of infection in hospitalized patients.

Overview of the Pathogenic Mechanism

Being an opportunistic pathogen, *P. aeruginosa* contaminates areas of the body with reduced numbers of normal flora. Disruption of the normal flora is almost always due to antimicrobial agents. Because *P. aeruginosa* is resistant to most commonly used antimicrobial agents, it will replace the normal flora. After breaching the first line of defense, it invades and causes tissue destruction by releasing toxins and enzymes. The infection potential of *P. aeruginosa* depends on the virulence factors, which are controlled by regulatory networks such as QS and T6SS.

Clinical Diseases in Animals

P. aeruginosa can cause infection of almost any location in the host if the site colonized is compromised or contiguous to a compromised site. It causes a wide range of opportunistic infections. *P. aeruginosa* is associated with wound infections such as any injury, trauma, surgery, or burns in all animal species.

It causes otitis externa, lower urinary tract infection, pyoderma, pneumonia, and occasional ocular infections in dogs and cats. An ear infection is commonly seen in breeds with long droopy ears covering the ear canal, creating a moist environment for the pathogen to grow. In horses, it is often associated with endometritis, keratitis, and conjunctivitis following prolonged antibiotic treatment. In cattle and small ruminants, it causes mastitis, enteritis, metritis, and pneumonia. Mastitis is often associated with a contaminated environment, such as bedding and housing, and unhygienic milking practices (see Figure 18.4). In the swine population, it causes respiratory infections. It is involved in secondary bacterial superinfection in birds following antibiotic treatment for other bacterial infections. It causes fleece rot/dermatitis in sheep, a condition associated with prolonged wetting of the skin. In captive reptiles, moist and contaminated bedding predisposes them to necrotic stomatitis. Stress predisposes farmed minks to

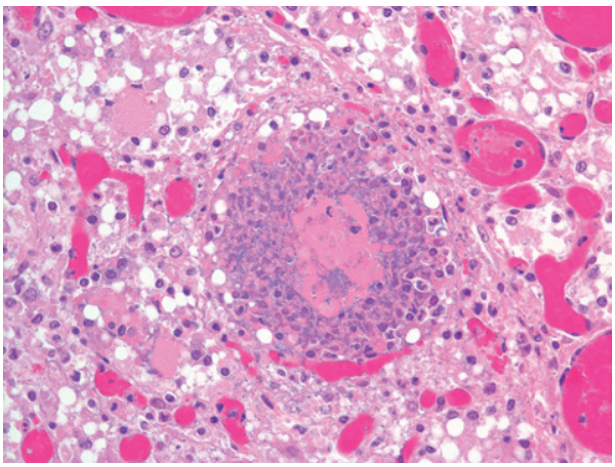


Figure 18.4 Histopathology of mastitis in a goat. Secretory units of mammae are multifocally necrotic with fibrin, degenerate neutrophils, cellular debris, and colonies of *Pseudomonas aeruginosa* (400×). *Source:* Courtesy of Dr. Nobuko Wakamatsu.

fatal and acute hemorrhagic pneumonia. In rabbits, it causes moist dermatitis where the skin from the affected area has a bluish-green discoloration (pyocyanin), and hence the condition is sometimes termed "blue fur disease."

Antimicrobial Resistance (AMR)

P. aeruginosa is resistant to commonly used antibiotics. It displays different resistance mechanisms to widely used antibiotics and can be classified into three categories: intrinsic, acquired, and adaptive resistance.

A) *Intrinsic resistance*
   a) OM impermeability: *P. aeruginosa* selectively restricts the permeability of OM, preventing the antibiotics from penetrating and reaching the target site to kill/inhibit the bacteria.
   b) Efflux pump resistance: The resistance-nodulation-division (RND) family of efflux pumps plays a significant role in the resistance mechanism.
   c) Antibiotic-inactivating enzymes: *P. aeruginosa* produces β-lactamase and an aminoglycoside-modifying enzyme.

B) *Acquired resistance*
   a) Resistance by mutation: In *P. aeruginosa*, mutations in DNA gyrase, a target site for quinolones, interfere with antibiotic uptake and binding to the target site, reducing susceptibility to this antibiotic.
   b) Resistance by horizontal gene transfer (HGT): *P. aeruginosa* carries aminoglycoside resistance genes and β-lactam resistance genes on the mobile genetic elements, allowing for HGT.

C) *Adaptive resistance*
   a) Biofilm-mediated resistance: *P. aeruginosa* tolerance to tobramycin, gentamicin, and ciprofloxacin antibiotics is explained by the expression of biofilm-specific characteristics.
   b) Persister-cells-mediated resistance: Persister cells in the *P. aeruginosa* biofilms tolerate aminoglycosides, quinolones, and the β-lactam group of antibiotics by altering antibiotic target sites. This state of drug tolerance is transient and reversible once the antibiotic is removed.

Treatment

Antibiotics

P. aeruginosa is one of the multi-drug resistant pathogens found in the veterinary hospital. Treatment involves correction of compromise and, if necessary, the use of an antimicrobial agent. It is usually susceptible to ceftazidime, tobramycin, marbofloxacin, ticarcillin, carbenicillin, and gentamicin, and these agents are used to treat soft tissue infections. In the canine urinary tract, tetracycline achieves concentrations sufficient to kill most isolates. Most pseudomonads are susceptible to levels achieved by antimicrobial agents in otic preparations: enrofloxacin, neomycin, polymyxin, chloramphenicol, and gentamicin. It should be noted that there are no *in vitro* tests that predict the susceptibility/resistance of an isolate from infectious processes that will be treated topically (e.g. the ear).

Non-antibiotic therapeutic approaches: Alternative therapeutic approaches are being considered in treating multidrug-resistant strains of *P. aeruginosa*. As this pathogen expresses several virulence factors, some of these approaches are directed toward the systems that control the expression of virulence factors. Approaches such as QS inhibitors, phage therapy, and nanoparticles have shown a significant antibacterial effect on resistant strains.

Vaccines: Vaccination can control *P. aeruginosa* infections. However, most *Pseudomonas* infections are incidental wound infections in veterinary medicine, except in farmed minks (highly contagious and fatal hemorrhagic pneumonia). Therefore, the only animal vaccine available to control and treat *P. aeruginosa* infections is for use in minks.

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19

Taylorella

Megan E. Jacob

Members of the genus *Taylorella* are gram-negative, nonmotile coccobacilli. Equid species appear to be the natural host of *Taylorella* organisms. The genus contains two known species: *Taylorella equigenitalis*, the highly contagious causative agent of contagious equine metritis (CEM), and *Taylorella asinigenitalis*, which has been associated with the genital tract of stallions or jacks. Although *T. equigenitalis* has significant clinical and economic importance, *T. asinigenitalis* has rarely been associated with natural infection.

T. equigenitalis

T. equigenitalis is the causative agent of an acute, suppurative, self-limiting disease of the uterus of mares called CEM. The disease was first described in Newmarket (United Kingdom) and Ireland in the spring 1977; in 1978, CEM was classified as a reportable disease in the United States. CEM can result in temporary infertility or rarely early abortion in mares. The organism is highly contagious, and long-term asymptomatic carriage of *T. equigenitalis* can follow initial infection. Stallions develop no clinical signs of disease but are important long-term carriers. Because of the costs associated with outbreak investigations, testing, and loss of reproductive efficiency, CEM has significant economic implications for the equine industry. The initial reports of CEM in 1977 led to the development of The Horserace Betting Levy Board of a Code of Practice for swabbing mares and stallions and was critical to preventive diagnostic practices still used today.

Descriptive Features

Morphology and Staining

T. equigenitalis is a gram-negative short rod or coccobacillus approximately 0.8 μm by 5–6 μm in size (Figure 19.1). The organism may exhibit bipolar staining and is nonmotile.

Structure and Composition

The cell wall structure of *T. equigenitalis* is typical of gram-negative bacteria, composed of lipopolysaccharide and protein. It is inconsistently fimbriated (typically in culture), and the outer membrane is covered by a capsule. The immunodominant outer membrane protein appears to resemble porin proteins from *Bordetella pertussis* and several *Neisseria* species.

Growth Characteristics

T. equigenitalis is a slow-growing, fastidious, capnophilic organism that grows optimally between 35 and 37 °C under microaerophilic conditions. The organism generally grows well on a rich base medium with 5% chocolate sheep blood, and antimicrobials can be added to the medium to improve the selectivity of the assay. Visible colonies can be seen after 48–72 hours of incubation, although growth may take up to 14 days. Current protocols recommend an incubation period of seven days before confirming that samples are negative. Overgrowth of other urogenital tract organisms may interfere with the culture and identification of

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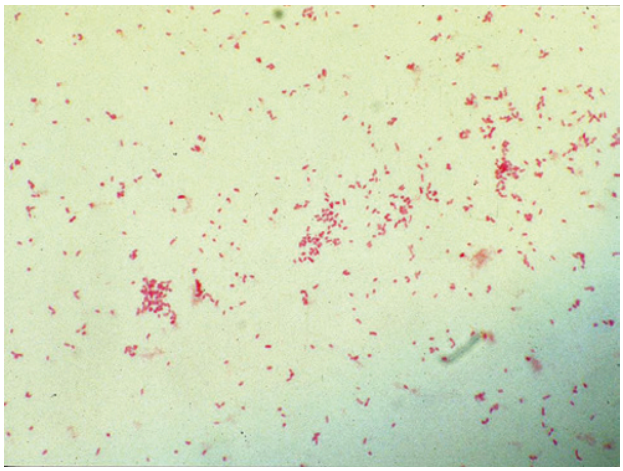


Figure 19.1 A Gram stain of *Taylorella equigenitalis*. *Source:* Courtesy of Peter Timoney and Mike Donahue.

T. equigenitalis; however, supplemental antimicrobials may improve recovery. In one recent laboratory-based study, transport media containing antibiotic and antifungal chemicals improved recovery of *T. equigenitalis*, which may also help reduce competing organisms. Colonies of *T. equigenitalis* are variable in size (2–3 mm in diameter) and are shiny, smooth, and grayish yellow. The organism is oxidase, catalase, and phosphatase positive and otherwise biochemically unreactive, producing no acid from carbohydrates.

Resistance

Although there are two biotypes of *T. equigenitalis* – one is susceptible to streptomycin, and another is resistant – *T. equigenitalis* isolates are generally susceptible to a broad range of antimicrobials with no obvious resistance patterns and are susceptible to various disinfectants.

Variability

Molecular methods such as repetitive extragenic palindromic polymerase chain reaction (PCR) and pulse-field gel electrophoresis of restriction endonuclease-digested DNA have been used to genotype *T. equigenitalis*. Some reports have suggested that differing strains vary in their ability to cause clinical disease in exposed mares; however, this association is not fully understood. In addition, strains can differ in their susceptibility to streptomycin; the clinical significance of this observation is unknown. Although different strains have been reported, genetic analysis suggests the organism is stable over time.

Ecology

Reservoir

The equine genital tract is the only known natural host of *T. equigenitalis*. Mares and stallions that carry the organism asymptomatically are the most important reservoirs. The foci of carriage in mares are the ventral clitoral fossa and sinuses; in stallions, the organism is typically a surface contaminant of the external genitalia or urogenital membranes including the urethra, urethral fossa, urethral sinus, and penile sheath. Stallions in particular can carry *T. equigenitalis* for an extended length of time, up to several years. Although antibodies to the agent have been found in species other than horses, no natural infections have been reported.

Transmission

T. equigenitalis is communicable, with an extremely high transmission rate. The organism is most commonly transmitted sexually, often through an asymptomatic carrier stallion. Foals born to infected mares can also be infected and become chronic carriers of *T. equigenitalis*. Indirect transmission can occur by contaminated fomites and hands during uterine or rectal exams, or through artificial insemination. There is no evidence to suggest that the organism can persist in the environment for an extended time and be transmitted independent of the horse.

Pathogenesis

Infection of mares with *T. equigenitalis* can result in variable clinical disease, ranging from asymptomatic to acute and overt. The incubation period for infection is generally 2–14 days. In acute clinical cases, a mucopurulent endometritis develops with variable amounts of discharge within days (2–12) of infection (Figure 19.2). The main damage is to uterine epithelium (exclusive of glands), which becomes covered by neutrophilic exudate. The cellular infiltrate in the endometrial stroma is predominantly mononuclear. The epithelium can be eroded or undergo severe degenerative changes. The uterine infection usually subsides spontaneously within several weeks. Endometrial repair is complete, and there is no lasting impairment of breeding performance. Infection has been demonstrated in placentas and newborn foals, but abortions are rare. There is no fever or other sign of illness. The only apparent effect may be failure of conception. In chronic cases, there is often less obvious



Figure 19.2 A mare with vaginal discharge associated with *Taylorella equigenitalis* infection. *Source:* Courtesy of Peter Timoney and Donald Simpson.

discharge and mild inflammation of the uterus. Carrier mares and stallions remain asymptomatic but are still highly infectious.

Epidemiology

The carrier state of stallions and mares has been described as the single most important factor in the dissemination and persistence of *T. equigenitalis* in horse populations. Dissemination of infection is most commonly tied to breeding operations and the movement and use of infected animals. CEM has been reported in horse populations throughout the world including Europe, Africa, Japan, Australia, and North and South America. Some countries have successfully eradicated the organism. As recently as 2013, the United States Department of Agriculture's (USDA) National Veterinary Services Laboratories confirmed a positive mare in California and Puerto Rico. Prior cases were identified in 2008, 2010, and 2011, with the 2008 source stallion being linked to 27 other *T. equigenitalis*–positive horses. Recent genetic analysis of *T. equigenitalis* isolates from US outbreaks showed little evidence of genetic recombination, suggesting the isolates are remaining stable over time.

Immunologic Aspects

Immunity to *T. equigenitalis* is weak and animals may become reinfected; however, recovered animals show increased resistance for several months, manifested by milder signs and a lower concentration of bacteria. The mechanism of resistance is not fully understood.

Serum antibodies can persist in mares for 3–7 weeks post acute infection; however, they may not be detectable until 15–21 days after recovery. Titers are unrelated to the carrier status. Antibody is present in vaginal mucus, but its relation to infection is unknown. Stallions will not mount an antibody response to colonization with *T. equigenitalis*.

Diagnosis

Different diagnostic approaches are reported for confirmation of *Taylorella*; however, federal regulations may require specific tests at approved laboratories for routine import and export purposes. In the United States, veterinary laboratories must be approved for USDA-authorized diagnostic procedures, and specimens must be submitted by an accredited veterinarian, federal veterinarian, or state animal health official. Confirmative diagnosis of CEM and *T. equigenitalis* carriers requires demonstration of the organism in the genital tract. Due to the fastidious nature of the organism, specific sampling and detection protocols are necessary to avoid false negative results. This includes not treating horses with antibiotics while they are undergoing testing.

In clinical cases, the agent can be demonstrated in the uterine exudate by Gram stain; however, this is only suggestive and not confirmatory for CEM disease. Most commonly, *T. equigenitalis* is diagnosed based on a positive bacterial culture on a selective medium. Confirmation of *Taylorella* species by biochemical, DNA sequencing, or other methods is needed. In the United States, all suspect isolates are required to be sent to the National Veterinary Services Laboratory for confirmation. Reports suggest that current MALDI-TOF databases poorly identify *Taylorella*.

In addition, several molecular tests have been reported that can directly detect and differentiate *T. equigenitalis* from other *Taylorella* species (*T. asinigenitalis*). A PCR-based assay has been developed and found to be more sensitive than traditional culture techniques used to identify *T. equigenitalis*–affected animals and isolates; however, it is not routinely used by CEM-approved laboratories. More recently, a real-time PCR assay was developed in Europe, and panels that distinguish *Taylorella*

species from other metritis-associated organisms have been described. These are promising new tests that should improve diagnosis of *T. equigenitalis*; however, further validation is required before their routine use. This validation should also include the impact of sample pooling, although one report suggests up to four samples can be pooled for molecular analysis without impacting the sensitivity of PCR-based assays.

In mares, the most appropriate direct detection sites include the uterus, cervix, clitoral sinuses and fossa, or vaginal exudate, if present. Appropriate samples from stallions include urethral fossa and sinus, distal urethra, and the external surface of the penis. Samples sent for *T. equigenitalis*, especially those utilizing culture, should be transported with ice packs and expedited in appropriate medium (i.e. Amies transport media with charcoal). Isolates exhibiting *Taylorella* characteristics (slow-growing, gram-negative coccobacilli that are oxidase positive) should be evaluated for reactivity with antiserum.

Other available testing strategies include serology (e.g. complement fixation test). These tests are often of limited value because they may detect only acutely infected mares; carrier mares and stallions will not demonstrate an antibody response. Serologic testing has not been reliable as a stand-alone test for diagnosis and control of *T. equigenitalis*.

Test breeding is a strategy to evaluate stallions for carriage of *T. equigenitalis*. Test breeding involves breeding a stallion to two pretested *T. equigenitalis*–negative mares. The mares would be sampled and evaluated for *T. equigenitalis* for up to 35 days post breeding to declare the stallion negative.

Treatment and Control

Treatment with antibiotics and disinfectants has been successful in eliminating *T. equigenitalis*. Topical treatment of affected carrier mares consists of a cleansing of the clitoral fossa with 4% chlorhexidine followed by liberal application of nitrofurazone ointment (0.2%) for five days. In stallions, a thorough cleansing of the urethral fossa and sinus, prepuce, and penis with a 2% chlorhexidine solution and 0.2% nitrofurazone ointment for five continuous days has been proposed. Surgery or ablation of the clitoral sinuses has been suggested in female carrier animals. Uterine infusions of disinfectants or antimicrobials and systemic antibiotic treatment have been used in attempts to reduce the severity and duration of illness and perhaps abort the carrier state. Currently, there is no effective vaccine available for controlling this organism; control is achieved by preventing transmission from infected animals.

In CEM-endemic countries, attempts to control CEM have included mandatory veterinary examinations, negative cultures of all animals intended for breeding, and supervision of the movement of horses. Because of subclinical infection or asymptomatic carriage by stallions, detection of the disease can be challenging. Biosecurity measures are very important in preventing the introduction and transmission of *T. equigenitalis*. The USDA has established import and export regulations for the United States, which include CEM quarantine, treatment and testing requirements on all sexually intact horses over 731 days of age imported from CEM-affected countries.

T. asinigenitalis

T. asinigenitalis is a gram-negative rod that is phenotypically similar to *T. equigenitalis*. The two organisms can be distinguished by PCR and differ after sequence analysis of 16S rRNA. *T. asinigenitalis* can cross-react with some of the identification tests for *T. equigenitalis*; however, it tends to grow more slowly in culture and may exhibit a slightly different colony appearance.

T. asinigenitalis has been reported in the genital tract of donkey jacks, a Persian onager, and a naturally infected stallion. It can be transmitted to mares by natural service or artificial insemination. Depending upon the strain of *T. asinigenitalis* that is used, some affected mares develop clinical disease that is similar to CEM including a transient metritis and cervicitis. However, mares do test positive when the complement fixation test is used to identify animals infected with *T. equigenitalis*. This, coupled with the fact that isolates of *T. asinigenitalis* and *T. equigenitalis* are similar phenotypically, makes identification of horses with *T. equigenitalis* (an economically devastating disease) difficult. At this time, the significance (other than making control of CEM more difficult) of *T. asinigenitalis* is not known.

Acknowledgment

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20

Spirilla I: Borrelia

Roman R. Ganta

Classification

Spirochetes include both aerobic and anaerobic species, all of which are *extracellular organisms*. Some species live in the rumen of cattle, where they help break down fiber and other difficult-to-digest plant polysaccharides. The order Spirochetes includes two families: Spirochaetaceae and Leptospiraceae. *Borrelia* and *Treponema* species belong to the Spirochaetaceae family, whereas the Leptospiraceae family has one major genus, *Leptospira*. *Borrelia* species are transmitted from an arthropod vector and are responsible for some important and major diseases in humans. *Treponema* species are transmitted directly from one host to another. *Treponema pallidum* is the causative agent of syphilis in people. *Leptospira* species are both pathogenic and free-living organisms. The filaments are visible in *Treponema* species because the organisms of this genus lack an outer membrane.

Borrelia Species

Borrelia species are long, slender, spiral-shaped bacteria, usually only a fraction of a micron (0.1–0.3 μm) in diameter and 5–250 μm in length (Figures 20.1 and 20.2). They are tightly coiled and look like miniature springs or telephone cords. Bacteria in this group have a unique arrangement of varying numbers of axial filaments (up to 100), which are otherwise like other bacterial flagella. The filaments run along the outside of the protoplasm, but inside an outer membrane (within the periplasmic space). The filaments enable the bacteria to move by rotation in their extracellular environment. One end of the filament is inserted near the pole of the organisms.

Pathogenesis

In unicellular organisms, the cell surface is critical, in that it interacts with its environment, mediates transport functions, and may serve as a sensor/transducer of signals that regulate the physiological pathways of the organism in response to environmental changes. In animal hosts, the surface of unicellular pathogenic organisms has the added role of avoiding recognition or resisting damage by the immune system. To escape the host immune responses, *Borrelia* species utilize one or more evasion techniques, like hiding in cells or in tissues where the immune attack is less effective, mimicry by absorption of host proteins or imitation of host surface proteins, blunting the attack by the immune suppression, and by varying surface-exposed antigens over time post infection in a host.

Antigenic variation is one of the most effective strategies to escape immune destruction by a host. It requires a pathogen to replace one type of surface protein with another. *Borrelia* species follow certain defined steps when using antigenic variation as a mechanism to escape its host immune response. They include making many different immunodominant surface antigens to evade the host immune response, switching the exposed antigens at least in a fraction of the population before antibodies wipe out the entire population, and expressing one or more surface antigens in a certain order to avoid gross population heterogeneity. Many sero varieties (serovars or sero types) found in *Borrelia* species may have resulted from continuous changes on the bacterial cell surface.

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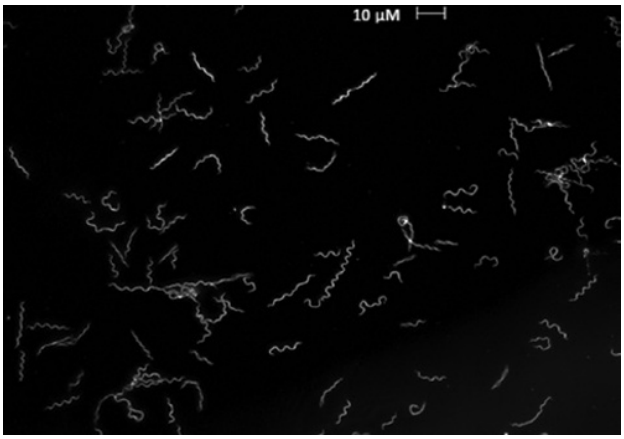


Figure 20.1 Dark-field micrograph of *Borrelia burgdorferi* organisms in growth media. Spiral bacteria that like miniature coils can be observed from the reflected light under field microscopy. *Source:* The image was shared by Dr. Md A. Motaleb, East Carolina University School of Medicine, Greenville, North Carolina.

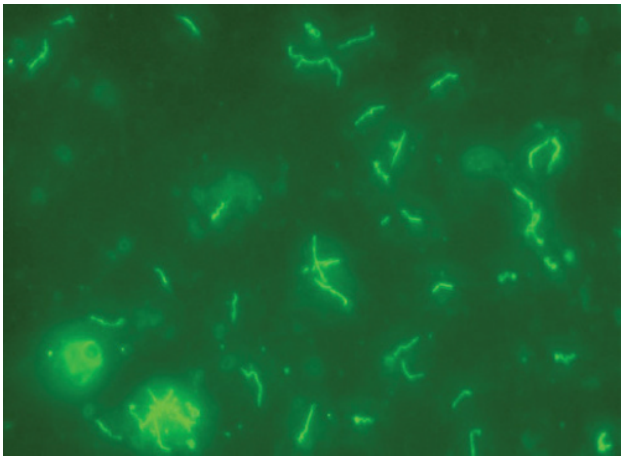


Figure 20.2 Immunofluorescence micrograph of *Borrelia burgdorferi* from an infected fed tick. Spiral bacteria detected with fluorescence marker tagged antibody. *Source:* The image was provided by Dr. Md A. Motaleb, East Carolina University School of Medicine, Greenville, North Carolina.

Diagnosis, Treatment, and Control

Some of the most effective methods of diagnosing *Borrelia* species in the early stages of infection are direct microscopic examination for the presence of spiral-shaped bacteria under a dark field (Figure 20.1), with Giemsa or Wright's stain of a specimen (organ or blood smear), or by immunofluorescence (Figure 20.2). A tick bite followed by a bull's-eye rash is typical for the Lyme disease pathogen (*Borrelia burgdorferi* in North America and *Borrelia afzelii* or *Borrelia garinii* in Europe and Asia). Lyme disease is the most frequently diagnosed in dogs and people in spring and summer months, when ticks are more prevalent. Lyme disease cases overlap with the distribution of *Ixodes* species ticks, which are more common in the north- and southeastern and western regions of the United States. Diagnosing *Borrelia* pathogens can often be a challenge following the early clinical phase as the pathogens hide in regions not easily accessible by standard diagnostic methods. For example, although several nucleic-acid-based molecular methods are available, often a blood or skin sample can test negative because of the presence of bacteria in low numbers. Serological methods are valuable to confirm the exposure of a host with *Borrelia* infections, but not to define the infection clearance. Tetracyclines are the most effective antibiotics.

Two types of vaccines have been established to be effective against *Borrelia* species: vaccines based on inactivated whole cell antigens and those having recombinant outer membrane proteins. Prevention of *Borrelia* infections requires appropriate precautions to avoid tick bites and control rodent populations. Careful use of insecticides to control ticks from both animals and humans is important. Protective clothing can reduce the risk for people from exposure to ticks when spending time outdoors in areas known to harbor ticks.

Important Diseases

Lyme Disease (Borreliosis)

Lyme disease was recognized in 1977 when arthritis was observed in a cluster of children in and around Lyme County, Connecticut. Lyme disease agents are transmitted from *Ixodes* species ticks: *B. burgdorferi* in the north America and *B. afzelii* and/or *B. garinii* in Europe and Asia. It is the most prevalent tick-borne disease in the United States, with thousands of cases reported each year. The pathogen also causes the disease in dogs, cats, cattle, and horses.

Clinical Description

Lyme disease is most often characterized by the *"bull's-eye" rash (erythema migrans)* accompanied by nonspecific symptoms such as fever, malaise, fatigue, headache, muscle aches (myalgia), and joint aches (arthralgia). The incubation period from infection to onset of erythema migrans is typically 7–14 days. Some infected individuals have no recognized illness while testing positive by serology, or manifest only nonspecific symptoms such as fever, headache, fatigue, and myalgia. Lyme disease spirochetes disseminate from the site of a tick bite by cutaneous, lymphatic, and blood routes. The signs of early disseminated infection usually occur days to weeks after the appearance of the solitary skin lesion.

During the second stage or chronic stage infection, the disease may be manifested with neurological signs, musculoskeletal system involvement, or cardiac abnormalities. *B. burgdorferi* infection in an untreated or inadequately treated patients may progress to late disseminated disease with symptoms detectable several months to years following the initial infection. The most common manifestation of late disseminated Lyme disease is intermittent swelling and pain of one or more joints, usually large, weight-bearing joints such as the knee. Some patients develop chronic polyneuropathy, or encephalopathy manifested by cognitive disorders, sleep disturbance, fatigue, or personality changes. All these symptoms are the result of the escape of the pathogen to sites where the antibodies are less effective in clearing the bacteria. Despite several complications resulting from Lyme disease, it is rarely fatal.

Lyme disease is also frequently reported in dogs and to a lesser extent in cats, cattle, and horses. In contrast to human cases, Lyme disease in dogs causes acute or subacute arthritis. The acute form may be transient and may recur in some cases. Lyme disease symptoms are difficult to detect in animals. For example, the characteristic skin rash is rarely seen in dogs or cats. Because the disease symptoms are like those of many other diseases, Lyme disease is often not considered until other diseases have been ruled out. Often, infected animals have high fever. Other common signs include lameness, joint swelling and pain, and swollen lymph nodes. Within days, weeks, or even months following the infection, more serious signs may develop that involve the heart, brain, and/or joints. Joint swelling and "shifting lameness" are the most common advanced signs in dogs. Affected animals are often described as "walking on eggshells." Dogs may have a fever, exhibit lethargy, and may become severely depressed. Lameness occurs two to five months after infection and may reoccur after several weeks. Some symptoms associated with the later stages of the disease include heart block, kidney failure, and neurological changes such as seizures, aggression, and other behavioral changes.

Small rodents, such as the white-footed mouse and other non-human vertebrate animals, serve as natural reservoirs for *B. burgdorferi*. Larvae and nymphal stage ticks typically become infected with *B. burgdorferi* when they feed on small animals that carry the bacteria. The bacteria remain in a tick as it changes from larva to nymph or from nymph to adult. Infected nymphs or adults serve as the transmitting vectors for *B. burgdorferi* to other small rodents, cats, dogs, horses, and mammals when taking a blood meal.

Diagnosis and Treatment

The diagnosis of Lyme disease is based primarily on clinical findings. Serologic testing by ELISA, IFA, or Western blot analysis may provide valuable supportive diagnostic information. *B. burgdorferi* can be cultured from 80% or more of biopsy specimens taken from early erythema migrans lesions. However, this procedure is rarely used because of the need for a special bacteriologic medium (modified Barbour-Stoenner-Kelly medium). Molecular methods, such as polymerase chain reaction (PCR) assays, have been used to amplify a segment of genomic DNA of *B. burgdorferi* in skin, blood, cerebrospinal fluid, and synovial fluid, although it can sometimes yield false negatives due to the low numbers of organisms in a sample. A history of exposure to ticks in an endemic area with typical clinical signs and/or positive antibody tests are considerations for antibiotic therapy. Tetracyclines, such as doxycycline, and penicillin-like antibiotics, including amoxicillin and ceftriaxone, are very effective if taken for three to four weeks.

Avian Borreliosis or Fowl Spirochetosis

Borrelia anserina, transmitted by the fowl tick, *Argas persicus,* is the causative agent for infections in fowls, including chickens, ducks, turkeys, and geese. It is a severe hemolytic disease that is responsible for causing intravascular hemolysis. Other symptoms include acute septicemia, fever, diarrhea, drowsiness, and emaciation (extreme weight loss). Morbidity and mortality can be high if appropriate treatment is not administered. Control measures should include the isolation of sick birds and de-ticking birds and the surrounding environment. Treatment with tetracyclines mixed with water is effective. A bacterin made from chicken embryo cultures is used for vaccination.

The morphology and ultrastructure analysis of *B. anserina* suggest that the cells are spiral and about 9–21 μm long and about 0.25 μm wide. This organism contains seven to eight flagella inserted at each end. It grows well in a liquid medium (Barbour-Stoenner-Kelly medium). Chickens infected with a virulent strain of *B. anserina* show acute enteritis, hemosiderosis in intestinal villi, and green diarrhea. Although spirochaetes in the intestine disintegrate within 96 hours after infection, enteritis and green diarrhea persist. The greenish discoloration may be a consequence of enteritis and hemosiderosis. Progressive enlargement of the spleen with mottling is the predominant gross finding. Spleen, liver, and small intestine of infected birds exhibit extensive erythrophagocytosis. Spirochaetes may be detected in the spleen, liver, intestine, kidney, and to a lesser extent in the lungs, but not in the heart and brain.

Relapsing Fever

Relapsing fever takes its name from the characteristic recurrences of elevated temperatures and flu-like symptoms once every few days. When a patient or an infected animal has a fever, numerous spirochetes are found in the blood that can be detected from a blood smear viewed microscopically. However, the spirochetes disappear in blood when the body temperature returns to normal. The disappearance of spirochetes and the decline in body temperature corresponds to the spike in antibodies against the spirochetes. The fever fluctuations are the result of antigenic variation of Spirochetes leading to new variants of bacteria to avoid immune clearance. Essentially, the pathogen stays one step ahead of antibodies by changing its surface proteins.

Tick-Borne Relapsing Fever (TBRF)

Relapsing fever in people has been recognized since ancient times. This tick-transmitted disease is caused by spirochetes entering from soft ticks of the genus *Ornithodoros*. There are many different *Borrelia* species can cause this disease. *Borrelia hermsii, Borrelia turicatae*, and *Borrelia parkeri* are the primary agents causing this disease in humans in the United States. Many rodents and small mammals serve as natural reservoirs, and the pathogens also persist in their tick vectors.

Louse-Borne Relapsing Fever (LBRF)

In LBRF, body lice acquire *Borrelia* species from humans who are already infected. The infected lice can then infect other humans. LBRF is said to be epidemic, as opposed to endemic, meaning that it can occur suddenly in large numbers in specific communities of people. A louse must be crushed or smashed in order for *Borrelia* pathogens to be released which then enter through areas where the person may have scratched him or herself.

Diagnosis is established for both types of relapsing fever by the demonstration of spirochetes in peripheral blood smears of febrile patients examined by dark-field microscopy or by examination of a stained blood smear. The recommended treatment is with doxycycline, although penicillin, erythromycin, or ceftriaxone may also be effective.

Further Reading

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21

Spiral-Curved Organisms II: *Brachyspira* and *Lawsonia*

Gerald E. Duhamel

Members of the genera *Brachyspira* and *Lawsonia* are gram-negative, curved or spiral-shaped bacilli that are associated with intestinal disease or are normal commensals in several species of wild and domestic mammals and birds. *Brachyspira* are oxygen-tolerant anaerobic bacteria that belong to the *Spirochaetes* phylum. *Lawsonia intracellularis*, the only member of the genus in the family *Desulfovibrionaceae*, is an obligate intracellular bacterium that is not phylogenetically related to any other species of pathogenic bacteria.

Brachyspira

Colonization by all members of the genus is restricted to the large intestine by mucin-induced chemotactic rotation of endoflagella, which allows motility of the bacteria within the intestinal lumen and localization within the mucosa. Nine species are recognized, and two are proposed (see Table 21.1). Of the 11 currently described *Brachyspira* species, only *B. aalborgi* and *B. canis* have not been recovered from pigs or birds. *Brachyspira aalborgi* has only been found in humans and nonhuman primates, while *Brachyspira pilosicoli* has a broad range of mammalian and avian host species.

The most important and by far the best-characterized species is *Brachyspira hyodysenteriae* (previously named *Treponema hyodysenteriae*, *Serpula hyodysenteriae*, and *Serpulina hyodysenteriae*), the cause of swine dysentery (SD). Clinically, SD is characterized by variable degree of mucohemorrhagic necrosis and inflammation restricted to the cecum and large intestine (typhlocolitis) mostly of weaned and growing pigs, but also finishing and breeding pigs, and is of worldwide welfare and economic significance. *B. hyodysenteriae* is also recognized as a cause of necrotizing typhlocolitis of the common rhea. Beside *B. hyodysenteriae, Brachyspira suanatina*, and *Brachyspira hampsonii* have been isolated and experimentally shown to produce typical clinical signs and lesions of SD in pigs. Isolation of *Brachyspira suanatina* is limited to pigs with SD and healthy

Table 21.1 Species of *Brachyspira,* their primary hosts, and major diseases with which they are associated.

Species	Primary host(s)	Major disease
Brachyspira hyodysenteriae	Swine, rheas	Swine dysentery
Brachyspira hampsonii	Swine, waterfowls	Swine dysentery
Brachyspira suanatina	Swine, mallards	Swine dysentery (Sweden and Denmark)
Brachyspira pilosicoli	Swine, birds, humans	Porcine, avian, and human spirochetosis
Brachyspira intermedia	Swine, birds	Avian spirochetosis
Brachyspira alvinipulli	Birds	Avian spirochetosis
Brachyspira aalborgi	Humans	Human spirochetosis
Brachyspira innocens	Swine, birds	Non-pathogenic commensal
Brachyspira murdochii	Swine, birds	Non-pathogenic commensal
"*Brachyspira pulli*"	Swine, birds	Non-pathogenic commensal
"*Brachyspira. canis*"	Dogs	Non-pathogenic commensal

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mallard ducks in Sweden and Denmark. In contrast, *B. hampsonii* has been isolated from pigs with SD in several countries, including the United States and Canada (not in Australia), and from healthy migrating waterfowls, including ducks and geese in North America and Europe. Based on their morphology and growth characteristics, *B. hyodysenteriae, B. suanatina,* and *B. hampsonii* are indistinguishable; however, each of these organisms is genetically distinct.

B. pilosicoli (*Anguillina coli, Serpula pilosicoli*, and *Serpulina pilosicoli*) is the cause of porcine intestinal/colonic spirochetosis (PCS) and avian intestinal/colonic spirochetosis (ACS), which are characterized by chronic mucoid diarrhea in pigs, and by diarrhea, egg production losses, and increased mortality in laying hens, and occasionally in turkeys and game birds. *Brachyspira intermedia* and *B. alvinipulli* also cause ACS. *B. pilosicoli* has also been isolated from humans, nonhuman primates, dogs, and mice, with and without diarrhea. In humans, *B. pilosicoli* causes mild, watery, or mucoid diarrhea, referred to as human intestinal/colonic spirochetosis (HCS). Rare cases of *B. pilosicoli* bacteremia in humans, mostly immunocompromised individuals, have been reported. The organism can be isolated from the stools of healthy people, especially those in economically underdeveloped and rural areas. HCS is also associated with *B. aalborgi* alone or together with *B. pilosicoli*, but *B. pilosicoli* is most common. Animals, particularly pigs and dogs living in close contact with humans, can serve as reservoirs of *B. pilosicoli* for human infection.

In addition to being associated with ACS, *B. pilosicoli* and *B. intermedia* are commonly isolated from healthy chickens. *Brachyspira innocens, B. murdochii*, and *B. pulli* are part of the normal, non-pathogenic commensal flora of the chicken large intestine. A few reports suggest an association between the presence of *B. intermedia* and *B. murdochii* with mild typhlocolitis in pigs, but neither species nor *B. innocens* is considered pathogenic species for pigs.

Brachyspira canis is the most common *Brachyspira* species identified in the stools of dogs with or without diarrhea, but most surveys do not find a significant association between this non-pathogenic commensal species and diarrhea. In contrast, diarrhea, colonic colonization, and fecal shedding are associated with *B. pilosicoli*.

Descriptive Features

Morphology and Staining

Brachyspira species are helical spirochetes with one or two loose serpentine curves. They range in size from 3 to 19 μm in length and 0.25 to 0.6 μm in width. They stain weakly gram-negative, but this characteristic is not used for routine identification or detection. Romanovsky's stains (e.g. Wright's, Giemsa), Victoria blue 4R, or crystal violet are more useful in demonstrating these organisms in histological examination of cultured organisms, or smears of stool sample, or cecal/colonic mucosa (see Figure 21.1).

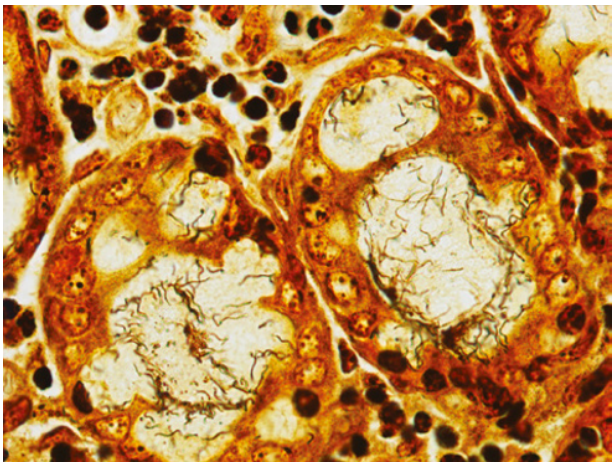


Figure 21.1 *Brachyspira hyodysenteriae* in colonic crypts of a pig with swine dysentery. Warthin–Starry silver stain.

Cellular Morphology and Composition

Ultrastructurally, cells are typical of spirochetes, consisting of a protoplasmic cylinder bounded by an inner cell membrane loosely attached to an outer cell membrane. The two membranes are separated by a periplasmic space that contains variable numbers of periplasmic flagella, also refer to as endoflagella or axial filaments, that vary in numbers with the species. The axial filaments of *B. hyodysenteriae* consist of up of 8–12 flagella inserted at each end that overlap in the center of the bacterial cell. *B. hampsonii* has 10 to 14 flagella, whereas *B. suanatina* has 14–18 flagella; *B. innocens* has 10–13 flagella, whereas *B. pilosicoli* has 4–5.

The genome of *B. hyodysenteriae* consists of a circular 3 000 694 base pair (bp) chromosome, and a 35 940 bp circular plasmid, while preliminary data for *B. hampsonii* reveals a 3 189 639 bp circular genome with a single plasmid. *B. pilosicoli* genome consists of a smaller circular 2 586 443 to 2 889 521 bp chromosome, with no evidence of an extrachromosomal element.

Cellular Products of Medical Interest

Cell Wall

Brachyspira species possess a gram-negative cell wall that differs somewhat from many gram-negative bacteria. The lipopolysaccharide (LPS) does not possess an outer layer of repeating polysaccharide side chains (O-antigens) or it only has an irregularly spaced, partial layer of polysaccharide side chains, referred to as lipooligosaccharide (LOS), and is a rough or semi-rough form of LPS. The LOS of different

species varies in that the LOS of *B. pilosicoli* has more complete O-antigen side chains, and thus, is smoother than that from *B. hyodysenteriae*. LOS has many of the same biological properties as LPS and is likely a virulence determinant. The lipid A component (endotoxin) is toxic and binds to the plasma protein LPS-binding protein (LBP), which then binds to CD14. The CD14-LPS complex binds to the Toll-like receptor 4 (TLR4)–MD-2 complex on the surface of macrophages and dendritic cells, triggering the release of pro-inflammatory cytokines. The length of O-antigen side chains is important in hindering the attachment of the attack complex of the complement system to the outer membrane of the spirochete. The LOS of *Brachyspira* species is strongly immunogenic and is the basis for serotyping.

Hemolysin/Cytotoxin. An important virulence factor of *B. hyodysenteriae* is its hemolytic and cytotoxic activity. *B. hyodysenteriae, B. hampsonii*, and *B. suanatina* are strongly beta-hemolytic, whereas all other members of the genus are weakly beta-hemolytic. *B. hyodysenteriae* produces a beta-hemolysin encoded by the *hlyA* gene that is cytotoxic for multiple continuous cell lines, primary cultures of porcine cells, and epithelial cells in ligated porcine intestinal loops. The bacterium also has three hemolysin regulatory genes, *tlyA*, *tlyB*, and *tlyC*. Isolates with mutations in either the *hlyA* or *tly* genes are not hemolytic and are significantly less virulent for pigs.

Flagella. Flagella are necessary for virulence. The periplasmic flagella allow motility and penetration of the intestinal mucosal mucus layer and access to target cells within the surface and crypt columns of the large intestine. Experimental mutations of the genes encoding the periplasmic flagella (*flaA* and *flaB*) of *B. hyodysenteriae* significantly reduce motility and virulence. Virulent, but not avirulent, strains of *B. hyodysenteriae* are attracted by pig intestinal mucin, and both motility and chemotaxis for mucin are necessary for full virulence. Strains of *B. pilosicoli* isolated from pigs, dogs, chickens, and humans possess varying attraction for pig intestinal mucin, but the effect of chemotaxis on virulence in the pathogenesis of disease is unknown.

NADH Oxidase. Although *Brachyspira* species are obligate anaerobes, they tolerate short exposure to oxygen. In fact, when cultivated in broth, addition of 1% oxygen enhances growth in the otherwise anaerobic medium. This resistance to oxygen is due to NADH oxidase, which also is an important *in vivo* virulence factor. NADH defective mutants are significantly less virulent for pigs than their wild-type parents. Oxygen tolerance likely assists colonization of the oxygenated mucosal surface of the large intestine and survival for extended periods in the environment (manure pits and lagoons) after being shed in feces.

Gene Transfer Agent of *B. hyodysenteriae*, VSH-1. The genome of *B. hyodysenteriae* codes for a mitomycin-C-induced bacteriophage-like gene transfer agent named VSH-1. Induced VSH-1 particles transfer random 7.5 kb genomic fragments between *B. hyodysenteriae* cells. Carbadox, an antimicrobial agent commonly used to prevent and treat diseases in pigs, and metronidazole, a compound commonly used to treat anaerobic infections in humans and small animals, recently have been shown to induce VSH-1. The induced VSH-1 particles transmitted tylosin and chloramphenicol resistance to susceptible strains of *B. hyodysenteriae*. VSH-1 is likely important in the continuing development of antibiotic resistance of *B. hyodysenteriae*. The gene for VSH-1 has also been identified in several other *Brachyspira* species.

Plasmid of *B. hyodysenteriae*. Virulent strains of *B. hyodysenteriae* possess a 35 940 bp circular plasmid. Strains that lack the plasmid are significantly less virulent for pigs, suggesting that the plasmid carries genes important in disease pathogenesis.

Growth Characteristics

Brachyspira species are slow-growing, obligate anaerobes that grow best in 10% H_2 :10% CO_2:80% N_2 atmosphere at 42 °C. After two to five days incubation on blood agar, growth appears as a thin flat haze on the surface of the agar and is surrounded by a zone of hemolysis (see Figure 21.2). *B. hyodysenteriae*, *B. hampsonii*, and *B. suanatina* are strongly beta-hemolytic, a trait that is used to differentiate them from other *Brachyspira* species isolated from pigs (*B. innocens*, *B. intermedia*, *B. murdochii*, and *B. pilosicoli*), which are weakly beta-hemolytic.

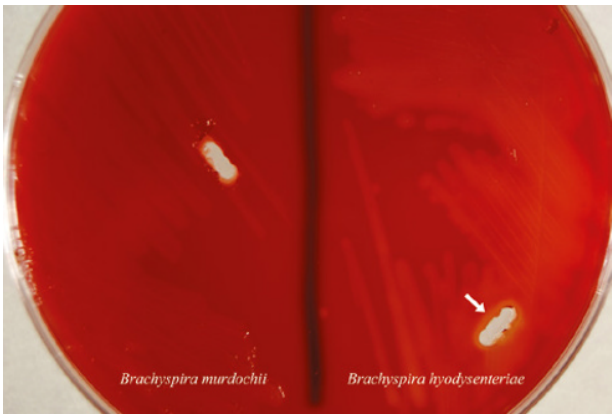


Figure 21.2 Strong beta-hemolysis by *Brachyspira hyodysenteriae* and weak beta-hemolysis by *B. murdochii* after four days of incubation. Note the ring of enhanced hemolysis (ring phenomenon) surrounding the cut in the agar with the *B. hyodysenteriae* isolate (arrow). *Source:* Photograph provided by Joann M. Kinyon and Dr. Kent Schwartz, Iowa State University, Ames, IA.

Brachyspira species are resistant to high concentrations of certain antibiotics, a useful characteristic for isolation from intestinal mucosal scrapings and fecal specimens. Enteropathogenic *Brachyspira* species, including *B. hyodysenteriae* and *B. pilosicoli*, remain infective for long periods within organic materials, especially feces, at temperatures of 5–25 °C. They do not withstand drying or direct sunlight, and are sensitive to most disinfectants.

Variability

At least 12 LOS serotypes of *B. hyodysenteriae* have been described. However, strains of the same serotype often vary greatly in virulence, and two strains of the same serotype can be quite different genetically. Consequently, serotyping is seldom used. Fingerprinting isolates by means of restriction-length polymorphisms of whole-cell DNA, comparison of DNA sequences of ribosomal RNA genes, multilocus enzyme electrophoresis, multilocus sequence typing, and multilocus variable-number tandem-repeat analysis demonstrate marked heterogeneity of *B. hyodysenteriae* and other members of this genus. For example, 111 isolates of *B. hyodysenteriae* were divided into 67 sequence types and 46 amino acid types by multilocus sequence typing. Using the same technique, 77 isolates of *B. intermedia* consisted of 71 sequence types and 64 amino acid types. Multilocus variable-number tandem-repeat analysis divided 172 isolates of *B. hyodysenteriae* into 44 types. In addition to demonstrating a high degree of genetic variability, fingerprinting allows isolates of a particular *Brachyspira* species to align into clonal complexes with each complex containing multiple sequence types. Over time, isolates of *B. hyodysenteriae* from individual pig farms tend to remain confined to a single clonal complex, but isolates of *B. pilosicoli* from chickens on a single farm often fall into multiple complexes.

Ecology

Reservoir and Transmission

The reservoir for *B. hyodysenteriae* is the large intestine of pigs, especially pigs that have recovered from SD and become asymptomatic carriers. Transmission of all *Brachyspira* species is by the fecal-oral route. The organism can survive in soil, lagoons, and pig feces for weeks to months depending on the environmental conditions, primarily temperature and moisture. Transfer of the organism between pens and buildings on the footwear of workers and farm equipment is an important means of spread. *B. hyodysenteriae* has been isolated from the feces of dogs, rats, and mice living on farms where the disease exists. Mice are an important concern because experimentally they can shed *B. hyodysenteriae* for up to six months, whereas rats shed for only two days. *B. hyodysenteriae*, *B. hampsonii*, and *B. suanatina* also have been isolated from wild waterfowls, but the contribution of birds to disease transmission is unknown. Asymptomatic carrier pigs and infected rodents on farms with endemic SD are by far the most important reservoirs. Farms that have eliminated SD can remain free of disease, as long as they do not reintroduce infected pigs or rodents into the herd.

B. pilosicoli has been isolated from many mammalian and bird species, including pigs, dogs, mice, chickens, turkeys, gamebirds, waterfowls, but also nonhuman primates, and humans. The concern is that humans can acquire *B. pilosicoli* from infected animals or animal products. Species normally associated with chickens, such as *B. alvinipulli, B. intermedia*, and *B. pulli*, are occasionally isolated from dogs, and dogs are possibly infected by ingestion of uncooked or undercooked poultry products. *Brachyspira* species that have been isolated from wild mallard ducks include *B. hyodysenteriae*, *B. suanatina*, *B. pilosicoli*, *B. intermedia*, *B. alvinipulli*, and *B. pulli*. Whether infection is associated with diarrhea or whether mallards serve as a reservoir of *Brachyspira* species for domestic animals is unknown.

Pathogenesis and Clinical Signs

SD can be experimentally reproduced by orally inoculating conventional pigs with pure cultures of either *B. hyodysenteriae*, *B. hampsonii*, or *B. suanatina*. In contrast, germ-free pigs inoculated with pure cultures of *B. hyodysenteriae* do not develop disease. Anaerobic bacteria that are part of the normal large intestinal flora, especially *Fusobacterium* species and *Bacteroides* species, are necessary along with *B. hyodysenteriae* for development of clinical disease. Diet plays a critical role in the development and severity of clinical signs of SD. Experimental studies show increased severity of disease in infected pigs fed diets low in fiber and high in highly fermentable carbohydrates. The enteropathogenic *Brachyspira* species, *B. hyodysenteriae*, *B. hampsonii*, *B. suanatina*, and *B. pilosicoli*, multiply and cause disease only in the large intestine. Flagella and chemotaxis to mucin produced by the goblet cells of the intestine allow the spirochete bacteria to penetrate the mucus layer that coats the mucosa and to closely associate with the epithelial cells that cover the luminal surface and line crypt columns. *B. hyodysenteriae*, *B. hampsonii*, and *B. suanatina* cause necrosis and loss of mucosal epithelial cells followed by hyperemia, edema, hemorrhage, fibrin exudation, and influx of neutrophils along the damaged mucosa and into the lumen of the large intestine. Alteration of colonic mucin production by goblet cells develops early after

infection, resulting in decreased expression of sulfated and MUC4 mucins, and concurrent increased expression of MUC5AC. Pigs with SD have mucoid loose stools that rapidly progress to mucohemorrhagic watery diarrhea. Spirochetes are present in large numbers along the damaged mucosal surface and in the lumen of the cecal and colonic crypts (see Figure 21.1) and extend to the lamina propria and within epithelial cells, especially goblet cells. Cellular attachment and invasion are not required for disease production. The diarrhea is attributable to a combination of decreased colonic absorption and loss of fluid and electrolytes from mucosal damage. Evidence of increased fluid secretion due to an enterotoxin or secondary to the inflammation has not been documented.

SD is most common in weaned pigs two to four months old, but can occur in pigs as young as two to three weeks or adult breeding stock; however, the disease is rare in nursing piglets and adults. The incubation period is usually about 10–14 days. Although pigs can die suddenly without evidence of diarrhea, the most consistent clinical sign is diarrhea of variable severity and duration. Pigs are febrile and hyporexic, and initially pass semisolid, yellow-to-gray loose feces that contain excessive amounts of mucus without blood. After a few days, feces become more liquid and contain large amounts of mucus and increasing amounts of fresh blood that gives a sweet putrid odor. Affected pigs become dehydrated and lose weight rapidly. Morbidity rates in susceptible pigs can be close to 90%, and mortality in untreated herds can be 20–40%. The duration of illness varies and can range from a few days to several weeks. With the exception of dehydration and emaciation, postmortem lesions are limited to the large intestine. The walls of the cecum and spiral colon are variably expanded by edema and hemorrhage, and the mucosa can have focally coalescing, superficial erosions to ulcerations. When ulcerated, the mucosa is covered by fibrin and mucus containing flecks of fresh blood. Large numbers of spirochetes can be demonstrated by silver impregnation stains of formalin-fixed and paraffin-embedded tissue sections, such as Warthin–Starry and modified Steiner stains. Further confirmation by using immunohistochemical staining or fluorescent *in situ* hybridization methods are available in specialized laboratories. Survivors may be permanently stunted and may remain asymptomatic fecal shedders.

Clinically, spirochetal infection of weaned pigs, chickens, nonhuman primates, and dogs associated with *B. pilosicoli* presents with variable degrees of persistent diarrhea, weight loss or decreased weight gain, and negligible mortality. Clinical disease has not been reported in broiler chickens, but broilers hatched from flocks with clinical ACS often grow more slowly than normal. Histologically, the affected large intestinal mucosa often has a layer of spirochetes closely adhered by one end to the apical membrane of the luminal epithelium, forming a false brush border (see Figure 21.3). This "end on" attachment occurs only with *B. pilosicoli* and *B. aalborgi*, and is demonstrated by silver impregnation stains of formalin-fixed and paraffin-embedded tissue sections, including Warthin–Starry and modified Steiner stains; by immunohistochemical staining with *Brachyspira*-specific antibody reagents; by fluorescent *in situ* hybridization methods with genus- and species-specific probes; or by ultrastructural studies using scanning or transmission electron microscopy. Diarrhea likely results from malabsorption due to disruption of the microvillus border of the apical membrane of superficial colonic epithelial cells. In cases of ACS due to *B. intermedia* and B. *alvinipulli*, the bacteria do not attach to the large intestinal epithelium, but are free in the lumen and the colonic crypts. Very little is known about the pathogenesis of disease caused by the latter two organisms.

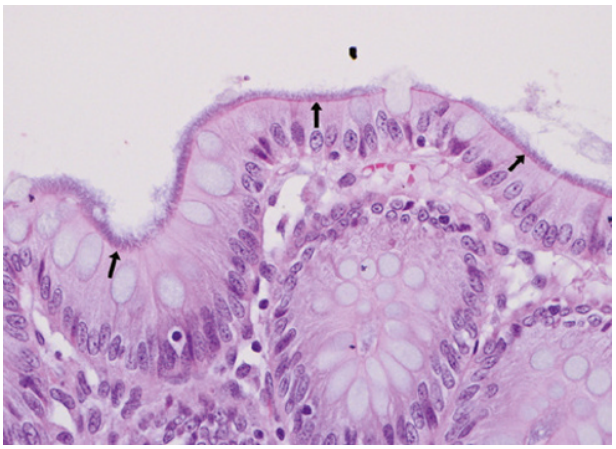


Figure 21.3 *Brachyspira pilosicoli* forming a false brush border (arrows) on the apical border of the colonic epithelium of a monkey. Hematoxylin and eosin stain.

Immunologic Aspects

The immunologic aspects of diseases caused by enteropathogenic *Brachyspira* species are poorly understood. Some pigs that have recovered from SD are resistant to reinfection for up to approximately four months, but many pigs remain susceptible. Infection induces *B. hyodysenteriae* specific serum IgG and colonic IgA, but neither is protective. Bacterins have limited efficacy, and the immunity that does develop is serotype specific, so vaccine must contain the serotype to which the pigs are exposed. Cell-mediated immune responses and changes in populations of $CD4^+$ and $CD8^+$ lymphocytes have been described, but their role in protective immunity and disease is unknown.

Poultry and mammals with spirochete infection can remain persistently infected, and if infection is eliminated by antimicrobial treatment, they are susceptible to reinfection.

Laboratory Diagnosis

Sample Collection

Cecal/colonic mucosal scrapings, segments of cecum/colon, or cecal/colonic contents taken from freshly necropsied animals are optimal, but fecal samples or swabs in transport medium taken from clinically affected animals are also satisfactory. Samples should be chilled, but not frozen, and transported to the laboratory as soon as possible. Preventing samples from drying out during shipping is also important.

Direct Examination

Wet mounts or smears of fecal material or scrapings of cecal/colonic mucosa can be examined by phase-contrast or dark-field microscopy or stained with a Romanovsky's stain (e.g. Wright's, Giemsa), carbol fuchsin, Victoria Blue 4R, or crystal violet and examined by conventional light microscopy (see Figure 21.4). Observation of loosely coiled spirochetes with one or two serpentine curves is presumptive evidence of infection with *Brachyspira* species, but additional tests are necessary to differentiate pathogenic from non-pathogenic species.

Isolation

Isolation of *Brachyspira* species from fecal samples is accomplished by inoculation onto blood agar plates containing antibiotics to prevent overgrowth by faster-growing enteric bacteria. Agar plates for isolation of *B. hyodysenteriae* typically contain 400 μg/ml spectinomycin (S400), or combinations of colistin, vancomycin, and spectinomycin (CVS), or CVS with additional rifampin, spiramycin, or polymixin. Because *Brachyspira* species differ in antibiotic sensitivity, blood agar containing 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (VCS) is routinely used for culture of *Brachyspira* species. The plates are incubated in an anaerobic environment containing 10% carbon dioxide. After two to five days incubation, *B. hyodysenteriae*, *B. hampsonii*, and *B. suanatina* form a thin surface film with a zone of strong beta-hemolysis. Other *Brachyspira* species form a zone of weak hemolysis. Making a trough the entire thickness of the agar with the inoculation loop that extends from the site of inoculation across the plate at the time of inoculation helps with identification of *B. hyodysenteriae, B. hampsonii*, and *B. suanatina* because a zone of enhanced hemolysis (referred to as the ring phenomenon) develops along the deep cut in the plate with strongly beta-hemolytic species, but not with the other weakly hemolytic species (see Figure 21.2). Plates without growth should be re-incubated and reexamined every two days for at least 10 days. For phenotypic identification, a pure culture is obtained by subculturing isolated areas of surface growth to fresh agar plates. This is difficult, especially with heavily contaminated plates, requires considerable experience, and may take two weeks or longer to accomplish. For antigen detection and nucleic-acid-based methods such as polymerase chain reaction (PCR) assays, subculturing the isolate is not as critical.

PCR assays for most *Brachyspira* species and multiplex PCR assays that simultaneously identify different combinations of *B. hyodysenteriae, B. pilosicoli*, *L. intracellularis*, and *Salmonella* species have been developed. In some instances, PCR assays can be performed directly on fecal specimens or mucosal scrapings, and test results are obtained within one to two days. In other instances, presumptive *Brachyspira* species isolated on blood agar are identified by performing PCR assays. The best results are obtained when bacteriological culture and PCR assays are used in combination. Unclassified *Brachyspira* isolates from pigs and poultry are not uncommon, and if only PCR assays are used for diagnosis, unclassified strains will not be detected. A case in point is the identification of the pathogenic *B. hampsonii* many years after the near eradication of *B. hyodysenteriae* from the North American pig industry, which only became possible when strongly beta-hemolytic *B. hyodysenteriae* PCR assay negative isolates were isolated by conventional culture.

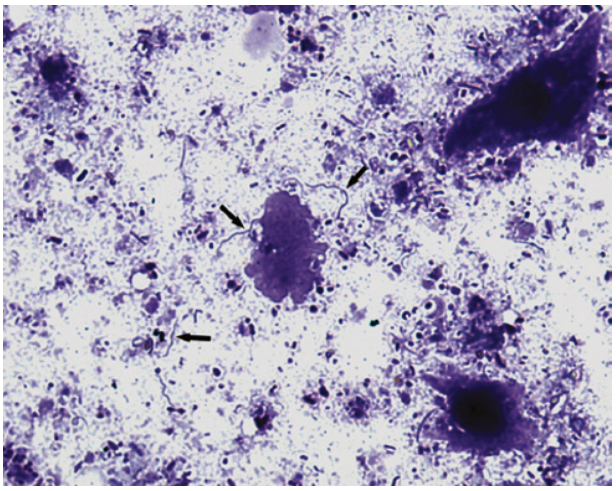


Figure 21.4 *Brachyspira* spp. (arrows) in a smear prepared by scraping the large intestinal mucosa of a pig without diarrhea. Crystal violet stain.

Identification

After isolation, *Brachyspira* species must be identified to differentiate pathogenic from non-pathogenic species. This is routinely done by performing PCR assays; however, observation of the strength of beta-hemolysis and results of

Table 21.2 Phenotypic characteristics of *Brachyspira* species.

Species	Hemolysis	Indole production	Hippurate hydrolysis	α-Galactosidase activity
Brachyspira hyodysenteriae	Strong	+[a]	−	−
Brachyspira hampsonii	Strong	+	−	−
Brachyspira suanatina	Strong	−	−	−
Brachyspira pilosicoli	Weak	−[b]	+[b]	±
Brachyspira intermedia	Weak	+	−	−
Brachyspira alvinipulli	Weak	−	+	−
Brachyspira aalborgi	Weak	−	+	−
Brachyspira innocens	Weak	−	−	±
Brachyspira murdochii	Weak	−	−	−
"*Brachyspira pulli*"	Weak	−	−	+
"*Brachyspira canis*"	Weak	−	−	−

[a] Indole-negative strains of *Brachyspira hyodysenteriae* and indole-positive strains of *B. pilosicoli* have been reported.
[b] Hippurate-negative strains of *B. pilosicoli* have been reported.

biochemical reactions may be used for preliminary classification. Biochemical tests commonly used are indole production, hippurate hydrolysis, and α-galactosidase assays with pure culture of test organisms (see Table 21.2), but there is some variability in biochemical results between strains of a given species, and misidentification using phenotypic criteria is possible. For instance, strongly beta-hemolytic, indole-positive isolates from pigs are identified as *B. hyodysenteriae* or *B. hampsonii*, whereas weakly beta-hemolytic, indole-positive isolates are identified as *B. intermedia*. However, *B. suanatina* and rare strains of *B. hyodysenteriae* are indole negative and strongly beta-hemolytic. Similarly, weakly hemolytic, hippurate positive isolates from pigs are identified as *B. pilosicoli*, but rare strains of *B. pilosicoli* are hippurate negative. Therefore, laboratories increasingly rely on *Brachyspira* species-specific PCR assays and sequencing. The most common targets for *Brachyspira* species-specific PCR assays are the 16S and 23S rDNA, NADH oxidase (*nox*), hemolysin (*tlyA*), and chaperonin (*cpn60*) genes.

Treatment, Control, and Prevention

Through the years many drugs have been used to treat SD, but several have been withdrawn from the market and others have limited effectiveness because of development of antimicrobial resistance. The most commonly used drugs are tiamulin, valnemulin, tylosin, and lincomycin. Because of resistance to tylosin and lincomycin, the pleuromutilins (tiamulin and valnemulin) are currently the most efficacious antimicrobials, but resistance to tiamulin and valnemulin is being reported from a few countries. Carbadox is used in the United States and it is usually effective, but it is approved for use only in pigs that weigh less than 34 kg (75 lb) and is not approved in Canada or Europe. Severely affected pigs sometimes require initial treatment with injectable antibiotics, but the preferred route of administration is usually through the drinking water for short-term therapy followed by medicated feed. The same medications that are used for SD are also used for treatment of pigs with *B. pilosicoli* infection. Metronidazole is the recommended treatment for dogs with intestinal/colonic spirochetosis. No drugs are approved for treatment of ACS. Recently 30 of 30 *Brachyspira* isolates from chickens were shown to be sensitive to tylosin, valnemulin, tiamulin, and doxycycline, while two isolates showed decreased sensitivity to lincomycin. A problem in treating chickens is that ACS occurs in mature laying hens and withdrawal times are required before the eggs can be used for human consumption. In addition, chickens are likely to be re-infected when drug therapy is stopped.

The best prevention for SD is strict biosecurity, so that only pigs from SD-free herds are brought onto disease-free farms. To date, vaccines have met with limited success and are not used. Vaccines for *B. pilosicoli* have so far proved ineffective.

Lawsonia

L. intracellularis is the only member of the genus *Lawsonia*, and it is the causative agent of proliferative enteropathy (PE), a diarrheal disease characterized by segmental thickening of the intestinal mucosa because of dysregulated expansion of infected epithelial cells lining intestinal villi and crypts. The bacterium is an obligate intracellular microorganism that can be demonstrated laying free within the apical cytoplasm of proliferating intestinal epithelial cells and also within phagocytic vacuoles of mucosal resident macrophages (see Figure 21.5). Porcine PE (PPE) is a worldwide disease of

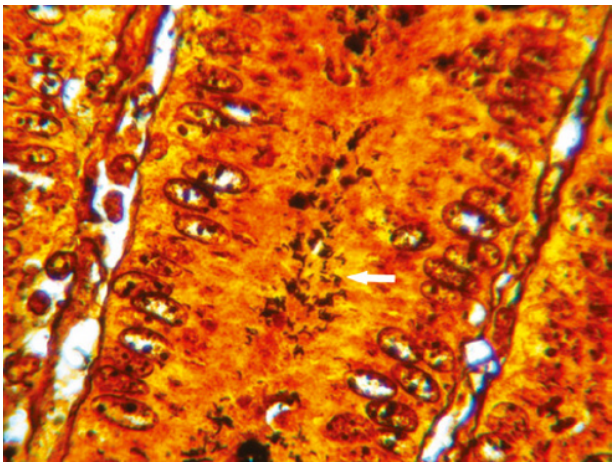


Figure 21.5 *Lawsonia intracellularis* (arrow) in the apical cytoplasm of enterocytes in the ileum of a weaned foal with equine proliferative enteropathy. Warthin–Starry silver stain.

major clinical, welfare, and economic significance. First recognized in pigs in the 1930s, a similar common and severe spontaneous disease with more than 90% mortality was recognized in the mid-1960s in weanling golden hamsters (*Mesocricetus auratus*; Syrian hamsters). The disease in hamsters has been variably referred to as wet-tail, proliferative ileitis, regional enteritis, terminal ileitis, atypical ileal hyperplasia, enzootic intestinal adenocarcinoma, and hamster enteritis. First recognized in 1982 by Duhamel and Wheeldon, equine PE (EPE), also associated with infection of enterocyte with *L. intracellularis*, has become an important emerging disease of yearling foals. Sporadic cases of PE attributed to intestinal infection with *L. intracellularis* have also been reported in ferrets, foxes, dogs, mice, rats, rabbits, sheep, white-tailed deer, emus, ostriches, and guinea pigs. Although classic PE has been reported in several species of nonhuman primates, PE has not been seen in humans.

In the 1970s, curved bacilli were first observed free within the apical cytoplasm of enterocytes by ultrastructural studies, and a variety of *Campylobacter* species were proposed as possible causes. In 1991, the organism was first isolated in cell culture and used to reproduce the disease. Until its formal recognition in 1995, *L. intracellularis* had the provisional vernacular name "ileal symbiont intracellularis."

Descriptive Features

Morphology and Cellular Anatomy

L. intracellularis cells are straight to curved bacilli 1.25–1.75 µm long by 0.25–0.45 µm wide. They have a single polar flagellum, do not possess fimbriae, or form spores. Extracellular bacteria grown in cell culture have darting motility.

Cellular Products of Medical Interest

Cell Wall

The cell wall of *L. intracellularis* is typical of gram-negative bacteria. Little is known about the biological effects of LPS from *L. intracellularis*, but typically LPS is an important virulence determinant. The lipid A component is toxic, and the length of the repeating polysaccharide side chains (O-antigens) hinders bacterial killing by the complement system. Serum LBP is an acute-phase protein that mediates innate immune response by binding to bacterial LPS and presenting it to CD14 and TLR4, which are cell surface pattern recognition receptors required for activation of monocytes and release of pro-inflammatory cytokines.

Type III Secretion System

Type III secretion systems are responsible for translocation of bacterial proteins into host cells. Protein components of a type III secretion system (IscN, IscO, and IscQ for *L. intracellularis* secretion component) have been identified in multiple strains of *L. intracellularis*. The role of the Isc proteins is unknown, but they are expressed during natural infection, and pigs react immunologically to the proteins.

L. intracellularis surface antigen A (LsaA):LsaA is a protein expressed *in vitro* and *in vivo*. The exact role of LsaA is unknown, but it is involved in adherence to and invasion of epithelial cells. Pigs respond immunologically, and LsaA has been used as antigen to develop enzyme-linked immunoassays (ELISAs) to identify infected pigs and rabbits.

Growth Characteristics

L. intracellularis has only been isolated in actively dividing eukaryotic cells maintained in a microaerobic atmosphere of 82.2% nitrogen, 8.8% carbon dioxide, and 8% oxygen.

Ecology

Reservoir

The reservoirs of *L. intracellularis* are the intestinal tracts and the environment of infected animals where organisms can remain viable in feces for several weeks at 5–15 °C. Mice and rats trapped on pig and horse farms are commonly infected and are potential reservoirs. Fecal shedding has also been reported in dogs and cats, as well as in wild cottontail rabbits, opossum, and skunks.

Transmission

Infection occurs following ingestion of material contaminated with *L. intracellularis* infected feces.

Pathogenesis

PE has been reproduced in conventional pigs, hamsters, and horses by oral inoculation with pure cultures of *L. intracellularis*. However, disease does not develop following oral inoculation of germ-free pigs. Interaction between *L. intracellularis* and unidentified normal intestinal flora is necessary for development of PE.

Following oral inoculation of pigs and hamsters and *in vitro* inoculation of cultured epithelial cells, *L. intracellularis* cells attach to the apical membrane of epithelial cells and are internalized into phagocytic vacuoles. Specific adhesions or attachment factors have not been identified. The bacteria do not have to be viable to invade cells, but treatments that inhibit cellular metabolism inhibit bacterial invasion. After internalization, the microorganisms escape from the vacuole into the cytoplasm where they multiply and induce the upregulation of host genes involved in cell cycle regulation, cell differentiation, and cell proliferation. Cellular division continues and postmitotic daughter cells contain bacteria, which contributes to the spread of bacteria throughout the length of the intestinal crypts. *L. intracellularis* also invades and spreads within the intestinal lamina propria, which likely contributes to infection of enterocytes throughout the intestinal mucosa. Immature enterocytes, which are normally restricted to the crypts, proliferate and replace normal goblet cells and absorptive villous enterocytes. This results in elongation of intestinal crypts, shortening of villi leading to increased fluid loss, decreased absorption, and loss of protein into the intestinal lumen. Inflammation is variable and can be absent or consists of variable numbers of neutrophils, macrophages, and lymphocytes.

Following experimental infection, bacterial invasion and multiplication initially occurs in the distal jejunum and ileum. Lesions in the proximal large intestine of experimentally infected pigs are believed to be extensions of the upper intestinal disease caused by bacteria that infect enterocytes in the large intestine.

Clinical Features and Lesions

PPE is a disease complex that can occur in four forms: (i) intestinal adenomatosis, a chronic form characterized by thickening of the intestinal mucosa due to hyperplasia of the crypt epithelium (see Figure 21.6); (ii) necrotic enteritis, a chronic form in which there is extensive necrosis and replacement of the mucosa by a necrotic membrane (see Figure 21.6); (iii) regional ileitis, a chronic form with thickening of the smooth muscle wall resulting in a contracted segment of distal small intestine (sometimes referred to as "garden-hose gut"); and (iv) proliferative hemorrhagic enteropathy (PHE), an acute form characterized by thickening of the small intestinal mucosa and acute hemorrhage into the intestinal lumen. Because the most consistent location of gross lesions in pigs with PPE is the distal jejunum and ileum, and mucosal inflammation can be prominent, PPE is also referred to as proliferative enteritis or proliferative ileitis. Gross lesions (EPE in horses and PE in hamsters) also consist of mucosal hyperplasia and thickening of the distal small intestine (see Figure 21.7). A necrotizing form of EPE similar to PHE, with a short clinical course of less than five days, has also been recognized in weanling foals.

Although PHE usually affects pigs 4- to 12-months-old, the other three forms usually affect younger pigs between

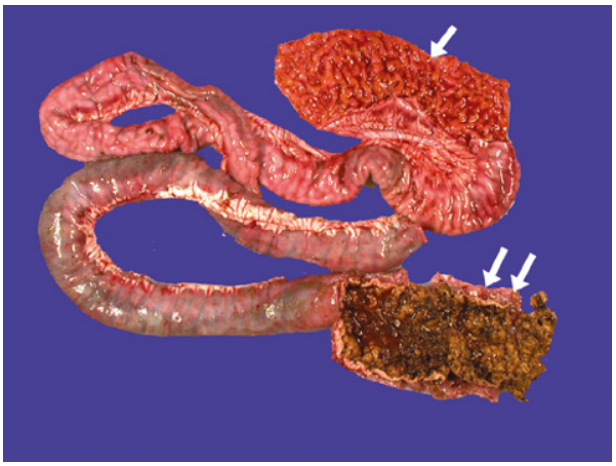


Figure 21.6 Small intestine from a pig with proliferative enteropathy. Note the diffuse rugose appearance of the serosa and the thickened and rugose appearance of the mucosa, which are characteristic of intestinal adenomatosis (single arrow). Also, note the replacement of the mucosa with a necrotic membrane, which is characteristic of necrotic enteritis (double arrows).



Figure 21.7 Small intestine from a foal with equine proliferative enteropathy.

six weeks and five months of age. In horses, EPE is primarily seen in weaned foals between four and seven months of age, but the disease can affect foals that are 2–13 months of age. Hamsters are usually affected soon after weaning and are experimentally resistant by 10–12 weeks of age. Clinically affected pigs, horses, and hamsters develop lethargy and diarrhea, become dehydrated, and lose weight. Hypoalbuminemia leading to dependent edema is common in foals. Poor weight gain is often the only manifestation of subclinical infection in pigs and possibly other species.

Epidemiology

L. intracellularis occurs worldwide in pigs and probably in horses. Clinical disease in horses has been reported from the United States, Canada, Europe, South Africa, and Australia. Serological surveys indicate that infection is widespread in horses in the United States, Canada, and Europe. The majority of pig herds in swine raising areas are infected. Isolates from pigs, horses, hamsters, and deer have greater than 98% DNA homology, but there is no evidence that transmission between species is important. Experimentally, pig isolates were infective for hamsters and horses, and a horse isolate was infective for hamsters. However, the target species had to be immunosuppressed, and infection was mild or subclinical.

Immunologic Aspects

Systemic humoral and cell-mediated immune responses are generated following infection of pigs and horses. Pigs, and most likely other species, develop specific IgA- and cell-mediated responses in the intestinal mucosa. Pigs that recover from PE are resistant to reinfection. An attenuated live *L. intracellularis* vaccine is available for pigs, and it is effective and widely used. Preliminary experiments with vaccines for foals show promising results.

Laboratory Diagnosis

Sample Collection

Fresh and formalin-fixed intestines, intestinal scrapings, and/or feces from affected animals are used for diagnosis.

Direct Examination

Modified acid-fast and Gimenez stains of intestinal mucosal smears will demonstrate small curved bacilli in enterocytes of animals with PE. The histologic intestinal changes are characteristic and can be used for a presumptive diagnosis. Silver and immunohistochemical stains are used to detect the bacteria in fixed tissue (see Figures 21.5 and 21.8).

Isolation

L. intracellularis can only be grown in living cells and has been isolated by only a few researchers in the United States and Scotland. Isolation is not feasible for diagnosis.

Identification

The combination of macroscopic and microscopic lesions and identification of intracellular bacteria in enterocytes are considered diagnostic of *L. intracellularis* infection. Identification of *L. intracellularis* in tissue sections can be made by using fluorescent antibody or immunohistochemical staining (see Figure 21.8) or by *in situ* hybridization with *L. intracellularis*–specific antibodies and rRNA probes, respectively. PCR assays that detect *L. intracellularis* alone

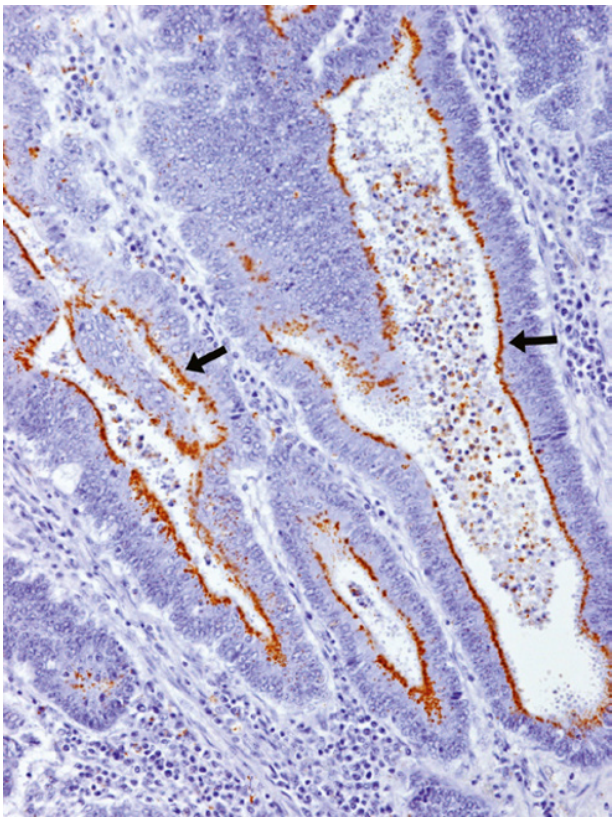


Figure 21.8 *Lawsonia intracellularis* in the apical cytoplasm of small intestinal enterocytes (arrows) of a pig with proliferative enteropathy. Immunohistochemical stain with a hematoxylin counter stain.

or in combination with one or more common bacterial causes of diarrhea in pigs (*B. hyodysenteriae*, *B. pilosicoli*, and *Salmonella* species) are used by many veterinary laboratories.

Treatment

Antimicrobials most commonly used to treat PPE are tetracyclines, tylosin, tiamulin, lincomycin, and, where available, carbadox. There is little evidence of development of antibiotic resistance. When properly diagnosed, EPE generally has a high treatment success; however, foals with the necrotizing form of EPE may require a more aggressive therapy with broad-spectrum antimicrobials. Generally, foals with EPE are treated with macrolide antibiotics alone or in combination with chloramphenicol, tetracyclines, or rifampin.

Excluding the organism from swine herds is very difficult as the disease occurs in many isolated "high health" herds. The most common method of control is oral vaccination with an attenuated, live vaccine. If vaccination is not possible, prevention is often by feeding antibiotics for two to three weeks beginning just before the expected onset of clinical disease. Experimental trials in horses with inactivated and attenuated vaccines approved for pigs have shown promise, but are used off-label.

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22

Spiral-Curved Organisms III

Campylobacter and *Arcobacter*

Gerald E. Duhamel

Members of the genera *Campylobacter* and *Arcobacter* (previously classified within the genus *Campylobacter*) are small, gram-negative, slender curved rods associated with diseases of the reproductive and intestinal tracts. Both genera are members of the family *Campylobacteraceae*.

Descriptive Features

Morphology and Staining

Members of the genera *Campylobacter* and *Arcobacter* are gram-negative, non-spore-forming, slender curved rods that are 0.2–0.9 µm wide by 0.5–5.0 µm long. They have a capsule and are motile by means of a polar flagellum at one or both ends. When two or more bacterial cells are held together, they can form S or gull-winged shapes that can appear as "spirals" (see Figure 22.1).

Campylobacter

The genus contains nine species, including two species divided into subspecies, and one species divided into three biovars (see Table 22.1). Most species of *Campylobacter* are pathogenic and cause reproductive and intestinal diseases in animals and humans. Non-pathogenic species are commensal bacteria of the intestinal tracts of humans, domestic and wild mammals and birds, and shellfish. The species of veterinary importance are *Campylobacter fetus*, *Campylobacter jejuni* ssp. *jejuni*, *Campylobacter coli*, *Campylobacter upsaliensis*, *Campylobacter lari*, *Campylobacter hyointestinalis*, *Campylobacter helveticus*, *Campylobacter sputorum*, and *Campylobacter mucosalis* (see Table 22.1). *C. fetus* and *C. jejuni* are important causes of reproductive failure in ruminants. Several species, especially *C. jejuni* and *C. coli*, are important causes of foodborne and waterborne enterocolitis in humans and minor causes in animals.

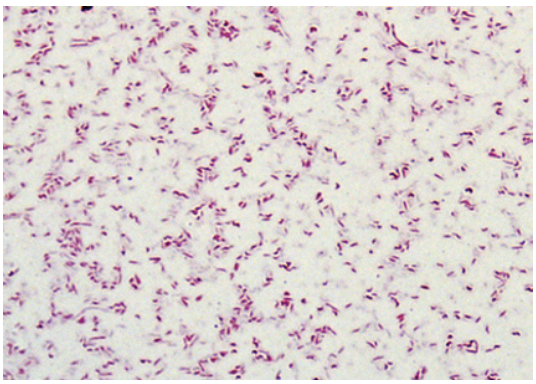


Figure 22.1 *Campylobacter jejuni* from a blood agar plate. Gram stain.

C. fetus has two subspecies: *venerealis* and *fetus*. Disease caused by the two organisms is often referred to as vibriosis because *C. fetus* was originally classified in the genus *Vibrio*. *C. fetus* ssp. *venerealis* is host adapted to cattle and causes bovine venereal or genital campylobacteriosis, which is a venereal disease characterized by early embryonic death and infertility. The organism is of major importance because there are regulations to prevent transmission by international trade. *C. fetus* ssp. *venerealis* also causes sporadic bovine abortions. *C. fetus* ssp. *fetus* and *C. jejuni* ssp. *jejuni* are worldwide causes of abortion outbreaks in sheep and sporadic abortions in goats and cattle. *C. fetus* ssp. *fetus* has been isolated from aborted alpaca fetuses. *C. coli* also causes abortions in sheep, but much less commonly than either *C. fetus* or *C. jejuni*. Intestinal colonization by *C. jejuni* occurs in many animal species. Infection is often accompanied by self-limiting diarrhea in young ruminants, puppies, and kittens, but in older animals, infection is usually asymptomatic. The prevalence of persistent, asymptomatic *C. jejuni* infection in poultry is high, but *C. jejuni* is not associated with naturally occurring clinical disease in this host species. *C. coli* is most commonly isolated from the intestine of healthy pigs and poultry.

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Table 22.1 Species of *Campylobacter*, and their corresponding primary host(s), and disease associations in animals and humans.

Species	Primary host(s)	Disease in animals[a]	Disease in humans[a]
Campylobacter fetus ssp. *fetus*	Ruminants	Abortion	Diarrhea, bacteremia
C. fetus ssp. *venerealis*	Cattle	Infertility, abortion	None reported
Campylobacter jejuni ssp. *jejuni*	Poultry, ruminants, pigs, dogs, cats, humans	Abortion, diarrhea, asymptomatic	Diarrhea, bacteremia
Campylobacter coli	Pigs, poultry, ruminants, humans	Asymptomatic, abortion	Diarrhea, bacteremia
Campylobacter upsaliensis	Dogs, cats, poultry	Asymptomatic, diarrhea	Diarrhea, bacteremia
Campylobacter lari	Wild birds, dogs	None reported	Diarrhea, bacteremia
Campylobacter hyointestinalis ssp. *hyointestinalis*	Ruminants	Diarrhea, asymptomatic	Diarrhea
C. hyointestinalis ssp. *lawsonii*	Pigs	Diarrhea, asymptomatic	Diarrhea
Campylobacter helveticus	Cats, dogs	Asymptomatic, diarrhea	None reported
Campylobacter sputorum biovar sputorum	Ruminants	None reported	Diarrhea
C. sputorum biovar faecalis	Ruminants	None reported	None reported
C. sputorum biovar paraureolyticus	Ruminants	None reported	Diarrhea
Campylobacter mucosalis	Pigs	None reported	None reported

[a] The first entry is the most common clinical presentation.

Certain *Campylobacter* species are leading causes of foodborne, and waterborne bacterial enterocolitis of humans. Greater than 90% of human cases of campylobacteriosis are caused by *C. jejuni* and *C. coli*, with *C. jejuni* being by far the more prevalent cause of disease. Other less common enteropathogenic *Campylobacter* species affecting people include *C. fetus* ssp. *fetus*, *C. upsaliensis*, *C. lari*, *C. hyointestinalis*, and *C. sputorum* (see Table 22.1). Most human infections result from ingestion of contaminated uncooked or undercooked food, but also unpasteurized milk, or contaminated water. Consumption of poultry meat or poultry products that have been contaminated during processing is the most important risk factor for campylobacteriosis in humans. Although infection of humans with *C. fetus* ssp. *fetus* is uncommon, it is the most likely species to cause bacteremia and extraintestinal infection in people, most commonly in immune-compromised individuals.

C. helveticus is most commonly isolated from feces of healthy dogs and cats, but in some studies isolation is more frequent from pets with diarrhea. The bacterium is not known to cause disease in humans.

C. hyointestinalis and *C. mucosalis* were proposed as causes of porcine proliferative enteropathy, which was later proved to be caused by *Lawsonia intracellularis* (see Chapter 21). *C. hyointestinalis* has two subspecies: *hyointestinalis* and *lawsonii*, which can be asymptomatic or cause diarrhea, primarily in ruminants and pigs, respectively. *C. hyointestinalis* is a rare cause of enterocolitis in humans, while *C. mucosalis* is not known to cause disease in animals or humans.

C. lari was originally isolated from the feces of asymptomatic gulls (from which it derives its name). Since then the organism has been isolated from the feces of dogs, wild birds, and horses. *C. lari* is considered to be a sporadic cause of diarrhea and a rare cause of bacteremia in humans, but it is not known to cause disease in animals.

C. sputorum has three biovars: *sputorum, faecalis*, and *paraureolyticus*, which are commensals of the oral cavity of humans, the intestinal tract of ruminants, and the reproductive tract of cattle. The biovars are not specific as to location. It is important to differentiate *C. sputorum* from *C. fetus*. Strains from the reproductive tract of cattle that were previously classified as *C. sputorum* biovar *bubulus* have been reclassified as biovar *sputorum*. *C. sputorum* is associated with enterocolitis in humans.

C. upsaliensis is the most common *Campylobacter* isolated from the feces of asymptomatic dogs and cats, but also dogs and cats with self-limiting mucoid to watery diarrhea. The organism has also been isolated from poultry. The bacterium is associated with diarrhea in puppies, but adult dogs and cats are usually asymptomatic. This microorganism has been reported to cause enteric disease, bacteremia, and abortion in humans.

Descriptive Features

Cellular Products of Medical Interest

Cell wall. Campylobacters have a gram-negative cell wall. Although the lipid A component of lipopolysaccharide

(LPS) can bind serum lipopolysaccharide-binding protein (LBP), triggering the host innate immune response, the LPS of *C. fetus* has lower biological activity when compared to other gram-negative organisms, such as members of the *Enterobacteriaceae*. This is likely important in allowing the organism to establish a persistent infection. The length of the repeating polysaccharide side chains (O-antigens) of LPS is important in resistance to complement killing in serum, and serum-resistant strains of *C. fetus* have long O-antigens. Isolates obtained from blood or other extraintestinal sites are often serum resistant, but that is because of their capsule and not because of the lipooligosaccharide (LOS).

The LPS of *C. jejuni* lacks repeating polysaccharide side chains and is a rough form of LPS referred to as LOS. Most isolates of *C. jejuni* are from the intestine and are serum sensitive. The LOS of *C. jejuni* is important in resistance to killing by cationic antimicrobial peptides (CAPs) or defensins, which are produced by intestinal epithelial cells as part of the innate immune response. Intestinal isolates of *C. jejuni* have truncated LOS and are more sensitive to killing by CAPs in comparison to isolates from the blood or extraintestinal sites. Mutations in the LOS genes also decrease adherence to and invasion of epithelial cells. Different forms of LOS are expressed by *C. jejuni* at 37 °C than at 42 °C, which likely contributes to adaptation to the different temperatures of mammalian and avian intestine.

Capsule. The outer membrane is covered by a highly variable polysaccharide capsule. Mutations in the capsule genes of serum-resistant strains of *C. jejuni* decrease susceptibility to the bactericidal effects of serum. The capsule helps shield the LOS from host cell receptors, such as Toll-like receptors, and thus prevents activation of the host innate immune response. The capsule plays a role in adherence of *C. jejuni* to epithelial cells, and isolates without a capsule do not efficiently colonize chick intestine. Capsular antigens are variable owing to a mechanism of phase variation associated with phase-variable gene expression, which helps the organism to rapidly change phenotype and adapt to the host environment, including colonizing the intestine and evading the host immune response. The capsular antigens are also used for serotyping.

Microcapsule or S-Layer of *C. fetus* ssp. *fetus*. A protein microcapsule called the S-layer (for surface layer) surrounds *C. fetus* ssp. *fetus* and is an important virulence factor. The S-layer allows resistance to the bactericidal effects of serum by inhibiting binding of complement factor C3b and phagocytosis. In sheep, the S-layer is required for systemic spread and subsequent abortion. Protective antibodies against S-layer antigens are produced, but phase variation in expression of S-layer proteins helps the organism evade the immune system.

Adhesins. CadF is an outer membrane protein highly conserved in *C. jejuni* and *C. coli* that binds the matrix protein fibronectin and mediates adherence to host cells. Mutations in the CadF gene reduce bacterial adhesion and virulence in animal models. Peb 1 (also known as CBF1) is an adhesin of *C. jejuni* located in the periplasmic space that binds to aspartate and glutamate. Peb 1–deficient mutants do not efficiently colonize mice intestine. CapA is a lipoprotein that contributes to adherence of *C. jejuni* to epithelial cells and is important in the colonization of and establishment of persistent infection in chick intestine.

Flagella. The flagella of *C. jejuni* are essential for motility and for chemotaxis to mucin and to amino acids present in high levels in chicken intestine and, thus, for intestinal colonization. The flagellar apparatus of *C. jejuni* also functions similar to a type III secretion system (T3SS), which is an assemblage of proteins that forms a hollow tube-like structure through which proteins are "injected" into host "target" cells. Mutations in the flagellin (fla) genes, which code for the major proteins that comprise the flagella, abolish motility, and secretion of Cia (Campylobacter invasion antigens) proteins. Exported Cia proteins are delivered into the cytosol of host epithelial cells, where they interact with signaling pathways involved in *C. jejuni* invasion.

Cytolethal distending toxin (CDT). This is a genotoxin, the only toxin proved to be produced by *Campylobacter* species. Whereas *C. jejuni* has high concentrations of cell-associated and secreted CDT, these levels of CDT are variable in *C. fetus* ssp. *fetus, C. fetus* ssp. *venerealis*, *C. upsaliensis*, *C. lari*, and *C. hyointestinalis*, and although *C. coli* carries the genes encoding CDT, the protein toxin is undetectable in this species. The toxin causes double-strand DNA breaks that block mammalian cell cycle progression at the transition between the G_2/M phase, leading to irreversible cell death by apoptosis. Although the role of CDT in disease production is uncertain, recovery from *C. jejuni* infection elicits an adaptive humoral immune response to the toxin. Experimentally, CDT induces production of interleukin-8 (CXCL8) by epithelial cells, which likely contributes to recruitment of neutrophils involved in the initial host innate defense, and subsequent mucosal inflammatory response. However, clinical disease in humans caused by non-CDT-producing strains of *C. jejuni* is indistinguishable from disease caused by CDT-producing strains.

Type II secretion system. *C. jejuni* possesses a type II secretion system that binds free DNA from the environment for transport into the cytoplasm. Once in the cytoplasm, the DNA is incorporated into the genome or, in the case of a plasmid, it can replicate freely. The ability to acquire DNA from the environment is known as natural competence.

Type IV secretion system (T4SS**)**. The genome of *C. fetus* ssp. *venerealis* contains a unique segment of DNA (known as a genomic island) that contains genes believed to be responsible for bacterial adaptation to the bovine reproductive tract. Within the genomic island are genes for a T4SS, which is an apparatus that mediates the transfer of DNA and/or proteins between bacteria and/or eukaryotic host cells. Mutations in the T4SS genes reduce the virulence of *C. fetus* ssp. *venerealis*. Mutations in genes encoding orthologues of a T4SS in a *C. jejuni* pVir plasmid also contributes to epithelial cell adherence, invasion, and virulence in a ferret model of campylobacteriosis.

Growth Characteristics

Campylobacter species are microaerobic, requiring an atmosphere containing 3–10% oxygen and 3–15% carbon dioxide. All species grow at 37 °C, and most species are also thermotolerant, and grow at 42 °C, a characteristic that is commonly used in isolation from diagnostic specimens and feces. Most species do not multiply below 30 °C, and their numbers are greatly reduced by freezing and thawing. However, they can remain viable for days to weeks in organic materials such as feces, meat, or milk, especially if chilled. Campylobacters are oxidase positive, do not ferment or oxidize carbohydrates, but generate energy from oxidation of amino acids or tricarboxylic acid intermediates through the respiratory pathway.

Variability

Campylobacters were originally classified on the basis of heat-stable LPS antigens A, B, and C. *C. fetus* ssp. *fetus* has serovars A-2 and B, and *C. fetus* ssp. *venerealis* has serovars A-1 and A-sub 1. Strains that express antigen C grow at 42 °C and are classified as *C. jejuni* and *C. coli*. The Penner serotyping system is based on extractable, heat-stable capsular antigens, and *C. jejuni* has 42 serotypes and *C. coli* 18. Over 100 serotypes of *C. jejuni*, *C. coli*, and *C. lari* are identified by a system proposed by Lior based on a heat-labile surface and flagellar antigens. Because of the need for a large number of typing sera, serological typing is limited to only a few reference laboratories. Currently, most typing is done by molecular methods such as ribotyping, restriction endonuclease analysis, pulse-field gel electrophoresis, amplified fragment length polymorphism, and PCR assays with genomic sequence analysis. Genetic analysis also indicates that there is widespread genetic variability in individual enteric *Campylobacter* species, such as *C. jejuni*. However, a 2008 study of *C. jejuni* isolates from sheep abortions from different parts of the Unites States found that 66 of 71 isolates were derived from a single clone.

Ecology

Reservoir

C. fetus* ssp. *venerealis. The primary reservoir is the preputial crypts of persistently infected carrier bulls. Approximately 1–2% of cows become persistent vaginal carriers and can serve as reservoir hosts.

C. fetus* ssp. *fetus. The reservoir is the intestinal tract and gall bladder of infected ruminants.

***C. jejuni* and enteric campylobacters**. The reservoir of *C. jejuni* for sheep is primarily the intestinal tract and gall bladder of carrier sheep. Poultry, various birds, cattle, sheep, dogs, cats, and swine also can be carriers of *C. jejuni*, and pigs and poultry are the primary reservoir for *C. coli*. The reservoir for other enteric campylobacters is presumed to be the intestinal tract of infected animals (see Table 22.1).

Transmission

Reproductive disease. Infection by *C. fetus* ssp. *venerealis* is predominately by coitus, but transmission by artificial insemination with contaminated semen and/or equipment is possible. Infection by *C. fetus* ssp. *fetus* and *C. jejuni* is by ingestion of organisms in food or water contaminated by feces of carrier ruminants or by fetal fluids and placental membranes from aborted fetuses.

Enteric disease. Infection by campylobacters associated with enteric disease (*C. jejuni*, *C. coli*, etc.) is acquired primarily by ingestion of contaminated food or water. For humans, consumption of contaminated poultry products is the single most important risk factor for enterocolitis caused by *C. jejuni*, but ingestion of other meats, unpasteurized milk, and contaminated water are also important risk factors. Multiple outbreaks of human infections associated with multidrug-resistant *Campylobacter* have been traced to contact with infected pet store puppies in the United States. Human-to-human transmission rarely occurs.

Pathogenesis and Clinical Signs

Reproductive disease (cattle). *C. fetus* ssp. *venerealis* is introduced into a susceptible female by an infected bull at coitus. The organisms multiply in the vagina, but do not enter the uterus until the end of estrus, possibly because of increased numbers of neutrophils in the uterus during estrus. In the uterus, further multiplication and possibly mucosal invasion results in endometritis. Infection does

not affect fertilization, but it creates an environment unsuitable for survival of the embryo. The female returns to estrus, usually at an interval of more than 25 days (21 days is normal). The process repeats itself until the female mounts a protective immune response that eliminates the organisms from the uterus. Subsequently, the endometritis subsides, the animal conceives, and the pregnancy proceeds to term. On average, infected cattle go through about five estrus cycles before carrying a fetus to term.

Clinically, the signs are repeat breeding, irregular estrus cycles, increased calving intervals, and increased nonpregnant cows. If the herd remains closed, herd immunity develops, and fertility and calving intervals gradually return to normal, but often do not become normal without intervention. Abortions in cattle and goats infected with *C. fetus* ssp. *fetus* and *C. jejuni* typically occur as single, sporadic cases.

Reproductive disease (sheep). Following ingestion, *C. fetus* ssp. *fetus* and *C. jejuni* reach the bloodstream, and in pregnant sheep, localize to the placenta. This results in placentitis, fetal infection, and abortion, usually three to four weeks after infection, but it can be as long as two months. The placenta, uterine fluids, and fetus contain large numbers of organisms, which is an important source of transmission to susceptible animals. Abortions are usually in the second half of pregnancy, and often occur in "storms" affecting up to 50% of pregnant animals, although approximately 25% is more typical. In the majority of cases, gross findings consist only of placentitis, which is not specific for *Campylobacter* infection, but is suggestive of bacterial infection (see Figure 22.2). Occasionally, the fetal liver contains tan-to-red foci of necrosis that often have a characteristic target- or donut-like appearance (see Figure 22.3).

Enteric disease. *C. jejuni* adheres to and invades the epithelial cells of the small and large intestine, where they translocate across the epithelium into the lamina propria.

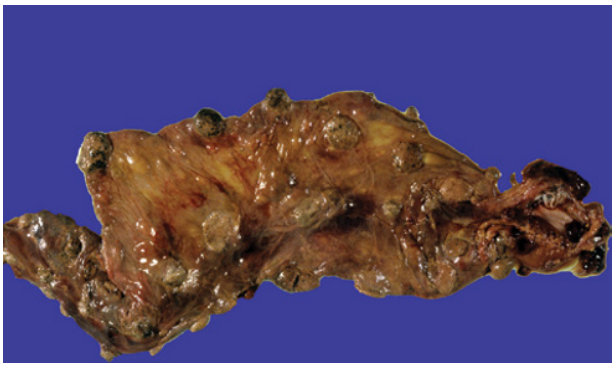


Figure 22.2 Inflamed placental membrane obtained from an aborted sheep fetus infected by *Campylobacter fetus* ssp. *fetus*.



Figure 22.3 Yellow round or target-shaped areas of parenchymal necrosis with red, depressed centers in the liver of an aborted ovine fetus infected by *Campylobacter jejuni*. *Campylobacter fetus* ssp. *fetus* causes similar lesions.

Invasive *C. jejuni* damage the epithelium, induce hemorrhage, and elicit the production of interleukins and prostaglandins that recruit neutrophils and macrophages, causing inflammation. Clinical signs include fever, abdominal cramps, and self-limiting watery to bloody diarrhea within a few days to a week in both animals and humans. The bactericidal effects of serum inactivate *Campylobacter* that reach the lymphatics and the bloodstream. Only a small minority of strains are serum resistant and can cause systemic infection with extraintestinal localization. The exception is *C. fetus* ssp. *fetus*, where nearly all strains are resistant to serum killing and to phagocytosis. Consequently *C. fetus* is more important as a cause of systemic disease than any other *Campylobacter* species.

Epidemiology

Reproductive disease (cattle). Venereal campylobacteriosis occurs worldwide, but primarily occurs in beef cattle bred by an infected bull. The use of artificial insemination has almost eliminated the agent from dairy cattle, because it is easily inactivated in semen and on insemination equipment.

Reproductive disease (sheep). Abortions caused by *Campylobacter* species occur wherever sheep are raised. Historically, *C. fetus* ssp. *fetus* was the more important cause, but since the late 1980s *C. jejuni* abortions have become more common, and in some parts of the world outnumber those caused by *C. fetus*. *C. coli* causes sporadic abortions in sheep, but is not common in most parts of the world. However, in a few areas, *C. coli* is isolated from up to 20% of *Campylobacter* abortions.

Enteric disease. Feces from infected animals are a source of infection for other animals and humans.

Uncooked, undercooked, and improperly handled contaminated poultry and other meats, raw milk, and contaminated water are sources for human infection. The ceca of approximately 50% of chickens contain *C. jejuni*. At slaughter the organism contaminates the environment, and as a consequence almost all chicken meat products in stores are contaminated. Infected pets and farm animals are potential sources of infection. Campylobacters are found in feces from healthy pets, but at lower prevalence and numbers than those with diarrhea. Dogs and cats from shelters and dogs fed homemade diets are more likely to carry *C. jejuni*. *C. jejuni* is commonly isolated from bovine feces, and occasionally from cattle with mastitis. Human outbreaks associated with ingestion of unpasteurized milk likely occur through fecal contamination during the milking process.

Immunologic Aspects

Reproductive disease (cattle). Protective immunity to *C. fetus* ssp. *venerealis* develops in the uterus and, although IgM is first produced, it is primarily IgG based. Antibodies coat the bacteria and activate the complement cascade, resulting in bacterial lysis. IgG antibodies also act as opsonizing agents and bind capsular antigens, which enhances phagocytosis and intracellular killing of *Campylobacter*. Secretory IgA, IgG, and IgM bind surface antigens and prevent adherence of the organisms to epithelial cells. All isotypes of antibodies specific for flagellar antigens prevent bacterial motility and, thus, movement from the vagina to the uterus. The response in the vagina is primarily non-opsonizing IgA, and it is less effective in clearing the organisms than the IgG response in the uterus. Most cattle eliminate *C. fetus* ssp. *venerealis* from the uterus in two to three months, but often require six months or more to eradicate the organisms from the vagina. Vaginal clearance rarely takes more than 10 months, but 1–2% of cattle become persistent vaginal carriers. Re-exposure to *C. fetus* ssp. *venerealis* results in vaginal infection that does not spread to the uterus in 30–70% of animals that eliminated the organism.

Bulls less than four years old rarely remain infected for longer than a few days, but older bulls can be infected for life, unless treated. The most commonly held explanation is that preputial crypts of older bulls are deeper and offer a more suitable environment for persistence than those of younger bulls. The immune response elicited by natural infection is ineffective in eliminating the bacteria from bulls. However, vaccination elicits specific IgG antibodies in serum and mucosal secretions that can prevent or eliminate infection from males and females.

Reproductive disease (sheep). Sheep are immune following abortion or vaccination, primarily through production of IgM and IgG antibodies. These opsonizing antibodies bind *Campylobacter* in the bloodstream and tissues, and initiate complement activation, leading to uptake by phagocytic cells and intracellular killing.

Enteric disease. Circulating and mucosal antibodies develop following adherence to and invasion of the intestinal mucosa. This results in clearance of campylobacters from the intestinal mucosa, but not from the intestinal lumen. The immune response does not prevent recolonization of the intestine, but it does help prevent clinical signs of disease.

Laboratory Diagnosis

Sample Collection

Reproductive disease (cattle). Samples from males are much more likely to be positive than samples from females. Smegma or preputial scrapings are collected by aspiration into the tip of an insemination pipette. Samples from females are collected using a tampon placed in the anterior vagina. Samples should be chilled, but not frozen, and if they cannot be delivered to the laboratory within six to eight hours, they should be placed into transport medium, such as Clark's or Lander's media, or thioglycolate broth. All bulls or 20 females or 10% of the herd, whichever is greater, should be sampled.

Reproductive disease (sheep). Samples of fetal stomach contents, lung, and liver are most appropriate. Environmental contamination of the placenta makes bacteriological culture and isolation more difficult from this sample.

Enteric disease. Fecal samples, segment of intestine, or intestinal mucosal scrapings are used for bacteriological culture and isolation of *Campylobacter*.

Direct Examination

Reproductive disease (cattle). Direct visualization of *C. fetus* ssp. *venerealis* in stained smears of samples taken from infected bulls and/or cows is unlikely, because of the low numbers of bacteria. Fluorescent antibody-stained preparations are useful, and sometimes used in combination with bacteriological culture to increase test sensitivity.

Reproductive disease (sheep). Direct microscopic examination of wet mounts or Gram- or Romanowsky-stained preparations of stomach contents from aborted fetuses often contain small curved rods. Campylobacters have a characteristic rapid "corkscrew or tumbling" motility that can be seen in wet-mount preparations of stomach

contents viewed with phase-contrast or dark-field microscopy. Finding yellow round or target-shaped areas of parenchymal necrosis with red, depressed centers in the liver of aborted fetus (see Figure 22.3) also supports a presumptive Diagnosis of *Campylobacter* abortion.

Enteric disease. Numerous motile, slender, curved, gram-negative rods, blood, mucus, neutrophils, and cell debris are seen in stained fecal smears.

Isolation

Reproductive disease (cattle). Smegma, preputial scrapings, vaginal fluid, or fetal stomach contents are plated onto selective media that contain antimicrobial agents to inhibit the growth of environmental contaminants. Vancomycin, polymyxin B or C, and trimethoprim are commonly used antimicrobial agents, and amphotericin B is sometimes included to inhibit fungal growth. It is important not to use selective media designed for isolation of *C. jejuni* and *C. coli* that contain cephalothin, such as Butzler's and Campy-BAP media, because both subspecies of *C. fetus* are inhibited by cephalothin. The plates are incubated at 37 °C in an atmosphere containing 6% oxygen and 5–10% carbon dioxide and examined in 48 hours.

Reproductive disease (sheep). Stomach content, lung, and/or liver from aborted fetus are plated onto blood agar plates (with or without antimicrobials, depending upon the degree of contamination) and incubated at 37 °C in an atmosphere containing 6% oxygen and 5–10% carbon dioxide. Plates are examined in 48 hours.

Enteric disease. Enteric campylobacters are best isolated from intestinal samples on selective media containing antimicrobial agents (e.g. Campy-CVA medium containing cefoperazone, vancomycin, and amphotericin B or Skirrow medium). Media that contain cephalothin (such as Butzler's and Campy-BAP media) are suitable for isolation of *C. jejuni*, *C. coli*, and *C. lari*, but not *C. fetus* or *C. upsaliensis*. Because of the small size of *Campylobacter* species, an alternate method is to filter samples with 0.45 or 0.65 μm pore-size filters to reduce contamination and plate the filtrate onto blood agar without antibiotics. The plates are incubated at 37 or 42 °C or both, in an atmosphere of 6% oxygen and 5–10% carbon dioxide.

Identification

Small, gram-negative, oxidase-positive, curved rods that grow at 37 or 42 °C in a microaerobic environment can be presumptively identified as *Campylobacter* or *Arcobacter* species. The two genera are difficult to differentiate based on phenotypic characteristics but isolates that grow in an aerobic atmosphere at less than 30 °C can be presumptively identified as *Arcobacter*. The ability to grow microaerobically on MacConkey agar at 37 °C is further evidence that an isolate is an *Arcobacter* species, but failure to grow on MacConkey agar does rule out *Arcobacter*. *Campylobacter* isolates that grow at 42 °C and are hippurate positive are identified as *C. jejuni*; however, hippurate-negative strains have been reported. Isolates can be identified as to species based on phenotypic characteristics (see Table 22.2), but there is variability between isolates of the same species. Because of the difficulty in differentiating *Campylobacter* species, molecular methods such as species-specific PCR assays and sequencing, genomic fingerprinting, and sequencing of the ribosomal RNA genes are also used. However, where available, rapid and reliable routine identification is accomplished by using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Serodiagnosis

C. fetus ssp. *venerealis* does not induce detectable serum antibodies, but antibodies in vaginal fluids are used for diagnosis. The vaginal mucus agglutination test identifies approximately 50% of infected cows, which is useful for herd, but not individual cow diagnosis. Agglutinating titers are highest 30–70 days after infection, and can persist for approximately seven months. Samples should be collected one to two days before or four to five days after estrus, to prevent excessive dilution of antibodies by the increased secretions during estrus. The presence of blood invalidates test results, and the test cannot differentiate between infected and vaccinated animals. Antibodies in vaginal mucus can also be detected by an enzyme-linked immunosorbent assay (ELISA) that has higher sensitivity and specificity. The ELISA test can be designed to detect IgA and differentiate between infected and vaccinated cattle.

Treatment

Reproductive disease (cattle). Vaccination of bulls with *C. fetus* bacterin eliminates *C. fetus* ssp. *venerealis* from persistently infected bulls. Systemic administration of streptomycin, topical streptomycin, and topical neomycin and erythromycin can be effective for treatment of bulls, but if not vaccinated they are susceptible to reinfection. Administration of bacterin vaccine followed by annual booster is an effective strategy for treatment and prevention of disease in breeding-age females.

Bovine venereal campylobacteriosis is best controlled by prevention. Use of virgin bulls from herds free of campylobacteriosis, avoiding replacement females from herds with

Table 22.2 Phenotypic properties of *Campylobacter* and *Arcobacter* species.

Species	Growth 25 °C	Growth 37 °C	Growth 42 °C	Aerobic growth	Catalase	Urease	Growth In 1% glycine	Hippurate hydrolysis	Indoxyl acetate hydrolysis	H_2S in TSI	Selenite reduction	Growth in 4% NaCl	Cephalothin sensitivity
Campylobacter fetus ssp. *fetus*	+	+	(−)[a]	−	+	−	+	−	−	−	V		S
C. fetus ssp. *venerealis*	+	+	(−)[a]	−	+	−	−	−	−	−	V		S
Campylobacter jejuni ssp. *jejuni*	−	+	+	−	+	−	+	+[b]	+	−	V		R
Campylobacter coli	−	+	+	−	+	−	+	−	+	V	+		R
Campylobacter upsaliensis	−	+	(+)[a]	−	−	−	+	−	+	−	+		S
Campylobacter lari	−	+	+	−	+	V	+	−	−	−	V		R
Campylobacter hyointestinalis ssp. *hyointestinalis*	−	+	+	−	+	−	+	−	−	+	+		S
C. hyointestinalis ssp. *lawsonii*	−	+	+	−	+	+	V	−	−	+	+		S
C. helveticus	−	+	+	−	−	−	V	−	+	−	−		S
Campylobacter sputorum biovar sputorum	−	+	+	−	−	−	+	−	−	+	V		S
C. sputorum biovar faecalis	−	+	+	−	+	−	+	−	−	+	V		S

C. sputorum biovar paraureolyticus	−	+	+	−	−	+	+	−	−	+	V		S
Campylobacter mucosalis	−	+	+	−	−	−	V	−	−	+	V		S
Arcobacter butzleri	+	+	(−)[a]	+	V	−	+	−	+	−	−	−	R
Arcobacter cryaerophilus	+	+	−	+	+	−	V	−	+	−	−	−	R
Arcobacter skirrowii	+	+	−	+	+	−	V	−	+	−	NT	+	S
Arcobacter thereius	+	+	−	+	+	−	+	−	+	−	−	−	NT

V, variable results; NT, not tested; R, resistant; S, susceptible; TSI, triple sugar iron.
[a] Most strains grow (+) or do not grow (−) at this temperature.
[b] Approximately 10% of *Campylobacter jejuni* isolates do not hydrolyze hippurate.

unknown health status, or sharing pastures with cattle of unknown health status are simple preventative methods. Artificial insemination is a highly effective method for control and elimination of the disease.

Reproductive disease (sheep and goats). Traditionally, treatment of *Campylobacter* abortion storms has consisted of parenteral tetracycline followed by tetracycline in the feed. However, resistance of *C. jejuni* to tetracycline has become increasingly common. A 2008 study in which a large majority of ovine abortion isolates of *C. jejuni* were from a single clone reported 100% of isolates resistant to tetracycline. Potential alternatives include tilmicosin, erythromycin, tylosin, and florfenicol. Vaccination with *C. fetus* ssp. *fetus* and *C. jejuni* bacterins in the face of an abortion storm helps reduce the number of abortions. Vaccination prior to breeding helps prevent the disease.

Enteric disease. Enterocolitis caused by *C. jejuni* and other enteropathogenic campylobacters is usually self-limiting, but occasionally requires treatment. Macrolide antibiotics are the drugs of choice. Tetracyclines are often effective when macrolides cannot be used, but tetracycline resistance is becoming more common. Most *Campylobacter* isolates from animals are susceptible to fluoroquinolone antibiotics. However, because of the high rate of resistance, fluoroquinolones are not the drugs of choice.

Control in the veterinary hospital and kennel requires meticulous adherence to hygienic measures, such as hand washing, cleaning, and disinfection protocols.

There are no vaccines effective in preventing enteritis or colonization by *C. jejuni*.

Arcobacter

Members of the genus *Arcobacter* are associated with diarrhea in livestock, mastitis in cattle, abortions in cattle and swine, and enterocolitis in humans. The genus was created in 1991 to accommodate two species of *Campylobacter* that grew in air, and it currently contains 29 species. The six species that have been isolated from animals are *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, *Arcobacter thereius*, *Arcobacter cibarius*, and *Arcobacter trophiarium*. The remaining species are from shellfish, water, plants, and sewage.

A. butzleri, *A. cryaerophilus*, and *A. skirrowii* are the only species isolated from both livestock feces and humans with diarrhea and are the species most likely to be animal pathogens. These species have been isolated from feces of pigs, cattle, sheep, and horses with and without diarrhea, and from dogs without diarrhea. Whereas *A. cryaerophilus* is commonly isolated from the feces and oral cavity of normal dogs, *A. butzleri* is only found in feces. Although cats have a higher prevalence of oral *A. butzleri* than *A. cryaerophilus*, a disease association is not found. In humans, *A. butzleri* is an emerging cause of food- and waterborne enterocolitis and has been occasionally isolated from the blood or from patients with endocarditis and peritonitis. *A. cryaerophilus* and *A. butzleri* have been isolated from cattle with mastitis, and experimentally an *Arcobacter* isolate from milk-caused clinical mastitis. Both species have also been isolated from the milk of cows without mastitis. All three species are isolated from aborted calves, lambs, and pigs, with isolation of *A. cryaerophilus* from aborted pigs being particularly common. However, the organisms are not associated with pathological changes, and the bacteria are also isolated from healthy pigs infected *in utero* and from amniotic fluid from normal litters.

A. thereius has been isolated from aborted piglets and the cloacae of healthy ducks. Its role in pig abortion is unproved. *A. cibarius* was isolated from the carcasses of broilers at slaughter and *A. trophiarium* from the feces of healthy pigs. Neither organism has been associated with disease. Arcobacters are commonly isolated from feces of healthy poultry and are not associated with disease. The role of all *Arcobacter* species as a cause of animal disease is uncertain.

Descriptive Features

Cellular Products of Medical Interest

Arcobacters have a gram-negative cell wall and polar flagella. The LPS, LOS, and flagella of *Campylobacter* species are known to play an important role in host–bacteria interactions and pathogenesis. However, the role of LPS, LOS, and flagella of *Arcobacter* in disease is poorly understood. Clear evidence of toxin production has not been found, and virtually nothing is known about which genes are involved in virulence. The genes encoding CDT have not been found in *A. butzleri*.

Growth Characteristics

Arcobacter species grow under both aerobic and microaerobic conditions and at temperatures as low as 15 °C. Except for a few strains of *A. butzleri*, they do not grow at 42 °C.

Ecology

Reservoir

The reservoirs of *Arcobacter* are presumably the intestinal tract and the environment of affected animals. Asymptomatic swine, cattle, poultry, and possibly other animals may serve as an important reservoir for humans.

Transmission

Most animals are likely infected by ingestion or entry of bacteria through mucous membranes. Some are infected *in utero* following bacteremia.

Pathogenesis

Very little is known regarding the interactions of *Arcobacter* with the host.

Epidemiology

Asymptomatic infection of the intestinal tract of domestic livestock and poultry is common. Asymptomatic infection of dogs and horses also occurs, probably at low prevalence. The bacteria are also isolated from the environment where animals are kept, raw milk, and water, all of which serve as a reservoir for infection of other animals and humans.

Immunologic Aspects

Arcobacter-specific antibodies were present in colostrum of naturally infected sows that farrowed piglets congenitally infected by *A. cryaerophilus*. Two weeks after birth, most congenitally infected piglets were no longer shedding *A. cryaerophilus* in their feces, but the role of colostral antibodies is unknown.

Laboratory Diagnosis

Sample Collection

Arcobacters have been isolated from the stomach contents, kidney, liver, and placenta of aborted fetuses and milk from cattle. Fecal samples are used for diagnosis of *Arcobacter*-associated diarrhea and identification of carrier animals.

Direct Examination

In studies of swine abortion where fetal samples were directly examined for *Arcobacter* species, bacteria were not seen. It is impossible to differentiate *Arcobacter* from *Campylobacter* without further testing, such as staining of samples with fluorescent antibody reagents.

Isolation

The same media used to isolate *Campylobacter* species support growth of *Arcobacter* species. Isolation from feces is enhanced by use of commercial *Arcobacter* enrichment broth and agar supplemented with antimicrobial agents to reduce growth by other bacteria and fungi. One combination contains cefoperazone, amphotericin B, and teicoplanin (CAT medium), and other procedures include 5-fluorouracil and cefoperazone, with or without amphotericin B, novobiocin, cycloheximide, and trimethoprim. Culture plates are incubated at 25–30 °C and at 37 °C and examined daily for three to five days. Whether it is better to incubate aerobically or microaerobically is unproved. Common practices are to incubate all plates microaerobically or to incubate aerobically at 25–30 °C and microaerobically at 37 °C.

Identification

Small gram-negative, curved rods that are positive for oxidase, catalase, and indoxyl acetate hydrolysis, and that grow aerobically and microaerobically, at 25–30 °C, are presumptively identified as *Arcobacter* species. Growth on MacConkey agar at 37 °C is further evidence that an isolate is an *Arcobacter* species. Fermentation reactions are used to speciate *Arcobacter* isolates, but the results are often not definitive (see Table 22.2). Molecular methods such as PCR assay and sequencing of the 16SrRNA gene are more reliable.

Treatment

Most *Arcobacter* infections in humans are self-limiting, but in cases of prolonged or severe disease, fluoroquinolones and tetracyclines are most commonly used for treatment. However, resistance to nalidixic acid and ciprofloxacin has been reported. Treatment of *Arcobacter*-associated conditions in animals is poorly documented.

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23

Spirilla IV: *Helicobacter* – the Spiral Microorganisms of the Gastrointestinal Tract and Liver

Megan E. Jacob

Spiral shaped microorganisms have been noted throughout the gastrointestinal tract of animals for more than a century. After *Helicobacter pylori* was isolated from human gastric tissue in the 1980s, *Helicobacter* species were identified in animals including ferrets, birds, nonhuman primates, dogs, cats, pandas, dolphins, and pigs, among others (Table 23.1). These organisms have generated a great deal of interest because of their causal role in gastric disease, and more recently in their association with extra-gastric manifestations associated with the gut-brain axis. In humans, *H. pylori* can cause persistent gastritis and peptic ulcer disease, and also has been linked to the development of gastric adenocarcinoma and gastric mucosal-associated lymphoma. Other *Helicobacter* species, often referred to as non-*H. pylori* Helicobacter species (NHPH), identified throughout the oral and gastrointestinal tract and/or liver of most animals, are associated with clinical signs ranging from cancer to mild gastritis and asymptomatic carriage. Neurodegenerative disorders have also been associated with infection with gastric *Helicobacter* infection, and *Helicobacter suis* has been shown to induce inflammation in the brain.

The taxonomy of *Helicobacter* species is complicated, at least partially because of the rapidly expanding list of members and improved identification methods. Members of the genus are taxonomically distinct from the genus *Campylobacter*, where they were originally classified and which they resemble morphologically. There appear to be two broad groups of *Helicobacter*: those that are associated with gastric tissue and direct disease, and those associated with the microbiome of the lower intestine and are opportunistic pathogens when translocated to hepatic regions.

Descriptive Features

Morphology and Staining

Helicobacter species are gram-negative bacteria with morphologies ranging from tightly coiled spirals (e.g. *H. pylori*, *H. felis*, and *H. suis*) to slightly bent rods (e.g. *Helicobacter mustelae* and *Helicobacter baculiformis*). Species vary in size, with a length between 1.5 and 10 μm and a width between 0.3 and 1.2 μm. All known *Helicobacter* species express flagella, making them motile; the number (range between 4 and 23) and the location (bipolar, monopolar, etc.) of flagella differ between species. Figure 23.1 depicts *Helicobacter* sp. in a case of hypertrophic gastropathy in a dog, showing typical spiral shaped morphology.

Virulence Factors

Helicobacter species possess several cellular products of importance; however, not all species produce all products. Some of the virulence factors represented by the genus include the following.

Flagella

Motility via multiple flagella is characteristic of *Helicobacter* species and vital for the organisms' ability to penetrate mucus and adhere to gastric epithelial cells. The number and location differ by species; however, all species possess flagella and most are sheathed.

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Table 23.1 Examples of selected *Helicobacter* species reported from animal hosts.

	Animal species						
	Canine	**Feline**	**Swine**	**Ovine**	**Bovine**	**Human**	**Other**
Helicobacter pylori		+		+		+	Nonhuman primates
Helicobacter felis	+	+				+	Rabbit
Helicobacter suis			+			+	
Helicobacter cinaedi	+	+				+	Hamsters, fox
Helicobacter canis	+	+				+	
Helicobacter salomonis	+	+					Rabbit
"Flexispira rappini" or Helicobacter rappini	+	+		+		+	
Helicobacter bizzozeronii	+	+	+			+	Nonhuman primates
Helicobacter mustelae							Ferret
Helicobacter bilis	+		+				Mice
Helicobacter hepaticus							Mice
Helicobacter bovis							
Helicobacter pullorum						+	Mice, poultry
Helicobacter cetorum							Dolphins, whales
Helicobacter brantae							Poultry

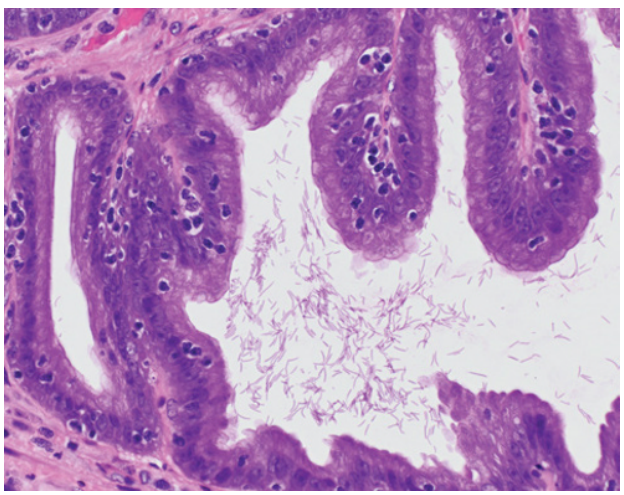


Figure 23.1 *Helicobacter* sp. showing typical spiral shaped morphology observed in a case of hypertrophic gastropathy in a dog. H&E stain, 600× magnification. *Source:* Courtesy of Dr. Brandon Plattner, Veterinary Diagnostic Laboratory, Kansas State University.

Periplasmic Fibrils

These fibers (present singly or in groups) wrap helically around the body of some *Helicobacter* (e.g. *H. felis* and *Helicobacter bilis*), but are not present on all species. Although associated with motility of the organisms, periplasmic fibrils are distinct from flagella and are located under the outer membrane.

Urease

Urease hydrolyzes urea to ammonium and carbon. Ammonium ions neutralize stomach acid, thereby allowing the bacterium to live in the gastric environment. Urease is also associated with inflammation. Most *Helicobacter* strains produce urease; however, it seems to be variably produced by some enterohepatic species.

Adhesins

Adhesins are proteins that mediate adherence to target cells in the gastrointestinal tract comprising the niche for the strain. *Helicobacter pylori* expresses at least two adhesins with specificity for gastric epithelia, sialic acid-binding adhesin (SabA), and blood group antigen-binding adhesin (BabA). Similar adhesins have not been identified in all *Helicobacter* species.

Lipopolysaccharide

The lipopolysaccharide (LPS) in the outer membrane is an important virulence determinant. Not only is the lipid A component toxic (endotoxin) but the length of the side

chain in the O-repeat unit also hinders the attachment of the membrane attack complex of the complement system to the outer membrane. *Helicobacter* LPS is important in the immune response as it is ultimately responsible for the release of pro-inflammatory cytokines and also has been associated with the adhesion process in gastric tissues.

cag Pathogenicity Island and CagA

The cag (for cytotoxin-associated gene) pathogenicity island (PAI) is associated with the pathogenesis of *H. pylori* strains. Specifically, the cag PAI encodes a type IV secretion system that mediates translocation of the CagA protein into host cells. Once inside the cell, CagA is phosphorylated, resulting in actin rearrangement. The PAI is heterogeneous between *H. pylori* strains, differing on the number of insertion sequences and related virulence. Various pieces of the cag PAI and type IV secretion systems have been found in other members of the genus.

Vacuolating Cytotoxin (Vac)

The vacuolating cytotoxin, well described in *H. pylori*, has been associated with the colonization and persistence of the organism in the gastric epithelial cell barrier and stimulating an inflammatory response. Previous studies have shown the association between VacA activity *in vitro* and disease status. The vacA gene encodes the cytotoxin that ensures urea availability among other functions. VacA has been the subject of vaccine intervention research.

Cytolethal Distending Toxin

The cytolethal distending toxin (CDT) observed in *H. hepaticus* and *Helicobacter pullorum* is highly homologous to the CDT toxin produced by *Campylobacter jejuni*, which is associated with cell cycle arrest in eukaryotic cells.

Other miscellaneous cellular products including superoxide dismutase and catalase have also been described in *Helicobacter* species.

Growth Characteristics

Helicobacter organisms grow under microaerophilic conditions at 37 °C and typically appear as flat, nonpigmented, grayish white, and nonhemolytic colonies, approximately 1–2 mm in size; however, some species do not form distinct colonies, but rather appear as a fine spreading film. Long incubation periods (up to one week) may be required for visible growth of *Helicobacter* colonies. Growth will not be observed under aerobic conditions; however, the growth of some species may be enhanced with atmospheric hydrogen, varying pH, or an enrichment step. The ability of *Helicobacter* species to grow at 42 °C is also variable and may aid in identification.

Variability

A large degree of variability exists between and within *Helicobacter* species. Related to a high genomic heterogenicity, variability in phenotypic and growth characteristics, as well as virulence factors and sensitivity to antimicrobials is common. Urease production, nitrate reduction, and indoxyl acetate hydrolysis all can vary between and within *Helicobacter* species; this variability is often but not always differentiated between species common to the gastrointestinal tract versus enterohepatic species. In addition, some *Helicobacter* species can grow at 42 °C, and some require the presence of hydrogen for growth. The most recognizable difference between *Helicobacter* species is the number and placement of flagella, and the presence of periplasmic fibrils. Genomically, the G + C moles percent content can vary between 30 and 48% in different *Helicobacter* species.

Ecology

Reservoirs

Helicobacter species have been identified from numerous sites within animals including the stomach, liver, bile duct, and small and large intestine. The degree to which *Helicobacter* species exclusively occupy specific niches within animals has not been well described, with the possible exception of enterohepatic species of mice. In general, gastric *Helicobacter* species occupy the mucus layer or adhere to the gastric epithelium tissue. Whereas some *Helicobacter* species can infect a number of different animal species, others are more specific, infecting only one known host. Multiple *Helicobacter* species have been shown to infect an individual animal.

It is estimated that a large proportion (>50%) of the human population carries *H. pylori* and potentially additional species within their gastrointestinal tract. *Helicobacter* organisms also appear to be highly prevalent in dogs and cats, with studies finding 80–100% of subjects

infected with at least one species. A number of NHPH species have been identified in dogs including *Helicobacter bizzozeronii*, *felis, heilmannii,* and *bilis* among others. Studies evaluating *Helicobacter suis* prevalence in slaughter pigs found at least 60% of the population colonized. The enterohepatic *Helicobacter* species (*H. hepaticus* and *H. bilis*) are particularly prevalent and important in the mice population (including laboratory animals), and reports of >60% prevalence of *H. pullorum* have been reported in poultry populations. *Helicobacter* species have been found in virtually every animal examined including sheep, monkeys, ferrets, whales, dolphins, geese, and numerous others, although the prevalence of *Helicobacter* and the distribution of specific species in these populations is less well established. Interestingly, even though NHPH are routinely detected in animals, and evidence of gastritis is found histologically, most colonized animals appear clinically healthy, and the organisms are thought to be opportunistic.

Transmission

Both oral–oral and fecal–oral are hypothesized transmission routes for *Helicobacter* species. There is some evidence to support spread by both routes. The ability of organisms to survive outside of the gastrointestinal environment and be spread through other means is controversial and has not been resolved. Although additional vehicles may exist, they are not considered the primary mode of transmission.

Zoonotic Potential

There are strong indications that *Helicobacter* species can be zoonotically transferred between humans and animals. Humans have been infected by numerous different *Helicobacter* species and contact with different animal species has been associated with infection, although there is little causal data. There is some evidence that similar strains are present in humans and their pets. *Helicobacter* species also have been isolated from milk products on several occasions, and there are reports of *H. pullorum,* an enteropathogen of poultry, as a foodborne pathogen associated with contaminated meat carcasses. Zoonotic transmission is most commonly considered in NHPH species, where human prevalence (estimated at 6%) is typically lower than *H. pylori* prevalence (estimated at 50%); however, cats harboring *H. pylori* have been reported as a zoonotic risk.

Pathogenesis

Numerous studies have shown that gastric *Helicobacter* species alter the gastric physiology. There also appear to be multiple mechanisms by which this can occur. Although the degree of pathology, the site of infection, and the *Helicobacter* species differ by animal species, one common theme of clinical infection is the induction of a chronic inflammatory response and ulceration of the gastric mucosa (Figure 23.1). Previous work with experimentally *H. felis*–infected dogs reported no correlation between the number of organisms colonizing and the degree of gastric inflammation. Gastric *Helicobacter*-like organisms in cats have demonstrated good correlation between the degree of colonization and the lymphoid follicles; however, the correlation was not obvious in dogs. The lack of standard criteria for evaluating histological changes makes interpretation of these results difficult; however, it seems likely that individual host factors are large contributors to the degree and type of pathogenesis, with some individuals remaining asymptomatic. Genes related to motility, acid neutralization/acclimation, chemotaxis, and adherence have been identified in numerous *Helicobacter* species. These, in addition to virulence factors like LPS, VacA, urease, and the cag PAI all likely contribute to the immune response, and subsequently, clinical manifestation. Since 2018, evidence of *H. suis* binding to gastric mucins and glycolipids have been reported, which seems to suggest that the bind structure may be different in neutral and acidic pH environments.

Immunologic Aspects

The immune system is an important mediator for *Helicobacter*-induced pathogenesis. A chronic inflammatory response is typically associated with infection; however, the regulation of the immune response can also break down. The immune response mechanism leading to pathology has been most commonly studied in mice; however, it likely differs between *Helicobacter* species and host animals. As an example, work with *H. felis* in mice revealed that cellular infiltrate was composed of neutrophils, B-cells, and CD4 T-cells. Of these, T-cells were shown to be the most critical in pathology. *H. hepaticus*, which can induce hepatitis in mice, was shown to be related to a Th1-mediated immune response. The precise response from infection with different species of *Helicobacter* in different animal species is not fully understood.

Animals infected with *Helicobacter* generally produce a significant IgG response against the organisms, and specific serum IgG antibodies to gastric *Helicobacters* in animals have been used to diagnose both naturally and experimentally infected animals. Analyses of serum and mucosal secretions by enzyme-linked immunosorbent assay in *H. pylori* naturally infected cats reveal an *H. pylori*–specific IgG response, and elevated IgA anti–*H. pylori* antibody levels in salivary and local gastric secretions. As in humans, though helpful in diagnosis, neither secretory nor serum antibody responses appear protective from infection.

Laboratory Diagnosis

Direct Examination

Diagnosis of *Helicobacter*-associated chronic gastritis cannot be made with gross visual endoscopic examination. Other means of diagnosis vary in their degree of sensitivity. Spiral bacteria can be observed with cytological examination of impression smears or with a Gram stain on homogenized gastric tissue. Gastric brushing cytology can be performed during routine endoscopy. Cells and mucus that adhere to the brush are applied to a glass slide, air-dried, and stained with a Giemsa stain. For visualization of gastric *Helicobacter* organisms, oil immersion magnification (100×) is used. The urease activity of these gastric bacteria is frequently utilized as a diagnostic test, particularly for *H. pylori*. The urease test is commercially available, detecting urease activity in gastric tissue within 15 minutes to 3 hours. In addition, a gastric biopsy can be minced and placed directly into urea broth, and a positive reaction obtained in one hour gives a presumptive diagnosis.

Isolation and Identification

Samples from gastric or intestinal biopsies are ideal submissions for *Helicobacter* detection by culture or molecular methods (see the later section titled "Molecular Methods"). Gastric or intestinal contents or feces can be submitted, although recovery is less predictable. Liver biopsies may also be submitted for detection in appropriate suspect patients. *Helicobacter* species are fastidious, microaerophilic, and biochemically unreactive. Higher hydrogen levels generally enhance recovery of the enterohepatic *Helicobacters* in culture; however, they can be isolated on the same medium as gastric *Helicobacters*. The organisms are typically isolated using a rich medium such as brucella blood agar with antibiotics including trimethoprim, vancomycin, and polymyxin B. Some species remain difficult to isolate, requiring specific pH or enrichment techniques, and some may even be uncultivable. Because *Helicobacter* species may have different antibiotic susceptibilities, selection of antibiotics in culture media may aid in successful isolation. Finally, the use of 0.45 or 0.65 μm filters to selectively filter feces has been recommended to help minimize contamination from other enteric organisms during primary culture on selective agar.

Molecular Methods

PCR-based detection methods specific to *Helicobacter* virulence proteins, as well as primers specific for segments of DNA encoding the 16S rRNA, have been described in the literature. Specialized polymerase chain reaction (PCR) protocols are increasingly used for detection of "*Helicobacter* DNA" from gut contents in various animal species. In addition, the complete genome of several *Helicobacter* species including *H. pylori, H. suis,* and *H. felis* have been sequenced and annotated, aiding in the rapid expansion of molecular identification techniques. New methods including FISH (fluorescence in situ hybridization) and Western blot analysis have recently been described as diagnostic techniques for human samples.

Treatment and Control

Treatment for *Helicobacter* infection is controversial, and most commonly infection is thought to be subclinical or in pets, asymptomatic. In addition, these organisms have been shown to quickly develop resistance to antimicrobials; therefore, treating *Helicobacter* infections should be based on lesions causing clinical illness. A combination of amoxicillin and metronidazole or clarithromycin with omeprazole or famotidine have been most commonly reported for treatment of dogs and cats with gastric *Helicobacter* infections; however, triple therapy may not result in long-term eradication of the organism from the host. Recent studies in pigs have shown that diet composition can impact the presence and concentration of NHPH, and therefore feeding strategies may be one means of controlling *Helicobacter* abundance.

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Further Reading

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24

Spirochetes V: *Leptospira*

Sreekumari Rajeev

Introduction

The pathogenic members of the spirochete bacteria belonging to the genus *Leptospira* are maintained in the renal tubules of many animal species including, cattle, dogs, horses, and pigs. *Leptospira* infection is generally acquired through contact with infected animal urine or after exposure to a contaminated environment and may result in mild to life-threatening disease in animals and humans. Symptomatic and asymptomatic infections may occur in animals. Leptospirosis is a widespread zoonotic disease, recognized by the World Health Organization (WHO) as a neglected re-emerging disease. It is estimated to be responsible for more than 50,000 human deaths and one million clinical cases each year.

Descriptive Features

The members of the genus *Leptospira* are 0.1 μm in diameter, 6–20 μm in length, motile, tightly coiled aerobic organisms with a distinctive hook-shaped appearance at the pointed ends. In common with other spirochetes, *Leptospira* have a typical double membrane. Their motility is facilitated by endoflagella or axial filaments located in the periplasmic space. They are very thin and hence not clearly visible under usual bacterial stains such as Gram stain. However, they can be visualized by dark-field microscopy (Figure 24.1). *Leptospira* was initially divided into two broad species: *Leptospira interrogans* sensu lato and *Leptospira biflexa* sensu lato containing pathogenic and saprophytic strains, respectively. Two taxonomic systems, one based on serology and the other based on DNA sequence, are widely used.

Characterization of *Leptospira* strains requires both species identification by molecular methods and serovar identification by traditional serological approaches. The traditional classification based on the serological response depends on the similarity of the carbohydrate component of the lipopolysaccharide (LPS). Although serovars with similar serological reactivity are conveniently placed together into serogroups, this classification has no official taxonomic status. The phylogenetic classification clusters the members of this genus into three clades: pathogens, nonpathogens, and an intermediate group. Molecular typing classifies *Leptospira* into several new species, and currently, at least 65 *Leptospira* species are described based on whole-genome sequencing and analysis. Some recognized pathogenic species include *L. interrogans, Leptospira borgpetersenii,* and *Leptospira kirschneri*. The divergent nature of the LPS present on the outer surface is the basis of serovar classification. The LPS composition of the members of this genus is similar to that of other gram-negative bacteria, but its endotoxin activity is lower. Genetic heterogeneity is common among strains belonging to the same serovar. Serovar identification is not correlated with species identification. Same serovars can be seen in multiple species, suggesting horizontal gene transfer among the members. Therefore, the serovar classification alone is no longer adequate to describe epidemiologically important strains.

Leptospira species have pathways for amino acid and nucleic acid metabolism. They derive their energy from beta-oxidation of long-chain fatty acids. They have genes encoding tricarboxylic acid cycle, and hence they can grow in aerobic or microaerophilic environments. They have large genomes compared to other spirochetes (3.9–4.7 MB), and they carry two circular chromosomes. Extrachromosomal elements such as plasmids and phages may be present.

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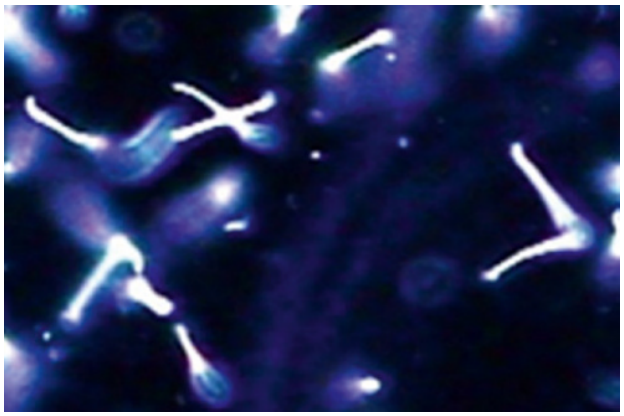


Figure 24.1 *Leptospira* observed under a dark-field microscope.

Epidemiology and Transmission and Zoonosis

Pathogenic *Leptospira* species colonize the proximal renal tubules of mammalian hosts and have global distribution. A broad range of mammalian hosts can be classified as maintenance or reservoir hosts that serve as asymptomatic carriers of the infection. The accidental hosts or incidental hosts develop clinical disease following infection. There are some known associations between some animal species and serovars. *Leptospira* infection usually occurs either by direct or indirect contact with the urine of an infected animal or from a contaminated environment. The incidence of infection is high in warmer, tropical countries with seasonal disease outbreaks during the rainy season. An outdoor lifestyle and close contact with domestic livestock animals in the tropics increase the risk of exposure. Transmission depends on the population density in an area, climate, and the nature and degree of contact between accidental and maintenance hosts. The typical route of entry is through cuts or abrasions in the skin or mucous membranes, including oral, respiratory tract, and genital surfaces. In recent years in humans, a significant risk has been associated with recreational activities in water, ecotourism, and global travel. *Leptospira* transmission can be influenced by a number of environmental factors, including high rainfall, natural disasters, population growth, flooding, poor sanitation and hygiene, and increased urbanization.

Pathogenesis and Diseases

On entering a susceptible host, *Leptospira* disseminate throughout the tissues, and they are cleared from all tissues, except the kidney, by the immune system. Once in the kidney, the they continue to multiply in the renal tubules, and they can be excreted in the urine. In maintenance hosts, in which chronic infection is present, there appears to exist an equilibrium between the host and certain *Leptospira* serovars. There is little understanding of the molecular changes that allow for the development of the carrier state. Common virulence factors include LPS, sphingomyelinases acting as hemolysins, cytotoxins, and flagella. Several outer membrane proteins (LipL32, LipL41) and immunoglobulin-like genes (ligA, ligB, and ligC) are seen in pathogenic *Leptospira* with potential virulence attributes that may play a role in virulence. The kidney is the principal site in both chronic and acute infection. The difference in susceptibility of disease in both animals and in humans may depend on host adaptation and genetics. *Leptospira* species can colonize the renal tubules of some hosts despite the presence of an active immune response. Biofilm formation in the renal tubules may help them evade the immune response. Leptospirosis has "protean manifestations" due to its large and extremely broad range of symptoms, ranging from mild febrile illness to severe life-threatening multiorgan disease. The clinical disease can be biphasic. An initial acute/septicemic phase lasts for around a week, followed by an immune phase characterized by the production of antibodies and the excretion of bacteria. Animal leptospirosis is different from human leptospirosis in a number of important features. Similar to human disease, acute clinical symptoms are seen, but there is also chronic infection that can result in reproductive wastage and hence economic losses, especially in livestock species.

Leptospirosis is an important disease in livestock and is reported worldwide, particularly in tropical countries. Leptospirosis in cattle is an important cause of decreased production, creating serious economic losses due to reproductive failure. Cattle are known to be maintenance hosts of *L. borgpetersenii* serovar Hardjo (Hardjobovis) in the western hemisphere. In contrast, the geographic prevalence of *L. interrogans* serovar Hardjo (Hardjoprajitno) is still a mystery. Subclinical persistent infection of the reproductive tract is a feature of *L. borgpetersenii* serovar Hardjo. Other serovars including Icterohaemorrhagiae, Autumnalis, Australis, Javanica, Tarassovi, Hebdomadis, Pyrogenes, and Grippotyphosa may cause incidental infection with more severe clinical signs. Animals may show pyrexia, hemoglobinuria, icterus, haemolytic anemia, meningitis, and death. Incidental infections in lactating cows are frequently associated with blood-tinged milk, a drop in milk production, and agalactia. Leptospirosis in small ruminants generally goes unnoticed, with most animals experiencing a subclinical infection. The number of studies on small ruminant leptospirosis is small. Leptospirosis in goats can take an acute or

chronic course. In acute cases, symptoms such as pyrexia, jaundice, anorexia, depression, and hemoglobinuria may be present. Seroprevalence studies have shown positive reactions to serogroups Grippotyphosa, Australis, Sejroe, and Hebdomadis. Sheep and goats can be an alternative reservoir host for the serovar Hardjo with most infections being subclinical, with the organism persisting for long periods in the kidneys. Leptospirosis in swine is recognized as an infectious disease resulting in reproductive disorders worldwide. Reproductive effects of infection with *Leptospira* spp. include an increase in the number of stillbirths, decreased number of piglets per litter, as well as a decrease in the number of weaning piglets and increased weaning-to-estrus interval.

Leptospirosis in dogs is generally associated with infections caused by *L. interrogans* and *L. kirschneri*. Serovars of the serogroup Icterohaemorrhagiae remain, and Grippotyphosa is the most important cause of infection in dogs. Seroprevalence studies have shown that the prevalence of Canicola is decreasing in many countries, with Grippotyphosa and Pomona now emerging as causes of disease in dogs in the United States and Grippotyphosa and Bratislava causing disease in dogs in Europe. The clinical presentation can be severe anicteric or icteric forms with high mortality. The hepatic involvement can range from mild elevations in liver enzymes to liver failure accompanied by hepatic encephalopathy. As in humans, the incidence of severe leptospiral pulmonary hemorrhage syndrome (LPHS) has increasingly been documented with high mortality rates. Occasionally uveitis and chronic interstitial nephritis have been documented as chronic outcomes.

Cats are generally considered resistant to *Leptospira* infection even though significant seroprevalences have been recorded in some cat populations. Clinical disease in cats has been rarely documented, and because cats appear to be resistant to experimental and natural infection, the clinical signs of feline leptospirosis are controversial. Clinical signs, when present, are usually mild despite leptospiruria, leptospiremia, and renal and hepatic inflammation. Recently, studies conducted in cat populations in Reunion Island and Taiwan using polymerase chain reaction (PCR) have found evidence of renal carriage in those populations.

Horses can be infected with a wide range of serovars, particularly those belonging to serogroups Grippotyphosa and Pomona. Globally the most common antibodies detected are to serovar Bratislava, and it has been suggested that in horses, both incidental and maintained infections could occur. As with other species, the majority of infections are subclinical with low-grade fever, depression, and anorexia. When acute infection arises, symptoms such as jaundice, depression, conjunctival suffusion, hemoglobinuria, and renal disease are seen. Typically, equine leptospirosis is associated with reproductive disorders and recurrent uveitis. With severe disease, abortion, stillbirth, and weak, sickly foals can result. In the United States, Kennewicki has been the serovar most frequently associated with disease and abortion, whereas in Europe, Grippotyphosa is more common. It has been documented that two to eight months after *Leptospira* infection, recurrent uveitis can occur, and *Leptospira*-associated uveitis forms an important subset of uveitis cases, with *Leptospira* spp. considered the most frequent cause of infectious recurrent uveitis. It seems to be an organ-specific autoimmune response and is accompanied by the presence of *Leptospira* and antibodies in the vitreous and aqueous humor.

Many wildlife species can be infected with various *Leptospira* species. However, the burden of disease among wild animals is under-documented in species other than rats and mice. Rats and mice are the major reservoir species for *L. interrogans* serovar Copenhageni and *L. borgpetersenii* serovar Ballum, respectively. Rodent control is a major strategy in preventing animal and human leptospirosis. Seroprevalence studies have recorded high reactivity in New World monkeys and macaques and among zoo animals, including a polar bear, wild dogs, and black rhinoceros. Leptospirosis is a common cause of stranding in the California sea lions. *Leptospira* infection has also been recorded in some bat populations. In addition, several studies have demonstrated infection in frogs, toads, turtles, and snakes.

Laboratory Diagnosis

General clinical laboratory findings include elevation of liver enzymes in hosts with hepatic involvement, the elevation of blood urea nitrogen and creatinine in animals with renal impairment, leukocytosis, and thrombocytopenia. Confirmation of *Leptospira* infection can be made by detecting the bacterial presence in various clinical specimens and the host serological response. When choosing the diagnostic methodology, one has to consider the animal species, whether clinical investigation or surveillance is targeted, and the stage of infection.

Direct Detection of *Leptospira*

Dark-field microscopy (DFM) can be used for presumptive demonstration of *Leptospira* in fresh clinical specimens such as blood and urine or tissues from the autopsy. A wet mount can be prepared from body fluids and can be examined using a dark-field microscope. Coiled motile bacteria with bright hook-shaped ends can be visualized. The method lacks

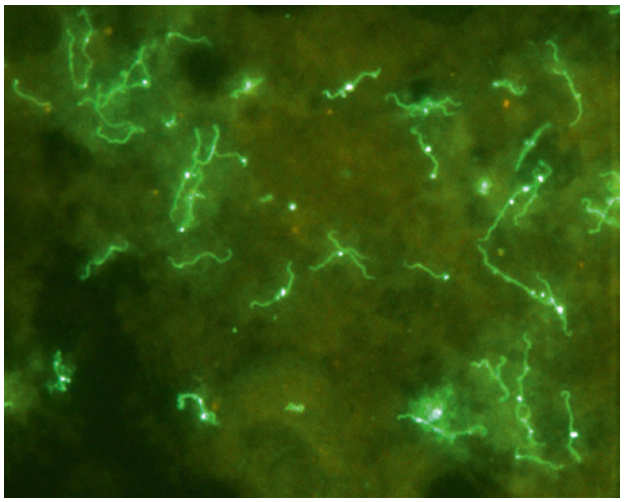


Figure 24.2 Morphology of *Leptospira* observed using immunofluorescence assay.

sensitivity and specificity and is subject to misinterpretation in the absence of technical expertise. At least 10^4 bacteria per milliliter of clinical specimen is required to visualize the organism.

Direct immunofluorescence assay (DFA) is commonly used in urine and tissue specimens. Smears prepared from clinical samples are fixed in acetone and treated with polyclonal antibodies conjugated with fluorescein isothiocyanate. Fluorescing organisms with specific morphology can be detected in positive cases (Figure 24.2). Other direct detection methods include Warthin starry staining and immunohistochemistry generally applied to histological specimens.

Bacterial Culture

Pathogenic *Leptospira* are BSL-2 level pathogens. Culture and isolation are not commonly practiced for diagnostic purposes, as it requires special media and prolonged incubation conditions. Primary cultures can be attempted from urine, kidney, blood, liver, and spleen. Ellinghausen-McCullough-Johnson-Harris (EMJH) media with enrichment is the most commonly used media for growing *Leptospira* under laboratory conditions. Other less commonly used growth media includes Fletcher's, Stuart's, and Kolthoff. Because *Leptospira* species do not survive long in clinical specimens, samples must be processed immediately after collection. Cultures are incubated at 28–30 °C for up to 12 weeks. Contamination can be prevented by passing the specimen through a 0.45 mm filter or by adding 5-fluorouracil or antibiotic cocktails to inhibit the growth of contaminant bacteria. Either liquid or semisolid media can be used for primary isolation. In the semisolid media, they form a dense growth just below the surface described

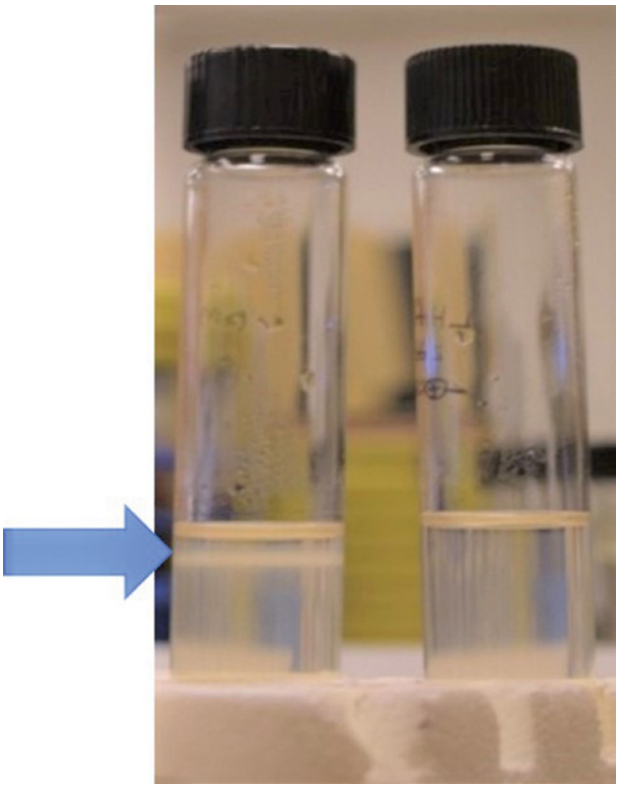


Figure 24.3 *Leptospira* growth in semisolid media. The blue arrow points to the Dinger zone.

as Dinger's zone (Figure 24.3). Primary culture of *Leptospira* on solid media is often difficult, but subculture can be performed for individual isolation of the bacterial strains. *Leptospira* isolates are serologically classified by microscopic agglutination test (MAT) and more specifically at the serovar level using the cross-agglutination absorption test (CAAT) using a panel of antibodies. Molecular techniques used for species identification are multilocus sequence typing (MLST) and whole-genome sequencing.

Serological Methods (Antibody Detection)

The MAT, a widely used serological test that detects agglutinating antibodies against *Leptospira* during infection, is complex and difficult to interpret at times. It is performed by incubating a patient's serum with various live *Leptospira* serovars. The test is considered the gold standard, but this definition should be revisited because the many limitations of this test often produce false-negative and false-positive test results depending on the stage of infection, previous exposure, and vaccination status of the animals. Cross-reactivity and agglutinating reactions to even non-infecting serovars, termed as paradoxical reactions, are common. A 50% agglutination of the organism with serum is considered positive, and a serum dilution of 1:100 is

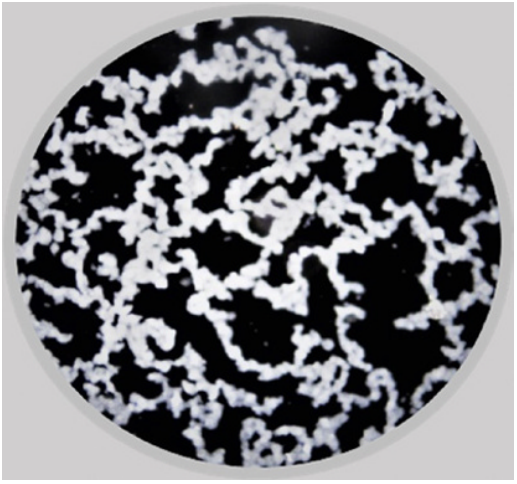


Figure 24.4 Microscopic agglutination test. *Leptospira* serovar Grippotyphosa agglutinated by a positive serum.

accepted generally as a cutoff titer. Figure 24.4 is representative of a complete agglutination observed in MAT testing. The MAT panel should consist of common serovars found in the area. This test is the most appropriate to use in epidemiological serosurveys.

ELISA assays have been designed to detect serovar Hardjo infection in sheep and serovar Pomona infection in sheep and cattle. Rapid point-of-care tests detecting IgM or IgG are commercially available for canine diagnosis. The results should be interpreted with caution in the absence of clinical signs. The PCR has been used to amplify *Leptospira* DNA from a number of clinical samples such as serum, cerebral spinal fluid (CSF), urine, and a variety of tissues post mortem. *Leptospira* DNA has also been detected by *in situ* hybridization and dot blotting. A number of quantitative PCR assays have been developed for diagnostic use that target either the *Leptospira* housekeeping genes *rrs*, *gyrB*, or *secY* or the pathogen-specific genes such as *lip*32, *lig,* or *lfb*1. PCR cannot identify the infecting serovar. Although not required for making clinical decisions, serovar identification is important in both public health and epidemiologic studies. In clinically ill animals, appropriate diagnostic tests or a combination must be chosen considering the biphasic nature of the disease. In reservoir animals such as cattle, multiple diagnostic tests will be required to confirm the positive status. There is very little correlation with MAT titers and the presence of organisms in the kidney.

Treatment

Standardized antimicrobial susceptibility testing is not available for *Leptospira*. Penicillin and doxycycline are the commonly used antibiotics to treat *Leptospira* infection. In acute leptospirosis in companion animals, treatment consist of antimicrobials combined with supportive therapy, including blood transfusions, fluid therapy, and in some cases, dialysis depending on the nature and severity of clinical signs. In the case of production herd animals, treatment consists of antibiotics and vaccination as a preventative measure. The treatment of choice for clinical leptospirosis in livestock can be a combination of streptomycin and penicillin; however, third-generation cephalosporins, tetracyclines, tulathromycin, and amoxicillin have also been recommended

Prevention/Control

Methods to prevent leptospirosis generally depend on regional epidemiology based on surveillance data from epidemiologic surveys and diagnostic services. A combination of vaccination programs, rodent control, and pre-or post-exposure chemoprophylaxis is used. Vaccines available to prevent *Leptospira* infection in animals are bacterins, but immune response to these vaccines is serovar specific and short term and often may not result in sterile immunity. Current vaccines protect against disease caused by the antigenically similar or homologous serovars, and therefore vaccines used must contain serovars representative of those in the region. Cattle, pig, and dog vaccines may contain from one to five serovars. Most of the bovine vaccines contain the serovar Hardjo, and serovar Pomona is present in porcine vaccines. Studies have shown that vaccines containing the serovars Hardjo and Pomona for the immunization of cattle can protect for up to a year. However, multivalent cattle vaccines are less efficacious when compared to monovalent vaccines. In dogs, vaccines more often contain the serovars Icterohaemorrhagiae and Canicola.

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25

Staphylococcus

George C. Stewart

Descriptive Features

The staphylococci are spherical, gram-positive bacteria that divide in multiple planes and remain associated post division to form irregular "grape-like" clusters. The cells are 0.5–1.5 µm in diameter. The staphylococci are facultative anaerobes, with the exception of *Staphylococcus aureus* subsp. *anaerobius* and *Staphylococcus saccharolyticus*, which are microaerophilic and anaerobic, respectively. The former is the causal agent of Morel's disease in sheep and goats, and culture of this pathogen requires reduced oxygen conditions. Staphylococci grow well on common laboratory media. On blood agar, colonies are round and relatively large (3–5 mm). *S. aureus* strains may produce carotenoid pigments that confer a golden color, whereas colonies of other species are generally nonpigmented (white). Selective media contains a high sodium chloride content, such as mannitol salt agar, which contains 7.5% NaCl. The high salt content is used to inhibit most other gram-positive and gram-negative bacteria. Biochemically, staphylococci (except for the two non-facultative anerobic species listed above) are distinguished from the streptococci by the production of catalase, the enzyme that breaks down hydrogen peroxide to water and oxygen. Differential characteristics of multiple cocci are listed in Table 25.1. Although they do not form spores, these bacteria tend to be resistant to environmental insults. They can survive on inanimate objects, especially in the presence of blood or pus, for long periods of time. Resistance to disinfectants and antibiotics are also seen in certain species.

The staphylococci are present as part of the skin and mucous membrane microbiomes of humans and other animals. The number of species in the genus *Staphylococcus* has been a wildly changing number over the years. Staphylococci isolated from different animal hosts often were considered unique species. Molecular techniques are now used to identify species, and this should provide some stability to the nomenclature. The genus *Staphylococcus* now includes at almost 60 recognized species. The staphylococci are considered to be opportunistic pathogens. However, severity of disease and virulence among species and even strains can differ markedly.

Coagulase-Positive and Coagulase-Negative Staphylococci

Production of the enzyme coagulase is the most significant correlate of virulence in staphylococci. Although this protein is not considered to be a major virulence factor, it is associated with the more virulent species of the genus *Staphylococcus*. Thus, we functionally classify the staphylococci as coagulase-positive and coagulase-negative species, with the former being the major pathogens of the genus. The coagulase-negative staphylococci, usually members of the normal flora of animals, are of lesser virulence. However, they are opportunistic pathogens and can, under the right conditions, cause serious disease. The coagulase-positive species include *S. aureus* (infects humans and a variety of animal hosts), *S. argenteus* (previously grouped with *S. aureus* but now recognized as a distinct species and has been identified in human and animal infections), *Staphylococcus pseudintermedius* (the *S. aureus* of dogs and cats), *Staphylococcus schleiferi* subsp. *coagulans* (causes skin and ear infections in dogs), and *Staphylococcus schweitzeri* (isolated from nonhuman primates). In addition, production of coagulase is a variable trait among strains of *Staphylococcus hyicus* (a bovine and porcine pathogen) and *Staphylococcus agnetis* (associated with infections in poultry and mastitis in cattle).

Virulence Properties

Virulence has been best characterized with *S. aureus*. This organism possesses a broad array of potential virulence factors. The virulence is said to be "multifactorial," meaning no

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Table 25.1 characteristics characteristics of aerobic and facultatively anaerobic cocci.

	Catalase	TSI	Coagulase	Hemolysis
Coagulase-positive *Staphylococcus*	+	A/A	+	+ (usually)
Coagulase-negative *Staphylococcus*	+	A/A	–	– (usually)
Streptococcus	–	A/A, K/A	–	+ (most pathogens)
Micrococcus	+	K/K	–	–

A, acid; K, alkaline; TSI, triple sugar iron agar.

single one has been shown to be essential for virulence and the relative importance of each depends on the animal model of infection being studied. The genes encoding many of the virulence traits are "accessory genetic elements," meaning that they are not part of the core genome of the bacterium. Instead, these reside on plasmids (extrachromosomal DNA elements), bacteriophage, transposons, or genomic islands (large segments of DNA inserted into the chromosome). These accessory genes are present in some strains, but not in others. Thus, different clinical isolates contain different constellations of virulence genes, which can dramatically change their capacity to cause disease. The following are well-characterized virulence properties of *S. aureus*, some of which are found in other *Staphylococcus* species.

Hemolytic Toxins

The coagulase-positive staphylococci frequently give rise to colonies that exhibit hemolytic zones on blood agar plates. The type of hemolytic zones depends on which hemolytic toxins are produced by a particular species or strain. The different hemolysis reactions are illustrated in Figure 25.1. The hemolysins were initially characterized by their effects on red blood cells (RBCs), but they primarily function as cytotoxins and their role in virulence is due their effects on leukocytes or other host cell types. The principal hemolysins are α-toxin (a pore-forming toxin that creates channels in the plasma membrane leading to an influx of water and cell lysis, thus producing a zone of complete hemolysis around the colony), β-toxin (a sphingomyelinase that damages the RBC membranes, resulting in a larger zone of incomplete hemolysis; because of the damage to the RBCs, if the plates are refrigerated, this zone of incomplete hemolysis will become complete due to cold stress on the RBCs – a hot-cold lysin), γ-toxins (two bicomponent, pore-forming protein toxins, HlgAB and HlgCB), and δ-toxin (a peptide toxin [one of the phenol-soluble modulins] that produces complete hemolysis). On sheep blood agar plates, strains that produce both α- and β-hemolysins produce the double zone pattern of hemolysis with the larger β-hemolysin partial hemolysis zone and the smaller α-hemolysin complete hemolysis zone (Figure 25.1). Human clinical isolates frequently lack the β-hemolysin zone due to the insertion of a prophage (bacteriophage genome) into the β-toxin gene. The β-toxin incomplete hemolysis zone is the basis of the CAMP factor test used to help identify *Streptococcus* species, as described in Chapter 26.

Leukocidins (Leukotoxins)

They are important for killing of phagocytes and avoidance of the host innate immune system. Staphylococcal

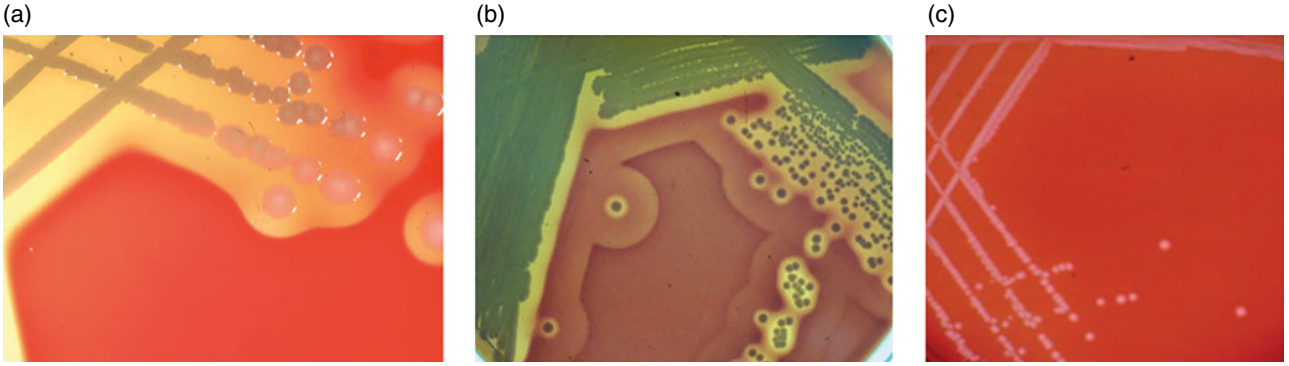


Figure 25.1 Sheep blood agar plate cultures of *Staphylococcus aureus* strains exhibiting the α-toxin mediated complete zone of hemolysis (panel A) or the double zones of hemolysis with the narrow complete zone from α-toxin production and the larger incomplete zone resulting from the production of β-toxin (panel B). In panel C is a culture of the non-hemolytic *Staphylococcus epidermidis*.

leukocidins comprise a class of bicomponent pore-forming toxins capable of damaging the host cell plasma membrane. The leukocidins characterized from human *S. aureus* isolates include Panton-Valentine Leukocidin (PVL) (LukSF), γ-hemolysins, LukED, and LukAB (also known as LukGH). The PVL gene is encoded on a prophage, so only bacterial cells lysogenic for that phage produce this toxin. LukMF′ is a toxin that was found in bovine *S. aureus* isolates and is active on bovine phagocytes, a property mediated by the targeting of CCR1, a chemokine receptor that is expressed on bovine neutrophils but not in human neutrophils. LukPQ is a leukocidin found in equine strains of *S. aureus* and whose receptor is CXCRA and CXCR2 on the surface on neutrophils. LukSF-I is produced by *S. pseudintermedius* and has substantial amino acid sequence similarity with LukED, and can target canine and human phagocytes. However, in contrast to LukED, LukSF-I has low hemolytic activity. *Staphylococcus delphini* encodes LukSF-I, which is a toxin similar to *S. pseudintermedius* LukSF-I. Bicomponent leukocidin-like toxins similar to the *S. aureus* LukAB toxin have been found in *S. argenteus* and *S. schweitzeri*. Species diversification of leukocidins suggests that the targeting of phagocytes by staphylococci is critical for host-specific adaptation.

Phenol-Soluble Modulins (PSMs)

These are small proteins that are cytotoxic to neutrophils and are commonly produced by highly virulent strains of methicillin-resistant *S. aureus* (MRSA). PSMs have surfactant activity *in vitro*, are involved in biofilm structuring, have antibacterial properties (may contribute to bacterial interference to outcompete other strains during an infection), are cytolytic, and promote release of pro-inflammatory cytokines. The *S. aureus* δ-hemolysin is a member of the PSM family of peptides.

Exfoliative Toxins

Epidermolytic toxins of *S. aureus* that produce a "scald"-like skin problem in infants (bacteria colonize the umbilical stump, and toxin gets into the bloodstream). The toxin causes an intra-epidermal cleavage through the granular layer, without an inflammatory response of the skin. Exfoliative toxin has been shown to cleave the human desmosomal adhesion molecule desmoglein-1, which when inactivated results in blistering of the skin. Similar toxins are associated with the skin lesions seen with *S. hyicus* infection in piglets resulting in exudative epidermitis (greasy pig disease). Six exfoliative toxins have been identified in *S. hyicus* strains (ExhA, ExhB, ExhC ExhD, SHETA, and SHETB). The principal clinical sign of exudative epidermitis is exfoliation of the skin in the upper stratum spinosum. The *S. hyicus* exfoliative toxins work by the same mechanism as their *S. aureus* counterparts.

Enterotoxins

These are secreted heat-stable (survives boiling for 30 minutes) protein toxins that when ingested by humans or non-human primates induce emesis. There are multiple serotypes (A through Y). Binding of enterotoxin to a receptor on the surface of the submucosal mast cells then induces 5-HT (5-hydroxytryptamine) release. Released 5-HT binds to the 5-HT3 receptor expressed on enteric nerves, and induces depolarization. Depolarization of the vagus afferent nerves stimulates the brainstem emetic loci to initiate the vomiting reflex. Staphylococcal food poisoning is an intoxication rather than an infection, and vomiting provides no advantage to the organism in terms of spread or survival. The real value of enterotoxin production to staphylococci is that these proteins, along with toxic shock syndrome toxin, are superantigens. Superantigens are proteins that bind to the outside of the MHC II molecules on antigen-presenting cells and T-cell receptors on T-cells and activate T-cells in a polyclonal fashion. This results in a cytokine storm as the activated cells release interleukin 2, tumor necrosis factor alpha, and interferon gamma. This leads to disruption of immune function and clinical signs that include high fever, headache, vomiting, hypotension, aches, and rash.

Coagulase and Clumping Factors

S. aureus secretes two proteins that promote coagulation, coagulase and von Willebrand factor binding protein, both of which function by activating prothrombin, thus initiating the coagulation pathway resulting in blood clot formation. This activity is thought to be involved in walling off the site of infection during abscess formation, a hallmark lesion of staphylococcal infections. Coagulase is detected with the tube coagulase test (Figure 25.2). There is also a slide coagulase test that detects "bound coagulase," which is now known as the bacterial cell bound clumping factors. Clumping factors are fibrinogen-binding proteins, and binding to fibrinogen in blood or plasma results in clumping of the bacteria. There are two clumping factors: ClfA and ClfB.

Protein A

A surface protein that has been well characterized from strains of *S. aureus* and *S. pseudintermedius* that can bind the Fc portion of immunoglobulins, especially certain IgG subtypes, from certain host species. The protein is anchored to the peptidoglycan cell wall, but some is released, especially in methicillin-resistant organisms.

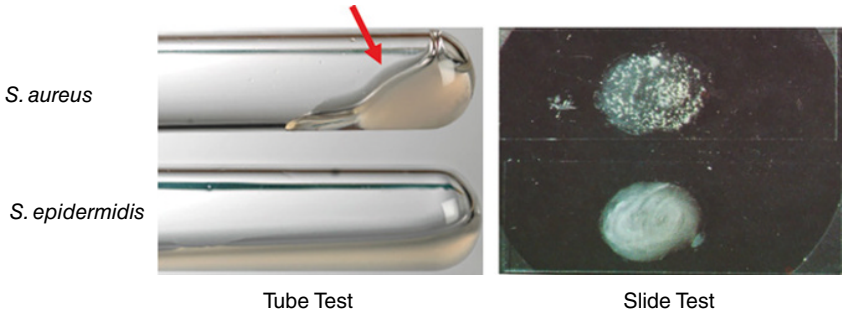


Figure 25.2 The "coagulase" tube and slide tests with the coagulase-positive *Staphylococcus aureus* and coagulase-negative *Staphylococcus epidermidis* strains. The left panel shows the results of the tube test, which measures production of coagulase or von Willebrand factor binding protein. The organisms are inoculated into tubes containing rabbit plasma, and following incubation, a positive result is indicated by the formation of a fibrin clot (denoted by the arrow in the upper tube). With the slide agglutination test (right-hand panel), the rabbit plasma is placed on a glass microscope slide and the bacteria are emulsified into the plasma. *S. aureus* expresses fibrinogen-binding proteins on its surface, and the binding to fibrinogen in plasma results in a visible clumping of the bacteria. The clumping-factor-negative *S. epidermidis* remains uniformly suspended in the plasma.

There is considerable sequence variability with Protein A, which has been exploited in strain typing schemes. Protein A is thought to play a number of roles in the infectious process. By binding the Fc portion of the antibodies, this effector end is not available for binding to the Fc receptors on host cells, which can inhibit opsonization of the bacteria. The binding of antibodies also coats the bacterial cell with host protein, which may be important in avoidance of the innate immune system early in the infectious process.

Capsule

Although highly encapsulated, mucoid *S. aureus* strains have been observed on rare occasions, most strains (as well as non-*aureus* staphylococci) do not produce large capsules characteristic of other bacterial pathogens. They do, however, produce a polysaccharide "microcapsule" that functions as an anti-phagocytic structure and is important for virulence. This capsule is not visible with India ink negative stains.

Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

Up to 24 surface proteins of *S. aureus* are covalently anchored to peptidoglycan. These proteins bind cell surface-associated ligands, often proteins of the extracellular matrix. These include proteins that bind fibrinogen (ClfA and ClfB), collagen (Cna), fibronectin, bone sialoprotein, desmoglein-1, and plasminogen. These proteins are important adhesins, and are also used for coating the bacterial cells with host proteins as a mechanism of immune avoidance. Individual MSCRAMM proteins frequently have been found to bind multiple host proteins.

Secreted Enzymes

Staphylococci secrete a variety of enzymes including deoxyribonuclease, proteases, lipases and esterases, and hyaluronidase. These may contribute to tissue destruction and may provide a source of nutrients for the bacteria. Some *S. aureus* strains produce staphylokinase, a fibrinolysin that can break down clots. This gene is encoded on a prophage, so only lysogens produce this enzyme. Lipases and esterases may also be important to neutralize toxic lipids in the skin, to promote skin colonization.

Other Virulence Attributes

Staphylococci can be associated with chronic infections, and some aspects of the resulting pathology are the result of delayed type hypersensitivity infections from staphylococcal antigens. For example, this undoubtedly plays a role in pyoderma cases in dogs infected with *S. pseudintermedius*. Staphylococcal infections can be refractive to treatment with antibiotics. There are multiple reasons for this, and the resistance contributes to the overall virulence properties. It has been shown that staphylococci can survive and replicate intracellularly in a variety of cell types. The intracellular organisms may be protected from the action of antibiotics that penetrate poorly into host cells. In addition, isolates from infected animals and humans have been shown to grow as respiratory-deficient small-colony variants (SCVs). SCVs are mutants arising during the infection that are defective in the electron transport chain, which result in cells that are slow growing and, as a consequence, are more resistant to the action of antibiotics or host cellular defense mechanisms. SCVs have been associated with unusually persistent, recurrent, and antibiotic-resistant infections. Staphylococci can form biofilms on biotic and abiotic surfaces, and these play an important

role in the infectious processes, especially with regard to colonizing surfaces such as indwelling catheters, prosthetic devices, and implants. Biofilms are a bacterial community that is metabolically heterogeneous and embedded in a self-produced extracellular matrix composed of polysaccharides (including polysaccharide intracellular adhesin [also known as poly-N-acetyl-β-(1–6)-glucosamine (PNAG)]), proteins, and extracellular DNA. Biofilms also contribute to antibiotic resistance properties of these pathogens, in part due to the presence of nongrowing subpopulations of cells within the biofilm community. Like most successful pathogens, staphylococci have iron acquisition systems to successfully compete with the host for iron. This includes heme iron acquisition systems involving peptidoglycan-anchored heme binding proteins and membrane transporters involved in heme translocation into the cell cytoplasm. *S. aureus* also produces the iron scavenging siderophores staphyloferrin A and B, aureochelin, and staphylobactin. The genes for one or more of these siderophores have been identified in genome sequences of both coagulase-positive and coagulase-negative species of staphylococci. Finally, many staphylococcal strains harbor plasmids, transposons, or genomic islands that encode resistance to antibiotics and disinfectants.

Regulation of Virulence

Early in the infectious process, when bacterial numbers are low, the staphylococcal pathogens need to bind to host tissues, go into stealth mode to hide from the innate immune system by coating themselves with host proteins, and replicate to increase their numbers. As the infection progresses and the bacterial numbers increase, the host innate immune system recognizes the infection and mounts an inflammatory response. The bacteria respond in kind by producing leukotoxins to kill of the phagocytes and downregulate the bacterial surface adhesins to promote release from the host tissue spread of the bacteria to other host sites. Staphylococcal regulation of virulence systems was first described for *S. aureus*, but have been found in other species as well. The first system was the Accessory Gene Regulator (Agr) quorum-sensing system. The Agr system is a quorum-sensing system in the staphylococci that regulates the expression of a number of virulence-associated genes. The *S. aureus agr* locus consists of two divergent transcripts (Figure 25.3). The *agrACDB* genes encode the two-component regulatory system. AgrD is a protein that is processed into a short peptide referred to as an autoinducing peptide (AIP) and exported from the cell by the AgrB protein. The 7- to 10-amino acid AIPs of different staphylococcal species have a conserved structure consisting of a 5

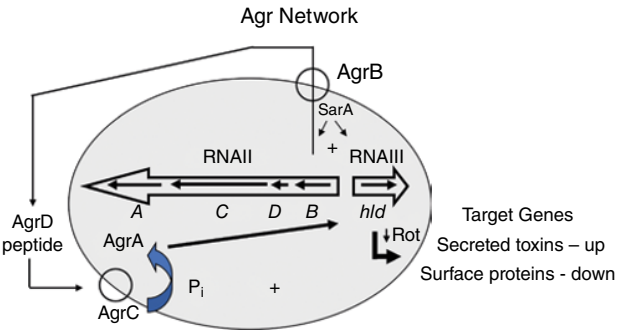


Figure 25.3 Agr system regulation of virulence genes of *S. aureus*. The polypeptide encoded by agrD is processed to a peptide and exported by the AgrB protein. As growth of the cells occurs, the AgrD peptide accumulates in the environment. When a threshold concentration is achieved, the peptide binds to the AgrC membrane protein (the sensory protein of the two-component regulatory system). Binding of the peptide activates the histidine kinase activity of AgrC, resulting in an autophosphorylation of AgrC. The phosphate moiety is then transferred to AgrA, the response regulator of the two-component regulatory system. When phosphorylated, AgrA is a transcriptional factor that binds to the agr promoter region, resulting in an increased production of RNAII and RNAIII. Elevation of RNAIII levels in the cells stimulates the activity of the Rot (repressor of toxins) transcriptional regulator protein, which results in an increased transcription and expression of genes encoding a variety of exotoxins, including many of the enterotoxins, and simultaneously effects a reduction in expression of genes encoding certain wall-associated surface proteins. The SarA protein is a transcriptional regulator that is a positive regulator of *agr* transcription and also affects expression of certain virulence genes in an Agr-independent fashion. Proper functioning of the Agr quorum-sensing system has been shown to be essential for virulence in *S. aureus*.

residue thiolactone ring (a lactone ring in one case) and a linear 2- to 5-amino acid tail. The AIP binds to the AgrC protein, which is the sensory protein of the two-component regulatory system. When a threshold concentration of AIP is bound, the histidine kinase activity of the AgrC membrane protein is induced, and AgrC becomes phosphorylated. The phosphate moiety is then transferred to the AgrA protein, the response regulator of the two-component system. The phosphorylated AgrA is a transcriptional factor that binds to the *agr* promoters and results in increased transcription of RNAII (encoding *agrACDB*) and RNAIII. The latter is a 514-nucleotide transcript that encodes the delta hemolysin (*hld*). However, it is the RNA molecule, not the Hld polypeptide, that is the effector molecule of the Agr system. When the concentration of RNAIII in the cell increases, transcription of a variety of toxins increases while the transcription of cell surface adhesin genes decreases. This is mediated by RNA–RNA interactions with both RNAIII and mRNAs that free up ribosome binding sites in the mRNA (such as that of α-toxin) to permit translation of the message or blocking of the ribosome

binding site or promoting RNase degradation of the mRNA (such as that of the *spa* [Protein A] mRNA) to prevent translation. RNAIII also blocks translation of the *rot* mRNA. Rot is a repressor of transcription of certain toxin genes and stimulates transcription of certain cell surface protein genes. Loss of Rot is associated with increased expression of toxins and reduced expression of adhesins. In addition to the Agr system, virulence genes are regulated by a complex network that integrates host- and environment-derived cues into a coordinated response. Most strains of *S. aureus* encode 16 different two-component regulatory systems including *saeRS*, *srrAB*, and *arlRS*. In addition, cytoplasmic transcriptional regulators, including the SarA protein family of transcriptional regulators (SarA, SarR, SarS, SarT, SarU, Rot, and MgrA) and alternative RNA polymerase sigma factors (SigB and SigH), contribute to the regulatory network. It is a complex, and highly effective, virulence controlling system.

Pathology and Pathogenesis

Staphylococci are opportunistic pathogens, including the more virulent coagulase-positive species. As such, predisposing conditions are necessary for the organism to transition from members of the skin and mucous membrane microbiome to pathogens. Among these are injury to skin to breach this innate immune system barrier, respiratory infections by other agents to weaken the immune defenses or provide a damaged environment, reduced levels of host resistance (including malnutrition), immunosuppression (including use of corticosteroids), disruption of the normal flora, presence of foreign bodies, a large exposure dose, and introduction of the bacteria into a new body site. Like streptococci, staphylococci cause pyogenic infections and abscess formation is common. The more virulent species can be invasive, leading to bacteremia and hematogenous spread to new body sites.

Hosts and Diseases

Cattle

Staphylococci are a leading cause of mastitis in dairy cattle. *S. aureus* is a contagious mastitis pathogen, and the source of the infection is mainly the udder from infected animals and is primarily acquired during the milking process. Intramammary infections with *S. aureus* result in moderate increases in somatic cell counts, is mostly associated with subclinical mastitis but can cause mild to severe clinical mastitis, results in chronic mastitis, and has a prevalence of approximately 66% of dairy herds in the United States. *S. aureus* is a cause of gangrenous mastitis (especially after calving). Alpha toxin is thought to be responsible for vasoconstriction and ischemia. *S. agnetis*, a coagulase-variable species, has been associated with subclinical or mild cases of clinical mastitis in dairy cattle. Most non-*aureus* isolates are coagulase-negative staphylococcal species and are the most prevalent bacteria isolated from milk in herds using recommended mastitis control measures. Some of these species are opportunistic, and others may be transmitted between cows. Common species identified include *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus simulans*, and *Staphylococcus xylosus*. Other types of *S. aureus* bovine infections include metritis, abscesses, pneumonia, and umbilical infections.

Horses

Usually the infections are with *S. aureus*. Diseases include secondary infections in wounds, botryomycosis (chronic pyogranulomatous disease characterized by granulation tissue enclosing multiple small abscesses) of the spermatic cord following castration, septic arthritis and tenosynovitis often following naval ill (local infection of the umbilicus), mastitis, and toxic shock syndrome (pneumonia associated with vasculitis and shock). Equine clinical isolates harboring the toxic shock syndrome toxin-1 have been identified. MRSA causes disease in horses, and the source of the infection can be humans or other horses. MRSA may become endemic in veterinary hospitals. As an example, a 2014 report from the Ohio State University veterinary hospital found that 8.6% of hospital surfaces and 5.8% of horses tested positive for MRSA (van Balen et al. 2014).

Swine

MRSA are common normal flora of pigs. *S. aureus* has been associated with cases of metritis, mastitis, arthritis in young pigs, and abscesses. Exudative epidermitis (greasy pig disease) of young pigs is an acute generalized dermatitis with sebaceous secretions, exudation, and exfoliation (Figure 25.4). This infection is somewhat common worldwide, occurring in roughly 30% of sites with young pigs in the United States. Infections arise from contamination of damaged skin (cuts, scratches, abrasions) by *S. hyicus*. In pigs up to seven weeks of age, the most common presentation of greasy pig disease is one of a brown to black development of scab appearance, starting usually around the shoulder and neck and spreading to part, or the whole, of the body. The infection is acquired through contact with older pigs. *S. hyicus* is part of the normal flora of the adult

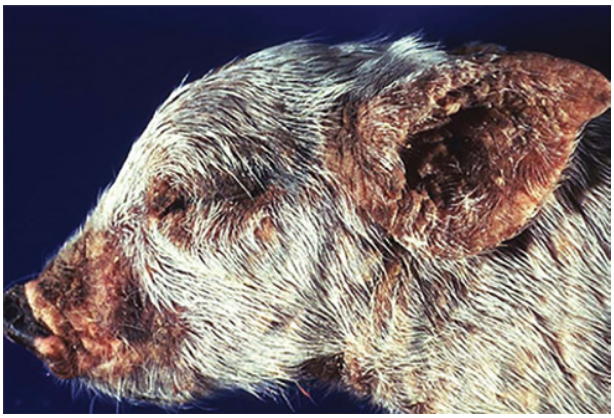


Figure 25.4 Porcine exudative epidermitis (greasy pig disease). Epidermal necrolysis is evident around the snout and ears of this affected piglet.

pigs. The *S. hyicus* produce an exfoliative toxin, which is responsible for the epidermal necrolysis. With severely affected young pigs, progressive dehydration and wasting of the affected pigs can occur. In herds facing severe outbreaks of the generalized form of exudative epidermitis, high mortality rates have been reported. Treatment is often complicated by the resistance of the *S. hyicus* strains to multiple antibiotics. Facility sanitation is important in prevention of greasy pig disease, and all-in/all out management of the facilities can reduce the incidence. Management practices that minimize skin abrasions are beneficial.

Poultry

Staphylococci are ubiquitous in the poultry farm environment and are part of the normal bacterial microbiota of the skin and mucous membranes of healthy birds, including poultry and pigeons. Infections, mainly by *S. aureus*, have resulted in septicemia with localization in joints and tendon sheaths and are often chronic in nature. Septicemia can develop following skin or mucous membrane infections. Common findings in ill birds are lameness and fever, which can be followed by depression and death. Diseases from *S. aureus* infections include arthritis, synovitis, chondronecrosis osteomyelitis, gangrenous dermatitis, green liver–osteomyelitis syndrome in turkeys, and omphalitis (infection of the navel and/or yolk sac in young poultry). Bumblefoot (pododermatitis) is a common inflammatory condition affecting the bottom of the bird's foot. The most frequent presentation is the presence of a hard, pus-filled abscess covered by a brown- to black-colored scab. As the severity of the infection increases, the birds become reluctant to walk. *S. agnetis* has been shown to be a pathological agent in broiler chickens, causing lameness induced by bacterial chondronecrosis with osteomyelitis, septicemia, and valvular endocarditis. Preliminary studies suggest that the poultry clinical isolates are distinct from the bovine mastitis isolates. A number of other *Staphylococcus* species are responsible for systemic infections including *S. hyicus*, *S. chromogenes*, *S. gallinarum*, *Staphylococcus warneri*, *S. xylosus*, and others.

Sheep

Staphylococci have been implicated in a variety of ovine infections including mastitis, dermatitis, wound infections, uterine infections, tick-bite-associated pyemia in lambs, osteomyelitis, neonatal arthritis, and epididymitis.

Goats

S. aureus is the most important cause of clinical mastitis in goats and non-*aureus* staphylococci are the most common isolates from subclinical mastitis. Staphylococcal skin infections may be secondary to chorioptic mange or contagious pustular dermatitis and usually involve *S. aureus*, but infections with *S. chromogenes* and *S. hyicus* have been documented.

Dogs

The principal staphylococcal pathogen of dogs is *S. pseudintermedius*, although *S. schleiferi* is increasingly seen as a cause of lesions. Methicillin resistance, frequently associated with resistance to multiple antibiotics, is found in clinical isolates, which complicates antibiotic therapy of chronic skin infections in dogs. Staphylococcal infections in dogs include pyoderma, otitis externa, otitis media, urinary tract infections, wound and soft tissue infections, bacteremia, osteomyelitis including discospondylitis, respiratory infections, and toxic shock syndrome. Pyoderma is usually triggered by an overgrowth of normal resident flora. The most common underlying triggers of superficial pyoderma include allergies (atopic dermatitis, food and flea bite allergies), endocrinopathies (hypothyroidism, hyperadrenocorticism), keratinization disorders, follicular dysplasias, ectoparasites, and poor grooming. *S. pseudintermedius* is usually the most serious infectious agent involved. *S. schleiferi* is often present in reoccurring cases.

Cats

Staphylococcal infections in cats are relatively uncommon. The most common species isolated from cats is the coagulase-negative *Staphylococcus felis*. This species is the most common skin isolate from cats and produces incomplete hemolysis on blood agar plates. Other coagulase-negative

species found include *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. warneri*, and *S. xylosus*. The coagulase-positive species *S. aureus* and *S. pseudintermedius* can also be commonly isolated from ill cats. Urinary tract infections, otitis externa, wound infections, pyoderma, ocular infections, and toxic shock syndrome have been reported in cats.

Zoonotic Potential

Historically, *Staphylococcus* strains, both coagulase-positive and especially coagulase-negative species, were considered to be highly host adapted, and not usually associated with infections in other host species. This situation is changing. Horizontal gene transfer between species of staphylococci has permitted the acquisition of colonization and virulence genes that enable strains to establish infections in new host species. For example, livestock-associated (LA) *S. aureus* isolates of multilocus sequence type ST398 were first recognized as part of the microbiome of pigs, but it has since been demonstrated that people working in close contact with livestock were at increased risk of infection with methicillin-resistant isolates of the ST398 clonal lineage. Human host adaptation and changes in virulence properties have resulted in infections in humans in the absence of livestock exposure. Human ST398 strains often carry virulence genes on accessory genetic elements. Prophages (often ϕSa3) harboring the human-specific immune evasion cluster genes (such as those encoding staphylokinase, enterotoxin A, chemotaxis inhibitory protein, and staphylococcal complement inhibitor) are frequently part of the genomes of these strains.

Animals that are in close contact with people (especially dogs, cats, and horses) can become colonized with human strains of *S. aureus* and serve as a reservoir for subsequent human exposure and infections. Humans can also serve as a source of infections, such as staphylococcal wound infections, for the animals. Because most infections, especially with the coagulase-negative species, are opportunistic and involve members of the normal microbiome, it is usually the host's own adapted strains that result in disease.

Resistance and Antibiotic Treatment

Penicillin G (benzyl penicillin) became available to treat infections in 1942 and was mostly effective for treating staphylococcal infections. However, stock cultures of *S. aureus* stored prior to this time indicated that a low level of penicillin resistance was present in *S. aureus* populations. This resistance was mediated by the production of the enzyme β-lactamase, the gene for which was often carried on plasmids. Exchange of plasmids between strains led to a rapid spread in penicillin resistance. To combat this problem, derivatives of penicillin were created that had bulky chemical substitutions on the β-lactam ring, and thus had reduced affinity for the inactivating enzyme. In 1960, methicillin was introduced for clinical use. Shortly thereafter, resistance to methicillin emerged, due to acquisition of a gene, *mecA*, that encoded a cell wall crosslinking enzyme (Pbp2a) that had a reduced affinity for methicillin and other β-lactam antibiotics. This permitted the MRSA to grow in the presence of methicillin. The *mecA* gene is encoded on an inserted element in MRSA strains. The incidence of MRSA strains increased, but at a much slower rate than that of penicillin resistance. Most of the MRSA problem was found in large urban hospitals, and the MRSA strains (referred to as HA-MRSA, for hospital-associated MRSA) were multiply antibiotic resistant. MRSA has also become a prominent pathogen outside the healthcare setting. In the late 1980s, reports surfaced of MRSA cases occurring in the community in patients without exposure to hospitals or nursing homes. These strains, called community-associated MRSA (CA-MRSA), cause necrotizing pneumonia and skin and soft tissue infections. CA-MRSA and LA-MRSA strains differ from HA-MRSA in that they tend to be not as multiply antibiotic resistant. Resistance to methicillin has emerged more slowly in veterinary medicine than in human medicine, largely due to less selective pressure to promote the resistance. However, methicillin resistance in canine pyoderma strains of *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* arising from protracted antibiotic treatment regimens has emerged naturally, indicating that the *mecA* element can be transmitted across species and across barriers of host adaptation. A second Pbp2a-encoding gene, *mecB*, originally identified in a transposon *mec* complex (Tn6045) in *Macrococcus caseolyticus*, a colonizer of animal skin, has been identified in *S. aureus*. Recently, a highly divergent *mec*A gene, *mec*C, was found in LA-MRSA *S. aureus* that causes bovine mastitis, and this strain was the causal agent of both human and animal infections. Overall, the incidence of veterinary *Staphylococcus* isolates resistant to methicillin has been trending upward. The higher incidence of MRSA, along with the increased zoonotic potential, is a growing public health concern.

Because of the increased incidence of MRSA and antibiotic resistance in general with the staphylococci, research into alternative treatment options has been under way. Among these approaches are an examination of compounds that interfere with the Agr quorum-sensing system that could prevent toxin gene expression, the use of lysostaphin (an enzyme that degrades the peptidoglycan cell

wall of *S. aureus*), and the use of staphylococcal bacteriophage as a therapeutic approach.

Products to boost the immune system as a treatment for chronic skin diseases in dogs are available. An example is the use of a bacteriophage-mediated lysate of *S. aureus* that is intended to boost immune responses to staphylococcal antigens. The lysate is prepared from a strain of *S. aureus*, and although the principal pathogen of canine pyoderma is *S. pseudintermedius*, the two species are antigenically related.

Vaccines

There is a rich history of evaluating a wide array of *S. aureus* virulence factors in immunization studies in mouse models of infection. The majority demonstrated protection. However, all vaccine candidates tested to date have failed human vaccine trials. Autogenous *S. aureus* bacterin vaccines, as well as specific protein vaccines, have been tested in dairy cows as a means of reducing mastitis levels. The results of these studies, often involving small numbers of animals, have yielded mixed results with no protection indicated in some and levels of efficacy in others. Currently, two whole-cell inactivated vaccines are licensed for protection against *S. aureus* mastitis: Lysigin® (Boehringer Ingelheim, Duluth, Georgia, United States) and Startvac® (Hipra, Girona, Spain). Although conflicting efficacy study results have been obtained, moderate decreases in the incidence of new *S. aureus* intramammary infections have been reported.

The use of autogenous *S. hyicus* bacterin vaccines given to sows at the end of gestation was shown to be efficacious in controlling exudative epidermitis outbreaks in their offspring during the nursery period in a 2018 study (Arsenakis et al. 2018). Pigs originating from the vaccinated sows had lower morbidity and mortality rates than those from the unvaccinated animals.

In general, because recurrent staphylococcal infections can occur, an effective vaccine approach to control infections in humans and other animals may be difficult to achieve.

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26

Streptococcus and *Enterococcus*

George C. Stewart

Streptococcus

Streptococci are gram-positive and catalase-negative cocci occurring in pairs and chains; they show considerable ecologic, physiologic, serologic, and genetic diversity. Currently there are 103 recognized species and 9 subspecies within the genus, but only a handful are significant pathogens. These are listed in Table 26.1.

On blood agar, the species exhibit various degrees of hemolysis, which can be used as an early step in identifying clinical isolates. Hemolysis produced by colonies on blood agar and Lancefield serological grouping are important factors in presumptive identification (Figure 26.1).

Alpha-hemolytic streptococci (α) do not lyse erythrocytes but produce a zone of green discoloration around the colonies (hydrogen peroxide oxidation of the hemoglobin to methemoglobin). Most commensal streptococci of animals are alpha-hemolytic. Streptococci that do this are sometimes referred to as viridans streptococci. Being alpha hemolytic does not mean avirulent, as *Streptococcus pneumoniae* and *Streptococcus suis*, important streptococcal pathogens, are alpha hemolytic.

Beta-hemolytic streptococci (β) lyse erythrocytes and produce a complete zone of hemolysis around the colonies. Pathogenic streptococci tend to be beta hemolytic.

Gamma streptococci (γ) are nonhemolytic. Most are nonpathogenic.

Older classification schemes grouped the streptococcal species based on biological properties:

Pyogenic group: The streptococci causing pyogenic infections in humans and animals; usually beta hemolytic.

Oral group: Primarily commensals on skin and mucous membranes; alpha or nonhemolytic.

Lactic group: Associated with milk and dairy products; now placed in the genus *Lactococcus*.

Enterococci group: Normal flora intestinal streptococci, opportunistically pathogenic; most now belong to the genus *Enterococcus*.

Anaerobic group: Anaerobic and microaerophilic species of *Streptococcus*. These organisms are found in the oral cavity, colon, and vagina of humans and animals and in the rumen of herbivores. This group includes organisms that have been found as part of mixed infections, but are not considered to be primary causes of disease.

Lancefield Grouping Scheme

Streptococci are divided into 20 groups (designated A through V, but with no I or J) using a precipitin test based on extractable group-specific carbohydrate antigens. The Lancefield groups are further subdivided into types based on protein antigens. The type-specific antigens are the M, R, and T proteins. Whereas antibodies to the Lancefield group antigens are not in general protective, those to the M, R, and T proteins are protective in a type-specific fashion. *Streptococcus pyogenes* has over 100 serotypes, based on M and other surface proteins, whereas *S. pneumoniae* has over 90 capsular polysaccharide types.

Descriptive Features

Morphology and Staining

Streptococci vary from spherical to ovoid cells, about 1 μm in diameter. Division occurs in one plane, producing pairs and chains, which is evident in liquid media or in clinical samples. Clumps of cells are often evident from cells taken for plate media. Some species, such as *S. pneumoniae*, are predominantly in pairs.

Young cultures stain gram positive. In exudates and older cultures (>18 hours), organisms often stain gram

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Table 26.1 Clinically important *Streptococcus* species.

Lancefield group	Species name	Type of hemolysis	Most important hosts	Diseases
A	*Streptococcus pyogenes*	β	Humans	Respiratory infections Rheumatic fever Glomerulonephritis
B	*Streptococcus agalactiae*	α, β, γ	Humans Cattle	Neonatal sepsis Mastitis
None	*S. pneumoniae*	α	Humans Horses NH primates Laboratory animals	Pneumonia
C	*Streptococcus equi* subsp. *equi*	β	Horses	Strangles
C	*S. equi* subsp. *zooepidemicus*	β	Horses Swine Cattle Humans Many others	Pyogenic infections
C, G	*Streptococcus dysgalactiae* subsp. *equisimilis*	β	Horses Swine Cattle Humans Dogs	Pyogenic infections
C, L	*S. dysgalactiae* subsp. *dysgalactiae*	α, β, γ	Cattle Humans Fish Swine Dogs	Pyogenic infections Mastitis
E	*Streptococcus porcinus*	β	Swine	Cervical lymphadenitis
G	*Streptococcus canis*	β	Dogs	Metritis, mastitis Neonatal infections
L	Group L *Streptococcus*	β	Dogs	Urogenital infections
D, R, S, T	*Streptococcus suis*	α	Swine Human	meningitis pneumonia septicemia
?	*Streptococcus uberis*	α, γ	Cattle	Mastitis
?	*Streptococcus parauberis*	α, γ	Cattle	Mastitis

negative, likely from effects of autolysins weakening the cell wall, thus leading to more efficient alcohol decolorization of the crystal violet primary stain.

Structure and Composition

Streptococci have a typical gram-positive cell wall structure. Capsules are produced by some species. In *S. pyogenes*, change from rough (matte) to smooth accompanies loss of M protein and virulence. Cell-wall-deficient forms (L forms) can also occur.

Growth Characteristics

Streptococci have fairly exacting growth needs that are best satisfied by media containing blood or serum. After overnight incubation at 37 °C, streptococci produce clear colonies, usually less than 1 mm in diameter. Encapsulated forms, such as *Streptococcus equi* subsp. *equi*, produce larger mucoid colonies. Pathogenic species grow best at 37 °C in the presence of elevated CO_2 levels, such as with a candle jar or CO_2 incubator.

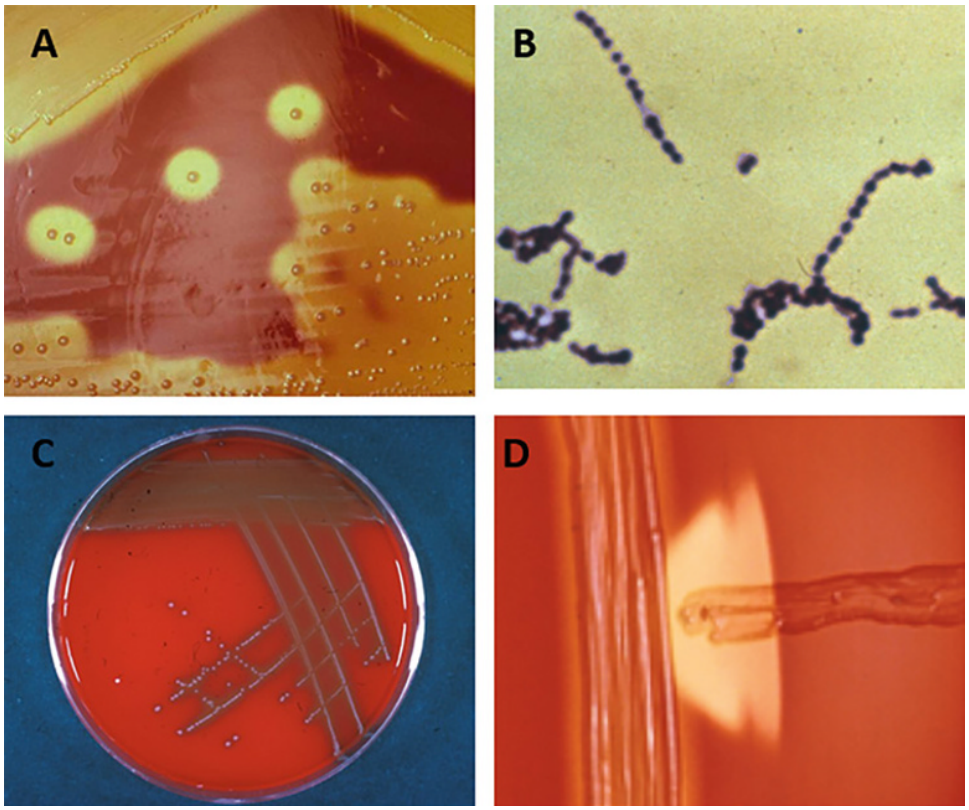


Figure 26.1 (A) *Streptococcus equi* subsp. *zooepidemicus* on sheep blood agar plate showing beta hemolysis. (B) Gram stain of *Streptococcus equi* subsp. *zooepidemicus*. (C) *Enterococcus faecalis* showing typical alpha hemolysis on sheep blood agar plate. (D) CAMP reaction on sheep blood agar. The vertical streak is growth of *Staphylococcus aureus* flanked by a zone of beta-toxin activity. Perpendicular to the *S. aureus* is a streak of *Streptococcus agalactiae*. Secretion of CAMP factor by the streptococci results in completion of the lysis of the beta-hemolysin-damaged erythrocytes producing the characteristic arrowhead zone of lysis within the incomplete hemolysis zone of the *S. aureus* streak. *Source:* Image courtesy of Dr. John Dustin Loy of the Nebraska Veterinary Diagnostic Center.

Biochemical Activities

Streptococci are catalase negative and obligately fermentative (can grow in the presence of oxygen but do not respire, deriving energy solely from fermentation).

Cellular Products and Activities of Medical Interest

A number of presumptive streptococcal virulence factors have been identified. The capsule of *S. pneumoniae* is an essential virulence factor, and M protein is an important virulence determinant; antibody to it is protective.

Adhesins Streptococci produce a number of surface proteins that bind to a variety of extracellular matrix proteins of the host (fibronectin, fibrinogen, collagen, vitronectin, laminin, decorin, and heparin sulfate-containing proteoglycans). These adhesins have been termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Some MSCRAMMs, specifically the fibrinogen-binding M protein, impart an antiphagocytic property to the streptococcal cell. Coating of streptococcal cells with host proteins through binding to MSCRAMMs is thought to result in the masking of sites for complement activation as well as those recognized by serum proteins (collectins/ficolins) that initiate the lectin pathway of complement activation. By doing so, the MSCRAMMs provide protection against opsonophagocytic killing.

M protein is an important virulence factor of *S. pyogenes* and S. equi subspecies *equi*. It binds fibrinogen, confers antiphagocytic properties, enhances adherence to the epithelial cells of the nasal mucosa, and may be associated with the post-infection immune disease in the horse (purpura hemorrhagica). The FbsA protein is the principal fibrinogen-binding protein of *Streptococcus agalactiae*, but unlike M protein, antibodies to FbsA are not protective. The FOG protein is the analogous protein in the group G *Streptococcus dysgalactiae* subsp. *equisimilis*, and the SzP protein is the M-like protein of S. equi subsp.

zooepidemicus (*Sez*). SzP was shown to bind to porcine host thioredoxin and regulate the alternative complement pathway. Thioredoxin can affect Complement C3 convertase and inhibit the conversion of C3 to C3a and C3b. This is a mechanism to evade host opsonophagocytosis.

S. equi subsp. *equi* produces a novel M-like protein, SeM, which has been linked to its increased virulence over its evolutionary parent, *Streptococcus zooepidemicus*. SeM actively binds fibrinogen and immunoglobulin G and inhibits the deposition of complement component C3b on the bacterial surface, resulting in an antiphagocytic action similar to that of the M proteins of group A streptococci.

The hyaluronic acid capsule of *S. pyogenes* is an adhesin (as well as imparting antiphagocytic effects) with an affinity for human epithelial cells via CD44, a hyaluronic acid–binding glycoprotein. Whether the hyaluronic acid capsules of streptococci of veterinary importance also act as adhesins is unclear.

The BibA protein of *S. agalactiae* specifically binds to human C4 binding protein, a regulator of the classic complement pathway, and deletion of the *bibA* gene severely reduces the capacity of group B streptococci to resist opsonophagocytic killing by neutrophils. BibA is an adhesin that is involved in binding to epithelial cells. BspA is another epithelial cell adhesin, but acts through binding to GP340 on the host cell surface.

Other adhesins are responsible for binding of streptococci to host cells. F protein, and other proteins such as Fnb and SFS, bind fibronectin and have been associated with bacterial adherence and internalization. PsaA (for pneumococcal surface adhesin) is a lipoprotein found on S. pneumoniae, *S. equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus* and is responsible for adherence to cells lining the upper and lower airways. E-cadherin, the cell junction protein in respiratory epithelial cells, has been shown to be a receptor for PsaA.

Capsule Some species of streptococci produce a capsule. Group A and C streptococcal capsules are composed of hyaluronic acid. Hyaluronic acid, also a constituent of mammalian connective tissue, is poorly antigenic and does not readily bind complement components (and is therefore antiphagocytic). The capsules described for Groups B, E, and G organisms are polysaccharide in nature, but not hyaluronic acid. The capsular polysaccharides of *S. suis* and *S. agalactiae* contain sialic acid.

Cell Wall The gram-positive cell wall contains proteins and polysaccharides that are of medical interest. The lipoteichoic acids and peptidoglycan of the gram-positive cell wall interact with macrophage cells, resulting in the release of pro-inflammatory cytokines.

Streptococcal Pyrogenic Toxin Superantigens (SPEs**)**. Superantigens simultaneously bind to major histocompatibility complex class II molecules and T-cell receptor molecules bearing a particular V-β region. This binding results in the activation of a large proportion of antigen-presenting cells and T cells, with subsequent release of high systemic levels of cytokines. Part of the systemic symptoms seen with streptococcal infections may be related to the cytokine storm that results from the large-scale T-cell stimulation effected by these toxins. The SPEs produced by *S. pyogenes* (the Group A streptococci) are the best studied. *S. pyogenes* produces several SPEs: SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SSA, and SmeZ (streptococcal mitogenic exotoxin Z). All known SPEs (with the exception of SmeZ, SpeG, and SpeJ) are localized on mobile DNA elements. As a consequence, each *S. pyogenes* isolate usually carries the genes for SMEZ, SpeG, and SpeJ, plus a variable combination of other SPE genes.

S. equi subsp. *equi* has been shown to produce SPEs including SeeH, SeeI, SeeL, and SeeM. These have been shown to stimulate proliferation of equine peripheral blood mononucleated cells (PMBCs) *in vitro*, but only SeeI showed pyrogenic activity in ponies. SeeH did not stimulate equine PMBCs, but was active with donkey PBMCs. These SPEs are prophage encoded. Three SPEs have been identified in *S. equi* subsp. *zooepidemicus*. These are SzeF, SzeN, and SzeP and have been shown to stimulate the proliferation of equine peripheral blood mononuclear cells, and stimulate tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) production.

BifA is a protein found to be critical for to cause meningitis in a mouse model of infection and to penetrate a human brain endothelial cell monolayer *in vitro*. BifA's Fic (filamentation induced by cyclic AMP) domain enables the protein to enter into endothelial monolayers and disrupt its barrier function. BifA acts by binding to moesin, a cytoskeletal regulatory protein, resulting in its phosphorylation and activation. Through its effects on moesin-dependent signaling, BifA enables *Sez* to efficiently penetrate the blood–brain barrier. The *bifA* gene is present in a pathogenicity island in certain *S. equi* subsp. *zooepidemicus* strains.

Miscellaneous Toxins and Enzymes The streptococci produce a number of proteins that potentially act as virulence factors.

Streptolysins O and S: oxygen-labile (O) and stable (S) hemolysins. These hemolysins are additionally cytolysins that cause lysis of neutrophils, macrophages, and platelets. Streptolysin S is responsible for the large zone of β hemolysis observed on sheep blood agar plates. The cytolytic spectrum

of streptolysin S is broad, including the membranes of erythrocytes, leukocytes, platelets, tissue-culture cells, and subcellular organelles such as lysosomes and mitochondria. The oxygen-labile and thiol-activated hemolysins, streptolysin O and the *S. suis* suilysin O, bind cholesterol in membranes. The toxin oligomerizes at or in the target membrane to form a hydrophobic protein complex integrated in the membrane with a hydrophilic channel-forming center. The resulting pores are relatively large (up to 30 nm in diameter).

Streptokinase. Encoded on a prophage and acts to activate plasminogen to plasmin. Plasmin is a protease that acts on host proteins, including fibrin, and thus degrades clots.

The streptococcal C5a-peptidases ScpA and ScpB of *S. pyogenes* and *S. agalactiae*, respectively, are multifunctional proteins found in Group A and B *Streptococcus* clinical isolates and are important for mucosal colonization. ScpB is reported to inhibit neutrophil chemotaxis by enzymatically cleaving the complement component C5a.

Enzymes with potential roles in virulence produced by streptococci include hyaluronidase and DNases (e.g. SpeF is DNAse B), NADases, and proteases.

Biofilm regulatory protein A (BrpA) is an important virulence regulator of *S. agalactiae*. In the mammary gland, biofilm production favors the establishment of the infection by enhancing resistance to antibiotics and clearance by host defenses.

Regulation of the Cellular Products of Medical Interest. Expression of virulence-related genes in *S. pyogenes* is regulated by at least three global systems. The first is a "growth-phase-related signal" that is undefined, but certain genes (including those encoding streptolysin S and DNases) are upregulated during the stationary phase, while others (including those encoding capsule, streptokinase, streptolysin O, and a protein called multigene regulator [Mga] in group A streptococci) are upregulated during the late exponential phase of growth.

Regulation occurs by both activation and repression of gene expression by transcriptional regulator proteins and by two-component regulatory systems. Major regulator proteins include Mga, responsible for the activation of expression during exponential growth of genes involved in adhesion, internalization and immune evasion; the RofA-like proteins including RofA and Nra, which regulate genes involved in persistence; FasA, which is involved in growth phase regulation of virulence gene expression; and Rgg (RopB), which activates expression of extracellular proteins such as the SpeB cysteine protease. Mga itself is regulated by growth phase as well as other undefined environmental cues.

The CovRS (control of virulence) two-component regulatory system regulates directly or indirectly 15% of the genome. Expression of several important virulence genes is directly regulated by the CovR response regulator protein, including those encoding the hyaluronic acid capsule operon, streptolysin S, streptokinase, *speB*, and *sda* (encoding the streptodornase DNase).

In the Group B streptococci, more than 20 distinct two-component systems (TCSs) have been classified to date, almost double the number found in *S. pyogenes* or *S. pneumoniae* clinical strains. The CovRS system is included.

Resistance

Beta-hemolytic streptococci can survive in dried pus for weeks. They are killed at 55–60 °C in 30 minutes, and inhibited by 6.5% sodium chloride and 40% bile (except *S. agalactiae*), 0.1% methylene blue, and low (≤10 °C) and high (≥45 °C) temperatures. Members of the genus *Enterococcus* tolerate these conditions. Viridans streptococci vary with respect to heat and bile resistance. Only *S. pneumoniae* is bile soluble. Streptococci tolerate 0.02% sodium azide, which is used in streptococcal isolation media.

Acquisition of antibiotic resistance by the streptococci has been slower than what has been observed with the *Enterobacteriaceae*, probably due to the much more limited horizontal spread of resistance genes. Nevertheless, transposons and integrative and conjugative elements (ICEs) can disseminate resistance genes among streptococci, and resistance is emerging. Antibiotic resistance is most problematic with *S. suis* infections. *S. suis* resistance to several antibiotics, including tetracycline, benzylpenicillin, clindamycin, tilmicosin, norfloxacin, streptomycin, kanamycin, tylosin, and erythromycin, has emerged over the last 40 or so years. Acquisition of resistance genes from members of the swine microbiome and fueled by the selective pressure of use of antibiotics has magnified the increased resistance patterns seen today.

Ecology

Reservoir

Most streptococci of veterinary interest live commensally in the upper respiratory, alimentary, and lower genital tract.

Transmission

Streptococci are transmitted by inhalation, ingestion, sexually, congenitally, or indirectly via hands and fomites.

Pathogenesis

Streptococci cause pyogenic infections primarily of the skin, respiratory tract, reproductive tract, umbilical stump, and mammary gland. Septicemia may result from hematogenous spread of the primary infection. Clinically, the streptococcal diseases are usually characterized at some stage by febrile symptoms, alone or associated with

symptoms of septicemia. The local areas exhibit pus formation that can be observed draining from the lesion. Abscesses form where this drainage is prevented. Toxemia and immune-mediated lesions are common sequela of the disease.

Pathology

The basic pathological process resembles that of staphylococcal infections; that is, the typical lesion is an abscess, an inflammatory focus in which participating cells have been destroyed by the combined effects of bacterial and inflammatory cell activity. This confrontation between leukocytes and microorganisms produces pus, a mixture of host cell debris and bacteria, living and dead. In an abscess, pus is surrounded by intact leukocytes and fibrin strands. Unless the pus is drained, a fibrous capsule will gradually be formed.

Disease Patterns

Equine Strangles is a highly contagious rhinopharyngitis caused by *S. equi* subsp. *equi* (*See*). After deposition on the mucous membranes of the upper respiratory tract, *See* adheres to epithelial cells by way of MSCRAMMs (M protein, fibronectin-binding protein, fibrinogen-binding protein, Psa) and the hyaluronic acid capsule. Adherence triggers internalization and subsequent localization in the subepithelial spaces. Cell wall constituents as well as pyrogenic exotoxins (SpeH and SpeI) initiate an acute inflammatory response. Capsule, M protein, and Scp protect *S. equi* from opsonization and phagocytosis. Streptolysins may act to destroy host cells by damaging their membranes. Systemic clinical signs may be due to the superantigen effects of pyrogenic exotoxins (SpeH and SpeI). Other enzymes and toxins may contribute to the process by digesting DNA (DNases), fibrin (streptokinase), and hyaluronic acid (hyaluronidase). The disease is marked by a serous or purulent nasal discharge, diphasic temperature rise, local pain, cough, and anorexia (Figure 26.2). In regional lymph nodes, abscesses develop, which typically rupture and drain within two weeks. Recovery follows. The overall mortality rate is under 2%. Complications include bastard (malignant) strangles, which result from metastasis of *See* to bronchial, mediastinal, or mesenteric lymph nodes where they can drain internally. Pyemic dissemination to meninges, lungs, pericardium, and abdominal viscera, or extension to the guttural pouches, can occur. Purpura hemorrhagica, a Type III hypersensitivity reaction manifested by subcutaneous swellings, mucosal hemorrhages, and fever, may follow the acute disease by about three weeks and is associated with significant (~50%) mortality (Figure 26.2).

Bacterial pneumonia/pyothorax in horses is almost always associated with a beta-hemolytic *Streptococcus* with *Sez* being the most commonly isolated. In addition, a gram-negative microorganism (*Actinobacillus* is the most common) is frequently found along with *Sez*. The infectious process is endogenous. The microorganisms involved are part of the normal upper respiratory tract microbiome, which then contaminate a compromised lung (e.g. following viral pneumonia). *Sez* (plus the others) are deposited in the lung and initiate or amplify a preexisting inflammatory process (due to cell wall constituents and pyrogenic exotoxins). The intensity of the inflammatory response is not as extreme as with the response initiated by *See*, nor are the constitutional signs as severe. However, the mechanisms involved in the pathogenesis are likely similar.

Pyothorax is probably an extension of the pneumonic process just described. Like pneumonia, *Sez* is the most common isolate, combined with a gram-negative species. Unlike pneumonia, an obligate anaerobe (*Bacteroides* or *Fusobacterium* are the most common) is frequently found as well.

Genital tract diseases in horses are associated with *Sez*, which is frequently associated with cervicitis and metritis. Infections in newborn foals, which are often umbilical infections (navel ill, pyosepticemia, joint ill, polyarthritis)



Figure 26.2 Equine strangles and purpura hemorrhagica clinical sign images. From left to right: inflammation of the nasal mucosa with discharges; acute pharyngitis with lymphadenopathy and abscess formation of the lymph nodes draining the head and neck (typically the submandibular and retropharyngeal lymph nodes); draining abscess (which usually leads to recovery); Purpura hemorrhagica-associated non-thrombocytopenic skin hemorrhages that occur 2-4 weeks post-sensitization [image from the Merck Veterinary Manual]; and purpura hemorrhagica-associated well-demarcated pitting, cold edema that can be found on the head, trunk, and extremities.

disseminate via the bloodstream to joints and the renal cortex. The microorganisms originate from the genital tract of the dam (part of her normal flora).

Beta-hemolytic streptococci (*Sez* is the most common) are associated with a variety of miscellaneous conditions in horses including osteomyelitis, arthritis, abscesses, and wounds. All are endogenous, with the infecting strain arising from the normal microbiome.

Swine Cervical lymphadenitis of swine (jowl abscess) is a contagious disease affecting swine. This condition is associated with *Streptococcus porcinus* (a Group E *Streptococcus*). The disease is analogous to strangles but clinically less dramatic and frequently not diagnosed until slaughter. Its most damaging aspect is carcass condemnation.

Secondary pneumonias in swine are sometimes associated with *S. dysgalactiae* subsp. *equisimilis*.

S. suis, *S. dysgalactiae* subsp. *equisimilis*, and streptococci belonging to Groups L and U cause neonatal septicemia, suppurative bronchopneumonia, arthritis, meningitis, polyserositis, endocarditis, reproductive problems, and abscesses. A fading pig syndrome has been reported in infected groups of pigs. Pigs usually have clinical signs and gross lesions referable to either the respiratory system or central nervous system, but not both. Transmission is from carrier pigs (upper respiratory tract and tonsils) with entry probably through the palatine tonsils. Disease is usually seen at 3–12 weeks of age, but all ages can be affected. Suilysin produced by *S. suis* may account for some of the tissue damage associated with this disease.

Of the 29 *S. suis* capsular serotypes, type 2 is considered the most virulent strain type of the species, is the serotype most frequently isolated from diseased animals, and has zoonotic potential. It causes serious infections in humans that may result in severe disability (deafness and ataxia). Association with swine is a predisposing factor.

Ruminants The leading agent of streptococcal mastitis is *S. agalactiae.* Less frequent causes are *S. dysgalactiae* subsp. *dysgalactiae*, and *Streptococcus uberis*. They produce a chronic subacute mastitis with periodic acute exacerbations. *S. equi* subsp. *ruminatorum* is a subspecies associated with clinical and subclinical mastitis in cattle, but has been reported to have a broader animal host range and has caused human infections, and thus is a concern as a zoonotic pathogen.

S. agalactiae (group B streptococci) are a leading cause of sepsis and meningitis in human neonates and pregnant women. However, the strains that cause disease in cattle are distinct from those that cause disease in humans.

Dogs and Cats Secondary pneumonias affecting dogs are sometimes associated with *Streptococcus canis*. *S. canis* is associated with septicemia in newborn puppies and a toxic shock–like syndrome (STSS) and necrotizing fasciitis (NF) in dogs. STSS is characterized by septic shock with multiple organ failure. NF may also be present. NF is a rapidly developing cellulitis of the soft tissues and fascia, usually in one limb, and characterized by necrosis of soft tissue. It is often followed by STSS.

Cats tend to be more resistant to streptococcal infections, and when they do occur, are most common in young kittens or immunocompromised cats. The infections seen are the same as those seen in dogs. Group G (*S. canis*) is the most common source of the infections.

Nonhuman Primates *S. pneumoniae* is a leading cause of pneumonia, septicemia, and meningitis in nonhuman primates. Pneumococcal pneumonia in monkeys runs an acute course with high mortality rates. The lesions are those of a fibrinous pleuropneumonia. Recent shipment and viral infection are common antecedents.

Miscellaneous Host Species

Cervical lymphadenitis in guinea pigs: *S. zooepidemicus* subsp. *zooepidemicus*.

Septicemic disease in fish from freshwater aquaculture farms and from salt-water environments: *Streptococcus iniae* (a beta-hemolytic *Streptococcus* without a Lancefield designation). Handling (cleaning/necropsy) affected fish is a risk factor for developing cellulitis, endocarditis, or arthritis presumably following self-inoculation.

Septicemic disease in seals: *Streptococcus phocae* (a beta-hemolytic *Streptococcus* that reacts to Lancefield group antisera C and F).

Septicemic disease and dermatitis in opossums: *Streptococcus didelphis* (a beta-hemolytic *Streptococcus* without affiliation with known Lancefield groups).

Epidemiology

Healthy individuals may carry all the streptococci discussed, and many infections are probably endogenous and stress related. Neonatal infections are commonly maternal in origin.

Strangles and porcine lymphadenitis are contagious diseases preferably affecting young animals (past infancy). *S. equi* and *S. porcinus* are spread by contaminated food, drinking water, or utensils and by recovered animals, which may remain clinically healthy shedders for months. Milking equipment, unskilled attempts at intramammary medication, and unsanitary milking practices often spread *S. agalactiae* among dairy cows.

Immunologic Aspects

Immune Mechanisms of Disease

Human poststreptococcal diseases (rheumatic fever, acute glomerulonephritis) are attributed to immunopathogenic mechanisms. Similarly, equine purpura hemorrhagica following strangles is immune complex mediated.

Recovery and Resistance

The main defenses against streptococcal infections are phagocytic, and the antiphagocytic M protein elicits protective antibodies. Animals recovered from strangles and cervical lymphadenitis are at least temporarily immune to reinfection.

Polysaccharide capsules of *S. agalactiae* and *S. pneumoniae* evoke the formation of opsonizing antibody. In streptococcal pneumonia, their appearance determines recovery from infection. In bovine mastitis, no useful immunity develops: cows, unless treated, remain infected.

All immunity is serotype specific.

Artificial Immunization

A whole-cell bacterin and an M protein vaccine are available for vaccination against strangles. Neither is uniformly effective, and often elicits local reactions at the injection site. An intranasal avirulent live vaccine that stimulates essential local antibody responses is available. Feeding live avirulent cultures has produced immunity to porcine jowl abscesses.

Laboratory Diagnosis

Sample Collection

Aspirates from unopened lesions, in sterile syringes or sterile containers, are preferred. Swabs in transport media are acceptable. Milk is collected into containers under sterile precautions.

Direct Examination

Smears of exudates or sediments of suspect fluids are fixed and gram stained. Streptococci appear as gram-positive cocci in pairs, short chains, and in some instances very long chains (typically seen in pus aspirated from cervical lymph nodes of horses infected with *S. equi*). Streptococci have a tendency to lose their gram-positivity and sometimes stain weakly gram positive or gram negative.

Culture

Exudates, milk, tissue, urine, transtracheal aspirates, and cerebrospinal fluid are cultured directly on cow or sheep blood agar. Incubation at 37 °C in 3–5% CO_2 is preferable. Streptococcal colonies, smooth or mucoid, will appear in 18–48 hours.

Identification relies on a combination of classical techniques (determination of Lancefield grouping and biochemical tests) and molecular techniques (e.g. determination of the sequence of DNA encoding the 16S ribosomal DNA, or via PCR using species specific primers). Commercial kits are available for both purposes. Other useful diagnostic tests include the following:

1) The CAMP phenomenon (named after Christie, Atkins, and Munch-Petersen) reflects hemolytic synergism between staphylococcal beta toxin (a sphingomyelinase) and a *S. agalactiae* toxin (CAMP protein sometimes referred to as cocytolysin). A beta-toxin-producing staphylococcus is inoculated across a sheep or bovine blood agar plate. At right angles to this line, and approximately 0.5 cm from it, a suspect S. agalactiae is inoculated. After incubation, hemolysis by CAMP-positive bacteria will be enhanced in the beta-toxin zone (Figure 26.1). The combined action of these two toxins on sheep or bovine blood agar produces larger and clearer zones of hemolysis than either agent alone.
2) Bacitracin sensitivity. Bacitracin disks (0.04 units) inhibit growth of *S. pyogenes* on blood agar. This reaction is not entirely consistent or specific.
3) Bile esculin agar tests the ability of 40% bile-salt-tolerant bacteria to hydrolyze esculin, a characteristic of those belonging to Lancefield Group D.
4) Optochin sensitivity. Growth of *S. pneumoniae*, but not other alpha-hemolytic streptococci, is inhibited around disks impregnated with optochin (ethyl hydrocuprein hydrochloride).

Treatment and Control

Localized suppurative conditions are drained of pus.

For systemic treatment, penicillin G and ampicillin are effective on most beta-hemolytic and viridans streptococci. Cephalosporins, chloramphenicol, and trimethoprim-sulfas are alternatives. Streptococcal endocarditis is treated with combined penicillin and gentamicin. Susceptibility to fluoroquinolones is unpredictable. Streptococcal toxic shock and NF are treated with penicillin G and clindamycin (clindamycin decreases toxin production, and penicillin G is bactericidal).

Penicillins (intramammary) are effective for treating mastitis due to *S. agalactiae* and most other streptococci. For the many available alternatives, a specialty text should be consulted. Important aspects of mastitis control lie in the areas of sanitation and herd management.

For strangles, it is most beneficial to treat exposed and affected animals prior to abscess formation and to continue treatment past the febrile stage. Early treatment, however,

likely results in the animal not developing protective immunity. Inappropriate or inadequate therapy of strangles is blamed for prolonging the illness and causing "bastard strangles" (widespread abscess formation with systemic manifestations). Populations at risk may be vaccinated. Affected or suspected horses should be rigorously isolated.

Enterococcus

Enterococci were once classified as Group D streptococci. Unlike most true members of the genus *Streptococcus*, these microorganisms possess phenotypic traits (resistance to salt, bile, methylene blue, and growth at increased temperatures) that set them apart. Molecular genetic analysis showed that they are unique, and prompted the establishment of a new genus, *Enterococcus*.

There are 60 species in the genus, most of which live in the intestinal tracts of mammals and birds. They are mainly opportunists that infect compromised sites to produce disease. There are some (*Enterococcus durans*, *Enterococcus hirae*, and *Enterococcus villorum*) that have been associated with intestinal disease in neonatal animals (piglets, foals, calves, puppies, kittens) as well as adult dogs and cats. Enterococci are either alpha or gamma hemolytic on sheep or cattle red blood cell–containing blood agar plates.

Descriptive Features

Morphology and Staining

Enterococci vary from spherical to short bacillary cells, about 1 μm in diameter. Division occurs in one plane, producing pairs and short chains.

Structure and Composition

Enterococci have a typical gram-positive cell wall structure. Polysaccharide capsules are made by some species. Most enterococci possess Lancefield Group D carbohydrate.

Cellular Products and Activities of Medical Interest

Much of what is known regarding cellular products and activities of medical interest comes from study of *Enterococcus faecalis* and *Enterococcus faecium*, the most common enterococci producing disease in human patients. Presumably, some of what is known about these two species also applies to the other members of the genus.

Aggregation Substance Aggregation substance is a surface protein that promotes adherence of enterococci to each other (to form aggregates) and to epithelial surfaces (an adhesin). It is an important component of the pheromone-inducible transfer of conjugative plasmids.

Capsule Some enterococci produce a polysaccharide capsule. The capsule discourages association with phagocytic cells by interfering with complement deposition and imparting a relative hydrophilicity to the microbe's surface.

Pili Pili of gram-positive bacteria have been implicated in adhesion to multiple types of human cells and in biofilm formation, two processes critical in the pathogenesis of many bacterial diseases. *E. faecalis* and *E. faecium* harbor pilin gene clusters.

Enterococcal MSCRAMMs Genome sequence analyses has revealed the presence of multiple MSCRAMMs, with seven enterococcal MSCRAMMs characterized in detail: Ace (adhesion of collagen of *E. faecalis*), Fss1, Fss2, and Fss3 (*E. faecalis* surface protein) and Acm (adhesin of collagen of *E. faecium*), Scm (second collagen adhesin of *E. faecium*) and EcbA (*E. faecium* collagen binding protein A). Ace was shown to bind to collagen types I and IV, laminin, and dentin and was shown to be involved in the pathogenesis of experimental endocarditis. Fss1, Fss2, and Fss3 bind to fibrinogen.

Cytolysin Cytolysin is a heat-labile, oxygen-stable, pore-forming exotoxin that lyses both bacterial and eukaryotic cells. It is a member of a large class of lanthionine-containing bacteriocins. It is also a hemolytic toxin, lysing human and horse red blood cells (but not sheep or bovine red blood cells, the red blood cells most often used in blood agar plates). Cytolysin production is under the control of a quorum-sensing system. Among cytolysin-producing strains, the cytolysin is either chromosomally encoded within a 150-kilobase pathogenicity island or on a conjugative plasmid. The functional cytolysin toxin is a binary complex of large (38 amino acids) and small (21 amino acids) peptide subunits.

Extracellular Superoxide Some enterococci secrete a superoxide that appears to afford some protection against killing by phagocytic cells.

Gelatinase Some enterococci produce a gelatinase that is expressed under the control of the *fsr* two-component regulatory system and is important in biofilm production and for virulence. It is a zinc-metallo-endopeptidase implicated in the hydrolysis of gelatin, collagen, β-insulin, hemoglobin, casein, and other bioactive peptides and is able to cleave fibrin and damage host tissue, potentially contributing to bacterial dissemination.

Iron Acquisition Enterococci produce a hydroxamate type of siderophore in response to low levels of iron.

Enterococcal surface protein (Esp) protein. The *esp* determinant is encoded on a pathogenicity island in the genome of both *E. faecalis* and *E. faecium*. It is important for biofilm formation, colonization of the urinary tract, and the pathogenesis of endocarditis and urinary tract infections.

Growth Characteristics

Enterococci produce clear to gray colonies 1–2 mm in diameter (either alpha or gamma hemolytic) after overnight incubation at 37 °C, though they will grow between 10 to 45 °C. Enterococci will grow in 6.5% sodium chloride, 40% bile, and in 0.1% methylene blue.

Biochemical Activities

Enterococci are catalase-negative facultative anaerobes, deriving energy from fermentation.

Resistance

Enterococci are hardy microorganisms able to survive in the environment for extended periods of time. They are able to grow in 6.5% sodium chloride and 40% bile, 0.1% methylene blue, and low (10 °C) and high (45 °C) temperatures. They are intrinsically resistant to the β-lactam antibiotics (including cephalosporins and penicillinase-resistant penicillins), aminoglycosides, clindamycin, fluoroquinolones, and the trimethoprim-sulfonamides (they are effective scavengers of thymidine found in exudates, thereby bypassing the effect of the trimethoprim-sulfonamides). They are able to acquire resistance to high levels of β-lactams, high levels of aminoglycosides, the glycopeptides (vancomycin), tetracycline, erythromycin, fluoroquinolones, rifampin, and chloramphenicol.

Ecology

Reservoir

Enterococci live in the intestinal tract of mammals and birds as part of the normal microbiome of these species.

Transmission

Enterococci that are associated with opportunistic disease are part of the normal flora of the host.

Pathogenesis

Except for *E. durans*, *E. hirae*, and *E. villorum*–associated disease, endogenous enterococci infect a compromised site (e.g. urinary bladder, moist external ear canal, catheter). The cell wall peptidoglycan and lipoteichoic acids initiate an inflammatory response. Capsule, cytolysin, and superoxide potentate the inflammatory processes.

E. durans, *E. hirae*, and *E. villorum* associate with the villi (from tip to crypt) of the small intestine of affected animals. Associated diarrhea does not appear to be due to an enterotoxin or epithelial cell damage.

Disease Patterns

Dogs and Cats Otitis externa is usually the result of infection (bacteria and a yeast [*Malassezia*]) of a compromised external ear canal. The bacteria involved are usually an environmental species (e.g. *Pseudomonas* and *Proteus* or a member of the patient's normal flora such as *Enterococcus* and *Staphylococcus pseudintermedius*).

Enterococcus is a common isolate from dogs with lower urinary tract infections. *Enterococcus* spp. (*E. durans*, *E. hirae*, and *E. villorum*) are associated with diarrhea in puppies, kittens, and adult dogs and cats. Almost any compromised site may be contaminated with an *Enterococcus*. Enterococci have been implicated in cases of bacterial endocarditis in dogs.

Horse *Enterococcus* spp. (*E. durans*, *E. hirae*, and *E. villorum*) are associated with diarrhea in foals. *Enterococcus* spp. should be expected in any condition that results from contamination of a compromised site with fecal material (e.g. street nail/sole abscess, wound).

Cattle *Enterococcus* spp. (*E. durans*, *E. hirae*, and *E. villorum*) are associated with diarrhea in calves. *Enterococcus* spp. should be expected in any condition that results from contamination of a compromised site with fecal material (e.g. wound).

Swine *Enterococcus* spp. (*E. durans*, *E. hirae*, and *E. villorum*) are associated with diarrhea in piglets. *Enterococcus* spp. should be expected in any condition that results from contamination of a compromised site with fecal material (e.g. wound).

Reptiles *Enterococcus lacertideformus* is an emerging pathogen of geckos, skinks, lizards, and anoles. Infections of wounds or perhaps colonization of the oral cavity followed by invasion result in gingival swelling and the formation of gelatinous subcutaneous nodules of the face and head. The infection may spread to other tissue sites and is ultimately fatal. The biofilm-encased and piliated bacteria replace the surrounding normal tissue and cause bone lysis. Inflammation is not generally seen. The course of the disease is slow, but progressive, with survival ranging from a few weeks to four months after the appearance of the lesions.

Rats *Enterococcus* spp. (*E. durans*, *E. hirae*, and *E. villorum*) are associated with diarrhea in infant rats.

Epidemiology

Most of the infectious processes from which enterococci are isolated are due to contamination with members of the normal flora. In hospital settings, nosocomial spread (e.g. fomites, hands of caregivers, soles of shoes) to compromised sites is an important issue.

Vancomycin-resistant strains of enterococci are a serious problem in human medicine, because members of this genus (especially *E. faecalis* and *E. faecium*) are major contributors to nosocomially acquired disease. The vancomycin resistance genes are encoded on a transposable element (Tn1546). Enterococci are very resistant to antimicrobial agents. Vancomycin (a glycopeptide antibiotic) is one of the few effective drugs for treatment of such infectious processes. Vancomycin-resistant strains of enterococci arose in Europe after the initiation of feeding of another glycopeptide antibiotic, avoparcin, as a growth promoter to food-producing animals. Though at first the vancomycin-resistant strains were limited to the intestinal tract of animals fed this antibiotic, they soon spread, as did the genes encoding vancomycin resistance (*vanA*), to the human intestinal tract. In the United States (where use of avoparcin was not permitted), injudicious use of vancomycin in human hospitals resulted in the same effect, that is, an increase in selective pressure resulting in an increase in colonization by vancomycin-resistant enterococci (especially in hospitals).

Laboratory Diagnosis

Sample Collection

Aspirates from unopened lesions, in sterile syringes or sterile containers, are preferred. Swabs in transport media are acceptable. Urine samples are obtained by antepubic cystocentesis (bladder tap), catherization, or midstream catch.

Direct Examination

Stained (Gram's or Romanowsky-type such as Wright's or Giemsa) smears of exudates are examined. Urine can be examined unstained or stained (Gram's or Romanowsky-type). Histopathologic sections of small intestine obtained from animals with associated enterococcal enteritis are needed to demonstrate characteristic adherence of coccal forms to villi.

Culture

Samples are streaked onto the surface of blood agar plates and incubated at 37°C overnight. Enterococci produce clear to gray colonies 1–2 mm in diameter (either alpha or gamma hemolytic). Preliminary identification entails testing for the production of catalase (negative), and ability to grow in 6.5% sodium chloride and 40% bile. Most isolates can be speciated using commercially available kits, sequencing the gene encoding the 16S ribosomal RNA, or a combination of both.

Treatment and Control

Correction of the underlying condition is the most important aspect of treatment of most situations from which enterococci are isolated. In some instances, removal of the compromise is enough to initiate host elimination of enterococci (because they do not have potent virulence determinants). This is an important concept because enterococci tend to be quite resistant to antimicrobials. In those infectious processes that have a polymicrobic etiology (along with the compromise), success can be achieved by correction of the compromise and antimicrobial therapy aimed at the other (usually more susceptible) microorganisms.

Enterococci isolated from the lower urinary tract of dogs are usually susceptible to urine concentrations of amoxicillin-clavulanate, chloramphenicol, and tetracycline. Even though most urinary strains of enterococci test susceptible to trimethoprim-sulfonamides, care should be taken in interpreting *in vitro* test results because of the thymidine-scavaging ability of this group of microorganisms.

Enterococci associated with otitis externa in dogs are dealt with by correcting the underlying compromise and use of any of the otic preparations that contain an antimicrobial (topical concentrations of most incorporated antimicrobics usually far exceed the minimal inhibitory concentration needed to inhibit growth of enterococci).

Abiotrophia and *Granulicatella* (Nutritionally Variant Streptococci)

Members of the genera *Abiotrophia* and *Granulicatella* were discovered as small colonies growing as satellites around other bacterial colonies when samples obtained from normal human mucosal surfaces (eyes, genital tract, mouth, respiratory tract) were inoculated onto blood agar plates. The bacteria comprising these colonies were gram-positive, catalase-negative cocci requiring vitamin B_6 for growth (satisfied by the addition of 0.002% pyridoxal hydrochloride to media). These microorganisms were provisionally termed "nutritionally variant streptococci," but later placed into the genera *Abiotrophia* and *Granulicatella* based upon the sequence of the gene encoding the 16S ribosomal RNA.

Clinically, members of these genera have been isolated from the bloodstream, abscesses, dental plaque, joints,

corneal ulcers, acute-onset postoperative endophthalmitis, and cardiac valve vegetations of human patients. They have been isolated from the genital tracts, respiratory tracts, abscesses, and eyes of horses and ruminants.

A fibronectin-binding protein, Cha, has been identified and characterized from *Granulicatella adiacens*. *Abiotrophia defectiva* and *G. adiacens* have been shown to be capable of biofilm formation *in vitro*.

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27

Trueperella

Tiruvoor G. Nagaraja

Species of the genus *Trueperella* (named after Hans Georg Truper, a German microbiologist) are gram-positive nonspore-forming nonmotile organisms ranging from short rods to coccobacilli that occur singly, in pairs, or in clusters. The members of the genus were once included in the genus *Arcanobacterium,* which included the following nine species: *A. abortisuis, A. bernardiae, A. bialowiezense, A. bonsai, A. haemolyticum, A. hippocoleae, A. phocae, A. pluranimalium*, and *A. pyogenes*. Based on 16S rRNA sequence analysis, phospholipid composition, and the presence of menaquinone (principally MK-K10[H_4]), the following five species – *abortisuis, bernardiae, bialowiezense, bonsai,* and *pyogenes* – are now included in the genus *Trueperella*.

Trueperella pyogenes is the main species of veterinary importance, and is involved in suppurative infections in all animals, but more common in cattle and swine.

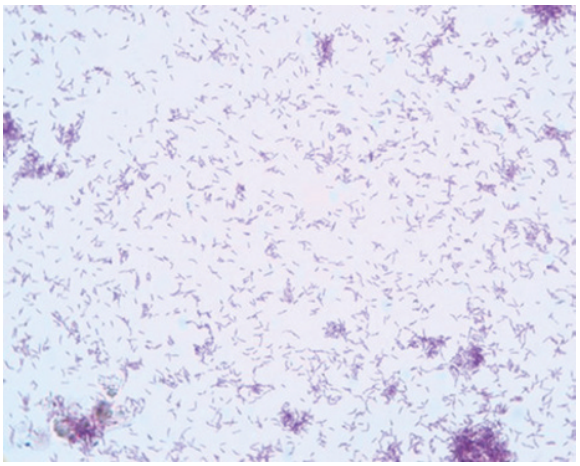


Figure 27.1 Gram-stained smear of *Trueperella pyogenes*. Note gram-positive coccobacillary to short rods. *Source:* Courtesy of Dr. J.D. Loy, University of Nebraska Lincoln, Nebraska Veterinary Diagnostic Center.

Trueperella pyogenes

The species *T. pyogenes,* previously classified as *Arcanobacterium pyogenes*, and before that, *Actinomyces pyogenes,* and *Corynebacterium pyogenes,* belongs to the family *Actinomycetaceae* in the phylum Actinobacteria. *T. pyogenes* is a normal inhabitant of the upper respiratory, urogenital, and gastrointestinal tracts of cattle, swine, and other domestic animals. In cattle, it has been isolated from the ruminal epithelium. The organism does not appear to be part of the normal flora in humans.

Morphology, Growth Characteristics, and Biochemical Activities

The morphology of *T. pyogenes* ranges from coccobacillary to short rods (0.5 μm by up to 2 μm) that occur singly or in pairs (Figure 27.1). Short diphtheroid forms with small clubs are also seen, particularly in young cultures. The cell wall peptidoglycan contains lysine, rhamnose, and glucose. Unlike members of the genera *Corynebacterium* and *Rhodococcus*, it does not contain mycolic acids. *T. pyogenes* is a facultative anaerobe with a fermentative metabolism and ferments proteins (gelatin, casein, coagulated serum) and sugars, such as lactose, to produce lactic acid as a major product. They require increased CO_2 for optimal growth. On blood agar, the organisms form pinpoint colonies with distinct zone of β-hemolysis. The zone of hemolysis is typically two to three times the diameter of the colony. The organism is catalase negative.

Virulence Factors

T. pyogenes produces a number of extracellular products and surface-exposed proteins that contribute to adherence, colonization, and host damage:

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1) **Cell wall macromolecules**. The lipoteichoic acids and peptidoglycan of the cell wall interact with macrophage cells, resulting in the release of pro-inflammatory cytokines.
2) **Pyolysin O (PLO)**. It is a hemolytic exotoxin and the primary virulence factor. The hemolysin is active against sheep, horse, human, rabbit, and guinea pig erythrocytes and is cytotoxic to polymorphonuclear leukocytes (PMN) also; in laboratory animals, it exerts dermonecrotic and lethal activities. It is a pore-forming, cholesterol-dependent toxin belonging to the thiol-activated family of cytolysins (CDC family) found in many gram-positive bacteria, such as pneumolysin of *Streptococcus pneumoniae*, suilysin of *Streptococcus suis*, streptolysin O of *Streptococcus pyogenes*, listeriolysin O of *Listeria monocytogenes*, and perfringolysin O of *Clostridium perfringens*. The family is named for the sensitivity of their activity to oxygen and restoration of their activity by reducing compounds (thiol activation). In contrast to the majority of thiol-activated hemolysins, the PLO is insensitive to thiols and other reducing agents, meaning the toxin is oxygen stable. The PLO is considered to be the major virulence factor in *A. pyogenes* infection, which is confirmed by reduction in virulence in PLO-deficient mutants.
3) **Neuraminidases**. Also called sialidases, they cleave terminal sialic acid from carbohydrates or glycoproteins from the host cell receptors. *T. pyogenes* produces two neuraminidases. One is a cell-wall-associated 107 kDA protein (NanH) encoded by the *nanH* gene, which is produced by all strains of *T. pyogenes* that have been tested. The protein is similar to a number of other bacterial neuraminidases. The second neuraminidase (NanP) is a 186.8 kDA protein encoded by *nanP* that is preferentially associated with bovine strains and is not present in all the strains tested. Neuraminidases play an important role in adhesion to the host cells.
4) **Extracellular matrix-binding proteins**. These are proteins on the surface of *T. pyogenes* that bind to the host extracellular matrix composed of structural glycoproteins such as collagen, fibronectin, fibrinogen, laminin, and elastin. These proteins belong to the microbial surface components recognizing the adhesive matrix molecules (MSCRAMM) family. A collagen binding protein, CbpA, has 50.4% similarity to Cna, a protein that binds to collagen, of *Staphylococcus aureus*. A 20 kDA cell-wall-associated, fibronectin-binding protein has been described. These binding proteins are involved on the adherence of *T. pyogenes* to epithelial cells of the host.
5) **Fimbriae**. Five fimbrial antigens have been described (FimA, FimB, FimC, FimE, and FimG). Fimbriae have filamentous structures and occur in a limited number of cells in a bacterial culture. Because the expression of fimbriae *in vitro* is poor, not much is known about their characteristics. They are believed to have a role in bacterial adherence, although the mechanisms of adhesions are not known.
6) **Other enzymes**. *T. pyogenes* secretes at least four distinct proteases, possibly serine proteases, and a DNase. Although some aspects of these enzymes have been studied, there is no evidence to support a role for any these enzymes in the pathogenesis of *T. pyogenes* infections.
7) **Biofilm**. A majority of *T. pyogenes* strains from different animal species and infections have been shown to produce biofilm.

Mode of Infection and Transmission

Because *T. pyogenes* is a commensal on skin and mucous membrane, most infections are endogenous. In "summer mastitis," cow-to-cow spread, mediated by flies, has been reported.

Pathogenesis

Mechanisms

Entry of *T. pyogenes* into the deeper tissues requires an insult or a stress of some kind such as a wound or a primary viral or bacterial infection. The organism can get disseminated and even cause abortion, but more often it results in suppurative infections involving the joints, skin, and visceral organs, usually complicated by other potentially pathogenic commensals, especially gram-negative, non-spore-forming anaerobes (*Bacteroides, Dichelobacter, Fusobacterium, Porphyromonas, Prevotella,* and *Peptostreptococcus*). The observation that *T. pyogenes* is often associated with *Fusobacterium necrophorum* has led to the suggestion of pathogenic synergy between the two species. Such synergistic interaction has been demonstrated in laboratory mice. The mechanisms involved in this synergism may be the supply of energy substrate (lactic acid by *T. pyogenes*), protection against phagocytosis (leukotoxin by *F. necrophorum*), and creation of anaerobiosis (oxygen use by *T. pyogenes*). Pyolysin is a major virulence determinant, as evidenced by the fact that mutants deficient in production of the toxin have reduced virulence and antibodies raised against the inactivated protein are host protective. The precise role of pyolysin in the pathogenesis of *T. pyogenes* infection is not known, but the membrane-damaging effect on host cells is probably a key mechanism. Neuraminidases play a role in bacterial adhesion to epithelial cells. The enzyme can also decrease the viscosity of mucus, thus facilitating colonization. Neuraminidases can also impair the host immune response

by desialylating IgA and increasing their susceptibility to proteolysis. The ability of *T. pyogenes* to bind to extracellular matrix proteins, mediated by surface proteins, may promote adherence and subsequent colonization. The proteases may play a role in tissue invasion and destruction, evasion of host defenses, and modulation of the host immune system during infection and inflammation. DNase may depolymerize viscous DNA released from lysed or disintegrating host cells in suppurative lesions. The exudate (pus) consists of bacteria, neutrophils (live or dead), and host cell debris.

Pathology

The lesions are abscesses, empyemas, or pyogranulomas. Abscesses are often heavily encapsulated. Any putrid odors are due to anaerobic bacteria.

Trueperella pyogenes Infections in Animals

T. pyogenes is involved in purulent infections, singly or in combination with other organisms, of traumatic or opportunistic origins, which may be local, regional, or metastatic. It is frequently isolated from a variety of pyogenic infections of the lung, pericardium, endocardium, pleura, peritoneum, liver, joints, uterus, renal cortex, brain, bones, and subcutaneous tissues of all animals, but most frequently in ruminants and swine.

Cattle

Infections of *T. pyogenes* in cattle include metritis, mastitis, liver abscesses, and a variety of pyogenic infections, such as pneumonia, encephalitis, pyelonephritis, endocarditis, lymphadenitis, and abscesses in a number of organs. The more common and economically significant infections are as follows.

Metritis and Endometritis in Dairy Cows

T. pyogenes is a member of the vaginal flora of dairy cows. As an opportunistic pathogen, the organism invades the uterus to cause infection, especially after parturition, when the protective epithelium of the endometrium is disrupted. The influx of inflammatory cells into the endometrium and the cytolytic activity of the pyolysin against the endometrial stromal cells are responsible for inflammatory lesions. Most often, the clinical infections with *T. pyogenes* are polymicrobial and other bacterial species frequently associated with them are *F. necrophorum, Escherichia coli, Streptococcus* spp., and so on. Such polymicrobial uterine infections, especially with *F. necrophorum* and other anaerobes, result in increased purulent discharge. The uterine inflammation that results in subclinical or clinical metritis and endometritis reduces milk yield and the reproductive performance of cows.

Liver Abscesses in Feedlot Cattle

T. pyogenes is the second most commonly isolated bacterium (next to *F. necrophorum)* from abscesses of liver in feedlot cattle. The organism originates from the rumen, more specifically from the ruminal wall, where oxygen is available from the blood circulation in an otherwise anaerobic environment of the rumen. The predisposing factor is ruminal acidosis in cattle fed high-grain diets, and acute or chronic acidity damages the ruminal epithelium, which permits the organisms to enter the portal circulation to reach the liver and cause abscesses. There is no evidence that *T. pyogenes* alone can cause liver abscesses. The synergistic interaction with *F. necrophorum*, whose leukotoxin provides protection, is required for *T. pyogenes* to cause abscesses. Generally, the occurrence of *T. pyogenes* in liver abscesses ranges from 2% to 25%, but in certain situations (cattle fed all-grain diet, abscesses in culled dairy cows, and cattle fed high-grain diets with tylosin), the prevalence becomes noticeably high (50–70% of liver abscesses).

Mastitis in Cows and Heifers

T. pyogenes causes severe mastitis characterized by thick, purulent secretions, frequently in dry cows and heifers before or at the time of calving, and occasionally in lactating cows, generally following teat or udder injury. The source of infection includes wound infections, abscesses, and genital tract infections, and infection spreads in a herd because of contact of teats with a contaminated environment, such as a calving area. In Europe, the disease is common in dry cows and heifers maintained on pasture, hence the name "summer mastitis." The seasonal prevalence is also associated with the fly season because biting flies are involved in the entry of the organism into the teats and spread of the organisms between animals. The infection caused by *T. pyogenes* is characterized by severe pyogenic lesions of the mammary tissue with foul smelling milk, particularly when co-infected with anaerobes. The anaerobic bacteria most often involved in such infections include *Peptoniphilus indolicus, F. necrophorum,* and *Prevotella melaninogenica*.

Foot Rot in Feedlot Cattle and Dairy Cows

T. pyogenes is commonly associated with *F. necrophorum* and *Porphyromonas levii* in necrotic infections of the laminae of the feet.

Swine

T. pyogenes is associated with pneumonia, pleuritic, endocarditis, osteoarthritis, polyarthritis, mastitis, metritis, and even septicemia. More often, abscesses located in a variety of organs may lead to systemic purulent infection involving lungs, liver, kidneys muscles, bones, joints, and so on. Again, many of these infections are polymicrobial, and involvement of bacterial species is similar to that in cattle. Septic arthritis in swine appears after farrowing, suggesting that the source of infection may be the uterus.

Sheep and Goats

T. pyogenes causes abscesses in different tissues and organs, including foot abscesses. In companion animals (horses, dogs, and cats), the infections caused by *T. pyogenes* are rare. In the United States and Canada, *T. pyogenes* is an emerging pathogen of captive or free-ranging white-tailed deer in which the organism causes suppurative meningoencephalitis complex characterized by intracranial abscesses. In humans, *T. pyogenes* infections are sporadic, mostly occurring in immunocompromised patients with occupational exposure to farm animals and their environment. Because *T. pyogenes* is not a commensal in humans, such infections are likely zoonotic.

Laboratory Diagnosis

Gram-stained smears from tissues or exudates reveal gram-positive, short to pleomorphic rods, often mixed with other bacteria. Material suspected of containing *T. pyogenes* should be cultured on blood agar in a 5% CO_2 incubator. Generally, minute colonies on blood agar with a distinct but narrow zone of hemolysis from a sample of pus are indicative of *T. pyogenes* involvement. Routine biochemical methods are used to identify this microorganism. Primers designed to amplify *T. pyogenes*–specific DNA by the polymerase chain reaction have been described.

Treatment and Control

Incision and drainage of abscesses are essential. *T. pyogenes* is susceptible to penicillin, amoxicillin, ampicillin, chloramphenicol, erythromycin, sulfamethazine, tylosin, and tetracyclines. The strains are becoming increasingly resistant to sulfonamides and tetracyclines.

Because of the variation in antimicrobial susceptibility of clinical isolates from cattle and swine, antimicrobial therapy should be based on antimicrobial susceptibility testing of clinical isolates. Regardless, the *in vivo* response to antimicrobials is poor because of the capsulation of the abscesses and antibiotic-binding proteins in pus. There are no specific control measures to prevent *T. pyogenes* infections in domestic animals.

Immune responses to *T. pyogenes* are not well understood. There have been attempts to vaccinate mice, cattle, and sheep with bacterins or formalin-inactivated crude *T. pyogenes* supernatant. The results of such studies have not been very convincing. In mice, vaccination with purified recombinant PLO resulted in neutralizing antibodies, which protected mice against challenge with *T. pyogenes*. Bacterin in combination with leukotoxoid of *F. necrophorum* was once available commercially for the prevention liver abscesses in cattle. A vaccine formulation containing inactivated bacterial components and proteins of *F. necrophorum, E. coli*, and *T. pyogenes* that has been shown to prevent puerperal metritis leading to improved reproduction in dairy cows has been reported. Because *T. pyogenes* infections are sporadic, vaccination is not a viable approach.

Other Species of *Trueperella*

Trueperella abortisuis was first reported as an unidentified species of *Arcanobacterium* from necrotizing splenitis in a slaughtered pig in 2006, and then in 2009 as *A. abortisuis* from the placenta of a sow after abortion in Japan. The species has been isolated in swine from the vagina, cervix, kidney, and urine of pigs and from umbilical and anal swabs of aborted fetuses. The species is likely to be present in other animals, including cattle, dogs, and cats.

Trueperella bernardiae was reported as a human pathogen in case reports of urinary tract infections, skin and soft tissue infections, septic arthritis, prosthetic joint infections, necrotizing fasciitis, and chronic osteitis with co-infection with *S. aureus*. The species has been isolated from a case of enteritis in piglets along with *C. perfringens* and *E. coli*. The species has been isolated along with beta-hemolytic streptococci, *Corynebacterium auriscanis* and anaerobic coccus from a case of purulent thelitis in a dog.

Trueperella bialowiezense and *T. bonsai* have been isolated from the prepuce of bison bulls suffering from balanoposthitis.

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28

Bacillus

George C. Stewart

Members of the genus *Bacillus* are gram-positive, endospore-forming, aerobic or facultatively anaerobic rods that typically inhabit soil and water. They are ubiquitous in nature, and are commonly isolated from a wide variety of surfaces, soils, and animal by-products. Average numbers of *Bacillus* spp. present in soil are between 10^6 to 10^7 per gram of soil. During periods of nutrient deprivation, a *Bacillus* cell undergoes a process known as sporulation by which it forms a dense, resistant endospore. Endospores are resistant to heat, desiccation, ultraviolet and ionizing irradiation, disinfectants, and a variety of other environmental stresses. They can remain viable in soil and water for decades, awaiting nutrients, or in the case of pathogenic members of *Bacillus*, entry of spores into their respective hosts.

Three species are considered pathogenic. *Bacillus anthracis* is a zoonotic pathogen and the causative agent of anthrax. *Bacillus cereus* is a causal agent of food poisoning, and *Bacillus thuringiensis* is a lepidopteran insect pathogen. Genomic studies, including DNA–DNA hybridization, 16S and 23S rRNA sequence comparisons, multilocus sequence typing, and amplified fragment length polymorphism analysis, have revealed a high degree of relatedness among these organisms, leading to the proposal that *B. anthracis*, *B. cereus*, and *B. thuringiensis* may be viewed as a single species. Their virulence and host range are determined by their virulence gene-encoding plasmid content. For example, virulence of *B. anthracis* requires the presence of the pXO1 and pXO2 plasmids, required for production of the anthrax toxins and capsule, respectively.

Bacillus anthracis

Descriptive Features

Morphology and Staining

Cells of *B. anthracis* are gram-positive, nonmotile, roughly rectangular rods with square ends (about 1 μm by 3–5 μm; Figure 28.1). Chains of rods are common. Chain formation is thought to contribute to the bacterium's ability to resist opsonophagocytic killing by making it difficult for the host cells to phagocytose the large chain of bacteria. Spores are produced within the cells during conditions of nutrient depravation and cause no cell swelling. A capsule is formed by the vegetative bacterial cells *in vivo*, or in bicarbonate-supplemented culture media and an elevated CO_2 atmosphere *in vitro*.

Cellular Composition

B. anthracis has a gram-positive cell wall structure. Covering the cell wall is a proteinaceous paracrystalline structure referred to as the S layer. The S layer is composed of the glycoprotein Sap during the exponential phase of growth and the glycoprotein EA1 during the stationary and sporulation phases. Although the S layer has not been shown to be essential for virulence, S-layer-deficient mutant strains were reported to be more sensitive to binding of the C3 component of the complement, and a *sap* mutant exhibits some defects in cell division, affecting cell separation and producing longer chains of cells. Studies in mouse models of inhalational anthrax suggest that EA1, but not Sap, may prove useful as a component in a vaccine against anthrax.

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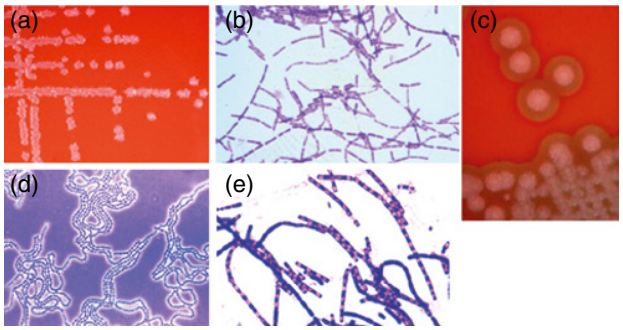


Figure 28.1 (a) *B. anthracis* colonies on a sheep blood agar plate; (b) Gram stain of *B. anthracis*; (c) *B. cereus* on a sheep blood agar plate with hemolysis evident; (d) string of pearls positive test with *B. anthracis*; (e) spore stain of *B. anthracis*.

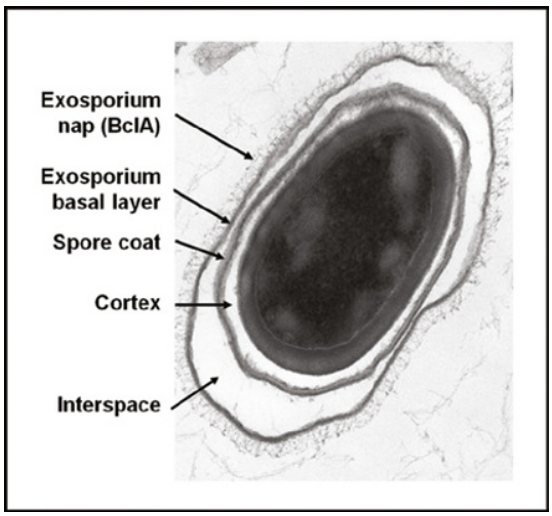


Figure 28.2 Transmission electron micrograph of a ruthenium red stained spore of *B. anthracis*. Ruthenium red enhances visualization of the BclA exosporium glycoprotein nap layer. Outer spore structures are labeled.

Spores are the infectious form of *B. anthracis*. They are immunogenic, and the addition of inactivated spores has been shown to increase the degree of immunity against highly virulent strains of *B. anthracis* in animal models of infection. The spores consist of multiple layers (Figure 28.2). The core is the inert, partially dehydrated bacterial cell. It is surrounded by the cortex, a layer of modified peptidoglycan. This is surrounded by the inner and outer coat layers, protein layers that confer many of the resistance properties on the spore. The outermost layer of the spore is the exosporium layer, which is separated from the coat by the interspace layer. The exosporium consists of a paracrystalline protein basal layer, and its outer surface is covered by filaments of the hair-like nap composed of the BclA collagen-like glycoprotein. Because the exosporium is the outermost structure of the spore, it is likely to play a major role in interactions with the environment and with the host immune system. The *B. anthracis* exosporium was reported to play a role in limiting access to inducers of cytokine responses *in vitro* in macrophages. The uptake of spores by macrophages has been shown to involve interactions between BclA and the integrin Mac-1 (CR3). Spores lacking the BclA glycoprotein do not specifically target these cells, but bind more generally to epithelial cells. Spores lacking BclA have been shown to bind to extracellular matrix components such as laminin and fibronectin. However, mutants lacking the BclA protein retain virulence in animal models of infection. The spore-surface BclA fibers may act to promote uptake by professional phagocytes and to inhibit nonspecific interactions between *B. anthracis* spores with nonprofessional phagocytic cells in the early stages of infection.

Cellular Products of Medical Interest

1) **Lethal toxin (LeTx)**. Two virulence-associated, pXO1-encoded toxins are made by *B. anthracis*. LeTx is a binary toxin composed of protective antigen (PA) and lethal factor (LF). PA is responsible for binding of LeTx to target cells, whereas LF is responsible for its toxic activity. PA and LF associate only after PA has bound to its receptor and then proteolytically processed by endopeptidases of the furin family. Processed PA forms a homoheptameric ring on the target cell surface. The PA can then serve as the binding component for the LF protein or the edema toxin. The toxin is endocytosed, and when the reduction of pH in the endosome occurs, conformational changes ensue and the PA forms a transmembrane channel to deliver LF into the cytosol. LF is a zinc metalloprotease that inactivates mitogen-activated protein kinase kinases (MAPK kinases) by cleaving off their N-terminal sequences, resulting in the disruption of signaling pathways within the affected cell and leading to inhibition of cell proliferation and ultimately to apoptosis of the affected cells. Macrophages from numerous species have been shown to react to LeTx exposure by undergoing apoptosis. LeTx derives its name for its lethal effects when injected into animal models including guinea pigs, rats, and mice. LeTx induces vascular collapse (pleural edema and rapid shock), and yet exposure to purified LeTx often results in no observable histopathology. Activity of the toxin on heart tissue is thought to lead to the pulmonary edema. In certain mouse strains, LeTx exposure results in rapid lysis of macrophages and production of inflammatory cytokines. However, other strains do not show this property. Macrophage

lysis is not a common feature in other animal models. LeTx exposure does result in apoptosis in a variety of cell types and a cell-death independent loss of barrier function in epithelial and endothelial cells *in vitro*. Production of LeTx is under control of the regulatory gene product, AtxA (for anthrax toxin activator), which responds to environmental cues. AtxA is also encoded on pXO1. LeTx production by cells cultured *in vitro* is upregulated by the presence of bicarbonate in the medium and an elevated CO_2 atmosphere in an AtxA-dependent manner, suggesting that this may be the cue recognized by AtxA. Lethal toxin is produced at levels five times greater than that of edema toxin.

2) **Edema toxin**. Edema factor (EF), the enzymatically active component of edema toxin (EdTx), is also encoded on pXO1. EdTx is a binary toxin composed of the PA receptor binding subunit and EF. PA delivers EF to the target cell's cytoplasm by the same mechanism as lethal toxin. EF is a calmodulin-dependent adenylate cyclase that increases levels of cAMP within the affected cell. This disrupts cell signaling pathways, resulting in cell-type-specific physiological changes. In animal models of infection, purified EdTx produces hemorrhagic lesions in multiple organs accompanied by hypotension and bradycardia. EdTx has not been shown to be cytotoxic for multiple cell types. It has been reported to suppress thrombin-induced platelet aggregation and clotting function. Production of EdTx is also under AtxA control.
3) **Capsule**. The vegetative form of *B. anthracis* produces a capsule composed of a polymer of poly-γ-D-glutamic acid. The genes encoding this structure are on the pXO2 plasmid. The expression of the genes encoding the capsule is under control of two regulatory gene products, AtxA and AcpA (for anthrax toxin activator and anthrax capsule activator, respectively), which respond to environmental cues. Mucoid colonies, indicative of capsule production, are formed in media containing bicarbonate when incubated in a CO_2-enriched atmosphere. Mucoid colonies are not produced on blood agar or other commonly used media in diagnostic laboratories. The capsule enables the bacterial cells to avoid phagocytosis. The Sterne vaccine strain is attenuated for virulence because it lacks the capsule-encoding pXO2 plasmid.
4) **Miscellaneous virulence-associated products**. Several homologues of proteins shown to be important in virulence of other microorganisms have been discovered in the DNA sequence of the genome of *B. anthracis*. These include genes encoding proteins important in survival within phagolysosomes and on mucosal surfaces (InhA and MprF), and in escape from phagolysosomes and phagocytic cells (anthrolysins).
   a) InhA. Immune inhibitor A (InhA) is a metalloprotease similar to that produced by *B. cereus*. It contributes to pathogenicity through several mechanisms, including the cleavage of antibacterial proteins, escape of bacteria from macrophages, control of blood coagulation, and degradation of matrix-associated proteins. InhA recently has been postulated to play a role in increasing blood–brain barrier permeability and contributing to cerebral hemorrhages.
   b) MprF. The *B. anthracis* genome includes the gene encoding the homologue of MprF (for multiple peptide resistance factor) of *Staphylococcus aureus*. It has been shown to confer resistance to defensins (found within the phagolysosome, and in secretions bathing mucosal surfaces) by lysinylation of phospholipids in the bacterial cell membrane.
   c) Anthrolysins. The genome of *B. anthracis* contains genes encoding anthrolysins (also referred to as anthralysins), three of which are phospholipase C homologues and anthrolysin O is a cholesterol-binding pore-forming cytolysin. Although each is inessential for virulence, loss of all four results in attenuation. It is hypothesized that these are important in escape of the organism from the phagolysosome following germination of spores in macrophages. Anthrolysin O is poorly expressed by *B. anthracis* (which is why *B. anthracis* is non-hemolytic). The reason is that its regulator, PlcR, is inactive owing to a truncation of its coding sequence. Recombinant anthrolysin O is lethal to human monocytes, neutrophils, macrophages, and lymphocytes.
   d) Nitric oxide synthase. *B. anthracis* bacilli and endospores exhibit nitric oxide synthase activity, which may protect germinating spores from nitric oxide production by host macrophages by using up available substrate pools of arginine.
5) **Regulation of the cellular products of medical interest**. Two proteins, AtxA and AcpA (for anthrax toxin activator and anthrax capsule activator, respectively), are produced in response to environmental cues. What these cues are *in vivo* is largely unknown. Under the appropriate conditions, however, AtxA increases the production of LeTx, EdTx, and proteins involved in the escape from phagolysosomes and macrophages. AcpA production is amplified *in vitro* under increased amounts of CO_2 (5% or greater). AtxA acts synergistically with AcpA in this regard. The genes encoding AtxA and AcpA are on the plasmids pXO1 and pXO2, respectively. The *B. anthracis* transcriptional regulator CodY senses the intracellular GTP and branched-chain

amino acid levels in the cells and induces appropriate changes in the transcriptome of the cell. Deletion of the *codY* gene attenuates virulence. CodY activates toxin gene expression by post-translationally regulating the accumulation of the AtxA protein, but does not affect levels of *atxA* mRNA. CodY is also required for heme utilization as an iron source.

Growth Characteristics

B. anthracis is a facultative anaerobe and grows on common media between 15 °C and 40 °C. Colonies reach a diameter of 2 mm or greater in 24 hours at 37 °C (Figure 28.1). Colonies grown in air have a dull surface and wavy margin formed by strands of bacterial chains (medusa-head appearance). Cells are nonencapsulated unless grown in greater than 5% carbon dioxide on media containing 0.7% bicarbonate, in which case the colonies are mucoid. No hemolytic zones are produced.

Sporulation occurs under conditions of nutrient depravation *in vitro*. The process is more efficient in the presence of oxygen, and does not occur inside a living animal host. Organisms in infected tissue or fluids exposed to air (such as rupturing of the carcass) sporulate after several hours.

Resistance

Although generally antibiotic sensitive, all sequenced strains of *B. anthracis* possess a latent β-lactamase gene, which is transcriptionally silent. Strains have been identified in which this gene is activated and inducible penicillin resistance is observed.

Vegetative cells in unopened carcasses may survive for up to one to two weeks, but spores can persist for decades in a stable, dry environment. Spores are killed by autoclaving (121 °C/15 minutes) and dry heat (150 °C/60 minutes), but not by boiling (100 °C) for less than 10 minutes. They are not highly susceptible to phenolic, alcoholic, and quaternary ammonium disinfectants. Aldehydes, oxidizing and chlorinating disinfectants, beta-propiolactone, and ethylene oxide are more useful. Spores are efficiently inactivated by exposure to 10% bleach. Heat fixation of smears does not kill spores.

Variability

The peracute nature of the infectious process and spore-based life cycle mean that *B. anthracis* replicates in infected hosts and does not appreciably replicate after the dormant spores are formed in the soil. Thus, these organisms have undergone far fewer replication cycles than other forms of pathogenic bacteria. This results in much less mutational alteration of their genomic sequences and very limited strain-to-strain variation. The genome is more homogeneous than is the case with other pathogens.

Ecology

Reservoir

The soil is the source of anthrax infection for herbivores. Other species, including humans, are exposed via infected animals and animal products.

Transmission

The spore is the infectious form. Infection usually takes place by ingestion of contaminated feed or water. Exposure via wound infection and arthropod bites can occur.

Human infections occur following exposure to spores on infected hides or other animal products, from soil, ingestion of contaminated meat, or exposure to infected animal blood or tissue.

Pathogenesis

Mechanisms. Spores are acquired from the environment (e.g. soil, animal products). They are phagocytosed by macrophages or dendritic cells (polymorphonuclear neutrophil leucocytes do not appear to play a prominent role in the disease process). The spores germinate within the phagolysosome compartment. Vegetative bacteria escape from the phagolysosome and later from the phagocytic cell. During the intracellular replication process, the phagocytic cells traffic to the regional lymph nodes. Release of the bacteria permits the organisms to access the bloodstream.

Pathology. In tissue, spores germinate and the vegetative form proliferates, producing gelatinous edema. Inflammatory reactions are minimal. Infection disseminates to reticuloendothelial sites. When these are saturated, a terminal bacteremia occurs, with enormous numbers of organisms in circulation. There are no consistent pathognomonic lesions, and considerable similarities are seen to other infectious and toxic causes of acute death. Postmortem findings are widespread hemorrhages; a black, engorged, friable spleen; tarry, nonclotting blood; and absence of rigor mortis. Bleeding at body orifices is common.

Experimental transmission of *B. anthracis* has revealed that cattle are resistant to spores introduced parenterally, but sensitive to sensitive to spores introduced by the oral route. The LD_{50} is likely to be $<10^7$ spores. With sheep, the LD_{50} is reported to be between 50 and 250 spores by the subcutaneous inoculation route. Swine are resistant to lethal infections by *B. anthracis* spores. Other species, including dogs, rabbits, and chickens, are highly resistant to anthrax infection as well.

Disease Pattern

Ruminants. The process described above is typical for the most susceptible species: cattle and sheep. The course, following an incubation period of one to five days, ranges from a few hours to two days. Some animals die without overt clinical signs. Others develop high fever and agalactia, and they may abort. There is congestion of mucous membranes, hematuria, hemorrhagic diarrhea, and often-regional edema. These forms are regularly fatal. Occasional animals show just localized edema or an ulcerative skin lesion and recover.

Horses. Anthrax in horses is usually acute, and clinical signs depend on the route of exposure. If ingested, clinical signs include loss of appetite, colic, enteritis, fever, trembling depression, and bloody diarrhea. Swelling often occurs around the neck and may be so severe that asphyxiation is possible. Edema in the chest or lower abdomen may occur. Death usually ensues within two to three days of the first signs.

Swine. In swine, localization in pharyngeal tissues is typical. An ulcerative lesion at the portal of entry is associated with regional lymphadenitis. Obstructive edema may cause death. Ulcerative hemorrhagic enteritis and mesenteric lymphadenitis sometimes occur.

Carnivores. Carnivores (including mink) are rarely affected; when they are, the disease pattern is similar to that in swine, although massive exposure through tainted meat may trigger septicemia.

Humans. Distinct disease forms arise depending the route of spore entry into the patient. Exposure through skin wounds or abrasions results in cutaneous anthrax, which constitutes 95% of naturally occurring human infections. The hallmark lesion is the malignant pustule, a local ulcerative inflammatory lesion covered by a black scab (eschar) (Figure 28.3). Possible complications are subcutaneous edema and septicemia. The case fatality rate is 10% to 20%. Inhalation of spores leads to inhalational anthrax or wool-sorter's disease, which is almost universally fatal if untreated and 75% to over 90% lethal even with antibiotic therapy. The poor prognosis is due to the initial nondescript influenza-like symptoms, which delays correct diagnosis, and the rapid time course of the infection. Radiographic evidence of mediastinal widening is characteristic. Pulmonary edema, hemorrhagic pneumonia, and meningitis have been described in affected patients. The lungs serve as the portal of entry of the spores, but the disease is not manifest as a pneumonia until late in the infectious process. Inhalational anthrax is the form of human disease of concern from a bioterrorism standpoint. Ingestion of *B. anthracis*–contaminated meat results in gastrointestinal anthrax, in which the portal of entry is the gastrointestinal tract. Bacterial proliferation occurs within the gastrointestinal tract and results in ulceration of the mucosa. This leads to bacteremia and toxemia. Clinical signs include vomiting, diarrhea, shock, and death, with the untreated mortality rate being 25–75%. This form of anthrax is rarely seen in the United States. A more recently identified form of human anthrax is injectional anthrax. It was first identified in 2009 in heroin-injecting drug users in northern Europe. The source of the infection was *B. anthracis*–contaminated drug. The disease was associated with a 37% case fatality rate.

Epidemiology

A soil rich in calcium and nitrate, with a pH range of 5.0–8.0, favors sporulation and bacterial proliferation at temperatures above 15.5 °C (60 °F), especially after flooding. The geography and seasonality of outbreaks reflect such circumstances. In cattle, sheep, and possibly horses, outbreaks begin with a few cases contracted from the soil. After excretions and postmortem discharges seed the area, secondary cases occur. Floods and industrial effluents from rendering works, tanneries, carpet mills, brush factories, or wherever else carcasses are salvaged may contaminate

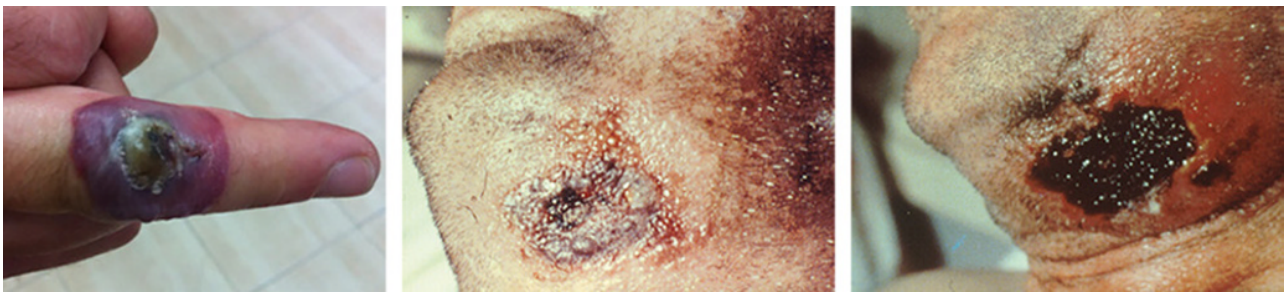


Figure 28.3 The malignant pustule lesion of human cases of cutaneous anthrax. Spores enter through abrasions in the skin, usually on the hands (left panel) or around the head and neck from self-inoculation of spores from the hands. The lesion consists of a blue-black edema fluid filled central area surrounded by a zone of induration and erythema. This form of anthrax does not usually progress to bacteremia (at least not rapidly), responds well to antibiotic treatment, and has a relatively low mortality rate. The center image shows the characteristic lesion. The right-hand image is the same patient at a later time point in the absence of antibiotic therapy. It is thought that a neutrophil response provides the protection against more invasive disease.

areas. Bone meal, an animal feed supplement, is a common vehicle in nonendemic areas. Carnivores (mink) are usually exposed via infected meat.

Human exposures are contracted in occupations dealing with animals and animal-derived material such as imported hides, wool, and bone. Anthrax occurring under industrial conditions is often the lethal airborne version. Non-industrial exposures have recently been associated with products such as decorative hide drums. Cutaneous anthrax infections are the usual form of non-industrial exposure.

Immunologic Aspects

Hyperimmune sera can prevent and alleviate disease. Antibacterial and antitoxic factors are thought to be involved. In most species, immunity is directed against the PA. Capsular polypeptide fails to stimulate protective antibody.

Immunization of livestock has utilized mostly modified live spore vaccines. Currently these are derived from avirulent (noncapsulated) mutants. The most widely used is the Sterne vaccine (a strain of *B. anthracis* that lacks the pXO2 plasmid). A cell-free vaccine consisting of concentrated culture filtrate from a pXO2-negative strain of *B. anthracis* has been used on humans exposed to industrial anthrax, researchers working with *B. anthracis*, or victims of suspected bioterrorism events involving *B. anthracis*. Five intramuscular injections over an 18-month schedule followed by an annual booster are recommended. Recombinant PA-based vaccines have shown promise, but are not currently available.

Laboratory Diagnosis

Sample Collection

During sample collection, precautions against contamination of the environment are important. Blood may be aspirated from a superficial vessel. Aqueous humor has the added advantage of remoteness from sources of early postmortem contamination. For direct examination, bloody discharges from orifices are sampled.

If the carcass has been opened, spleen material may be collected.

B. anthracis is categorized as a Tier 1 Select Agent and once identified is subject to strict regulations for possession or transport. Only registered and approved individuals can possess or work with Select Agent pathogens or toxins, and this must be done in Biosafety Level Three facilities. The regulations are available on the Center for Disease Control and Prevention website (http://www.cdc.gov/phpr/dsat.htm and https://www.selectagents.gov/sat/list.htm).

Direct Examination

Blood and organ smears are stained by Gram stain and a capsule stain such as McFadyean's methylene blue. Chains of encapsulated, gram-positive, non-spore-forming rods suggest *B. anthracis*. Contaminant *Bacillus* species are usually not encapsulated and lack the clipped, squared-off appearance of anthrax bacilli. Fluorescent antibody helps in the differentiation.

Isolation and Identification

B. anthracis grows on common laboratory media. No hemolytic zones are evident around the colonies, although this feature is true of certain other *Bacillus* contaminants, and the "string of pearls test" (the characteristic cell rounding that occurs when *B. anthracis* is exposed to penicillin producing a chain of spherical cells as the cell walls become weakened [Figure 28.1]). Definitive identification is by sensitivity to the gamma bacteriophage or use of bioluminescent indicator phage.

Experimental animals (mice, guinea pigs) are injected subcutaneously with suspect material. Death from anthrax occurs after 24 hours. Lesions include hemorrhages, gelatinous exudate near the inoculation site, and an engorged spleen. The encapsulated agent is demonstrable in blood and tissue. This requires Biosafety Level Three laboratory facilities.

Immunodiagnosis

B. anthracis antigens can be demonstrated in extracts of contaminated products by a precipitation test using antiserum prepared in rabbits by subcutaneous immunization with the Sterne strain (Ascoli test). The test lacks high specificity, in that the thermostable antigens of *B. anthracis* are shared by other *Bacillus* spp., and is dependent on the probability that only *B. anthracis* would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction.

Molecular Techniques

PCR assays targeting pXO1, pXO2, and chromosome-specific gene targets have been developed.

Treatment, Prevention, and Control

B. anthracis does not normally exhibit antibiotic resistance, although resistance to penicillin can emerge, as described above. Treatment should continue for at least five days. In some areas, antiserum is given simultaneously. Antiserum is not available in the United States. In acute anthrax, antimicrobial treatment is often unsuccessful.

Populations at risk are vaccinated annually.

When an outbreak or a case of anthrax has occurred, animal health authorities are notified to supervise control

measures. Carcass disposal involves incineration (preferred) or deep burial (>6.5 ft) under a layer of quicklime (anhydrous calcium oxide). Surviving sick animals are isolated and treated. Susceptible livestock are vaccinated. The premises are quarantined for three weeks subsequent to the last established case. Milk from infected animals is discarded under appropriate precautions. Barns and fences are disinfected with lye (10% sodium hydroxide). Boiling for 30 minutes will kill spores on utensils. Surface soil is cleared of spores by treatment with 3% peracetic acid solution at the rate of 8 l (2 gal) per square meter. Some other material can be gas-sterilized with ethylene oxide.

Prevention of anthrax exposure through animal products imported from endemic areas requires disinfection of such material as hair and wool by formaldehyde. Bone meal is sterilized by dry heat (150 °C/3 hours) or steam (115 °C/15 minutes).

Bacillus cereus

B. cereus is a spore-former commonly found in soil and produces cells and colonies morphologically similar to those of *B. anthracis*. *B. cereus* cells tend to be motile and produce colonies with zones of complete hemolysis on sheep blood agar plates. Capsule formation is a variable trait in this species. Resistance to beta lactam antibiotics occurs frequently.

Strains of *B. cereus* vary in their virulence potential to the extent that some strains have been included in probiotics for human consumption and others cause opportunistic infections in animals or people. Virulence factors encoded on plasmids gives rise to the different virotypes. Examples of infections include abortions and bovine mastitis. The latter is often acutely gangrenous and rapidly fatal or destructive to entire quarters. Frequent initiators are udder surgery and intramammary medications. There was a recent report of an uncommon epizootic outbreak of *B. cereus* that caused the sudden death of 12 psittacines belonging to the species *Anodorhynchus hyacinthinus*, *Diopsittaca nobilis*, *Ara severa*, and *Ara ararauna* in a Brazilian zoo. Postmortem examination revealed extensive areas of lung hemorrhage, hepatic congestion, hemorrhagic enteritis, and cardiac congestion. Histopathological examination of the organs and PCR analysis showed the presence of *B. cereus*.

B. cereus is also responsible for several forms of human food poisoning manifested by diarrhea and/or vomiting, the former being associated with various foods, the latter mostly with rice. Toxins are involved: an emetic toxin (cereulide) and three secretory enterotoxins (hemolysin BL, nonhemolytic enterotoxin, and cytotoxin K). Spores survive cooking and are kept at temperatures that permit bacterial replication and toxin production.

B. cereus is responsible for endophthalmitis, usually following traumatic eye injury. Spores introduced into the vitreous germinate and replicate. Severe pain accompanies a rapid decline in visual acuity, exacerbated by corneal ring abscess formation, periorbital edema, and proptosis. Systemic symptoms include fever, leukocytosis, and general malaise. Several clinical outcomes include the loss of all light perception and the need for enucleation or evisceration.

Since 2004, there have been reports of atypical *B. cereus* strains causing anthrax-like disease (severe hemorrhage in the lungs, intestines and other internal organs, and characteristic edema and damage to the lungs) in humans and other mammals. There are two variants of *B. cereus* that cause this anthrax-like disease, the so-called atypical strains (such as G9241) and *B. cereus* biovar *anthracis* (Bcbva) strains. Bcbva strains have been responsible for animal infections (gorillas, chimpanzees, zebras, monkeys, elephants, and various livestock), and the atypical strains caused human infections. A risk factor for human infections is being a welder. These have been predisposed to *B. cereus* G9241 infections due to work-related physical lung injuries due to the inhalation of hot or toxic fumes. Human cases (atypical strains) have been reported from the United States and animal cases (Bcva strains) from Africa. The anthrax-like strains are endowed with two virulence plasmids: (i) pBCXO1, which harbors the anthrax toxin genes (PA, LF, and EF), the regulatory protein AtxA, and the genes responsible for the synthesis of hyaluronic acid capsule, and (ii) pBC210 (in the atypical strains) or pBCXO2 (in Bcbva strains) that harbor genes for the *B. cereus* exopolysaccharide (BPS) or the polyglutamate capsule, respectively. *B. anthracis* does not make the polysaccharide capsule due to a frame-shift mutation in the pXO1 plasmid. *B. cereus* atypical strains form a large capsule from two polysaccharides, BPS and hyaluronic acid. Each capsular structure contributes to the pathogenesis of *B. cereus* G9241 anthrax-like disease in a mouse model of infection. Mutants lacking both are highly sensitive to phagocytic killing by macrophages and are unable to cause anthrax disease in animal challenge experiments. Atypical *B. cereus* strains with a missing or incomplete pBC210 plasmid have been associated with fatal inhalational anthrax-like disease and characteristic cutaneous anthrax-like disease, suggesting that the BPS capsule is not required for virulence in humans. No human clinical isolates have been obtained that fail to produce the hyaluronic acid capsule, suggesting it is required for virulence.

The emerging *B. cereus* strains possess other potential virulence factors not found in *B. anthracis*. For example, the pBC210 plasmid encodes a mono-ADP-ribosyltransferase that has been designated certhrax toxin. The target for

certhrax is vinculin, part of the cytoskeletal complex and involved in focal adhesion. However, a role for certhrax in virulence is currently uncertain.

Bacillus subtilis

Prior to the 1950s, aerobic spore-forming bacilli obtained from soil were frequently identified as *Bacillus subtilis*. As such, many of the early infections associated with *B. subtilis* most likely resulted from infections by other pathogenic *Bacillus* members. *B. subtilis* is a common in soils and thus a common contaminate of clinical samples. Rare cases of *B. subtilis* isolation from cases of bovine and ovine abortions as well as in mastitis have been reported, but *B. subtilis* has never been determined to be the actual causative agent. In humans, rare infections with *B. subtilis* have led to bacteremia, endocarditis, pneumonia, and septicemia, but each infection was associated with an immunocompromised individual.

B. subtilis spores have been studied as a potential immune stimulator following oral administration in pigs. Potentiating effectors were thought to result from upregulation of memory $CD4^+/CD8^+$ T cells in a porcine study. Oral administration of *B. subtilis* spores have been reported to provide protection against challenge by enteric pathogens such as *Salmonella enteritidis* and *Clostridium perfringens* in chickens in a number of studies. Thus, *B. subtilis* spores may be effective adjunct control agents in the management of infectious diseases.

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Corynebacterium

Tiruvoor G. Nagaraja

The genus *Corynebacterium* (from the Greek word *koryne*, meaning "club") includes species that are pleomorphic, non-spore forming, nonmotile gram-positive rods. Microscopically, the shape ranges from coccoid to rods, with some appearing club shaped, and have characteristic cell arrangements described as a "V," stacked in packets of parallel (called "palisades") or crisscrossing cells that appear like "Chinese letters." The shape and arrangement are called "diphtheroid" after the type species *Corynebacterium diphtheriae*, the most well-known species of the genus and a human pathogen that causes diphtheria in children and young adults. Corynebacterial cells also contain inclusion bodies, called metachromatic granules, which are composed of inorganic polyphosphates that can be visualized by staining with methylene blue.

Corynebacterium has some similarity with the genera *Mycobacterium* and *Nocardia* in that they have a high guanine and cytosine content and have specific cell wall organization composed of peptidoglycan, arabinogalactan, and mycolic acids. The chain lengths of mycolic acids in *Corynebacterium* are considerably shorter (28–40 carbons) than those of *Mycobacterium* (60–90 carbons) and *Nocardia* (40–56 carbons), and are usually saturated or have a single double bond.

Species of the genus *Corynebacterium* constitute a very heterogenous group and have been isolated from a variety of habitats, such as soil, water, plants, and animal surfaces. The genus contains as many as 110 species that are of medical and veterinary importance and have industrial applications (*Corynebacterium gluatmicum* in the amino acid fermentation industry). The species of medical and veterinary importance include a number of zoonotic and nosocomial human pathogens. Among the species that cause human infections, two exotoxin-producing species are the most important: *C. diphtheriae* and *Corynebacterium ulcerans*. The exotoxin produced by these two species is diphtheria toxin, a classic A-B toxin and a major virulence factor responsible for the respiratory and cutaneous forms of diphtheria, characterized by pharyngitis, skin ulceration, and the formation of an inflammatory pseudomembrane. The toxin is encoded by the *tox* gene carried on a prophage, and cytotoxicity is due to protein synthesis inhibition. Diphtheria in children caused by *C. diphtheriae* has practically disappeared in the developed countries because of the use of an effective vaccine targeting the diphtheria toxin. Diphtheria toxin is also produced by lysogenized strains of *C. ulcerans* and *Corynebacterium pseudotuberculosis*, and their infections in humans are more often of zoonotic origin. Most human diphtheria cases in developed countries are caused by *C. ulcerans,* which has become an emerging pathogen. The major species associated with diseases in animals include *C. pseudotuberculosis*, which causes abscesses in ruminants and horses, and the *Corynebacterium renale* group, composed of three species (*renale, cystitidis,* and *pilosum*) that cause urinary tract and genital organs infections in ruminants. Other *Corynebacterium* species associated with sporadic animal diseases include *Corynebacterium amycolatum, Corynebacterium aquilae, Corynebacterium auriscanis, Corynebacterium belfantii, Corynebacterium bovis, Corynebacterium camporealensis, Corynebacterium canis,* and *Corynebacterium rouxii*.

Also included in this chapter is *Actinobaculum suis,* the cause of urinary tract infection of sows. At one time, the species was in the genus *Corynebacterium*.

C. pseudotuberculosis

The species, with a worldwide distribution, is a major animal pathogen for sheep, goats, and horses and occasionally a zoonotic pathogen in humans. The species causes caseous lymphadenitis (CLA) in sheep and goats, and ulcerative lymphangitis and abscesses in the ventral thoracic region (pigeon fever) and ventral abdomen in horses. The other animals affected are cattle, buffaloes, swine, and camels.

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Morphology, Growth Characteristics, and Biochemical Activities

C. pseudotuberculosis (Figure 29.1) is a typical diphtheroid that exhibits pleomorphic forms ranging from coccoid to filamentous and from 0.5 µm to >3.0 µm in length. They are nonencapsulated; nonmotile; often contain metachromatic granules, particularly in rods; and are fimbriated. *C. pseudotuberculosis* is a facultative anaerobe that grows best on media containing blood or serum at a pH of 7.0–7.2. At 48 hours on blood agar (35–37 °C), colonies are off-white, dull, faintly *β*-hemolytic, and about 1 mm in diameter. Because of the waxy cell wall, the colony can be pushed across the agar surface without disintegrating (described as pushing a "hockey puck"), and disperse poorly in liquids. The organism is catalase positive and urease positive, and all strains produce acid but not gas from glucose, fructose, maltose, mannose, and sucrose.

Biotypes

The species is distinguished into two biotypes (or biovars), ovis and equi, based on the biochemical activity of nitrate reduction. Ovis is nitrate reduction negative, and equi is nitrate reduction positive. A PCR assay for nitrate reductase gene can be used to identify the biotype. In sheep and goats, disease is caused by the ovis biotype, and in horses and buffaloes, the biotype is equi.

Virulence Factors

The organism is a facultatively intracellular pathogen. The two virulence factors that play a major role in the pathogenesis are phospholipase D (PLD) and the mycolic acids of the cell wall. Both enhance the organism's ability to survive phagocytosis and multiply within macrophages. The waxy coat also contributes to the organism's high resistance to environmental conditions. Other factors contributing virulence may be the iron acquisition system and a secreted 40 kDa protease enzyme. Some strains of *C. pseudotuberculosis* produce diphtheria toxin by acquiring lysogenic β-phage carrying the *tox* gene.

Phospholipase D (PLD)

The secreted enzyme or exotoxin, encoded by the *pld* gene, hydrolyses ester bonds of sphingomyelin in host cell membranes to produce ceramide phosphate and choline. The damage or destruction of host cell membranes contributes to the spread of infection. The other biological activities of PLD include increased vascular endothelial cell membrane permeability, lysis of erythrocytes of sheep and cattle, synergistic lysis of erythrocytes in the presence of an extracellular *Rhodococcus equi* factor (Figure 29.2), inhibition of staphylococcal beta hemolysin-induced lysis of erythrocytes (reverse CAMP test; Figure 29.3), inhibition of *α*-toxin of *Clostridium perfringens*, dermonecrosis, and lethality to a number of laboratory animals. The PLD assists in the establishment of the organism at the initial site of infection by impairing the chemotaxis of neutrophils and decreasing the chance for phagocytosis. The importance of PLD as a major virulence factor is supported by several pieces of experimental evidence. A mutant that lacks the *pld* gene is incapable of inducing the classic lymph node abscesses in sheep, and antitoxin to PLD limits the spread of infection.

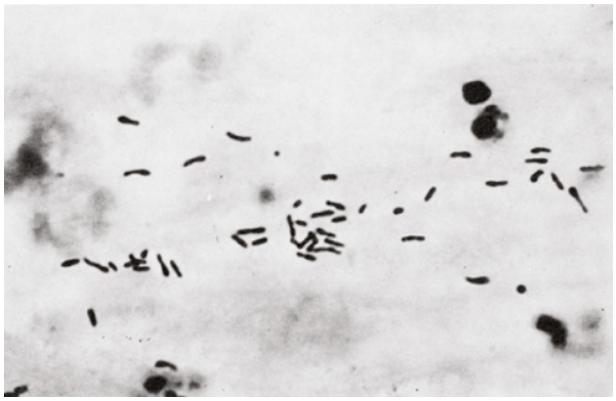


Figure 29.1 Corynebacterium pseudotuberculosis in pus from an equine chest abscess. Note club shapes, palisading, "Chinese letter" patterns, and pleomorphism (Gram stain, 1000×).

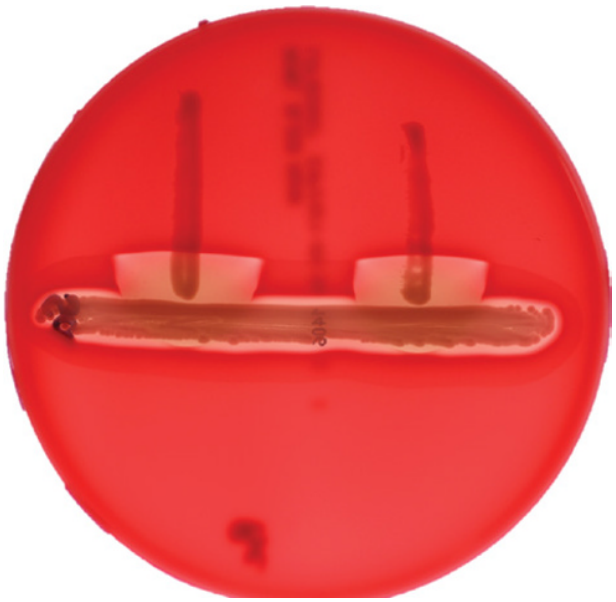


Figure 29.2 Synergistic hemolysis of *Corynebacterium pseudotuberculosis* with *Rhodococcus equi*.

Mycolic Acids

The waxy mycolic acid coat on the cell wall surface has well-established cytotoxic activities and is a major factor contributing to pathogenesis. The toxicity of extracted lipid material has been demonstrated by the induction of hemorrhagic necrosis by subcutaneous injection in mice and guinea pigs. The waxy coat provides the organism with physical, and possibly biochemical, protection against lysosomal enzymes within phagocytic cells. The protection allows the organism to survive as a facultative intracellular pathogen and to facilitate spread of the organism from the site of entry to the eventual site of abscess formation. In fact, the toxicity of mycolic acid is a contributing factor in the formation of abscesses. There is direct relationship between the amount of mycolic acid in isolates and their ability to induce abscesses in mice.

Iron Acquisition

An iron uptake gene operon, downstream from the *pld* gene, designated the Fe acquisition gene operon, has been shown to play a role in virulence. The operon has four genes, *fagA*, *fagB*, *fagC*, and *fagD,* that encode for proteins involved with iron uptake. Mutants lacking these genes exhibit reduced virulence compared to wild type in a goat model of caseous lymphadenitis.

Protease

A secreted protease of 40 kDa (CP40) has been shown to be a protective antigen. The antigen was identified by probing whole cell antigens of *C. pseudotuberculosis* with antibodies from antibody secreting cells obtained from induced infections in sheep. The CP40 antigen was highly protective against experimentally induced CLA. The role of CP40 in virulence has not been studied.

Ecology

Reservoir

The ovis biotype *C. pseudotuberculosis* is found in the gastrointestinal tract of normal sheep and in the soil of sheep pens. The reservoir of the equine type is not known.

Transmission

The entry of infection in a disease-free flock is generally via the introduction of a clinically or subclinically infected carrier animal. Sheep become infected through breaks in the skin (e.g. wounds acquired during shearing, castration, ear tagging, and docking), goats probably following trauma related to butting, and horses following breaks in the skin (e.g. biting flies). Not infrequently, sheep can cough up bacteria onto skin cuts of other sheep. The organism has the ability to survive for several weeks in the environment because of cell wall lipids, which contributes to the spread within a herd or flock. In cattle, houseflies have been shown to mechanically transmit the organism.

Pathogenesis

Mechanisms

C. pseudotuberculosis is a facultatively intracellular pathogen, and its survival within phagocytes is because of the protection afforded by cell wall mycolic acids. PLD increases the permeability of vascular endothelial cells, activates the complementary pathway of the innate immune system, and impairs chemotaxis of neutrophils. Following infection, abscesses form but remain localized when exotoxin and/or protease are absent or neutralized.

Pathology

Neutrophilic infiltration and endothelial damage characterize the early changes. The lesions are abscesses. Distribution, progress, and appearance differ with species and routes of inoculation, but lymphatic involvement is a consistent feature. The nature of the pus varies largely with the age of the lesion, which grossly appears creamy to dry and crumbly ("cheesy"). Old abscesses consist of dead macrophages with peripheral neutrophils, giant cells, and fibrous tissue. Lesions almost always contain *C. pseudotuberculosis*.

Disease Patterns

Sheep and Goats

C. pseudotuberculosis causes caseous lymphadenitis, a chronic and contagious disease of sheep and goats in North America, Europe, Australia, and New Zealand. The disease causes significant economic losses to sheep and goat producers, particularly in countries such as Australia and New Zealand where sheep are a dominant livestock. The economic losses are because of the reduction in wool, meat, and milk production, decreased reproductive efficiencies of affected animals, and condemnation of skins in abattoirs. The disease is characterized by abscess formation in the skin, internal and external lymph nodes, and internal organs. The organism most commonly enters the body through breaks in the skin or mucous membranes. After entry, the organism elicits a diffuse inflammation, followed by the formation of an abscess that coalesces and undergoes encapsulation. Inflammatory cells traverse the capsule peripherally, adding a layer of suppuration and a new capsule. Several such cycles give the lesions, especially in sheep, an "onion ring" appearance. Old lesions acquire thick fibrous capsules. There are two forms of the disease: (i) an "external form" characterized by infection, at the site of entry, of the subcutaneous tissue and the regional lymph nodes (parotid, mandibular, superficial cervical, subiliac,

popliteal, and mammary) and (ii) an "internal form" characterized by abscess development in internal organs such as the lung, liver, kidneys, uterus, spleen, and internal organs (mediastinal, bronchial, and lumbar). Both forms can occur in the same animal. The internal form is subclinical and is commonly associated with progressive weight loss and ill thrift, a condition called thin ewe syndrome. Most forms of infection are chronic. The visceral form is more common in sheep, and the external abscess is more common in goats. Abscesses occur most commonly in the external lymph nodes of the head and neck in goats and in the subiliac and superficial cervical lymph nodes of sheep. Differences in abscess content have also been described between sheep and goats. In sheep, the content has a laminar form when cut that is caused by the concentric layers of fibrous tissue. In goats, the content consists of thick pasty material

Equine

C. pseudotuberculosis infections in horses can occur in three forms: ulcerative lymphangitis, external abscessation, and internal abscessation. The external form is the most common. In the case of ulcerative lymphangitis, which is reported rarely, infection is cellulitis that ascends the lymphatics, usually of the hind limbs, starting at the fetlock. Its progress toward the inguinal region is marked by swelling and abscesses, which rupture leaving ulcers along its course. Hematogenous dissemination is rare. These horses are usually febrile, and exhibit pain and lameness of the affected limb. In the external abscess form, generally a single, often large abscess occurs underneath the abdomen or the pectoral region. The abscess tends to have a thick (few centimeters) capsule filled with a thick yellow to tan pus. The terms "pigeon fever" and "breast bone fever" are often used for the pectoral abscess because of its pigeon breast appearance. The internal abscesses are difficult to recognize, and the most common symptoms are weight loss, fever, depression, and colic. The most common organ to have an internal abscess is the liver, but abscess can also occur on the kidneys, lungs, and spleen. Septicemia occurs rarely, but may result in abortions, renal abscesses, debilitation, and death. The superficial lesions resolve slowly after drainage. Environmental conditions that may contributed to the incidence include dry season, which promotes survival of the organism in soil. In addition, studies have shown transmission of the pathogen by houseflies, stable flies, and horn flies.

Cattle

Cattle occasionally develop skin infections with lymph node involvement. Such episodes are often acute and can be epidemic. The most common site is the lateral body wall, suggesting that trauma initiates the disease by producing breaks in the skin.

Humans

Human infections with *C. pseudotuberculosis* occur rarely, and many of the reported cases are related to occupational exposure (e.g. shepherds, sheep shearers, abattoir workers, butchers). Human infections tend to be chronic, and infected humans generally have lymphadenitis affecting the axillary, inguinal, and cervical lymph nodes and abscesses. Certain strains that carry the prophage with *tox* gene and produce diphtheria toxin can cause the classic diphtheria in humans.

Laboratory Diagnosis

Although the presence of external abscesses in sheep and goats is highly suggestive of caseous lymphadenitis, a bacteriologic culture is needed to confirm and rule out other abscess-causing pathogens such as *Trueperella pyogenes* and *Pseudomonas aeruginosa*. Intracellular or extracellular diphtheroid-shaped organisms may be demonstrable in stained direct smears of material from lesions (see Figure 29.1). Inhibition of staphylococcal *β*-toxin (see Figure 29.2) and synergistic hemolysis with *R. equi* (see Figure 29.3) are phenotypic tests used to confirm identification of *C. pseudotuberculosis*. A multiplex PCR assay targeting 16S rDNA, *rpoB* (codes for RNA polymerase β-subunit) and *pld* genes can be used to confirm the species and differentiate it from other corynebacterial species.

Animals with internal abscesses are difficult to diagnose, although animals with lung abscesses can be diagnosed by radiography and transtracheal aspiration and abscesses in other organs can be visualized by ultrasonography. The



Figure 29.3 Inhibition of hemolysis of *Staphylococcus aureus* with *Corynebacterium pseudotuberculosis* (reverse CAMP test).

synergistic hemolysin inhibition (SHI test) test to detect antibodies directed against the PLD exotoxin can be useful. High titers are highly correlated with internal abscesses. Several tests have been developed to detect anti-PLD antibodies, but most lack either sensitivity or specificity. The usefulness of many of those tests as diagnostic or culling tools is debatable. An ELISA test to detect gamma interferon (IFN-γ) as a marker for cell-mediated immunity has been developed and is reported to be effective in control and eradication programs.

Treatment and Control

The organism is susceptible to beta-lactams (including ceftiofur), erythromycin, chloramphenicol, lincomycin, tetracycline, enrofloxacin, and trimethoprim–sulfonamide. The organism is resistant to aminoglycosides. Generally, the caseous lymphadenitis abscesses are refractory to antibiotic therapy because of thick encapsulation. However, in sheep, goats and horses, long-term (four to six weeks) systemic antibiotic (penicillins or erythromycin, often in combination with lipid-soluble rifampin) treatment can be effective in treating internal abscesses and reducing the chances of recurrence of external abscesses.

Equine abscesses, if external, are handled surgically. Once the abscess is lanced and allowed to drain, the horse heals without complications. Prolonged penicillin therapy is used to prevent or treat ulcerative lymphangitis or the form with internal abscesses.

Vaccines

Bacterins, culture supernatants, or their combinations provide some protection from the disease. The efficacy of the culture supernatant is attributed to the protective effects of antibodies generated against the PLD exotoxin. Vaccination does not eliminate an established infection but prevents the onset of infection and inhibits the dissemination of an established infection. A vaccine composed of inactivated whole cell wall antigen and PLD toxoid is commercially available for use in sheep. The vaccine is given subcutaneously in sheep after three months of age followed by a booster dose in four weeks. Adult sheep are vaccinated annually. Most of the vaccines are generally combined with vaccines for other pathogens, particularly *Clostridium*. Currently, no vaccine is available for horses.

Control and preventive measures include using disinfectants, improving sanitary conditions in the farm or of the stables and beddings, isolating and segregating infected animals to minimize environmental contamination with purulent exudates, fly control, and proper wound management.

C. renale Group

The group consists of three species, *renale*, *pilosum*, and *cystitidis*. The species *renale,* as a bovine pathogen, has been known for over 100 years. Originally classified as serological types of *C. renale*, I, II, and III were later identified to include two new species. All three species colonize the lower urogenital tract of cattle and sometimes sheep. All three cause bovine cystitis and pyelonephritis, but *C. renale* is the most common of the three species and causes the more severe infection. In sheep, *C. renale* can occasionally cause cystitis and pyelonephritis and osteomyelitis in goats. More often, *C. renale* causes ulcerative balanoposthitis ("pizzle rot") in sheep and goats.

Morphology, Growth Characteristics, and Biochemical Activities

The species of the *C. renale* group are typical diphtheroids. The cells range from coccoid to filamentous, are non-acid-fast, nonencapsulated, and often contain granules. Members of the *C. renale* group are facultative anaerobes capable of growing on most common laboratory media as nonhemolytic, opaque, off-white colonies that develop within 48 hours at 37 °C on blood agar. Members of the *C. renale* group have potent urease activity, which is demonstrable in most strains within minutes of contact with urea. Glucose is slowly fermented, and other carbohydrates are fermented variably. All strains are catalase and urease positive. Members of the *C. renale* group produce an extracellular protein, renalin, which lyses erythrocytes in synergy with *β*-hemolysin of *Staphylococcus aureus* (positive CAMP test; see *Streptococcus agalactiae*; Chapter 26).

Virulence Factors

Fimbriae

All three species of the *C. renale* group are fimbriated. Strains of *C. renale* and *Corynebacterium cystitidis* possess a small number of fimbriae, whereas the species *Corynebacterium pilosum* possess a large number – frequently long and thick bundles – of fimbriae.

Urease

All three species of the *C. renale* group produce urease, which is considered to be an important virulence factor. In a rat model, the enzyme has been shown to play a crucial role in the establishment of pyelonephritis.

Cell Wall

The gram-positive cell wall contains peptidoglycan, teichoic acid, and lipids of medical interest. The lipoteichoic

acids and peptidoglycan of the gram-positive cell wall interact with macrophage cells, resulting in the release of pro-inflammatory cytokines.

Ecology

Reservoir

Members of the *C. renale* group inhabit the lower genital tract, particularly of the vulval region, of apparently healthy cows and the prepuce of steers and bulls, and small ruminants, particularly of rams and male goats. Occasionally they are implicated in urinary tract infections of sheep, horses, dogs, and nonhuman primates. No human infections have been reported.

Transmission

Organisms are transmitted between animals by direct and indirect contact. Many infections are probably endogenous. Organisms shed in the urine of an infected cow may survive outside and invade a new host, most likely by attaching to the epithelial cells of the vulva. They can survive in soil for a long time, and it is possible that pasture could be a source of infection or reinfection.

Pathogenesis

Infection begins with attachment of the bacteria to the epithelium of the lower urinary tract. Fimbriae-mediated attachments to epithelium of the urinary bladder and ammonia production from urea hydrolysis are considered critical in the pathogenesis.

Pathology

Severe hemorrhage, epithelial degeneration, and ulceration of the mucous membrane of the urinary bladder is a common feature. A chronic inflammatory process successively involves the bladder, ureter(s), renal pelvis, and renal parenchyma in pyelonephritis in cattle. A similar inflammatory response occurs in small ruminants, but is usually localized to the distal urethra.

Disease Patterns

Cattle

Bovine pyelonephritis is an ascending urinary tract infection, beginning with hemorrhagic cystitis, proceeding to ureteritis and pyelonephritis. Rectal palpation reveals thickened bladder and ureteral walls, distended ureters, and enlarged kidneys with obscured lobulations. Clinical signs include fever, anorexia, with an arched back. Urine is voided in small amounts at frequent intervals and contains large amounts of albumin, leukocytes, fibrin, and usually small blood clots. Chronic infections progress to debilitation and death due to uremia. *Corynebacterium cystitidis* causes severe hemorrhagic cystitis with ulceration of the bladder, also progressing to ureteritis and pyelonephritis. *C. pilosum* is less pathogenic, and infection results in mild cystitis and rarely ascends to cause pyelonephritis.

Sheep and Goats

Ovine posthitis ("pizzle rot" or "sheath rot"), the more common form of infection in sheep, is a necrotizing inflammation of the prepuce and adjacent tissues in wethers or rams. Disease develops in the presence of the urease-producing bacteria in an area constantly irrigated with urine. Ammonia is believed to initiate the inflammatory process. A similar condition occurs in goats. Only *C. renale* and *C. pilosum* have been found in ovine posthitis. Pizzle rot occurs typically in animals on high-protein diets (>16% of the diet) or grazing legume-rich pasture that is high in proteins, which increases urea excretion and when hydrolyzed by the bacterial urease produces ammonia to cause irritation, inflammation, and ulceration of the skin. Preputial swelling and urine retention in the sheath may be seen. Pooled urine and purulent exudates inside the prepuce may lead to necrosis. In ewes, the disease is ulcerative vulvovaginitis.

Epidemiology

Bovine pyelonephritis is found mostly in cows near parturition, appearing as an opportunistic infection by a commensal organism. Bulls are rarely affected, but are commensal hosts of all three species and the sole commensal source of *C. cystitidis*.

Laboratory Diagnosis

Gross examination of urine may reveal the presence of red blood cells and high alkalinity (pH 9.0). Microscopically, packets of pleomorphic gram-positive diphtheroid rods are seen (Figure 29.4). The agent is readily cultured from sediment. A generous inoculum of colonial growth planted in one spot on a urea agar slant will produce an alkaline shift, indicating urea hydrolysis, within minutes of inoculation. A diphtheroid isolate from urine capable of producing this reaction and fermenting glucose probably belongs to the *C. renale* group.

Treatment and Control

Members of the *C. renale* group are susceptible to penicillins, but antibiotic therapy is successful only in the early stage of the infection. Treatment is more effective in cows with cystitis alone than with cystitis and pyelonephritis.

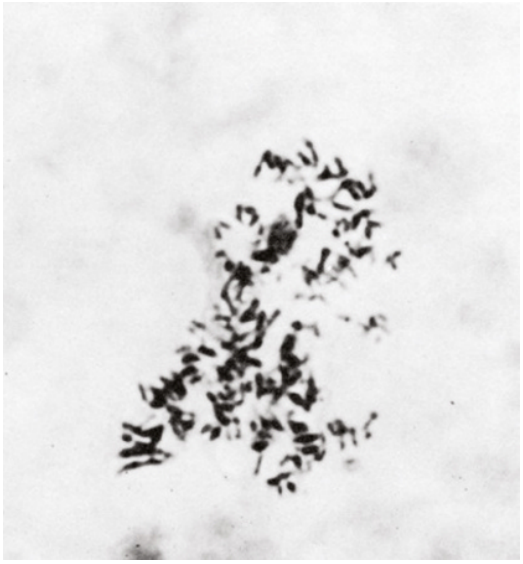


Figure 29.4 *Corynebacterium renale* in urinary sediment of a cow with pyelonephritis. Note "diphtheroid" configurations, including palisades and "Chinese letters" (Gram stain, 3000×).

Ovine posthitis is treated by surgical care of lesions, local antiseptic applications, dietary restriction, and testosterone administration.

Other Species of *Corynebacterium*

Corynebacterium ulcerans

The species is closely related to *C. diphtheriae*, and the very first reported isolation was from the throat of a human patient with diphtheria-like lesions. The species is a commensal of domestic and wild animals, and the range of hosts that may serve as a reservoir include cattle, goats, pigs, dogs, and cats. The species is a causative agent of several infections in a variety of animals, including mastitis in dairy cows, and in other animals, lymphadenitis in sheep and goats, nasal discharge in cats and dogs, and respiratory infection in macaques. In humans, it is most often involved in pharyngitis, which in some cases is linked to contaminated milk from cows, and contacts with pigs, dogs, and cats. Strains of *C. ulcerans* that are lysogenized with β-phage containing the *tox* gene produce diphtheria toxin and cause diphtheria-like disease in humans. The species is considered as an emerging human pathogen, and in some European countries, cases of respiratory diphtheria caused by *C. ulcerans* are more common than infections caused by *C. diphtheriae*. Besides diphtheria toxin, another exotoxin produced is phospholipase D, similar to *C. pseudotuberculosis*. In contrast to diphtheria toxin, the gene for PLD is present in the chromosome of all strains of *C. ulcerans*. PLD from *C. pseudotuberculosis* and *C. ulcerans* shares 80% homology in the nucleotide sequences and 87% homology in the amino acid sequences. Other potential virulence factors include urease, corynebacterial protease (CP40), neuraminidase, and iron scavenging systems.

Corynebacterium auriscanis

C. auriscanis is a typical diphtheroid isolated from various disease processes in dogs, mainly otitis externa and pyoderma. It has been isolated from the vagina of healthy dogs.

Corynebacterium rouxii

The species is reported cause purulent dermatitis in dogs.

Actinobaculum suis

The genus *Actinobaculum* was created to accommodate bacterial species previously named *Corynebacterium suis*, *Eubacterium suis*, or *Actinomyces suis* and some *Actinomyces*-like organisms isolated from human clinical samples. *A. suis* was initially placed in the genus *Corynebacterium* because of diphtheroid morphology. The human strains are now in a different genus called *Actinotignum* (for example, *Actinotignum schaalii*). Based on a systematic phylogenetic study of the genus *Actinomyces,* the species *suis* displayed 10–14% 16S rRNA sequence divergence with the *Actinomyces bovis* cluster of species, which necessitated creation of *Actinobaculum* genus. The members of the genus *Actinobaculum* have been isolated predominantly from veterinary clinical sources.

Morphology, Growth Characteristics, and Biochemical Activities

The organisms are slender, pleomorphic rods (1–3 × 0.5 μm), arranged singly, in pairs (often at an angle to each other or in palisades), or in small clusters. The cells stain gram positive, but old cultures are easily decolorized. It is not acid-fast, does not form spores, or has capsules. Colonies on blood agar plates are small, 0.5–3 mm in diameter after 48 hours of incubation. After one week, colonies are slightly larger (3–5 mm) and flatter; often show slightly raised centers that are denser than the rest of the colony, giving the colony a "fried-egg" appearance; and are nonhemolytic. The organism grows best at pH 7–8, but does not grow at pH 5 or below. It is anaerobic and has a strictly fermentative metabolism. Of the carbohydrates, only maltose, starch, and glycogen are fermented. The organism is urease positive but is negative for other common biochemical tests.

Virulence Factors

The organism is piliated and produces urease.

Transmission

The organism is a normal inhabitant of the preputial sac of the male pig. A high percentage of boars can be asymptomatic carriers. Therefore, the organism is sexually transmitted to sows.

Pathogenesis

The organism attaches to the epithelium of the urinary tract via pili. The ammonia production from ureolytic activity and subsequent elevation of urine pH may promote bacterial growth.

Disease Patterns

A. suis is a swine pathogen and causes cystitis and pyelonephritis in sows, producing acute or chronic renal failure. The disease occurs worldwide and is recognized not only in Britain and other European countries but also in North America, Australia, and Hong Kong. The disease is more prevalent in older sows housed in gestation stalls possibly because of increased chance for fecal contamination of the perineal region, lack of exercise, reduced water intake, and infrequent urination. Boars are frequent carriers in their preputial diverticulum, and occasionally, the organism may be associated with epididymitis. Urine stagnation promotes bacterial growth. Like bovine pyelonephritis, the disease is an apparently ascending infection. In the acute phase of the infection, the animal is anorexic and unwilling to rise. Pus or blood may be present in the urine. The urine is usually red-brown and has a foul odor. Mortality associated with untreated infections can be high, up to 100%. If the animal survives the infection, it can suffer weight loss and display polyuria and polydipsia. Typically, the sow becomes a repeat breeder with subsequent poor reproductive performance and is culled from the herd.

Diagnosis is generally based on clinical signs. Microscopic examination of purulent material from the urine or from bladder wall, ureter, and kidney pelvis will show a large number of organisms. The urine or other samples should be cultured under anaerobic conditions, and incubation should be for 48–72 hours to see colonies.

The organism is susceptible to gram-positive or broad-spectrum antibiotics (penicillins, cephalosporins, erythromycin, clindamycin, and tetracyclines). However, treatment is rarely successful.

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30

Erysipelothrix

Timothy Frana and Axel Neubauer

The genus *Erysipelothrix* consists of six species and 28 serotypes. Of veterinary relevance is primarily *Erysipelothrix rhusiopathiae,* which causes erysipelas, an important disease of swine and poultry and a sporadic disease of sheep and lambs. Clinical presentations include septicemia, arthritis, vegetative endocarditis, and generalized skin lesions. The organism is commonly isolated from alimentary and lymphoid tissues of healthy animals as well as the exterior slime layer on fish. *E. rhusiopathiae* can survive for long periods without replication in soil and marine environments. In humans *E. rhusiopathiae* causes erysipeloid, a generally self-limiting infection of the hands, which is considered an occupational disease of fish handlers, butchers, and veterinarians.

Some strains previously designated as serotypes of *E. rhusiopathiae* are now classified as *Erysipelothrix tonsillarum*, the other species of clinical relevance. *E. tonsillarum* is biochemically and morphologically similar to *E. rhusiopathiae* but is genetically distinct by DNA–DNA homology. *E. tonsillarum* causes occasional clinical disease in dogs and is nonpathogenic for swine.

Descriptive Features

Morphology and Staining

E. rhusiopathiae is a gram-positive, nonmotile, non-acid-fast, non-spore-forming bacillus that measures 0.2–0.4 μm by 0.8–2.5 μm in size. On subculture, rough colonies may develop and produce filamentous forms ≥60 μm in length.

Structure and Composition

E. rhusiopathiae exhibits a cell wall typical of gram-positive organisms. It contains murein of the B1 delta type. The diamino acid of cell wall peptidoglycan is lysine. The DNA base composition is 35–40 mol% G + C.

Cellular Products of Medical Interest

Spa Proteins

Surface protective antigens (Spa) were described in *E. rhusiopathiae*, but not in *E. tonsillarum*. The Spa proteins SpaA, SpaB1, SpaB2, and SpaC were associated with virulence and the induction of an antibody response during infection. SpA has, because of its presence in the clinically most relevant serotypes, been the subject of intense research and was shown to be a protective protein. SpaA shares similarities in the C-terminal region with choline-binding proteins of *Streptococcus pneumoniae*.

Adhesion

Two adhesive surface proteins (RspA and RspB) have been identified and shown a high degree of binding to fibronectin and type I and IV collagens. It is believed that these proteins participate in initiation of biofilm formation and therefore enhance virulence.

Capsule

E. rhusiopathiae produces a heat-labile polysaccharide capsule, which provides resistance to phagocytosis. Capsule-deficient *E. rhusiopathiae* mutant organisms were susceptible to phagocytosis in murine polymorphonuclear leukocytes and did not cause disease in mice.

Cell Wall

The cell wall of the members of this genus is typical of gram-positive bacteria. The lipoteichoic acids and peptidoglycan of the gram-positive cell wall interact with macrophage cells, resulting in the release of pro-inflammatory cytokines.

Neuraminidase

Neuraminidases are enzymes that cleave the terminal sialic acids from sialo-glycoconjugates on host cells, leading to a disruption in many functions as well as facilitating biofilm

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formation. Neuraminidase production varies between strains of *E. rhusiopathiae*, and virulence correlates directly with the amount of enzymes produced.

Hyaluronidase

It is thought that hyaluronidase destroys the polysaccharide matrix holding animal cells together, which allows spreading of a pathogen into tissues. Most strains of *E. rhusiopathiae* will produce hyaluronidase, but there does not appear to be a relationship between virulence and this enzyme.

Other Products

In addition, a 16 kDa hemolysin and 64–66 kDa antigen were identified. It was suggested that antibodies to the latter protein have protective activity.

Growth Characteristics

E. rhusiopathiae grows readily on most standard media. However, growth is enhanced in slightly alkaline media (pH of 7.2–7.6) with the addition of serum and glucose. *E. rhusiopathiae* is a facultative anaerobe preferring a reduced oxygen environment containing 5–10% CO_2. Optimal growth occurs in 24–48 hours at 30–37 °C; however, it is capable of growing over a temperature range of 5–42 °C and a pH range of 6.7–9.2.

Resistance

E. rhusiopathiae is resistant to drying and withstands salting, pickling, and smoking. It survives for up to six months in swine feces and fish slime at cool temperatures. It is killed by moist heat (55 °C) in 15 minutes but grows in the presence of potassium tellurite (0.05%), crystal violet (0.001%), phenol (0.2%), and sodium azide (0.1%). *E. rhusiopathiae* is generally susceptible to penicillin, cephalosporin, clindamycin, and fluoroquinolones, but is resistant to novobiocin, sulfonamides, and aminoglycosides. Resistance to oxytetracycline, erythromycin, lincomycin, ofloxacin, and enrofloxacin has been observed. Resistance is apparently not plasmid mediated.

Variability

Common heat-labile antigens account for cross-reactions between *E. rhusiopathiae* strains. Heat-stable somatic antigens account for the existence of at least 25 serotypes. No relationship between host species and serotype has been recognized. Serotypes 3, 7, 10, 14, 20, 22, and 23 exhibit a higher degree of DNA–DNA homology with the type strain of *E. tonsillarum* than with the type strain of *E. rhusiopathiae*. Cultures dissociate on passage from convex, circular, smooth colonies with entire edges to rough colonies with undulate edges. L-forms have been reported.

Ecology

Reservoir

E. rhusiopathiae is widely distributed in nature and is often recovered from sewage effluent, abattoirs, surface slime of fresh and saltwater fish, and soil. It has been recovered from over 50 species of mammals including swine, sheep, lambs, cattle, horses, dogs, cats, mice, rabbits, and various marine mammals. It has also been isolated from over 30 species of wild birds such as turkeys, chickens, geese, pheasants, and pigeons. *E. rhusiopathiae* can be isolated from the tonsils and gastrointestinal tracts of apparently healthy pigs, considered the most prominent reservoir.

Transmission

Transmission of *E. rhusiopathiae* among animals is mostly by ingestion of contaminated material (food, soil, water, and feces). Wound infections and arthropod bites are other possible routes.

Pathogenesis

Mechanisms

Strains of *E. rhusiopathiae* vary in virulence for reasons not fully understood. Neuraminidase is considered an important virulent factor, and strains that produce high levels of this factor in the logarithmic growth phase are more likely to result in acute septicemic infections versus strains that do not. Neuraminidase cleaves sialic acid present on cell surfaces, leading to vascular damage and hyaline thrombus formation. Neuraminidase also plays a role in bacterial attachment and invasion into cells. Antibodies to neuraminidase are protective against experimental infections in mice. In addition, the presence of capsules has been described and appears to play a role in resistance to phagocytosis by polymorphonuclear leukocytes. Survival inside professional phagocytes is important for pathogenicity. In the presence of normal serum, acapsular mutants do not survive inside phagocytes, whereas encapsulated organisms survive and multiply. In the presence of immune sera, opsonized bacteria are readily eliminated by macrophages and polymorphonuclear leukocytes.

Cutaneous lesions may be seen in animals during the acute septicemia phase or following recovery. These are due

to swelling of the endothelium leading to vasculitis, thrombosis, diapedesis, and fibrin deposition. Skin lesions may also appear with a non-systemic infection due to partial immunity of the host and attack from low-virulence strains.

Localization of *E. rhusiopathiae* in joints of swine leads to fibrinous exudation and pannus formation. Subsequent proliferative changes are primarily due to immunological responses including the formation of immune complexes, activation of complement, and the presence of neutrophils, which damages the auricular cartilage and synovial tissues. Joint deterioration becomes chronic and progresses for years. Although *E. rhusiopathiae* can no longer be cultured from joint fluid, the organism or bacterial antigens may persist. Valvular endocarditis is less common than joint involvement and is believed to be initiated by bacterial emboli and vascular inflammation, resulting in chronic changes and damage to the heart valves. Vegetative lesions can lead to valvular insufficiency and congestive heart failure or release emboli that may cause sudden death.

Pathology

Pigs dying from acute erysipelas infections exhibit hemorrhages of the gastric serosa, skeletal and cardiac muscles, and renal cortex. Congestion of lungs, liver, spleen, skin, and urinary bladder is frequent. Vascular damage with microthrombi is observed microscopically. A mononuclear infiltrate predominates in most cases. Raised, pink to purple cutaneous lesions result from vasculitis, thrombosis, and ischemia.

In joints, acute synovitis often proceeds to more chronic articular changes. Synovial membranes become hyperplastic and villous and are infiltrated with mononuclear cells. Spreading of granulation tissue over articular surfaces and erosion of articular cartilage may occur. Ankylosis of the joint may be the ultimate outcome. Localization to intervertebral disks leads to a destructive diskospondylitis.

In valvular endocarditis (Figure 30.1), the mitral valve is most commonly involved, with development of large, valvular vegetations due to fibrin deposition and connective tissue proliferation. Emboli may produce infarcts in the spleen, kidney, brain, and other internal organs.

In turkeys, the pathology associated with erysipelas infections is generally marked by congestion and intramuscular and subpleural hemorrhages, particularly affecting breast and leg muscles. Hemorrhages are also found in the mucosa of the gizzard and small intestine as well as serosal surfaces of the heart. The liver and spleen are often swollen, and the abdominal fat is petechiated. A swollen, cyanotic snood and diffuse reddening of the skin are frequent.

In lambs with polyarthritis, affected joints are swollen and thickened with granulation tissue present on the inner surface of the joint capsule. A clear to cloudy fluid is present with variable numbers of infiltrating polymorphonuclear leukocytes.

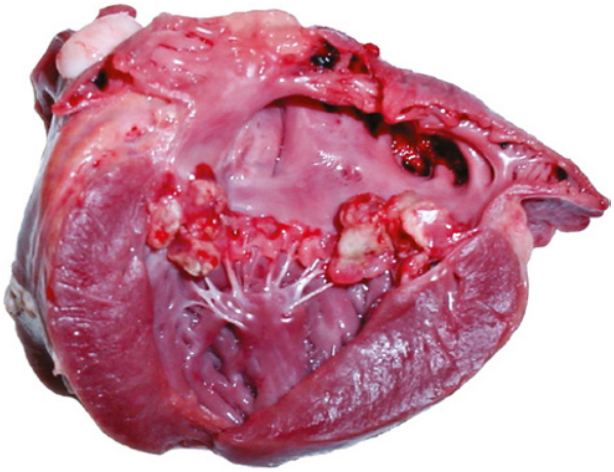


Figure 30.1 Valvular endocarditis lesions sometimes seen with *Erysipelothrix* infection in pigs. *Source:* Courtesy of Iowa State University Veterinary Diagnostic Laboratory.

Disease Patterns

Swine

Swine with the septicemic form present with fever, anorexia, depression, vomition, stiff gait, and reluctance to walk. Urticarial lesions in the skin may be palpable before becoming visible. They may be pink or, in severe cases, purplish, especially on the abdomen, thighs, ears, and tail. In severe cases, the skin becomes necrotic and is sloughed. If untreated, this form has a high mortality rate.

In a less severe form of erysipelas in swine, lesions are limited to the skin but may be accompanied by a mild fever. Skin lesions are red to purple rhomboids ("diamond skin disease") (Figure 30.2). Lesions may progress to necrosis or resolve, leaving a mild scruffiness to the skin. Mortality is seldom associated with this form.

Localization to certain tissues leads to chronic forms, which may occur as sequelae to the acute stages or without previous illness. A vegetative endocarditis is manifested by signs of cardiac insufficiency or sudden death. Arthritis is the other chronic form seen in swine. Signs include limping, stiff gait, and enlargement of the affected joints. Infrequently, sows abort due to *Erysipelothrix* infection.

Birds

Erysipelas in birds, especially turkeys, is usually a septicemia. Turkeys develop a cyanotic skin, become droopy, and

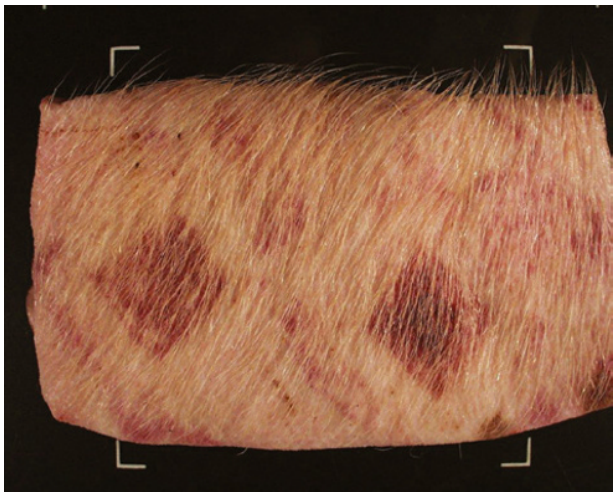


Figure 30.2 Rhomboid, diamond-shaped skin lesions of erysipelas as seen in pigs. *Source:* Courtesy of Iowa State University Veterinary Diagnostic Laboratory.

may subsequently die. A swollen cyanotic snood, if present, is considered almost pathognomonic. Mortality rates range from 2% to 25%. Chronic manifestations include vegetative endocarditis and arthritis. Turkeys with endocarditis appear weak and emaciated or die suddenly without prior signs. Other affected avian species include chickens, chukars, ducks, emus, geese, parrots, peacocks, pheasants, and pigeons.

Sheep

Polyarthritis is the most common presentation of *E. rhusiopathiae* infection in sheep. Entry is thought to be through the umbilicus or wounds associated with castration, docking, or shearing. Affected animals have a stiff gait and, often, swollen joints. Any joint may be affected, but most commonly the knee, elbow, hock, and stifle joints are involved. They may have trouble getting up and down. A cutaneous infection following dipping also occurs in sheep. Septicemia, pneumonia, and abortion have also been described in sheep.

Miscellaneous Species

E. rhusiopathiae causes arthritis and endocarditis in dogs. *E. tonsillarum* can also be a canine pathogen and has been isolated from dogs with endocarditis. Septicemia and urticaria due to *E. rhusiopathiae* have been reported in dolphins. Marine *E. rhusiopathiae* isolates appear to have greater genetic and antigenic diversity than pig isolates and are capable of inducing classical skin lesions in pigs.

Epidemiology

Pigs less than three months and over three years of age are least susceptible. Variable passive and active immunity probably accounts for the age-related susceptibility. Predisposing factors include environmental stress, dietary change, fatigue, and subclinical aflatoxicosis.

In turkeys, the male is most frequently infected, possibly through fight wounds. Insemination of hens with contaminated semen is an important source of infection. Outbreaks have been reported after contact with sheep or sheep ranges.

In sheep, lambs between two and six months of age are most commonly affected. The condition is usually associated with poor hygiene at lambing, castration, or docking. A particular form of erysipelas associated with fecal contamination of tank fluids used to dip sheep has been described.

Immunologic Aspects

Immune Mechanisms in Pathogenesis

Persistence of antigen in the joint tissue acts as a chronic stimulus for immune reaction and development of arthritis. In addition, an autoimmune process secondary to the erysipelas infection may be responsible for some of the chronic joint changes.

Mechanisms of Resistance and Recovery

Cell-mediated and humoral responses occur, directed at neuraminidase, protective surface proteins, and other cell wall components. Serum opsonins apparently play a decisive role. Phagocytosis is carried out primarily by mononuclear phagocytes.

Artificial Immunization

Attenuated live vaccines and bacterins have been used for vaccination in swine and turkeys. The duration of immunity varies from 6 to 12 months for these vaccines, and the efficacy is variable. Although effective against the acute forms, neither type appears to be highly protective against chronic erysipelas. Attenuated vaccines are given orally, parenterally, or, in some countries, by aerosol. Whole-cell bacterins and soluble antigen are given subcutaneously or intramuscularly. Most commercial vaccines are prepared from serotype 2. Cross-protection to acute disease caused by serotypes 1 and 2 has been demonstrated. However, certain strains have been refractory to vaccine-induced immunity. Formalin-inactivated, aluminum-hydroxide-absorbed bacterins are

available for turkeys and appear to be effective. Recent studies have focused on the Spa as potential vaccine candidates, but to date no commercial products are available.

Passive Immunity

Temporary protection (<2 weeks) with administration of specific immune serum has been utilized in the past. However, treatment must be initiated early in the course of the disease, and antibodies may affect vaccine efficacy if given concurrently. This approach is seldom used any longer.

Laboratory Diagnosis

Specimens

Specimens are collected from appropriate sites according to signs. Blood cultures from several affected animals are useful in diagnosing septicemia. Necropsy specimens include liver, spleen, kidney, heart, and synovial tissue. Recovery of the organisms from skin lesions is also possible. In the more chronic forms, cultures from joints or heart valves are less successful.

Direct Examination

Specimens are examined by Gram stain for the presence of gram-positive rods. A negative result does not preclude infection.

Culture and Isolation

Culture methods for the isolation of *E. rhusiopathiae* have traditionally involved the use of selective and enrichment media. One commonly used enrichment broth is *Erysipelothrix* selective broth (ESB), which contains horse serum, kanamycin, neomycin, and vancomycin. Other enrichment broths described include Bohm's medium containing sodium azide, kanamycin, phenol, and water blue and Shimoji's enrichment broth containing tryptic soy broth, Tween 80, Tris-aminomethane, crystal violet, and sodium azide. In addition, selective agar media including sodium azide crystal violet, nalidixic acid medium, and a modified blood azide agar have been described. These media take advantage of the organism's resistance to various antimicrobials and chemicals. ESB is perhaps the most commonly used enrichment broth in conjunction with selective media. One study found that ESB combined with selective agar medium (colistin nalidixic acid or sodium azide crystal violet) markedly increased detection of *E. rhusiopathiae* from tissues versus direct culture. On blood agar incubated at 37 °C in 10% CO_2, colonies often appear nonhemolytic and pinpoint after 24 hours incubation. At 48 hours, a greenish hemolysis may be apparent. *E. rhusiopathiae* is catalase and oxidase negative and nonmotile. Inoculation of triple sugar iron agar slants will show an acid reaction and H_2S production along the stab line (Figure 30.3). A "pipe cleaner" type of growth occurs in gelatin stab cultures of rough colonies held at room temperature for three to five days. *E. rhusiopathiae* does not hydrolyze esculin or urea, reduce nitrates, or produce indole. Fermentative activity is weak. Fermentable carbohydrates include glucose, lactose, levulose, and dextrin. *E. tonsillarum* usually ferments saccharose, whereas *E. rhusiopathiae* does not. The use of MALDI-TOF mass spectrometry has become common for identification of *E. rhusiopathiae* following isolation.

Serology

Serological tests to diagnose erysipelas have been developed. These include plate, tube, and microtitration agglutination, hemagglutination inhibition, complement fixation, enzyme-linked immunosorbent assay, and indirect immunofluorescence assay. However, none are conclusive in acute infections and may only be of value as herd tests in chronic infection cases. Serotyping is useful as a research tool and most commonly utilizes a double agar gel precipitation test. In swine, 76–80% of isolates are either serotype 1 or 2; serotype 1a has been associated with acute infections, whereas serotype 2a is more prevalent in chronic forms.

Molecular Identification and Characterization

Molecular identification and characterization of *E. rhusiopathiae* can support varying objectives ranging from



Figure 30.3 Hydrogen sulfide production by *E. rhusiopathiae* along stab line in Kligler's Iron Agar slant. *Source:* Courtesy of Iowa State University Veterinary Diagnostic Laboratory.

making or confirming a clinical diagnosis to aiding in epidemiologic work such as determining if outbreaks at different sites are related or how the prevalence of different serotypes changes over time. PCR is the most frequently used molecular assay to identify *E. rhusiopathiae* from clinical specimen for diagnostic purposes. Although early *E. rhusiopathiae*–specific PCRs exhibited significant limitations such as the need for prior culture enrichment, low detection limits, or the inability to differentiate between *E. rhusiopathiae* and *E. tonsillarum*, newer real-time assays do not face these constraints and can obtain specific results directly and with low detection limits from clinical specimens. Emerging technologies such as loop-mediated isothermal amplification (LAMP) have been adapted for *E. rhusiopathiae*, but PCR is currently the gold standard. Molecular tools can also aid in the identification of *E. rhusiopathiae* isolates; the objective here is to circumvent potentially time-consuming biochemical characterizations before a diagnosis can be made. Mass spectrometry (MALDI-TOF) as well as PCR and LAMP can be used. For epidemiologic studies, next-generation sequencing has at this point to be considered the gold standard; however, PCR-based assays that allow for rapid serotyping or strain differentiation as well as multilocus sequence typing schemes are still being used for certain applications.

Treatment, Control, and Prevention

Treatment with penicillin for at least five days is effective against the acute forms of erysipelas in swine and usually results in dramatic improvement within 24–36 hours. *E. rhusiopathiae* appears to be also highly sensitive to ampicillin, ceftiofur, enrofloxacin, tiamulin, and tilmicosin. Intermediate sensitivity is seen with clindamycin and spectinomycin. Resistance to chlortetracycline, florfenicol, gentamicin, neomycin, oxytetracycline, sulfadimethoxine, sulfachlorpyridazine, and sulfathiazole appears very high. Antiserum (equine origin) is sometimes used in conjunction with antibiotic therapy. Treatment of chronic forms is much less successful.

Good sanitation and nutrition are beneficial in preventing outbreaks. Infected carcasses should be disposed of in a proper manner and replacement animals isolated for at least 30 days before introduction into the herd. A number of single or combination *E. rhusiopathiae* products are available. Immunization with live attenuated vaccines or killed bacterins is recommended in areas with previous history of erysipelas.

In turkeys, penicillin is the drug of choice. Subcutaneous injection of penicillin and vaccination with erysipelas bacterin are recommended, if practicable. Penicillin in the drinking water for four to five days has been effective in controlling some outbreaks. Injectable erythromycin is a recommended alternative treatment.

Good management practices including preventing fighting among toms, ensuring proper insemination practices of turkey hens, rotating turkey ranges away from contaminated areas, and using vaccination in areas with a history of erysipelas are useful preventive and control measures.

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31

Listeria

Sanjeev Narayanan

Listeriosis is a sporadic disease affecting many species of animals and birds and is of zoonotic importance. It is most widely geographically distributed as a foodborne pathogen and in a multitude of foods in the world (partially due to its capability to grow at refrigeration temperatures) and is responsible for severe infections in immunocompromised persons, pregnant women, and neonates. The mortality rate in infected individuals is high despite the relatively low incidence. Disease manifestation could range from mild gastroenteritis to severe cases of sepsis, meningitis, encephalitis, or abortion. The 10 recognized species of *Listeria* – *Listeria grayi*, *Listeria innocua*, *Listeria ivanovii*, *Listeria marthii*, *Listeria monocytogenes*, *Listeria recourtiae*, *Listeria seeligeri*, *Listeria welshimeri*, *L. monocytogenes*, and *L. ivanovii* – are pathogens of veterinary importance. Two subspecies of *L. ivanovii* are recognized: subspecies *ivanovii* and subspecies *londoniensis*. Detection of the genus in samples, especially foods, is more important than speciation, although PCR and real-time PCR are available for accurate speciation.

Ruminants are the most frequently affected domestic species. The principal forms of listeriosis include septicemia, encephalitis, and abortion. In sheep and cattle, abortion is the usual manifestation of *L. ivanovii* infections. Listeriosis occurs worldwide, especially in temperate climates.

Descriptive Features

Morphology and Staining

Listeria is gram-positive, non-acid-fast, non-spore-forming, facultatively intracellular, acapsular rod-shaped bacteria that measures 0.5–2 μm by 0.4–0.5 μm.

Structure and Composition

Listeria has a typical gram-positive cell wall. Meso-diaminopimelic acid is the major diamino acid. Cell-wall polysaccharides determine the O-antigen. Peritrichous flagella and motility are present at 22 °C. Motility is poor at 37 °C.

Cellular Products of Medical Interest

Adhesins. Many adhesins, including Listeria adhesion protein (LAP that binds to host heat shock protein Hsp60) and internalin (inl1 and inl2, which bind to host E-cadherin and hepatocyte growth factor Met, facilitating entry into cells), have been suggested to serve an important role in attachment and invasion. However, two protein adhesins are considered critical.

ActA. ActA protein is important for intracellular movement by actin polymerization and is also thought to play a role in cell tropism (adhesion) and invasion.

Cell wall. The cell wall of the members of this genus is typical of gram-positive bacteria. The lipoteichoic acids and peptidoglycan of the gram-positive cell wall interact with macrophage cells, resulting in the release of pro-inflammatory cytokines. The peptidoglycan layer acts as an anchor for an array of surface-expressed proteins that have a conserved LPxTG motif in their C-terminus. A transpeptidase called sortase is essential for the covalent immobilization of such proteins. Some surface adhesins, including LAP (see above), are anchored by this enzyme.

Listeriolysin O (LLO), with hemolytic properties, is a pH-dependent pore-forming, cholesterol-dependent cytolysin that is a major virulence determinant for this species (mutants deficient in this protein have reduced virulence; antibodies to it are protective). The major role of LLO is in

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the release of *L. monocytogenes* from the phagosome into the cytosol following phagosome acidification, under which conditions LLO is most active. Other roles include the lysis of ferritin vacuoles and its effect on secondary vesicles formed during *L. monocytogenes* movement from cell to cell. LLO induces apoptosis by modulating mitochondrial dynamics and releasing host-cell granzymes. Ivanolysin, another cholesterol-dependent cytolysin, is the counterpart in *L. ivanovii*. See also streptococcal streptolysin O in Chapter 26 and 27 clostridial perfringolysin O in Chapter 34 and *Trueperella pyolysin* in Chapter 27.

Phospholipases C. Phosphatidylinositol-specific phospholipase C and a lecithinase are important in mediating membrane lysis.

Miscellaneous products. A bile salt hydrolase may promote the survival and persistence of *Listeria* in the intestinal lumen. A protein termed p60 (encoded by invasion associate protein (iap) gene secreted in large quantities into the growth media) may play a role in adherence to target cells.

Growth Characteristics

Listeria is a facultative anaerobe that grows best under reduced oxygen and with increased carbon dioxide concentration. Growth occurs at 4–45 °C, with an optimum at 30–37 °C. Simple laboratory media support growth, preferably an alkaline or neutral pH. On sheep blood agar, most strains of *L. monocytogenes* produce a narrow zone of hemolysis. Colonies are usually 1–2 mm in diameter and appear blue-green in obliquely transmitted light on solid media such as tryptose agar. Colonies of *L. ivanovii* typically produce a larger and more intense zone of hemolysis. Many commercial *Listeria* selective media are available. Most contain inhibitory compounds for non-*Listeria* organisms. The compounds include cycloheximide, colistin, acriflavine, amphotericin B, and cefotetan.

Listeria tolerates 0.04% potassium tellurite, 0.025% thallium acetate, 3.75% potassium thiocyanate, 10% NaCl, and 40% bile in media. Most strains grow over a pH range of 5.5–9.6. It has greater heat tolerance than other non-spore-forming bacteria; however, short-time high-temperature pasteurization is effective in killing *Listeria*.

Variability

There are 13 recognized serovars in the *L. monocytogenes* based on somatic (O) and flagellar (H) antigens. Most clinical isolates belong to serovars 1/2a, 1/2b, and 4b; and most food strains belong to serovar 1/2c. Although there is no correlation between serovars and species, strains of serovar 5 are *L. ivanovii*. No relationship between serovars and host specificity has been recognized. Various nucleic-acid-based methods have been used to further discriminate between *Listeria* strains for epidemiologic analysis and strain tracking purposes. Whole-genome sequencing of species within the genus has identified numerous genes found in virulent species but absent in avirulent species.

Smooth and rough colonial variants occur. In rough colonies, filaments 20 µm or more in length may be observed. L-forms develop on media containing penicillin and have been isolated from clinical cases in humans.

Ecology

Reservoir

Listeria has a worldwide distribution, but more frequently in temperate and colder climates, and have been isolated from soil, silage, sewage effluent, stream water, and over 50 species of animals, including ruminants, swine, horses, dogs, cats, and various species of birds. In some areas, up to 70% of humans are reported to be asymptomatic fecal carriers. Many isolates from environmental samples, previously called *L. monocytogenes*, would now be identified as one of the nonpathogenic species based on current taxonomic criteria. Biofilm formation (contributed by flagella-related *flaA*, *motB*, and the two-component chemotactic system *cheA/Y* genes) plays a critical role in environmental and host persistence and resistance to antimicrobial compounds.

Transmission

Ingestion of contaminated feed and inhalation are the primary modes of transmission of *Listeria*. Poor-quality silage, with a pH greater than 5.5, is commonly implicated and accounts for listeriosis often being referred to as "silage disease." An asymptomatic carrier can be a source of further contamination of the environment and, therefore, an indirect source of infection.

Pathogenesis

Mechanisms. Exposure to *Listeria* occurs via oral or, less commonly, the nasal route. Most *Listeria* is destroyed by gastric acids. The use of antacids and H2 blockers increases survival rates and are considered risk factors for developing listeriosis. Intestinal translocation appears to be a passive process that can involve both intestinal epithelial cells and M-cells overlying Peyer's patches. Internalins A and B, two surface proteins, interact with

host-cell receptors to mediate entry. Internalin A interacts with the cell adhesion molecule E-cadherin, and internalin B interacts with the hepatocyte growth factor receptor Met. After passage through the intestinal barrier, *Listeria* can be observed in phagocytic cells within the lamina propria. Further dissemination occurs via the bloodstream. Various bacterial ligands have been identified for adherence and include the internalin family, ActA, and p60. Nonphagocytic cells can internalize *Listeria* through a zipper-type mechanism. After internalization, *Listeria* escapes from the phagosome, becomes associated with actin filaments in the cytoplasm, and propels itself to the cell's plasma membrane via polar assembly of actin filaments (ActA). In this way, it is able to pass to neighboring cells in plasma membrane protrusions and thus avoid host defense mechanisms.

An alternative route of entry into the host has been proposed for central nervous system infections through damaged oral, nasal, or ocular mucosal surfaces via the neural sheath of peripheral nerve endings, particularly the trigeminal nerve. It is postulated that centripetal migration along cranial nerves leads to infection of the central nervous system. Organisms have been demonstrated in the myelinated axons of the trigeminal nerve and cytoplasm of medullary neurons. The lack of visceral involvement supports a route other than hematogenous, although a primary hematogenous route cannot be discounted.

In vivo selection studies demonstrate the importance of genes involved in redox regulation, including *gshF*, *spxA1*, *yjbH*, and *ohrA*. Mutants that failed to activate the master virulence regulator PrfA were also defective in intracellular survival and overall virulence.

Pathology. The brain stem is the most commonly involved area in the encephalitic form of the infection. The cerebrospinal fluid may be cloudy, and the meningeal vessels are congested. Occasionally, areas of hemorrhage and softening (malacia) in the medulla and brainstem are seen (Figure 31.1). Histologically, multifocal perivascular cuffing in which lymphocytes and histiocytes are predominant is commonly observed (Figure 31.2). Focal necrosis and microglial and neutrophilic infiltrates are seen in parenchymal tissue. The resulting microabscesses are characterized by liquefaction of the neuropil (Figure 31.3). Lesions may be present and distributed throughout the brain stem or, more frequently, are present unilaterally, further supporting the neural migration to the brain.

In the septicemic form, multifocal to diffuse necrosis in the liver (Figure 31.4) and, less frequently, in the spleen may be noted.

In the aborted fetus of ruminants, gross lesions are rare. Autolysis is usually present as a result of the dead fetus being retained for a period before being expelled.

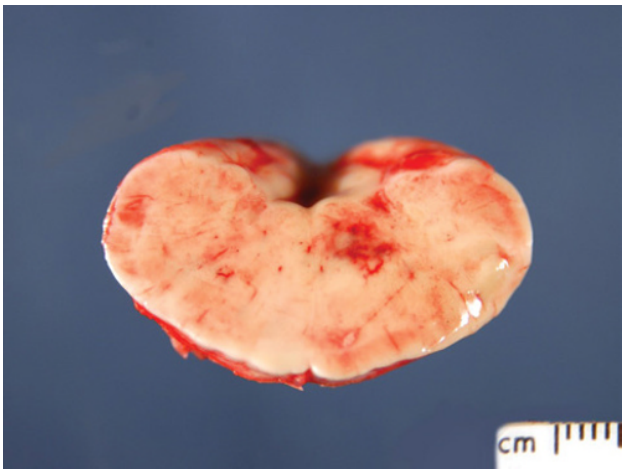


Figure 31.1 Cross section of the brainstem with areas of hemorrhage and perivascular congestion. Note that the lesions are frequently unilateral, explaining the clinical sign of circling in one direction. *Source:* Image courtesy: Dr. Margaret Miller, Purdue University.

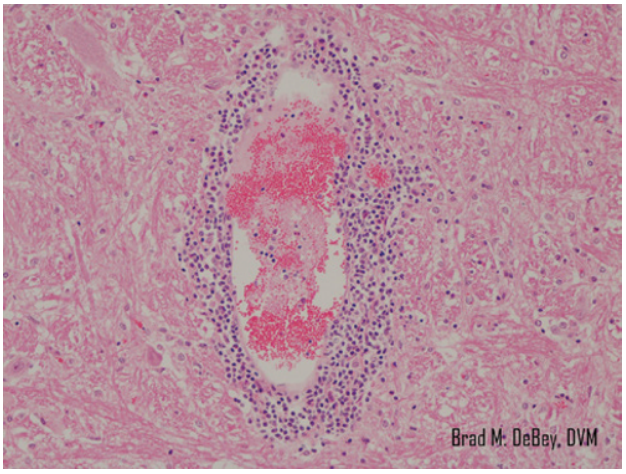


Figure 31.2 Section from the brain of a cow with the encephalitic form of listeriosis. Perivascular cuffing is present (H&E). *Listeria monocytogenes* was isolated. *Source:* Image courtesy: Drs. Brad DeBey and T.G. Nagaraja, Kansas State University.

Disease Patterns

The clinical outcome depends on the number of organisms ingested, the pathogenic properties of the strain of *Listeria*, and the immune status of the host. Septicemia, encephalitis, and abortion are the major disease forms.

Ruminants. The encephalitic form sometimes called "circling disease," is the most common form in ruminants. In cattle, it is subacute to chronic. Signs include depression, anorexia, a tendency to circle in one direction, head pressing or turning of the head to one side, unilateral facial and trigeminal nerve paralysis, and bilateral

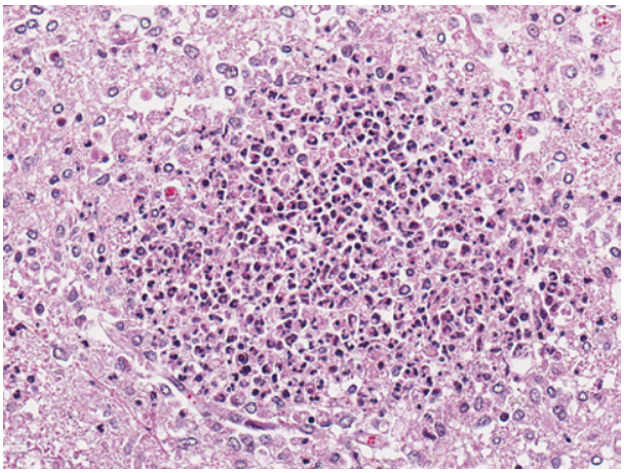


Figure 31.3 Section from the brain of a cow with encephalitic form of listeriosis. Formation of micro abscesses containing intact and degenerate neutrophils with loss of neuropil (H&E). *Source:* Image courtesy: Drs. Brad DeBey and T.G. Nagaraja, Kansas State University.

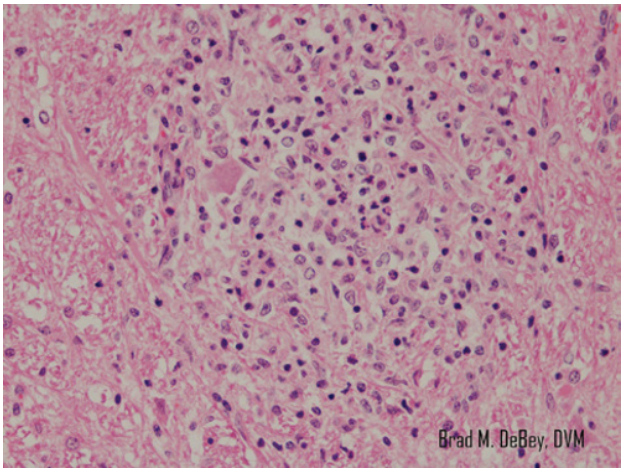


Figure 31.4 Section of liver from a five-week-old foal that died of *Listeria monocytogenes* septicemia. A severe, diffuse necrotizing hepatitis was present. *Source:* Image courtesy: Image courtesy: Drs. Brad DeBey and T.G. Nagaraja, Kansas State University.

keratoconjunctivitis. Similar signs are seen in sheep and goats, but the course is more acute and frequently fatal.

Abortion. Abortion is common in ruminants but also occurs in other species. Abortion is usually late term – after 7 months in cattle and 12 weeks in sheep. The fetus may be macerated or delivered weak and moribund. Retained placenta and metritis may also be the result. Systemic signs are rare in the cow unless the fetus is retained and triggers fatal septicemia. Although abortion is usually sporadic, abortion rates of up to 10% have been noted. It is uncommon to find the encephalitic form and abortions occurring in a single outbreak.

Conjunctivitis. Conjunctivitis in ruminants without associated abortions has been related to feeding on contaminated silage in elevated feed bunkers.

Localized infections such as acute or chronic mastitis are frequently subclinical, and their incidence may therefore be underestimated. Early diagnosis and treatment of listerial mastitis is an important measure to prevent transmission through milk.

Monogastrics and neonates. The septicemic form marked by depression, inappetence, fever, and death is the most common in monogastric animals, pre-ruminants, and neonates. Multifocal hepatic necrosis is the most common lesion in this form of the disease.

Chinchillas. Chinchillas are particularly susceptible to listerial septicemia.

Horses. Septicemia in neonates is the most common presentation in horses.

Humans. In humans, meningitis (or meningoencephalitis) is the most common form of listeriosis. Reproductive manifestations include abortion, stillbirth, premature birth, or septicemia in the newborn. Hydrocephalus is a frequent sequela of meningitis in newborns. Other clinical conditions include infective endocarditis, oculoglandular disease, and dermatitis.

Epidemiology

The widespread distribution of environmental and animal-associated occurrences of *Listeria* makes localizing the source of a particular outbreak difficult. A wide variety of vertebrates, including mammals, birds, reptiles, amphibians, and fish, can develop subclinical infections and shed *Listeria* in the feces.

Contaminated silage is a classic source of infection. Other sources include particularly organic refuse (e.g. poultry litter). Stress factors predisposing to the clinical disease include nutritional deficiencies, environmental conditions (including elevated iron concentrations), underlying disease, and pregnancy. Cases are usually sporadic and may involve up to 5% of cattle herds or 10% of sheep flocks over a two-month duration. Listeriosis in animals usually occurs in the winter and spring.

Most human cases occur in urban environments in the summer. There are occasional reports of listerial dermatitis in veterinarians and others after handling tissues from listerial abortions. Otherwise, animals are unlikely direct sources of human infections. Human epidemics have been traced to food sources of animal origin, including milk, Latin-style cheeses, hot dogs, and liver pâté. Coleslaw made from cabbage originating on a farm with a recent history of ovine listeriosis was the source of one outbreak. Another recent outbreak was linked to the consumption of

whole cantaloupes. In many instances, postprocessing contamination is found to be the source of *Listeria* contamination. Frequently there is also the opportunity for selective growth of *L. monocytogenes* to occur during long periods of refrigeration.

Immunologic Aspects

The majority of human cases of listeriosis are associated with immunosuppressed individuals, the elderly, neonates, and pregnant women. Likewise, in animals, neonates and pregnant animals are predisposed; however, a predisposing immunosuppressive factor is not apparent in some cases.

As a facultatively intracellular parasite, *Listeria* is primarily contained by cell-mediated responses. Humoral factors may play a limited role in host defense.

No immunizing preparations have met with significant success in domestic animals. Vaccines based on listeriolysin O and p60 have provided significant protection against infection in laboratory animals. Killed preparations have been ineffective, while live attenuated vaccines afforded some protection in sheep. Interestingly, live attenuated strains are capable of causing rare systemic abscesses in vaccinated animals. The sporadic nature of the disease has not warranted vaccination as a primary medium for disease prevention.

Laboratory Diagnosis

Specimens

Laboratory diagnosis is based upon the isolation of the organism. Spinal fluid, blood, brain tissue, spleen, liver, abomasal fluid, and/or meconium are cultured, depending on clinical signs, lesions, and tissues available.

Direct Examination

A direct smear of infected tissue may reveal numerous gram-positive rods in septicemias and abortions; however, fewer numbers of organisms are typically observed in the encephalitic form. Negative findings are inconclusive. Immunohistochemical staining with specific antisera is also useful in diagnosing encephalitic listeriosis (Figure 31.5).

Isolation

Samples are plated on sheep blood agar and incubated at 35 °C in 10% CO_2. Isolation of *L. monocytogenes* from brain tissue may be enhanced by pour plate methods. After the initial isolation attempts, the remaining tissue is stored at 4 °C for "cold enrichment." Such tissue is subcultured

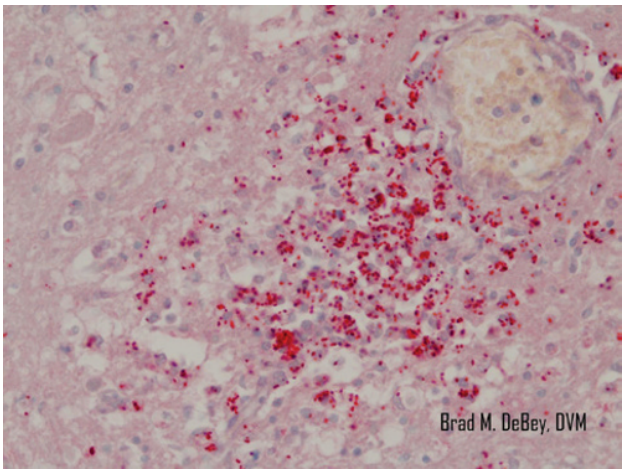


Figure 31.5 Immunohistochemistry-stained H&E section from the brain stem of a goat with listeriosis. Positive staining rods are present. *Source:* Image courtesy: Dr. Brad DeBey, Kansas State University.

weekly for up to 12 weeks. Cold enrichment is not necessary for isolation from listerial abortions or septicemias.

For samples where contamination is likely, enrichment and the use of selective media (lithium chloride-phenylethanol-moxalactam medium, Oxford medium, or PALCAM *Listeria* selective medium) are advisable. Modified University of Vermont broth, MOPS-buffered Listeria enrichment broth, Fraser broth, and Modified Oxford agar are essential components for USDA-recommended isolation methods from food products. Various DNA-based and antigen capture methods for detection of *Listeria* have been described, especially for detection in food products.

Identification

Typical colonies consisting of gram-positive, regular rods are suggestive. *Listeria* is catalase positive, motile at 25 °C, and hydrolyzes esculin. *Listeria monocytogenes* is CAMP positive when cross-streaked with a beta-toxin-producing *Staphylococcus aureus* on 5% washed sheep blood agar. A similar phenomenon is observed when *L. ivanovii* is cross-streaked with *Rhodococcus equi*. A weak CAMP-like reaction is sometimes observed between *L. monocytogenes* and *R. equi*. (Figure 31.6). In semisolid motility media incubated at room temperature, a characteristic umbrella pattern of motility develops 3–4 mm below the surface due to the microaerophilic nature of *Listeria* (Figure 31.7). An end-over-end tumbling type of motility with intermittent periods of quiescence is seen in hanging drop preparations. Acid is produced from glucose and l-rhamnose but not D-mannitol or d-xylose by *L. monocytogenes*. *L. ivanovii* differs by fermenting d-xylose but not l-rhamnose. Fluorescent antibody staining or agglutination with specific antiserum is helpful.

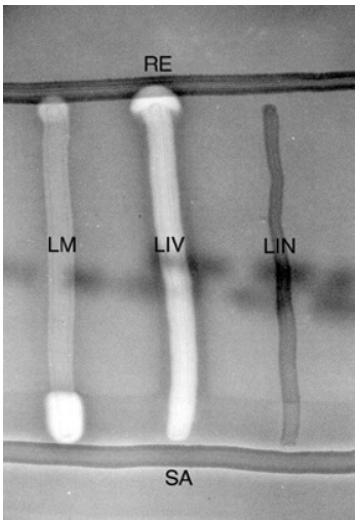


Figure 31.6 Positive CAMP reactions of *Listeria monocytogenes* (LM) with *Staphylococcus aureus* (SA) and *L. ivanovii* (LIV) with *Rhodococcus equi* (RE). A weak reaction is seen between *L. monocytogenes* and *R. equi*. No reaction is detected with *L. innocua* (LIN). The variation in the degree of intensity of hemolysis of *L. monocytogenes* compared to *L. ivanovii* is apparent. *Listeria innocua* is not hemolytic.

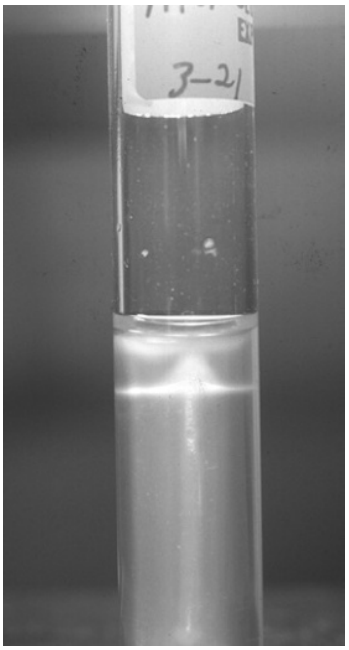


Figure 31.7 Umbrella-type motility of *Listeria monocytogenes* in semisolid motility media incubated at room temperature.

Mouse inoculation causes death within five days, with necrotic foci present in the liver. This procedure differentiates *L. monocytogenes* from nonpathogenic species of *Listeria*; however, it is rarely necessary for definitive identification.

Immunodiagnosis

Serology has not been useful for diagnosis due to the prevalence of positive titers in apparently normal animals and cross-reactions with *S. aureus, Enterococcus faecalis, and Trueperella pyogenes*.

Treatment, Control, and Prevention

L. monocytogenes are susceptible *in vitro* to penicillin, ampicillin, chloramphenicol, erythromycin, enrofloxacin, lincomycin, nosiheptide, rifampin, salinomycin, tetracycline, vancomycin, and virginiamycin. Recent studies report that some isolates are resistant to tetracyclines, sulfonamides and trimethoprim, erythromycin and fluoroquinolones, and penicillin minimum inhibitory concentrations are on the rise. Chlortetracycline and penicillin may be effective in the timely treatment of cattle with meningoencephalitis. Treatment of sheep has been less successful.

Control measures include reduction or elimination of feeding of silage, particularly poor-quality silage. All forms of stress should be minimized. Affected animals should be isolated and infected material disposed of properly.

Vaccination has not proved to be highly successful and may not be warranted due to the sporadic nature of the disease.

Further Reading

Bacterial taxonomy browser at http://www.ncbi.nlm.nih.gov/Taxonomy

Center for Food Security and Public Health. Listeriosis. Fact Sheet. https://www.cfsph.iastate.edu/Factsheets/pdfs/listeriosis.pdf

Maxie, G.M. (ed.) (2015). *Pathology of Domestic Animals*, 6e, vol. 1. Nervous System, 250–406. Saunders-Elsevier.

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32

Rhodococcus

Seth P. Harris and Joshua Daniels

Rhodococcus is a genus of facultative intracellular bacteria that are classified within the Nocardiaceae family. Of the many species of bacteria within *Rhodococcus*, *Rhodococcus equi* is the only one that is routinely encountered as a pathogen in veterinary medicine. Clinical disease presents as pyogranulomatous pneumonia or enteritis in foals less than six months of age, and other organ systems can also be affected. Occasionally, immunosuppressed adult horses and other animal species including humans can develop disease. The bacteria are naturally found within the environment, and foals are exposed within the first few days of life. Foals are uniquely susceptible due to the functional limitations of their immune system. Infection is generally seasonal and typically occurs during the dry summer months. Resistance to relevant antimicrobial agents has been detected on horse farms that actively monitor and treat early and subclinical infections.

Rhodococcus equi

Descriptive Features

Morphology and Staining

R. equi are strongly gram-positive-staining pleomorphic coccobacilli (Figure 32.1). From solid growth media, cellular forms appear predominantly coccoid, measuring approximately 1–5 μM. *R. equi* may stain weakly acid fast with both the Ziehl-Neelsen and Kinyoun techniques, and the degree of acid fast staining varies with the age of the culture and growth medium. Direct visualization of the organism in tracheal aspirates and bronchoalveolar lavage specimens may be accomplished with the Gram stain, Fite's acid fast stain, and Grocott's methenamine silver (GMS) stain. Colonies of *R. equi* have a characteristic salmon color.

Structure and Composition

Many of the genes encoded in the bacterial DNA are devoted to lipid metabolism, which is evident in the bacterial structure. The lipid-rich cell envelope of *Rhodococcus* is relatively resistant to drying, allows the bacterium to survive for months within soil, and confers the acid-fast staining characteristic. The basic cell wall structure is similar to other members of the supra-generic taxon Mycolata and consists of fatty acids and mycolic acids that are perpendicularly linked to an arabinogalactan polysaccharide. The outer part of the wall contains a variety of lipids including mycolic acids, lipoarabinomannans (LAMs), trehalose monomycolate, trehalose dimycolate, and cardiolipin. The *R. equi* mycolic acids are covalently bound to the cell wall. The VapA lipoprotein that is associated with virulence in equine isolates is located along the surface of the outer membrane. The structure of VapA allows it to anchor within the cell envelope, and it can be secreted to interact with the phagosome and prevent acidification. VapA can be isolated in conjunction with other surface antigens.

Cellular Products of Medical Interest

Virulence-associated proteins (Vaps). Pathogenic equine strains of *R. equi* contain an approximately 85–90 kb virulence plasmid. Bacteria lacking this plasmid are considered non-infectious to foals. Avirulent *R. equi* are effectively cleared by the immune system of adult horses and foals. Loss of the virulence plasmid can be induced by serial passage of the bacterium within culture. Plasmid-negative strains can regain the virulence plasmid through conjugation with a pathogenic strain.

The virulence plasmid contains genes for Vaps, of which VapA is immunodominant in virulent equine isolates. VapA is a 15–17 kDa lipoprotein that is anchored to the cell membrane; it enables survival of the bacteria within

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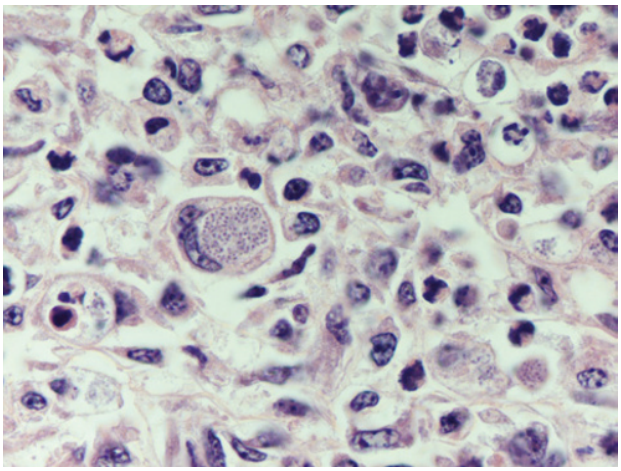


Figure 32.1 Histopathology of pneumonia in a foal. Note that the cytoplasm of this macrophage is distended by gram-positive coccobacilli (Gram stain, 600×).

macrophages and is released from the plasma membrane in the first few hours of infection. After release, it prevents acidification of the phagosome by preventing fusion of phagosomes with lysosomes. The VapA protein can traffic between cellular compartments, likely in vesicles, and can transfer to lysosomes and increase membrane permeability. Phagosomes associated with virulent *R. equi* have a pH of around 7.2, which is conducive to bacterial survival. The *vapA* gene is not sufficient to confer pathogenicity alone and requires expression of other virulence factors from the plasmid, such as the positive regulators *virR* and *orf8*. VapA is a ligand for TLR2 in macrophages and dendritic cells. Purified VapA has been shown to induce production of the pro-inflammatory cytokines TNF, IL-12p40, and NO in macrophages. Purified VapA in dendritic cells upregulates CD40 and CD86, which are surface expressed costimulatory molecules.

VapB is a Vap protein that is related to VapA. However, VapB is slightly larger, at approximately 20 kDa, and is typically isolated from pig strains instead of equine strains of *R. equi*. The *vapA* and *vapB* genes are never encoded in the same isolates of *R. equi*, indicating that they represent distinct plasmid subpopulations.

Bovine isolates of *R. equi* harbor a virulence plasmid called VapN, which lacks both the *vapA* and *vapB* genes, a finding consistent with a third plasmid subpopulation. The VapN plasmid is 120 kb and contains six *vap* genes (*vap*N through *vap*S). VapN appears to be required for pathogenicity in bovines, similar to VapA in equines.

Additional *vapA* homologues that have been discovered include the functional *vap*C, -D, -E, -G, and -H and the non-functional pseudogenes *vapF*, -I, and –X, as well as *vapN* through *vapS*. The proteins VapC, -D, and –E are secreted proteins, and similar to VapA have significantly elevated expression at a temperature of 38 °C and a pH <8. These conditions favoring expression of the Vap proteins are associated with the environment encountered inside a host phagosome.

LAM is a cell envelope lipid that functions as a virulence factor. The LAM in *R. equi* is structurally unique compared to other Nocardiaceae such as *Mycobacterium tuberculosis,* due to its smaller size and reduced arabinose content. Although the exact *in vivo* function of *R. equi* LAM has not been determined, it is speculated that it influences the route of phagocytosis by macrophages and the subsequent production of early macrophage cytokines. With the related bacteria *M. tuberculosis,* LAM is needed for bacterial growth and viability and is recognized by the host innate immune system through Toll-like receptors (TLR). A similar function may occur with *R. equi*.

Cell wall lipids. Major extractable cell wall lipids of *R. equi* using a chloroform-methanol technique include trehalose monomycolate, trehalose dimycolate, and cardiolipin. Trehalose monomycolate has a chemical structure of $C_{48}H_{92}O_{13}$, trehalose dimycolate has a structure of $C_{84}H_{162}O_{15}$, and cardiolipin has a structure of $C_{79}H_{154}O_{17}P_2$. Of these cell wall lipids, cytotoxic T-lymphocytes have been shown to recognize and lyse blood-derived macrophages that present trehalose monomycolate and cardiolipin, but not trehalose dimycolate. All three of these lipid fractions can induce upregulation of IFN-ɣ production in lymphocytes. This suggests that they may be recognized by CD4+ T-cells, or possibly ɤδ T-cells or NKT cells, in addition to aforementioned cytotoxic T-cells. The presentation of *R. equi* lipids antigens is not restricted by equine leukocyte antigen (equine MHC molecules) and is thought to occur through CD1 molecules. The equine CD1 family includes seven homologues of CD1a, two of CD1b, one of CD1c, one of CD1d, and two of CD1E. The length of the carbon chains in cell wall lipids varies between *R. equi* and other environmental *Rhodococcus* species, which may affect orientation of lipids within CD1 molecules and subsequent recognition by the immune system.

Growth Characteristics

An obligate aerobe, *R. equi* grows readily on many types of non-selective growth media (e.g. Trypticase soy agar with 5% sheep's blood) when incubated in ambient air. At 35–37 °C, pinpoint colonies will typically be visible at 24 hours, but after 48 hours develop a characteristic mucoid "milk-drop" appearance (Figure 32.2) with colonies that typically range from 3 to 10 mm in diameter and frequently coalesce in areas of dense growth. Colonial growth of most *R. equi* isolates develops a salmon coloration after three to four days due to the production of γ-carotene pigment. In

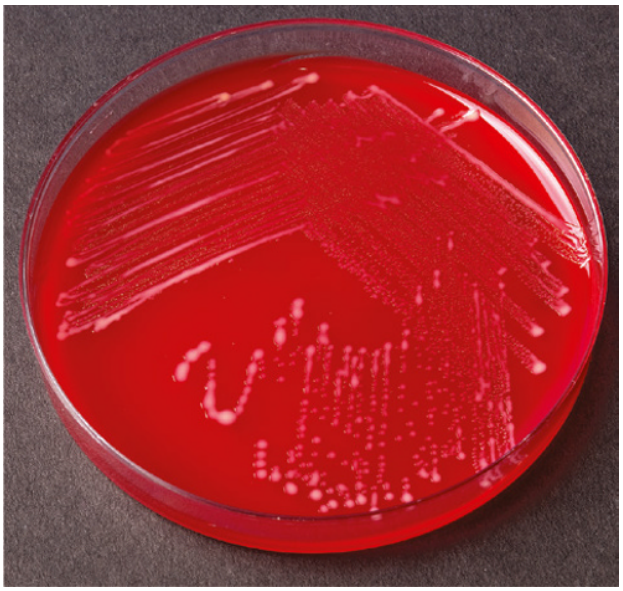


Figure 32.2 "Milk-drop" appearance of *R. equi* colonies on culture plate.

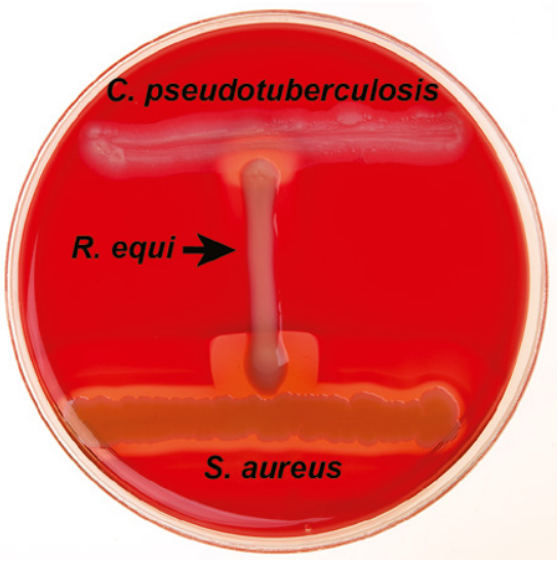


Figure 32.3 Phospholipases from *R. equi* enhance the hemolytic activities of *S. aureus* β-toxin and *C. pseudotuberculosis* phospholipase C (CAMP plate).

animal feces and soil, the organism may replicate at temperatures as low as 10 °C.

Biochemical Activities

The organism is catalase and urease positive and typically reduces nitrate. *R. equi* does not ferment carbohydrates (asaccharolytic) and is oxidase negative. An additional phenotypic characteristic that is helpful for laboratory identification is the activity of *R. equi* on a CAMP test. *R. equi* produces phospholipases ("equi factors") that enhance the hemolytic activities of *Staphylococcus aureus* β-toxin and *Corynebacterium pseudotuberculosis* phospholipase C (Figure 32.3).

Resistance

R. equi is remarkably resistant to acid. This is consistent with the organism's ability to persist in phagolysosomes of alveolar macrophages – an environment with low pH and considerable oxidative stress. Its resistance to acid may be exploited when attempting to isolate the organism from a heavily contaminated substrate, such as soil or feces. *R. equi* also tolerates ultraviolet light exposure from direct sunlight and is resistant to desiccation, characteristics that help it to persist in equine environments.

In vitro, *R. equi* is susceptible to most antimicrobial agents with gram-positive spectra, with the exception of the β-lactams and tetracycline. *In vivo*, lipophilic antimicrobial agents are most effective, particularly rifampin and the macrolides: erythromycin, clarithromycin, and azithromycin. Synergistic killing is achieved when rifampin is combined with a macrolide. Resistance to rifampin and macrolides has been emerging and has been documented on farms that routinely treat subclinical animals.

Variability

Over 27 antigenically distinct capsular types of *R. equi* have been described.

Ecology

Reservoir

R. equi is found within the soil wherever horses are present, and can become endemic in high-density horse farms. It has been found in all continents, with the exception of Antarctica. In horse breeding farms, the concentration of the bacteria in the soil varies from 10^2 to 10^5 CFU per gram with less than a quarter of the isolates being pathogenic. Infected foals are the major source of environmental contamination and can shed 10^6–10^8 bacteria per gram of feces, which is not surprising given that the bacteria can replicate in the intestinal tract. Although adult horses also can shed the bacteria within their feces, it tends to occur more sporadically and with a far lower quantity of bacteria. Adult shedding of less than 2000 colony forming units per gram of feces is common. Bacteria shed by horses may be environmental strains that lack a virulence plasmid encoding for the *vapA* gene. Pneumonic foals can exhale the bacteria, but it is doubtful that is a significant route of environmental contamination. Earthworms have been shown to harbor the bacteria, but it is questionable whether they have any significant role in the spread of pathogenic strains. Given

the shedding by horses, survival within worms, and general resistance to environmental factors, it is unlikely that an attempt at environmental disinfection could be successful.

Transmission

Transmission of bacteria within the environment typically occurs either via ingestion or inhalation. As *R. equi* can survive within soil and foals are naturally coprophagic, it is likely that many neonatal foals are initially exposed following ingestion of manure or contaminated soil. However, ingested strains can encompass either virulent or avirulent bacteria, and the bacteria can become part of the normal enteric flora. Ingested *R. equi* from the environment can colonize the gastrointestinal tract rapidly and have been detected in foal feces during their first week of life. Inhalation is considered to be the major route of infection in foals, and challenge studies in foals often use a respiratory route of infection to induce respiratory disease. The time between infection and disease varies based on the age of the foal and the quantity of the infectious bacterial dose. Heavy bacterial doses can result in pneumonia developing within a few days, and low doses can prolong the disease-free period for a few weeks; but, in experimental infections, the average incubation time is slightly over a week. In natural settings, infection via inhalation normally occurs during the summer months when manure contaminated with virulent *R. equi* becomes desiccated, leading to aerosolization and subsequent inhalation. However, it has also been found in colder and wetter environments, such as Japan and Sweden, and in all continents except for Antarctica. In natural settings, the disease is usually not seen after foals reach six months of age. The route of infection (ingestion versus inhalation) coupled with the infectious dose and immunologic factors, essentially representing the epidemiological triangle of infectious disease, influences whether a foal will develop disease and the severity of the clinical signs. Many cases are subclinical and respond without treatment, but these foals likely serve as a vector of spread.

Pathogenesis

Mechanisms. *R. equi* is a facultative intracellular pathogen that can survive within macrophages after phagocytosis. Phagocytosis is mediated by complement or antibody opsonization, and this plays a role in intracellular survival of the bacteria. Bacteria that are opsonized with antibody enter macrophages via the Fcγ-receptor and are killed as the phagolysosome matures. However, bacteria that are opsonized with the complement component C3b enter the cell through the Mac-1 receptor (CD11b/CD18) and are able to survive intracellularly by impeding phagosomal maturation and acidification. The ability to survive intracellularly is dependent on the presence of the Vap expressing the VapA surface lipoprotein, which confers partial lysozyme resistance. Thus, *R. equi* that lack the virulence plasmid and cannot prevent phagosomal maturation are avirulent in horses and infections are cleared without pathology, even in foals.

Pathology. *R. equi* is a parasite of macrophages that leads to the development of pyogranulomatous pneumonia (Figure 32.4). The severity of the disease can be highly variable, with some naturally infected foals developing subclinical infections and other succumbing to disease. The abscesses and granulomas are characterized by centers of neutrophils and necrotic debris that are surrounded by macrophages, multinucleate giant cells, lymphocytes, and a fibrous capsule. The more acute lesions are less organized, tend to have higher numbers of neutrophils, and lack a well-developed fibrous capsule. Grossly, the chronic lesions form well-defined granulomas with caseating centers. Abscesses that rupture can result in severe fibrinous pleuritis or peritonitis, septicemia, shock, and death. Extrapulmonary infections in a variety of organ systems are common, likely due to a period of septicemic spread.

Disease Patterns

Equine. Infected horses are almost always foals less than six months of age, with a majority of cases occurring in foals between one and four months. Affected foals develop pyogranulomatous bronchopneumonia as the most common lesion. The percentage of the lung affected is highly variable. The reported survival rate for rhodococcal pneumonia can vary based on the age of the foal, interval between infection and diagnosis, and treatment protocol. Extrapulmonary infections are estimated to occur in approximately 74% of foals with rhodococcal infections, and multiple extrapulmonary

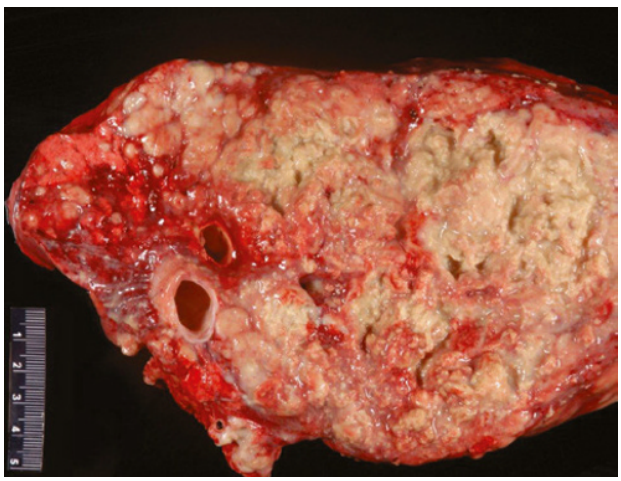


Figure 32.4 Pyogranulomatous pneumonia from a foal. The lesions vary from caseating to solid. *Source:* Courtesy of Duncan Russell, Ohio State University.

infections can occur simultaneously. These can develop complementary to or independent of a respiratory infection. Many of the extrapulmonary infections are subclinical but should be evaluated at necropsy in foals that succumb to respiratory disease; in these terminal foals, development of extrapulmonary infections are associated with significantly higher mortality rates than infections restricted to only the lung. The most common extrapulmonary manifestations include diarrhea, immune-mediated polysynovitis, ulcerative enterotyphlocolitis, intra-abdominal abscesses, and abdominal lymphadenitis, and those infections are found in approximately half of foals that succumb to disease. Polysynovitis is reported to occur in up to one third of infected foals, but it is unclear whether that is due to septicemia, an immune-mediated consequence, or a combination of both. Other extrapulmonary disease such as uveitis, hepatitis, and osteomyelitis are an uncommon occurrence, and there are rare reports of infections in most other organ systems. However, it is not clear if these are truly uncommon or simply have not been evaluated. Development of severe anterior uveitis is thought to be a poor prognostic indicator. Immunocompetent adult horses are nearly universally considered to be protected from disease and mount an effective immune response when experimentally infected, even when exposed to high bacterial challenges. Adult horses with no known immunological impairment rarely develop pneumonia. However, clinically affected adult horses may have an underlying undiagnosed immunodeficiency as the isolated strains do not always contain VapA. Foal isolates from pneumonic animals universally contain a virulence plasmid encoding for VapA.

Swine. *R. equi* has been recovered from lymph nodes collected from wild and domestic swine. The submaxillary lymph node is the most common site. Swine isolates are considered to be of intermediate virulence and contain a virulence plasmid encoding for VapB.

Cattle. Bovine isolates of *R. equi* are rare and are associated with a low degree of pathogenicity. Cattle isolates typically cause granulomas in a single lymph node that grossly resemble the lesion of bovine tuberculosis and should be distinguished with additional diagnostic testing. The retropharyngeal, bronchial, and mediastinal nodes are the most common lymph nodes affected. Cows have a lower incidence of infection than either steers or heifers, although an explanation for this epidemiological pattern is lacking. Cattle isolates were thought to not contain a virulence plasmid, although now they are known to contain a plasmid that does not code for either VapA or VapB, but VapN. This is in contrast to isolates from horses and pigs.

Goats. Infections in goats are rare, and positive identification requires culture. Multiple hepatic abscesses are the most common presentation. Other affected sites include the lung, lymph nodes, and rarely vertebral bodies. The pattern of abscesses in the liver and lung suggests that the bacterial pathogenesis in this species may involve accessing the enterohepatic circulation. This access may be due to damage within the gastrointestinal tract (e.g. compromised ruminal mucosal barrier due to ruminal acidosis). This has yet to be confirmed. Once within the blood supply, the bacteria can then disseminate to other organ systems. *C. pseudotuberculosis* is the main differential diagnosis as it is a common cause of abscesses in goats. Culture should be used for confirmation in suspected cases. No association with a virulence plasmid has been reported.

Humans. The incidence of *R. equi* in humans has increased with the growing numbers of immunosuppressed people. The AIDS epidemic, organ transplant recipients, and people undergoing chemotherapy are particularly at risk. Very rare reports of *R. equi* infections in immunocompetent people exist. However, immunocompetent individuals tend to have more localized infections and have a far lower mortality rate than the immunocompromised population. Most infections in immunosuppressed people are respiratory infections, but extrapulmonary coinfections occur and are reported to develop in approximately 20% of HIV-infected individuals. Pulmonary infections in humans can mimic tuberculosis, and the upper lung lobes are a more common site of pathology. Culture can be used for differentiation of the organisms. Unlike some of the other animal species previously described, there is no required association of a virulence plasmid with human isolates of *R. equi*. Some human isolates contain a virulence plasmid coding for either VapA or VapB, whereas other isolates are plasmid free.

Other. *R. equi* infections are reported sporadically in a number of animal species including cat, dog, sheep, deer, llama, buffalo, koala, seal, marmoset, alligator, and crocodile. *R. equi* may be an emerging disease in adult dromedary camels as all of their isolates have an association with the VapA containing virulence plasmid. Peccaries in a breeding farm had a low detection rate of *R. equi* from clinically normal lymph nodes at slaughter. *R. equi* has been found in the feces of many clinically normal animal species and may represent part of their normal enteric flora or passage of ingested environmental bacteria.

Epidemiology

R. equi is present in the environment wherever herbivore manure is present. High-concentration equine breeding farms have elevated bacterial concentrations within the soil. The disease is usually seasonal, and dry and dusty conditions during the late spring to summer predispose to aerosolization of desiccated manure and soil, leading to increased exposure to foals. However, the disease is not exclusively restricted to that seasonality or environment and is occasionally seen in other conditions. Young foals are particularly susceptible due to their inherent deficiency

in mounting a protective type I immune response. A retrospective study evaluating risk factors for the development of disease did not find any correlation regarding disease and examined factors such as the birth year, spring or fall birth season, gestational length, mare age, foal gender, or whether foaling took place in a stall or pasture.

Immunologic Aspects

Most of the features of a protective immune response to *R. equi* have been elucidated through studies in mice, as well as by comparing the immune responses of adult horse to foals. These studies demonstrate that the type of immune response developed directly affects the course of infection. A type I immune response (leads to cell-mediated immunity) is protective, whereas a type II immune response (leads to humoral immunity) is detrimental. The protective type I immune response encompasses both CD4+ and CD8+ T-lymphocytes. The CD4+ T-lymphocytes function as the predominant producers of IFN-ɣ, whereas CD8+ T-lymphocytes function as cytotoxic T-lymphocytes. Production of IFN-ɣ by CD4+ T-lymphocytes enhances phagosomal maturation and acidification, which kills intracellular bacteria without lysing the host macrophage. Cytotoxic T-lymphocytes kill intracellular bacteria by directly lysing *R. equi*–infected macrophages.

Rhodococcal pneumonia is considered to be an immune-based disease in which foals are uniquely susceptible. Foals and neonates in general are considered to have functionally impaired immune response during the neonatal period. This impairment is likely due to a combination of lack of previous pathogen exposure and subsequent memory response (immunologic naivety) as well as differences in the ability of their immune system to recognize and react appropriately to encountered pathogens. For example, under most circumstances, neonates (as opposed to adult horses) develop a type II rather than a type I immune response, which is detrimental to *R. equi* infections. The differences in the neonatal immune system spans both antigen-presenting cells (macrophages and dendritic cells) and lymphocytes. Antigen-presenting cells lack cytokines and costimulatory molecules, and are unable to properly prime lymphocytes during the neonatal period. Foals have less expression of MHC II and CD1 molecules than adult horses. Therefore, differing antigen doses may be necessary to prime an early immune response. Likewise, neonatal CD4+ T-lymphocytes tend to produce lower levels of IFN-ɣ than adults, and *R. equi*–specific CD8+ cytotoxic T-lymphocytes are lacking early in life. The level of IFN-ɣ produced by CD4+ T-lymphocytes gradually increases as a foal ages, whereas *R. equi*–specific cytotoxic T-lymphocytes are absent and appear at adult levels when foals reach six to eight weeks of age. Despite these impairments, under select experimental conditions, foals are able to overcome some of their immune deficiencies. Foals intrabronchially challenged with virulent *R. equi* are able to produce IFN-ɣ at near-adult levels. Foals orally inoculated with virulent *R. equi* during the first two weeks of life can develop *R. equi*–specific CTL by the time they reach three weeks of life. Oral exposure to inactivated *R. equi* in neonatal foals leads to increased production of IFN-ɣ and IgA. Therefore, a number of environmental factors (e.g. age when exposed, route of exposure, and bacterial dose) may influence the course of disease in individual animals.

Although antibody is thought to play a role in protection, it alone is not considered to be sufficient to completely protect against disease. Antibody can function by altering the route of phagocytosis due to utilization of the Fc receptor. Phagocytosis via the Fc receptor is associated with poor bacterial survival in comparison to bacteria that are opsonized with complement that utilizes the Mac-1 receptor. Based on this knowledge, hyperimmune plasma has been commercially marketed as a preventative for foals in high-risk areas. Hyperimmune plasma contains a variety of antibody types and other components that can enhance the humoral immune system. However, the efficacy of hyperimmune plasma can be highly variable, and it is considered to only be partially effective at preventing disease. There is some indication that hyperimmune plasma may also decrease the amount of bacterial shedding in feces, which would be expected if it also confers partial protection from extrapulmonary infections. The variability in the protection by hyperimmune plasma may be attributed to product differences among the manufacturers as well as a lack of uniformity in product use by the consumers. Companies producing hyperimmune plasma use undisclosed proprietary techniques that impede independent evaluation of the quality of their product. Therefore, factors such as bacterial strain, adjuvant type, route of vaccination, and so on, which could influence the effectiveness of hyperimmune plasma, remain an unknown variable. Vaccination of mares with the goal of producing protective colostral antibodies has not been shown to be effective at preventing disease in their foals.

To date no effective commercial vaccine for *R. equi* in foals has been produced. It is possible that the immature neonatal immune system in conjunction with early exposure to environmental bacteria may be an obstacle that cannot be overcome with traditional vaccination techniques. There may also be some degree of interference with vaccination due to colostral-derived antibodies. Alternative means of vaccination have been attempted including the use of DNA vaccines and oral vaccination with virulent and inactivated bacteria. Although DNA vaccinations show promise in inducing a type I immune response during the perinatal period in mice, they do not appear to be

effective in foals. Likewise, utilization of triton X-extracted antigen to vaccinate mares results in elevation of opsonizing IgG antibody titers, but does not confer protection against *R. equi* pneumonia in foals that have consumed the colostrum. One technique that currently is showing the most promise is vaccination through the oral route. Oral vaccinations have been successfully utilized to protect against pathogens in other animal species such as polio in humans. As foals are often initially exposed to environmental *R. equi* through ingestion, an oral vaccine could mimic natural exposure and induce a more appropriate immune response when compared to parenteral vaccination. Foals experimentally vaccinated orally during the first two weeks of life with live virulent *R. equi* were shown to not be susceptible to disease from a subsequent airborne challenge. However, they may have been at risk for development of subclinical extrapulmonary infections and could shed virulent bacteria into the environment. To address those issues, a vaccine candidate was made using bacteria that were inactivated and unable to replicate, but preserving immunogenic epitopes. Foals orally inoculated were able to develop elevated IFN-ϑ levels, which is indicative of a cell-mediated immune response. Mucosal IgA was also elevated, which may be partially protective against inhaled bacteria. For the time being it is unknown whether an oral strategy will prove practical and efficacious, as there are still a number of experimental questions to answer and practical implications to appraise before an oral vaccine can be considered.

Laboratory Diagnosis

There is no gold standard for laboratory diagnosis. Transtracheal washes are considered the best means of diagnosis. Fluid from the wash may be examined with cytology, cultured, or undergo polymerase chain reaction (PCR) using *R. equi*–specific primers. With cytology the bacteria can be found predominately intracellularly within foamy macrophages; they have a watermelon seed shape. In some instances, the shape of the bacteria can be difficult to differentiate from that of cocci. Identification of the organism in culture may be accomplished by Gram stain and CAMP test of isolates with consistent colonial morphology, as well as by MALDI-TOF mass spectrometry. Culture can have false negative results depending on the quality of the specimen, and results can be complicated if contaminating bacteria are present. PCR is a more sensitive means of detection than culture, although it carries a risk of false positive results and needs to be evaluated in conjunction with the clinical history. In cases where transtracheal washes are negative, thoracic radiographs can be used to help make a diagnosis. Initial infections demonstrate an alveolar pattern with poorly defined regions of consolidation. Chronic infections may display nodular masses within the lungs and lymphadenopathy. Serological tests such as enzyme-linked immunoassay (ELISA) or agar gel immunodiffusion assay (AGID) do not adequately differentiate between diseased and healthy foals. Fecal shedding is known to occur in foals with clinical respiratory disease, and fecal PCR has been used to positively corroborate respiratory infections. Fecal PCR has obvious advantages of being a non-invasive technique with ease of sampling, but there is variability in the reported sensitivity and specificity of the test in clinical and asymptomatic animals.

Treatment and Control

The prognosis of *R. equi* pneumonia in foals is guarded. Approximately 44% of subclinically infected foals in endemic farms will recover without antimicrobial therapy. Foals with clinical disease are treated with a combination of a macrolide antibiotic and rifampin. Clarithromycin is a more effective macrolide than azithromycin or erythromycin, when combined with rifampin; other macrolides such as tilmicosin, tulathromycin, spiramycin, and tylosin are not effective treatments. Isolates of *R. equi* can be resistant to macrolides and/or rifampin due to an exogenously acquired *erm* gene (macrolides) and an acquired mutation of the *rpoB* gene (rifampin), and foals infected with these isolates have up to a sevenfold increase mortality rate. The resistance to erythromycin and rifampin appears to be developing slowly and has been accentuated in farms that are utilizing ultrasound for early detection of disease in foals. This is thought to be occurring due to selective pressure on the bacteria from more widespread antibiotic treatment of animals that would be otherwise subclinical and recover without medical intervention. Antimicrobial resistant *R. equi* isolates are expectedly associated with a higher mortality rate in clinically diseased foals. Although there are reports that enrofloxacin has resulted in successful treatment, it carries a risk of articular cartilage toxicity and should not be used in young animals. In endemic farms, attempts should be made at early detection of infection, but treatment of subclinical foals needs to be weighed against the risk of antimicrobial resistance developing, especially as antimicrobial resistance is linked with an elevated mortality rate. Studies have demonstrated that treatment later in the disease course does not alter the prognosis, but delaying treatment will result in fewer subclinical animals being treated and an overall decrease in antibiotic usage. Early signs of infection include pyrexia and hyperfibrinogenemia, which can be monitored with rectal temperature and a complete blood count with fibrinogen. Some foals display nonspecific clinical signs such as diarrhea and weight loss, with the latter occurring more commonly in the chronic cases. Early cases can go undetected for weeks or longer.

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33

Gram-Negative, Non-Spore-Forming Anaerobes

Tiruvoor G. Nagaraja

Anaerobic bacteria use inorganic or organic molecules – rather than oxygen – as a final electron acceptor to generate energy for metabolism. Anaerobes are broadly categorized as obligate and aerotolerant based on the degree of susceptibility to molecular oxygen. Obligate anaerobes are adversely affected by oxygen, and the degree of oxygen toxicity varies with species and even strains within a species. When exposed to molecular oxygen, bacteria generate powerful oxidants, such as hydrogen peroxide, superoxide anions, singlet oxygen, and other oxygen radicals. These oxygen radicals interact with cell macromolecules, such as proteins and nucleic acids, to disrupt their structures and cause lethal damage to cells. Obligate anaerobes lack mechanisms and enzyme systems, such as superoxide dismutase and catalase, to neutralize toxic products. Gram-negative anaerobes of clinical importance are generally aerotolerant because many of them do contain low levels of superoxide dismutase and catalase or peroxidase and can resist exposure to oxygen, but the duration of tolerance varies with species. Because of aerotolerance, many of the clinical anaerobes can be grown in culture medium that is not pre-reduced and are able to grow on agar surfaces as long as incubation is carried out under an oxygen-free atmosphere.

A number of genera and species of gram-negative, non-spore-forming anaerobes are part of the normal flora of the mucosal lining of the mouth, intestine, and respiratory, urinary, and genital tracts of animals and humans. These organisms are more often opportunistic pathogens and generally cause infections following the breakdown in mucosal or epithelial barriers and the entry of the bacteria into normally sterile sites of the body.

Gram-negative, non-spore-forming anaerobes have undergone pronounced reorganization in taxonomy and nomenclature because of phylogeny-oriented taxonomy approaches, primarily based on 16S rRNA nucleotide sequences. Clinically important gram-negative, non-spore-forming anaerobic bacilli, other than those under *Fusobacterium*, were once grouped primarily in the genus *Bacteroides*, and the genus was regarded as phenotypically heterogeneous. In fact, the taxonomy of gram-negative anaerobes is in a state of constant revision, with new genera and species being described. Existing taxa have been reclassified and old species have been renamed. In recent years, 16S rRNA amplicon-based or shotgun metagenomics studies have identified a number of gram-negative anaerobic bacterial genera, many of which have not been cultured yet.

Gram-negative, non-spore-forming anaerobes include rods, cocci, and spiral organisms. Clinically, the rods are the most common. Gram-negative, non-spore-forming bacteria of clinical importance in animals belong to the genera *Bacteroides*, *Dichelobacter*, *Fusobacterium*, *Porphyromonas*, and *Prevotella*.

Virulence Factors and Virulence Mechanisms

The virulence factors of gram-negative non-spore-forming anaerobes are not as well studied as those of gram-negative aerobic or facultative bacteria. Although various virulence factors have been identified, in many instances the precise role of virulence factors and pathogenic mechanisms by which gram-negative anaerobes initiate infection and cause disease are not well defined. However, similar to aerobic or facultative bacteria, gram-negative anaerobes contain cell structures (e.g. capsule, fimbriae, flagella, agglutinins, adhesins, lipopolysaccharide (LPS), and outer membrane proteins) and secrete exotoxins (e.g. enterotoxin, hemolysin, and leukotoxin) and enzymes (e.g. neuraminidase, proteases, DNases, and lipases) that facilitate adherence, colonization, immune system evasion, invasion, and tissue destruction. In addition, a few fermentation products of anaerobic metabolism (e.g. lactic acid, butyric acid, succinic acid, ammonia, and amines) have inflammatory and cytotoxic effects and contribute to pathogenesis.

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Capsule

Capsular substances protect the outer membrane from the membrane attack complex of the complement cascade, mediate bacterial from attachment to the host cells, and inhibit ingestion by phagocytic cells. The capsules of *Bacteroides fragilis*, pigmented *Prevotella*, and *Porphyromonas* incite an intense inflammatory response. Capsular polysaccharide of *B. fragilis* can produce abscesses even in the absence of live cells.

Cell Wall

Gram-negative anaerobes contain a typical wall composed of lipoteichoic acids, peptidoglycan, LPS, and proteins. The LPS in the outer membrane is an important virulence determinant. The LPS of gram-negative anaerobes is generally considered biologically less potent than typical LPS of aerobic or facultative gram-negative bacteria, such as *Enterobacteriaceae*. Several conflicting explanations have been suggested, including differences in lipid A composition. However, the LPS of *Fusobacterium* sp. has been reported to be as potent as that of *Escherichia coli* LPS. The interactions of lipoteichoic acids and peptidoglycan with macrophage cells have been shown to release pro-inflammatory cytokines. The composition of inner and outer membrane proteins of certain gram-negative anaerobes (*B. fragilis, Fusobacterium necrophorum*) has been described.

Fimbriae

The fimbriae, if present, play an important role in adhesion, an initial event in the bacteria–host interaction. A good example of a fimbriated gram-negative anaerobe is *Dichelobacter nodosus*, the primary causative agent of foot rot in sheep.

Extracellular Products

Exotoxins, enzymes, and metabolic fermentation products with toxic activity have been demonstrated. The two well-characterized exotoxins are leukotoxin of *F. necrophorum* and an enterotoxin of *B. fragilis*. Many produce proteases, DNases, and other enzymes that may play a role in their pathogenic activities. IgA proteases, which have been shown to be produced by highly pathogenic species of facultative gram-negative bacteria, are produced by *Porphyromonas levii*. Short-chain fatty acids, which are produced in great quantities by anaerobes and accumulate at the infected site (contribute to putrid smell), have been shown to impair host phagocytic functions.

The ability of pathogenic anaerobes to create an anaerobic microenvironment (low redox potential) or to tolerate oxygen exposure is a prerequisite for their ability to establish infection. An anaerobic microenvironment can be created by the action of certain virulence factors (e.g. LPS, hemolysin, and platelet aggregation factor) or because of synergy with facultative bacteria. The mechanisms involved in this synergism are the supply of energy substrate, supply of essential growth factors, or in the case of facultative bacteria the production of low redox potential. A classic example of a synergistic pathogenic interaction is between *F. necrophorum* – an anaerobe – and *Trueperella pyogenes* – a facultative bacterium – both of which frequently occur together in infections such as liver abscesses, foot rot, and metritis in cattle and other animals (Table 33.1).

Oxygen tolerance allows anaerobes to survive in infected tissues until conditions become more conducive for growth and invasion. *B. fragilis* and *F. necrophorum*, for example, not only survive but can grow at low oxygen tensions.

The most common form of infection caused by gram-negative, non-spore-forming anaerobes is abscesses, although other infections, including septicemia can occur. Generally, abscesses are formed closer to the mucosal surfaces or at sites of direct contact, although distant abscesses can be formed because of hematogenous spread. Suppurative infections caused by anaerobic gram-negative bacilli are often polymicrobial, including both anaerobic and facultative bacteria, suggesting that anaerobic gram-negative bacteria may be of low virulence and that the virulence factors may be of low potency. The association of certain organisms may contribute to synergy, resulting in some cases of enhanced infectivity of anaerobes.

Table 33.1 Synergy between *Fusobacterium necrophorum* and *Trueperella pyogenes*.

Factor	*Trueperella pyogenes*	*Fusobacterium necrophorum*
Oxygen relationship	Facultative: Uses oxygen and creates anaerobiosis	Anaerobic, but aerotolerant
Substrate utilized or product produced	Lactic acid is a major product of substrate utilization	Utilizes lactic acid as an energy substrate
Toxin production	Produces hemolysin: Lyses erythrocytes and releases iron	Produces leukotoxin, which protects bacterial cells from being phagocytosed

Mode of Infection

The non-spore-forming gram-negative anaerobes that initiate pyogenic or necrotic lesions are usually part of the normal flora; hence, they are often an endogenous infection. Sometimes they are transmitted by bites or other trauma involving contaminated fomites.

Pathogenesis

Disease results from the extension of the normal flora (both obligate and facultative anaerobic bacteria) into a compromised site, either by contamination of a wound with nearby normal flora or from inoculation into tissue with biting or contaminated instruments. The kinds of anaerobes found in samples of such material reflect the site of injury or the bacterial population of the source of inoculation. Initiation of infection requires proliferation of anaerobes, which would be facilitated by the establishment of anaerobic conditions by trauma, vascular breakdown, or coinfection with facultative or aerobic bacteria. Synergistic interactions occur between facultative aerobic and anaerobic bacteria. The facultative species scavenge oxygen, curtail phagocytosis of the anaerobic component, provide nutrients (e.g. lactic acid), and may produce enzymes (e.g. β-lactamase) that might protect a penicillin-susceptible facultative or obligate anaerobic partner (and vice versa). Also, in compromised tissue, inflammatory cells and coinoculated facultative bacteria lower the redox potential (Eh) sufficiently for anaerobes to grow. Some anaerobes (*B. fragilis*) produce capsules that, due to their chemistry, are potent inducers of abscess formation.

Diagnosis

Clinical conditions that are indicative of anaerobic infections include putrid-smelling lesions and discharges, gas in the tissue, deep infections of mucosal or skin surfaces, and necrotic or gangrenous tissues. Specimens that exhibit bacteria on direct examination but are culture-negative by routine cultivation and infections that do not respond to aminoglycoside therapy are also suggestive of anaerobic bacterial involvement.

Sample Collection

Conventional anaerobic culture and identification methods are labor-intensive, time-consuming, expensive, and require some degree of expertise in handling samples and in isolation and identification. Samples obtained from sites that possess a normal anaerobic flora (feces, oral cavity, and vagina) are not usually cultured anaerobically. Routine anaerobic culture of urine specimens or ear, conjunctival, or nasal swabs is rarely justified. Suppurative and necrotic processes are the most promising sources of clinically significant anaerobic bacteria. Samples of fluids for anaerobic culture are collected in containers with little if any air space or oxygen. The easiest way is to collect the sample directly into a syringe and expel all the air. Materials collected onto swabs or bronchial brushes must be placed in culture immediately or into an anaerobic environment (anaerobic transport medium). A number of commercial sample collection or transport systems for anaerobic bacteria are available.

Direct Examination

Examination of stained smears prepared directly from the collected material may give valuable clues regarding the presence of anaerobic bacteria, particularly if aerobic culture comes out negative. Many obligate anaerobes have typical, unique morphologies: rods are usually narrow and thread-like in appearance, with some having pointed ends or bulges. Most of the gram-negative species stain poorly with the safranin used in the Gram stain (thus will be pale staining in gram-stained smears).

Isolation

Generally, anaerobic techniques for bacteriological analysis are based on the following principles:

1) Specimens and samples should be shielded from oxygen (exposure to air should be minimized) prior to bacteriological analysis.
2) Culture media used should have low redox potential. The media generally used include cooked meat broth, sodium thioglycolate medium, and brain heart infusion (BHI) or *Brucella* medium containing reducing agents (pre-reduced, anaerobically sterilized medium).
3) Cultures should be incubated in an oxygen-free atmosphere. The two most commonly used incubation methods are the anaerobic jar and the anaerobic glove box. An anaerobic environment is established by the reaction of a hydrogen-containing gas with the oxygen in air in the presence of a palladium catalyst in a closed container, such as in an anaerobic jar or in a glove box. The major advantages of an anaerobic glove box with a built-in incubator are that samples can be processed, inoculated, and incubated under anaerobic conditions, and inoculated plates can be examined at any time without being exposed to oxygen.

It is a good laboratory practice to process samples intended for anaerobic culture with minimal delay. If the sample is not processed immediately after collection, it should be held in an oxygen-free atmosphere, usually a container into which oxygen-free gas (CO_2 or nitrogen) is flowing. The sample is plated onto a blood agar (usually with a BHI or *Brucella* agar base) that has been stored in an anaerobic environment. After the plate has been inoculated, it is placed into an anaerobic environment and incubated at 37 °C. Most obligate anaerobes grow slowly, especially during the early stages, and plates are not examined for the first 48 hours unless they can be examined in an O_2-free environment (e.g. in a glove box). Because facultative species can grow anaerobically, colonies growing in an anaerobic environment must be tested for aerotolerance.

Identification

After an isolate has been shown to be an obligate anaerobe, the genus to which it belongs is determined by shape, gram-staining characteristics, and metabolic end products formed from an assortment of substrates, as determined by liquid–gas chromatography. Reactions in pre-reduced, anaerobically sterilized media containing different substrates help determine the species. Rapid and miniaturized systems are commercially available for identification of anaerobes of clinical importance. In recent years, there is evidence that most anaerobes of clinical importance can be identified by matrix-associated laser desorption ionization time of flight mass spectrometry (MALDI-TOF). Because MALDI-TOF is rapid, not labor intensive, inexpensive after the initial investment in the instrument, and allows direct identification from cultured plates, the method has replaced conventional identification systems in diagnostic laboratories.

Treatment, Control, and Prevention

Treatment of infections caused by anaerobes typically involves drainage of abscesses and the use of antimicrobial agents. Susceptibility data are usually not available for at least 48–72 hours after the sample is collected. Prior to this time, if the presence of anaerobes is suggested by the clinical presentation, direct smear, and other circumstances (odor), one of the following can be used: penicillin (ampicillin and amoxicillin), chloramphenicol, tetracyclines, metronidazole, and clindamycin. Although most anaerobes will test "susceptible" to trimethoprim–sulfonamides *in vitro*, this combination has unpredictable activity *in vivo* due to the presence of thymidine in necrotic material. The obligate anaerobes are resistant to all the aminoglycoside antimicrobial agents as well as to most fluoroquinolones (trovafloxacin is the exception). Approximately 10–20% of the isolates, usually members of the *B. fragilis* group, will be resistant to the penicillins (penicillin G, ampicillin, and amoxicillin) and first- and second-generation cephalosporins due to the production of a cephalosporinase, and often to tetracycline as well. Resistant isolates are susceptible to clavulanic acid–amoxicillin, clindamycin, metronidazole, and chloramphenicol. Antimicrobial therapy should be aimed at both the facultative and obligate anaerobic bacteria.

Dichelobacter nodosus

The species, the only member of the genus in the family *Cardiobacteriaceae* of the phylum Proteobacteria, is the etiologic agent of foot rot in sheep. Other animals, such as goats, cattle, and camelids, may carry the organisms, but rarely get infected to exhibit clinical signs. Although *D. nodosus* has not been shown to fulfill Koch's postulates, it is considered as the primary etiologic agent because vaccination against *D. nodosus* reduces disease occurrence. In addition to *D. nodosus*, another gram-negative anaerobe, *F. necrophorum* (described later), and a gram-positive facultative bacterium, *T. pyogenes* (see Chapter 27), are implicated. In experimental infections, *F. necrophorum* has been shown to be required for infection of the foot to be initiated by *D. nodosus*, and a strong association of the two bacteria has been shown in field cases of foot rot. There is some indication that *F. necrophorum* associated with sheep foot rot may be a distinct variant of *F. necrophorum* involved in bovine foot rot. In the early stages of infection, the population density of *D. nodosus* is highest in the lesions and decreases subsequently, possibly because of the entry of other bacteria.

Ovine foot rot is a contagious and debilitating hoof disease that begins as an interdigital dermatitis and progresses into formation of lesions on the interdigital wall of the hoof, leading to separation of the keratinous hoof from the underlying tissue. The obvious clinical signs are lameness and loss of body condition because of reduced feed intake and in some cases death from a combination of starvation, thirst, and systemic bacterial infections in sheep that are recumbent for prolonged periods.

Morphology, Growth Characteristics, and Biochemical Activities

D. nodosus is a straight or slightly curved rod measuring 1–1.7 to 3–10 µm. In smears from lesions, the rods exhibit terminal swellings, giving the appearance of a "dumbbell" (Figure 33.1); however, the feature is less discernible when

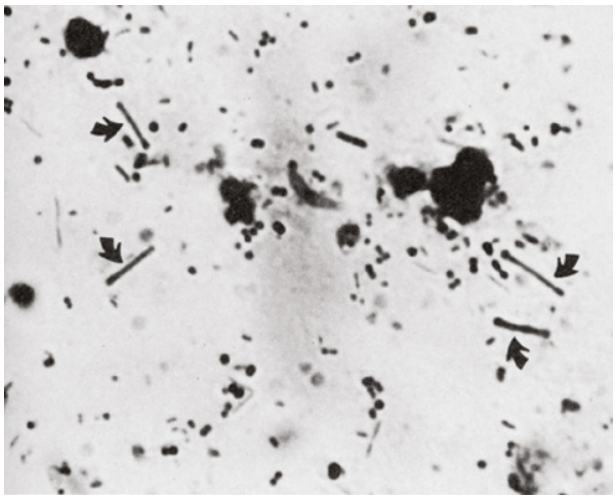


Figure 33.1 Exudate from ovine foot rot. Mixture of bacterial species, with *D. nodosus* recognizable as large rods with swollen ends: "dumbbells" (arrows) (Gram stain, 1000×).

clinical isolates are subcultured in broth. The organism is not flagellated but possesses long filamentous appendages called type IV fimbriae, because of their polar location and the conserved structure of the major fimbrial subunit protein (similar to type IV of *Moraxella bovis*, *Neisseria gonorrhoeae*, *Pasteurella multocida*, and *Pseudomonas aeruginosa*). Type IV fimbriae confer flagella-independent motility, called twitching motility, which leads to spreading of colonies on a plate, revealing a fuzzy appearance.

D. nodosus requires carbon dioxide and a rich medium, preferably containing protein, for growth. Although colonies may be visible on plates after two days, at least four to five days of incubation are required to see smooth colonies of about 1–2 mm in diameter. The species is described as a "strict" anaerobe; the organism is highly aerotolerant and can survive on plates exposed to air for up to 10 days. *D. nodosus* survives in the environment for two to three days and is killed by disinfectants and many antibiotics. The organism can survive in and is transmitted via soil and can also persist for months as a subclinical infection in the interdigital skin or in small cryptic lesions within the hoof.

The genome of *D. nodosus* is relatively small, with 1.4 MB and a GC content of 44.4%. Genome sequences of globally distributed strains have revealed that they are highly conserved with >95% sequence identity and form two distinct clades of benign and virulent phenotypes and genotypes.

Virulence Factors

Among the virulence factors identified, the two major contributors to the pathogenesis of foot rot are fimbriae and extracellular serine proteases.

Fimbriae

The type IV fimbriae, encoded by *fimA*, are involved in adherence of *D. nodosus* to the epithelial cells of the interdigital epidermis. The role of fimbriae in causing foot rot initially involves the promotion of close contact between *D. nodosus* and host cells. The twitching motility then enables translocation of the organism into a more anaerobic microenvironment, which is required for the bacterial growth and extracellular protease production, ultimately leading to lesion formation. Therefore, the twitching motility and production of extracellular proteases are key processes for virulence.

Serine Proteases

Strains of *D. nodosus* secrete three closely related serine proteases that have been postulated to be responsible for the tissue damage in foot rot infections. All proteases have a similar structure and are synthesized with a preproregion at the N-terminus of the mature protease and a C-terminal extension. The cleavage of both of these regions results in the active proteases. Highly virulent strains produce two acidic proteases, AprV2 and AprV5, and one basic protease, BprV. Only AprV2 has been shown to be essential for virulence because a mutant lacking the *aprV2* gene does not cause the disease. Strains that carry *aprV2* gene, but not those for the other two proteases, are associated with virulent foot rot. Strains that cause benign lesions produce similar proteases designated AprB2, AprB5, and BprB, which only show minor differences in amino acid sequences compared to the proteases of the virulent strains. The protease genes are located on pathogenicity islands (a cluster of genes encoding virulence determinant(s), an integrase protein, a specific insertion site, and mobility), which suggests that they may have originated from extrachromosomal sources.

Cell Wall

The chemical and biological characteristics of LPS of *D. nodosus* are similar to the LPS of other gram-negative bacteria.

Outer Membrane Proteins

These are not known to play a direct role in the pathogenesis but are thought to interfere with the host's immune response. The outer membrane proteins are highly antigenic and phase variant as a result of site-specific inversions within the outer membrane protein genes. The phase variance allows *D. nodosus* to undergo antigenic shifts to evade the immune system during the course of infection.

Variations in Virulence

The strains of *D. nodosus* have a wide range of virulence. The virulence can be tested *in vitro* for the presence and

activity of certain virulence factors (e.g. proteases and twitching motility caused by type IV fimbriae). The fimbrial proteins, which are highly immunogenic, are the basis of serotyping and classification of strains into 10 serogroups, named A to I and M. Based on the structural variation within the fimbrial protein FimA and the genetic organization of the *fimA* gene, the isolates of *D. nodosus* from the 10 serogroups are divided into two major classes. The isolates in serogroups A–C, E–G, I, and M that have the *fimB* gene (downstream to *fimA* gene), which is not required for fimbrial synthesis, belong to class I. The isolates in serogroups D and H that contain three genes adjacent to *fimA*, *fimC*, *fimD*, and *fimZ* of unknown functions belong to class II. However, no specific serotypes are associated with strain virulence.

Mode of Infection, Transmission, and Pathogenesis

Infection typically occurs because of physical injury, poor claw hygiene, and moist environmental conditions. Infection is initiated because virulent strains outcompete benign strains, suggesting that benign strains may be part of the normal microbiome of the hoof. Transmission is by direct or indirect contact. The brief environmental survival time of the agent requires prompt colonization of new hosts. Pathogenic mechanisms include the fimbriae-mediated attachment to host cells, proteolytic activity, and synergy with *F. necrophorum*.

Disease Patterns

The severity of the disease depends on the virulence of the strain. Foot rot is characterized by an exudative inflammation, followed by necrosis, of the epidermal tissues of the hoof. Three different forms of the disease have been recognized: virulent, intermediate, and benign. The virulence of a specific outbreak is governed by the population type of *D. nodosus* and the various factors that affect the infections, including environmental factors. The virulent form of foot rot is characterized by destruction of the horn and erosion of the skin–horn junction that penetrates the underlying tissues, causing delamination. The virulent form is also highly contagious and has significant impact on productivity. The benign form causes inflammation of the interdigital skin with dermatitis, but no destruction of the underlying tissues and delamination occurs. The sequence of events is typically as follows:

1) The interdigital epidermis is softened and easily damaged because of persistent soaking.
2) *F. necrophorum*, a soil organism, infects the damaged skin and produces superficial inflammation, hyperkeratosis, parakeratosis, and necrosis.
3) *D. nodosus* colonizes (with the aid of fimbriae) and proliferates in the lesion initiated by *F. necrophorum*, producing interdigital swelling. Invasion of epidermal structures begins at the medial aspect of the claw and, probably with the help of secreted proteases, advances to the epidermal matrix of the hoof, eventually separating it from the underlying dermal tissues ("underrunning").

Secondary invaders help maintain or aggravate the process. The result is extreme lameness. Affected sheep typically stand on three legs and exhibit lameness on ambulation, and sometimes graze while resting on the knee joints. When two or more feet are involved, sheep become immobile. Virulent foot rot is extremely painful; therefore, the disease is an animal welfare issue.

Foot rot causes substantial economic losses to the sheep industry. Direct costs are caused by reduced reproduction in ewes, increased fattening time for lambs, and reduction in milk yield and wool quality.

Epidemiology

Although *D. nodosus* is specific to sheep and goats, the organism is reported in foot lesions of other animals, including cattle, horses, pigs, and deer. The disease occurs on all continents. Cattle grazing with foot-rot-affected sheep can become carriers of *D. nodosus*. It is most serious in regions with a mild climate and periods of abundant rainfall (>20 in. (500 mm)). Dissemination of *D. nodosus* essentially ceases at ambient mean temperatures of less than 50 °F (10 °C), and foot rot does not occur in arid regions and improves during dry periods in endemic areas. All ages of animals beyond nursing stages are susceptible, but genetic differences in susceptibility exist. There is evidence that Merinos, a popular breed in Australia and New Zealand for wool production, are more susceptible than others. The environmental and management factors are believed to be more important than host genetics.

Diagnosis

Diagnosis is often based on obvious clinical signs. Direct smears from the foot lesion may reveal stout rods with terminal swellings (see Figure 33.1). Culture-based diagnosis is not routinely done because of fastidious growth requirements and the slow growth of the organism. Agar medium with hoof powder has shown to be beneficial for primary isolation. Once isolated, the organism is easy to culture. The isolates can be readily identified or confirmed by MALDI-TOF. Virulent strains can be phenotypically identified based on the gelatin gel thermostability test, which differentiates heat-stable and heat-labile extracellular proteases of APrV2 and AprB2, respectively. Polymerase chain

reaction (PCR) is often the method of choice for rapid diagnosis. Several PCR assays targeting 16S rRNA, *fimA,* or *intA* have been described. A competitive real-time PCR assay has been developed to discriminate virulent from benign strains based on the presence of *aprv2* and *aprB2,* respectively. The differentiation between the virulent and benign strains is based on a two-nucleotide difference resulting in a single amino acid difference in the AprV2 and AprV2 proteins. In epidemiological investigations, serotyping of the clinical isolates becomes important. Molecular serotyping based on type IV pili encoded by *fimA* can be done by multiplex PCR assay from cultured bacteria. Also, a multilocus sequence typing scheme based on seven loci has been established and is publicly available at pubMLST.org.

Treatment and Control

Treatment begins with removal and exposure of diseased tissue by hoof trimming (foot paring), followed by topical application of disinfectants or antibiotics, such as repeated treatment with 5–10% formalin, 5% copper sulfate, 10–20% zinc sulfate, or 5% tetracycline tincture. The use of foot baths is one of the common methods of control for foot rot. Formalin, copper sulfate, and zinc sulfate are used in footbaths. Three 1 hour 20% zinc sulfate soaks at weekly intervals have proved effective. Copper sulfate solution as foot bath is effective and inexpensive and is active even in the presence of organic matter. Antimicrobials such as oxytetracycline, florfenicol, amoxicillin, penicillin and streptomycin, lincomycin and spectinomycin, enrofloxacin, gamithromycin, and erythromycin have shown to achieve clinical cure.

Vaccines have been shown to be effective in treating and preventing foot rot. Vaccination is extensively used in sheep-producing countries, particularly Australia and New Zealand, and is an integral part of the eradication programs. Vaccination also has some therapeutic benefit in accelerating healing. The effective vaccines are based on fimbrial proteins as the protective antigen. There is reasonable correlation between serum antifimbrial agglutinin titers and resistance of vaccinated sheep to homologous infection. The limitation of the vaccine is the need to protect multistrain infections, and the inclusion of multistrains in a vaccine reduces the effectiveness because of antigenic competition. Commercially available vaccines contain 8–10 strains of the common serogroups. Use of monovalent vaccine is useful, but requires isolation and identification of the serogroup. The immunity developed from vaccination lasts 12–16 weeks, which is long enough to control during the foot rot season.

Control at a farm level is achieved by a combination of repeated examination, regular foot bathing and feet trimming, vaccination, systemic or topical antimicrobial use in active cases, and segregation of active cases from the healthy flock. Care must be taken to avoid adding infected animals to the flock. Contaminated lots should not be restocked for two weeks. Control programs should be instituted during dry weather.

Fusobacterium necrophorum

The name, *Fusobacterium*, comes from the Latin word *fusus* meaning "spindle." However, not all fusobacterial species have the characteristic spindle-shaped cells. A common biochemical feature of members of the genus is the production of butyric acid as a major fermentation product. Although fusobacteria stain gram negative, the organisms are related more to the gram-positive bacteria. For example, fusobacterial species are susceptible to typically gram-positive antibacterial spectrum antibiotics such as penicillins, tylosin, and virginiamycin. Currently, the genus *Fusobacterium* includes the following 17 species: *Fusobacterium canifelinum*, *Fusobacterium equinum*, *Fusobacterium gastrosuis, Fusobacterium gonidiaformans*, *Fusobacterium mortiferum*, *Fusobacterium naviforme*, *Fusobacterium necrogenes*, *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Fusobacterium perfoetans*, *Fusobacterium periodonticum*, *Fusobacterium plautii*, *Fusobacterium polysaccharolyticum*, *Fusobacterium russi*, *Fusobacterium simae*, *Fusobacterium ulcerans*, and *Fusobacterium varium*. Of these, *F. nucleatum* and *F. necrophorum* are the two most prevalent species of *Fusobacterium* in clinical samples. Both species are further categorized into two or more subspecies.

F. canifelinum, *F. mortiferum*, *F. naviforme*, *F. nucleatum*, *F. periodonticum*, *F. ulcerans*, and *F. varium* have been isolated mainly from human clinical samples. *F. canifelinum* and *F. russi* are members of the canine and feline flora and have been isolated from infected dog and cat bite wounds in humans. *F. gastrosuis* has been isolated from the stomach of pigs. *F. nucleatum* is one of the predominant organisms involved in gingivitis and periodontal diseases, particularly in children and young adults. *F. equinum*, *F. gastrosuis, F. necrogenes*, and *Fusobacterium simiae* are isolated mainly from animal infections. *F. equinum* is a relatively new species, which is phenotypically similar to *F. necrophorum,* and has been isolated from the normal oral cavity and oral-associated diseases of horses.

F. necrophorum is a major human and animal pathogen and is part of the bacterial flora of the oral cavities, gastrointestinal tracts, and genitourinary tracts of animals and humans. The species name is derived from the organism's frequent association with necrotic lesions in humans and animals. In animals, *F. necrophorum* is considered a major

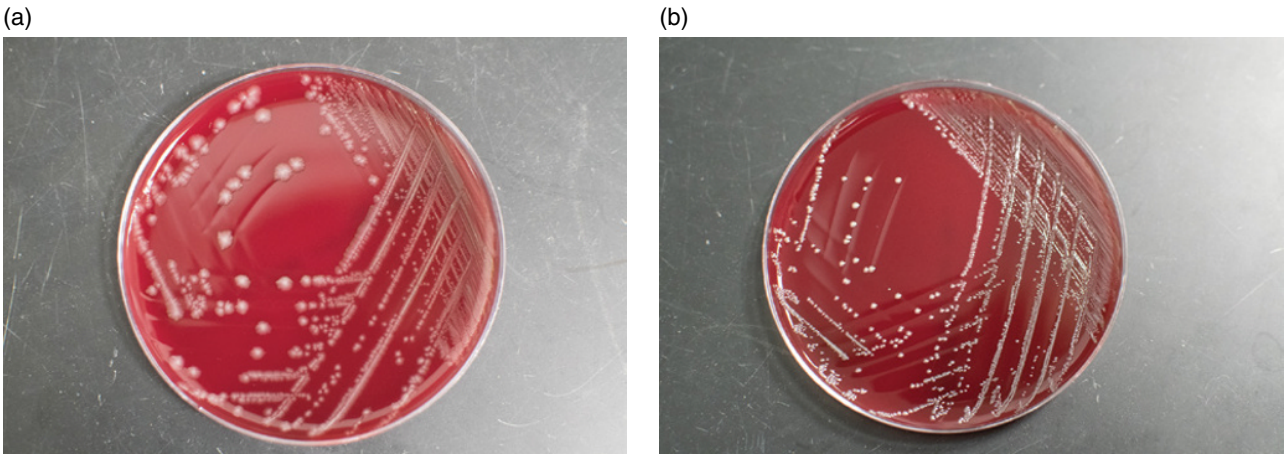


Figure 33.2 Colony morphologies of ssp. *necrophorum* (a) and ssp. *funduliforme* (b).

bovine pathogen because of its involvement in certain economically important diseases. It is one of the most common anaerobes isolated from abscesses in the abdominal area and respiratory tract infections in animals.

Morphology, Growth Characteristics, and Biochemical Activities

F. necrophorum is a gram-negative, nonmotile, nonsporulating, short to filamentous rod-shaped (pleomorphic) bacterium. The species is classified into two subspecies: ssp. *necrophorum* (formerly biotype A) and ssp. *funduliforme* (formerly biotype B). These two subspecies differ in colony characteristics (Figure 33.2), cell morphology (Figure 33.3), growth patterns in broth, extracellular enzymes, hemagglutination properties, virulence factors, and virulence in laboratory mice (Table 33.2). The organism is anaerobic, but aerotolerant. Primary isolates from clinical samples grow well on blood agar, BHI, or *Brucella* agar, as long as incubation is under anaerobic conditions. Although the organism secretes a hemolysin, a zone of hemolysis is usually not observed, but is often seen when frozen isolates stored at −80 °C are streaked onto blood agar plates. When cultured in a broth (it grows well in BHI broth that is pre-reduced and anaerobically sterilized), cells of ssp. *funduliforme* sediment to the bottom of the tube, whereas growth of ssp. *necrophorum* is uniformly turbid (Figure 33.4). The organism generally does not ferment any carbohydrates, although some strains ferment glucose weakly. The major energy substrate is lactic acid, which is fermented mainly to acetate, butyrate, and small amounts of propionate. An important biochemical characteristic that is useful in the identification is the production of indole from tryptophan.

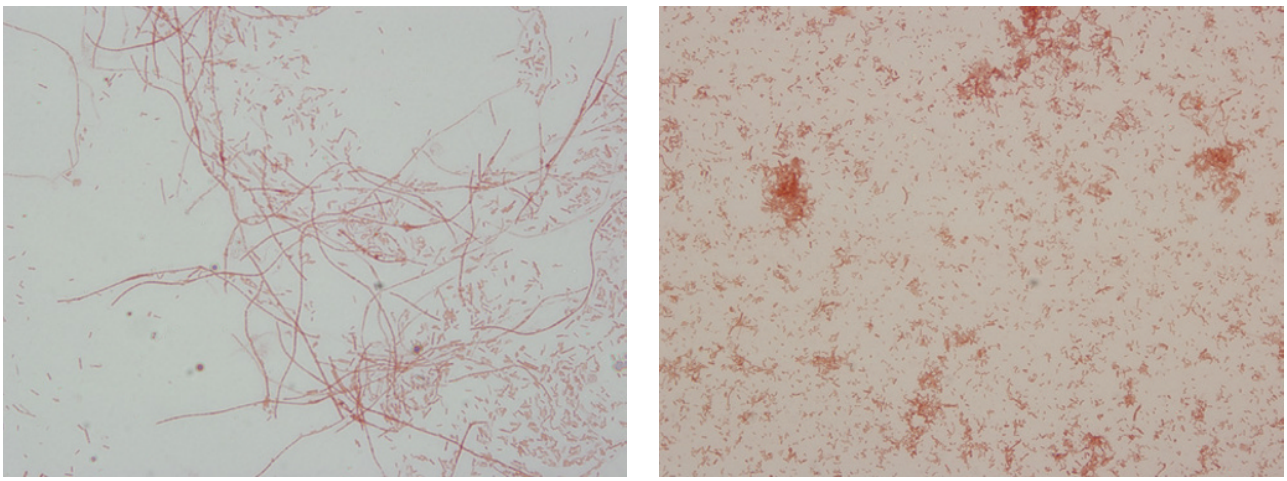


Figure 33.3 Microscopic morphologies of ssp. *necrophorum* (a) and ssp. *funduliforme* (b).

Table 33.2 Growth and biochemical, biological, and molecular characteristics of *F. necrophorum* ssp. *necrophorum* and ssp. *funduliforme*.

Characteristics	ssp. *necrophorum*	ssp. *funduliforme*
Colony morphology	Umbonate with irregular edges, opaque, and grayish to white in color	Small, smooth, round, waxy, and yellow in color.
Microscopic morphology	Highly pleomorphic, short to long rods, and many of the rods are filamentous (2–100 μm)	Short rods and typically more uniform (not pleomorphic)
Growth in broth, sedimentation	−	+
Biochemical characteristics		
Indole production	+	+
Phosphatase	+	−
Proteases	++	+
DNase	+	−
Lipase	+	−
Virulence in mice, % mortality	92–97	8–10
Biological activities		
Leukotoxin production	+++	+ or −
Hemagglutinin titer	+++	+ or −
Lipid A, % of LPS	15	4
Molecular characteristics[a]		
rpoB gene	+	+
Hemagglutinin (*haem*) gene	+	−
Leukotoxin (*lkt*) operon promoter length (bp)	548	337

[a] PCR amplification methods.

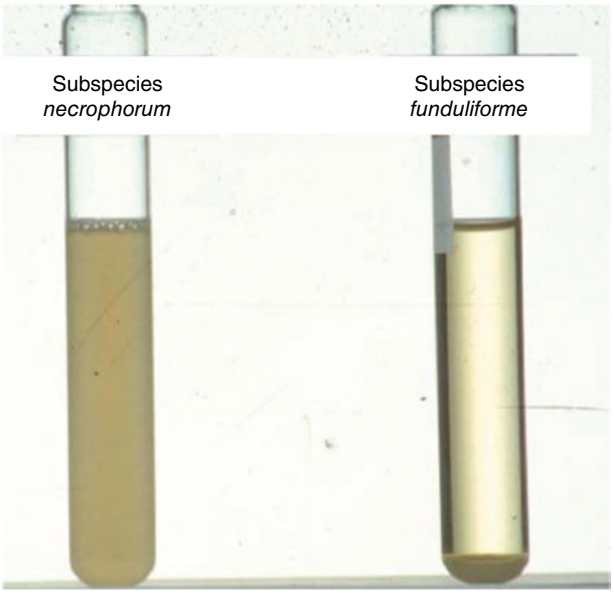


Figure 33.4 No sedimentation of broth culture of ssp. *necrophorum* (left) and sedimentation of broth culture of ssp. *funduliforme*

Virulence Factors

Cellular structures or products implicated in the pathogenesis of *F. necrophorum* include leukotoxin (or leukocidin), endotoxic LPS, hemolysin, hemagglutinin, capsule, adhesins, platelet aggregation factor, dermonecrotic toxin, and several extracellular enzymes, including proteases and deoxyribonucleases (Table 33.3). These factors contribute to entry, colonization, proliferation, and establishment of the organism, and to the development of pyonecrotic lesions. Similar to other gram-negative bacteria, the outer membrane of *F. necrophorum* contains endotoxic LPS. The chemical composition of *F. necrophorum* LPS varies depending on the subspecies. Also, LPS from ssp. *necrophorum* has been shown to biologically more potent than that from ssp. *funduliforme*. Leukotoxin is considered to be the major virulence factor involved in fusobacterial infections in animals, and is also a protective antigen,

Leukotoxin

It is a secreted protein that exerts cytotoxic activity on neutrophils, macrophages, hepatocytes, and possibly cells of the mucous membrane, skin, and ruminal epithelial cells. The

Table 33.3 Virulence factors of *Fusobacterium necrophorum*.

Factor	Characteristics	Mechanism of action.	Role in infections.
Leukotoxin	Extracellular protein (molecular weight 336 kDa).	Cytotoxic to neutrophils, macrophages, hepatocytes, and possibly to ruminal epithelial cells.	Protects against phagocytosis by neutrophils and macrophages, damages tissues by the release of cytolytic products.
Endotoxin	Outer membrane component, lipopolysaccharide.	Inflammatory and necrotic effects; induces disseminated intravascular coagulation.	Creates anaerobic microenvironment conducive to anaerobic growth.
Hemolysin	Extracellular but cell-associated protein.	Lyses erythrocytes of various animals.	Helps acquire iron, which stimulates bacterial growth, from the host; creates an anaerobic microenvironment.
Hemagglutinin	Cell-wall-associated protein.	Agglutinates erythrocytes of various animals.	Mediates attachment to epithelial cells.
Adhesins	Outer membrane proteins.	Affinity binding to host cells.	Mediates attachment to epithelial and endothelial cells.
Dermonecrotic toxin	Cell wall associated.	Causes necrosis in the epithelial cells.	Helps penetrate the epithelium or skin.
Proteases	Extracellular protein.	Breaks down cell protein.	Helps penetrate the ruminal epithelium or skin.

toxin is a high-molecular-weight (336 kDa) and heat-labile protein and is somewhat unique in lacking the amino acid cysteine, being considerably larger than leukotoxins of other bacteria with no significant sequence similarity to any other bacterial toxin. The leukotoxin operon consists of three genes – *lktB*, *lktA*, and *lktC* – of which *lktA* is the structural gene. The LktB protein is probably involved in secretion, and the function of LktC is not known. The importance of leukotoxin as a virulence factor is indicated by a correlation between toxin production and ability to induce abscesses in laboratory animals, the inability of leukotoxin-negative strains to induce foot abscesses in cattle following intradermal inoculation, and the relationship between antileukotoxin antibody titers and protection against infection in experimental challenge studies. *F. necrophorum* ssp. *necrophorum* produces more leukotoxin than ssp. *funduliforme*, which may explain why ssp. *necrophorum* is more frequently associated with infections, particularly in liver abscesses of cattle, than ssp. *funduliforme*. The role of leukotoxin in the pathogenesis includes thwarting attempts to contain the infection, modulation of the host immune system by its toxicity, including cellular activation of polymorphonuclear neutrophils and apoptosis-mediated killing of phagocytes and immune effector cells. The release of inflammatory mediators and the activated immune effector cells play the central role in the pathophysiology of fusobacterial infections.

Ecology

Reservoir

F. necrophorum is a normal inhabitant of the gastrointestinal tract of animals and humans. The bacterium is frequently isolated from healthy hosts, although at a relatively low concentration. Normal human feces contain relatively high concentrations of *Fusobacterium*, of which *F. necrophorum* is the most predominant species. The organism is also a member of the normal flora of the oral cavity and female genital tract of animals and humans. In cattle, *F. necrophorum* is a normal inhabitant of the rumen and has been isolated from ruminal contents of cattle fed a varied diet. The concentration in the rumen ranges from 10^5 to 10^7/g of ruminal content, with concentrations higher in grain-fed cattle compared to forage-based diet. Because *F. necrophorum* uses lactate as its major substrate and not sugars, the increased population in cattle fed high-grain diet is probably due to increased lactate availability. Both subspecies of *F. necrophorum* have been isolated from ruminal content.

In the rumen, fusobacteria are present as free-floating organisms or they are attached to the ruminal epithelial wall. Fusobacteria are ideally suited to the ruminal wall niche because of their aerotolerance and ability to grow best at physiological pH (7.4). Possibly, the attachment is mediated via outer membrane proteins. Reports on isolations of *F. necrophorum* from the ruminal wall are limited and are always in association with ruminal lesions.

Pathogenesis

Mode of Infection

Fusobacterial infections in animals are endogenous, generally initiated by entry into compromised tissues, such as the oral mucosa or ruminal wall. In the case of foot rot, the source of infection could be soil, which probably got contaminated with feces.

Table 33.4 Diseases caused by *Fusobacterium necrophorum* in animals and humans.

Host	Disease	Subspecies	Other commonly associated bacteria
Beef cattle	Hepatic necrobacillosis (liver abscesses)	*necrophorum* and/or *funduliforme*	*Trueperella pyogenes, Salmonella enterica*
Beef and dairy cattle	Interdigital necrobacillosis (foot rot)	*necrophorum* and/or *funduliforme*	*Trueperella pyogenes, Porphyromonas levii, Dichelobacter nodosus*
Calves (< 1 year old)	Necrotic laryngitis (calf diphtheria)	*necrophorum* and/or *funduliforme*	*Trueperella pyogenes, Pasteurella multocida*
Dairy cows	Metritis	*necrophorum*	*Trueperella pyogenes, Escherichia coli*
Dairy cows	Summer mastitis	ND	*Trueperella pyogenes, Peptostreptococcus indolicus*
Horses	Thrush	ND	
Antelopes	Mandibular abscesses Respiratory infections	*necrophorum* and/or *funduliforme*	*Trueperella pyogenes*
Humans	Pharyngitis (sore throat), Pharyngotonsillitis, Peritonsillar abscesses, Lemierre's syndrome and soft tissue abscesses	*funduliforme*	

ND = Not determined.

Fusobacterium Infections in Animals

F. necrophorum is commonly associated with numerous clinically significant anaerobic infections (Table 33.4). However, the prevalence of *F. necrophorum* infection is still believed to be underestimated. *F. necrophorum* is associated with numerous necrotic disease conditions, generally termed "necrobacillosis," and most often the infection is common in the liver (hepatic abscesses), feet (interdigital necrobacillosis or foot rot), mammary gland (mastitis), uterus (metritis and pyometra), and oropharyngeal mucosa (necrotic laryngitis or calf diphtheria in cattle and mandibular abscesses in antelopes, marsupials, and wild animals). Most often fusobacterial infections are polymicrobial, with a multitude of facultative and anaerobic species involved, but the most common ones are *T. pyogenes*, *D. nodosus*, and *P. levii*.

F. necrophorum is a major pathogen of cattle, and causes three economically important diseases: hepatic necrobacillosis, interdigital necrobacillosis, and necrotic laryngitis.

Liver Abscesses (Hepatic Necrobacillosis)

Liver abscesses occur in all ages and in all types of cattle and in different parts of the world, but are most common in intensively fed beef cattle in the United States, Canada, Japan, and South Africa. Abscesses found in the liver at the time of slaughter are often well encapsulated, possessing thick fibrotic walls (Figure 33.5). Abscesses in the liver are secondary to the primary foci of infection on the ruminal wall. Because of the close correlation between the incidence of ruminal pathology and liver abscesses in cattle, the term "rumenitis-liver abscess complex" is often used. Although the precise pathogenesis is not proved, it is accepted that rapid fermentation of grain by ruminal microbes and the consequent accumulation of organic acids result in ruminal acidosis. Acid-induced rumenitis and damage of the protective surface, often aggravated by foreign objects (e.g. sharp feed particles and hair), predispose the ruminal wall to invasion and colonization by *F. necrophorum*. The organism causes ruminal wall abscesses, which subsequently shed bacterial emboli into the portal circulation. Bacteria from the portal circulation are filtered by the liver, resulting in infection and abscess formation (Figure 33.6).



Figure 33.5 An abscessed liver at the time of slaughter.

Histologically, a typical abscess is pyogranulomatous, with a necrotic center, encapsulated, and often surrounded

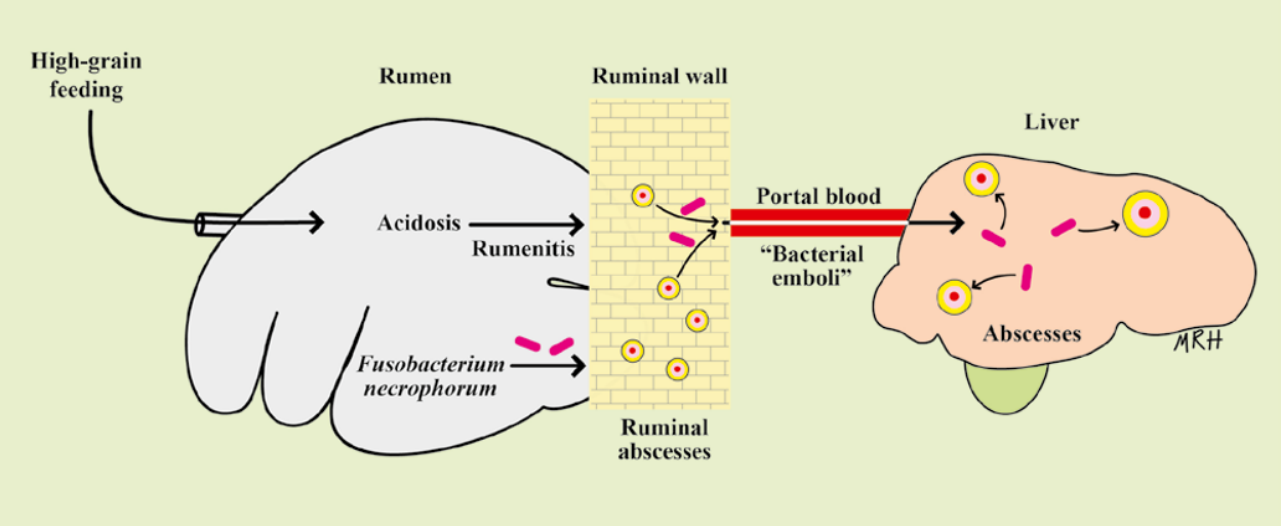


Figure 33.6 Pathogenesis of liver abscesses in feedlot cattle.

by an inflammatory zone. Of the two subspecies of *F. necrophorum*, ssp. *necrophorum* is more frequently encountered in liver abscesses than ssp. *funduliforme*. The difference in prevalence reflects the difference in virulence, particularly with regard to leukotoxin production. In most liver abscesses, *T. pyogenes* is the second most frequent pathogen isolated. The origin of *T. pyogenes* is also the rumen, and there is evidence of pathogenic synergy between *T. pyogenes* and *F. necrophorum*.

Necrotic Laryngitis (Calf Diphtheria)

The infection occurs in cattle up to three years of age and is characterized by necrosis of the mucous membrane of the larynx, particularly in the lateral arytenoid cartilage, and adjacent structures. The lesions appear as erosions progressing to ulcers and abscesses. Because *F. necrophorum* is a normal inhabitant of the respiratory tract of cattle, the source of infection is endogenous. The infection can be acute or chronic and is noncontagious. Clinically, an initial fever is followed by dyspnea that causes a roaring noise on inspiration ("hard breathers") and, in severe cases, painful swallowing and cough. Necropsy lesions include necrosis of the larynx and vocal cords and a mucous membrane covered by inflammatory exudate. Occasionally, bronchopneumonia may be evident.

Interdigital Necrobacillosis (Interdigital Phlegmon or Foot Rot)

This form of necrobacillosis (foot rot or foot abscesses) is characterized by acute or subacute necrotizing infection, involving the skin and adjacent underlying soft tissues of the feet. The infection is the major cause of lameness in dairy and beef cattle. The economic impact is from loss in productivity (milk production and weight gain). Predisposing factors involved in the pathogenesis of foot rot are damp soil and injury to the skin of the interdigital area. Fecal excretion of *F. necrophorum* is believed to provide the primary source of infection in foot rot and abscesses. In addition to *F. necrophorum*, *P. levii*, *Porphyromonas asaccharolytica*, *Prevotella melaninogenica*, and *Prevotella intermedia* are commonly isolated foot rot lesions. The lesions are characterized initially by mild cellulitis and swelling between the digits. Within a few days, fissuring with scabby exudate that eventually becomes pus in the margin of the fissure is observed. Fever and lameness are common clinical signs. Usually, healing is rapid once the abscess has discharged. Diagnosis is based on recognition of the characteristic interdigital necrotic lesion accompanied by foul-smelling discharge.

Metritis in Dairy Cows

Metritis, an inflammatory disease of the uterus in dairy cows, is associated with dysbiosis of the uterine microbiome, and one of the dominant species is *F. necrophorum*. Other bacterial species frequently isolated include *T. pyogenes*, *Bacteroides pyogenes*, *P. levii*, and *E. coli*. The infection is observed typically during the first two weeks post-partum and is diagnosed based on fetid uterine discharge with a reddish brown color, often associated with systemic signs of fever and decreased milk production. In North America, the incidence of mastitis is 10–20%. The role of *Fusobacterium* in metritis is evidenced by a marked reduction in the *Fusobacterium* population when metritis is resolved with antimicrobial treatment, such as ceftiofur, a third-generation cephalosporins. Also, vaccine formulations containing inactivated bacterial components or protein subunits of *F.*

necrophorum, T. pyogenes, and *E. coli* have been shown to prevent metritis during the first lactation of dairy cows.

Diagnosis

Necrobacillosis, particularly foot rot and necrotic laryngitis, can be diagnosed based on clinical signs. However, liver abscesses are detected only at the time of slaughter because cattle, even those that carry multitude of small abscesses or several large abscesses, seldom show any clinical signs. Moreover, hematology and liver function tests have not proved to be good indicators of liver abscesses. The usefulness of serum enzymes as indicators of liver dysfunction in cattle with naturally developed abscesses is minimal, because of lack of specificity as well as large variation in serum activity in clinically normal cattle. Ultrasonography that permits visualization of the liver has limited application because the scanning cannot visualize the whole liver, particularly of the visceral side, and the parts of the lobes that are covered by the lungs and kidneys. The involvement of *F. necrophorum* requires isolation and identification. *F. necrophorum* is one of the gram-negative anaerobes that can easily be isolated and identified from clinical samples. The presumptive identification can be made based on the sample source and colony morphology on blood agar. Commercial rapid identification kits (e.g. RapID ANA II, Innovative Diagnostic Systems, Atlanta, Georgia) have proved to be useful in identification of the species and even of the subspecies of *F. necrophorum*. MALDI-TOF identifies ssp. *necrophorum* correctly, but not ssp. *funduliforme*. The subspecies identification can be done with a couple of PCR assays.

Treatment and Control

F. necrophorum is susceptible to β-lactam antibiotics (penicillins and cephalosporins); tetracyclines (chlortetracycline and oxytetracycline); macrolides (erythromycin, tylosin, and tilmicosin); lincomycins (clindamycin and lincomycin); and chloramphenicol, novobiocin, and virginiamycin. The organism is insensitive to aminoglycosides (gentamicin, kanamycin, neomycin, and streptomycin, which are less active against anaerobes in general), ionophore antibiotics (monensin, lasalocid). The *in vitro* activity of penicillins and cephalosporins against *F. necrophorum* isolates is interesting considering that the organism is gram-negative, even based on the cell wall architecture. The susceptibility of *F. necrophorum* to virginiamycin and tylosin is anomalous because both are principally active against gram-positive bacteria.

Treatment of necrotic laryngitis and interdigital necrobacillosis is generally based on systemic administration of antibiotics. Sulfonamides and tetracyclines alone or in combination are commonly used to treat necrotic laryngitis. In the case of interdigital necrobacillosis, systemic administration of penicillin or tetracyclines is effective, particularly in the early stages of infection. Treatment of liver abscesses is not an option because of the insidious nature of the infection. Prevention is generally based on the use of antimicrobial feed additives. The antibiotic that is most commonly used in the feedlot industry is tylosin, administered at 10 g/ton of feed (90–100 mg/head per day). The extent of reduction in prevalence of liver abscesses varies from 30% to 70%. A bacterin is commercially available for the control of liver abscesses and foot rot, but field studies have not shown it to be efficacious. Because leukotoxin is a major virulence factor, a leukotoxoid-based (inactivated leukotoxin) vaccine has been developed for the prevention of liver abscesses, but it is not commercially available. In addition to the inclusion of antimicrobial compounds in the feed, proper feed bunk management to minimize ruminal imbalance is well accepted as a key factor for effective control of liver abscesses.

F. necrophorum Infections in Humans

Strains of *F. necrophorum* causing human infections appear to be distinct from the ssp. *necrophorum* of animal infections and seem to resemble ssp. *funduliforme* more closely. In humans, *F. necrophorum* is an important cause of pharyngitis, pharyngotonsillitis, and peritonsillar abscesses, especially in young adults, being second only to group A streptococci. It is associated with a rare complication called Lemierre's syndrome that primarily affects young and healthy persons. The infection starts out as an acute sore throat with purulent exudate, high fever, cervical, and submandibular lymphadenopathy, followed by secondary septic thrombophlebitis of the internal jugular vein and often leads to disseminated metastatic abscesses at various sites. The thrombosis is likely because of the ability of the organism to induce platelet aggregation. Liver abscesses caused by *F. necrophorum* have also been reported, and the source is generally thought to be disruption of the colonic barrier related to colorectal cancer.

Fusobacterium equinum

F. equinum is indistinguishable from *F. necrophorum* (both subspecies) based on morphological and biochemical characteristics. A PCR-based assay targeting 16S rDNA sequence can be used to distinguish between *F. equinum* and *F. necrophorum. F. equinum* is a normal inhabitant of the gastrointestinal, respiratory, and genitourinary tracts of

horses. It is an opportunistic pathogen and is generally associated with abscesses and various necrotic infections in horses, particularly oral, paraoral, and lower respiratory tract infections. Very little is known about the virulence factors associated with *F. equinum* infections in horses, but the species carries the leukotoxin gene and the culture supernatant exhibits leukotoxic activity. The species is not involved in thrush of the equine hoof, which is an infection of the epidermal tissue of the frog of the foot. The *Fusobacterium* species associated with equine thrush is more often *necrophorum*.

Fusobacterium gastrosuis

The species is often found to colonize the oral cavity, tonsils, and upper nonglandular region of the stomach of pigs. There is indication that the species may contribute to the pathogenesis of gastric ulceration, a polymicrobial infection, mainly initiated by *Helicobacter suis*. The gastric environment altered by *H. suis* promotes colonization and invasion by *F. gastrosuis*. Gastric ulceration is a common disease of pigs, which may result in decreased feed intake, body weight gain, and even death.

Bacteroides

The genus *Bacteroides* includes several species, but only one species, *B. fragilis*, is clinically important. *B. fragilis* is part of the normal colonic flora of humans and animals and is the most common anaerobe isolated from human colonic content samples. It is associated with abscesses, generally intraabdominal, soft tissue infections in animals. The capsular polysaccharide is a major virulence factor in the cause of abscesses. An abscess can be induced in laboratory animals by injecting purified capsular polysaccharide in the absence of bacteria. Some strains of *B. fragilis* produce an enterotoxin that has been shown to cause diarrhea in lambs, calves, piglets, foals, and infant rabbits. The strains have also been associated with diarrhea in humans, particularly in children.

Enterotoxigenic *B. fragilis*

Strains of *B. fragilis* that are enterotoxigenic (stimulate secretion in ligated ileal loops of lamb) were first identified in a diarrheic disease of newborn lambs. Subsequently enterotoxigenic *B. fragilis* (ETBF) has been isolated from cases of enteritis in calves, foals, and piglets. The ETBF strains are also associated with inflammatory disease in children older than one year and in adults. The enterotoxin, called *B. fragilis* toxin or fragilysin, is a heat-labile, approximately 20 kDa, protein that causes fluid secretion in intestinal loops, increases bacterial internalization in enterocytes, and modulates epithelial permeability. The toxin is a zinc metalloprotease, suggesting that the toxic properties are due to the proteolytic activity. The ETBF strains can be identified by amplifying the *bft* gene (by PCR), and diagnosis of ETBF infections can also be made by direct detection of the *bft* gene in DNA extracted from feces.

Prevotella and *Porphyromonas*

These two genera of gram-negative, non-spore-forming anaerobes include saccharolytic (*Prevotella*) and asaccharolytic (*Porphyromonas*), pigmented and nonpigmented species, previously included in the genus *Bacteroides*. Members of both genera are part of the normal flora of the oral cavity and gastrointestinal tract of animals and humans.

The genus *Prevotella* currently includes about 50 species, and the species associated with human or animal infections are recovered mainly from the oral cavity and the upper respiratory and urogenital tracts. A majority of *Prevotella* spp. have been isolated from the human oral cavity. About six species found in animals have been isolated from the rumen or oral cavity. *P. melaninogenica,* so named because of black pigmentation, is often isolated in pure or mixed culture from suppurative lesions in a variety of animals. It is often associated with *F. necrophorum* and *T. pyogenes* in foot rot lesions of cattle.

The genus *Porphyromonas* includes 17 species, many of which are of animal origin. The strains isolated from the gingival sulcus of various animals, which are distinct from related human strains of *Porphyromonas gingivalis*, are now included under a new species, *Porphyromonas gulae*. The organism is asaccharolytic and forms black-pigmented colonies and, similar to *P. gingivalis*, contains a 41 kDa fimbrial protein, which is an important colonizing factor in the periodontal disease. In addition to *P. gulae*, other *Porphyromonas* species that have been identified include *Porphyromonas canoris*, *Porphyromonas cangingivalis*, *Porphyromonas canis*, *Porphyromonas cansulci*, *P. gingivicanis*, *P. levii*, and *Porphyromonas crevioricanis. P. levii* is one of the species involved in summer mastitis.

Diseases

Because *Prevotella* and *Porphyromonas* species are members of the oral flora of all animals, they play a prominent role in oral and bite wound infections. Periodontal disease is the most common oral disease of adult animals, and includes gingivitis, periodontitis, and periodontal abscesses

initiated by bacteria in the dental plaque. Periodontal diseases affect a wide range of animals, such as dogs, cats, sheep, cattle, and captive and free-roaming wild animals. It is estimated that approximately 80% of dogs and cats have some degree of periodontal disease by four years of age. Periodontitis in dogs and cats can be a serious infection that could lead to anorexia, weight loss, swollen gums, tooth decay, loose teeth, breakage, or loss of teeth and even breakage of the mandibular bone. If left untreated, periodontal bacteria may spread to other sites in the body and lead to renal, coronary, or hepatic infections. Not surprisingly, *Prevotella* and *Porphyromonas* are frequently isolated anaerobes from infected dog and cat bite wounds in humans.

P. levii is often the predominant organism associated with cases of bovine foot rot. However, whether isolated strains fulfill Koch's postulates has not been determined. Not much is known about the source of the organism and virulence factors involved. It is believed that the organism works in synergy with *F. necrophorum* to produce the infection.

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34

Clostridium

Iman Mehdizadeh Gohari and John F. Prescott

The genus *Clostridium* is part of the family Clostridiaceae that currently has ~300 validly described species. Approximately one fifth are pathogenic to animals and humans. Recently, *Clostridium difficile* and *Clostridium sordellii* have been moved into the genera *Clostridioides* and *Paeniclostridium,* respectively. However, these two pathogens will be discussed in this chapter under the genus name *Clostridium* (sensu stricto). Although some clostridia stain gram negative and can grow in the presence of oxygen, most members of the genus *Clostridium* are gram-positive, spore-forming, anaerobic rods characterized also by the production of powerful extracellular toxins. In this chapter, the diseases produced by members of this genus (Table 34.1) will be discussed under three categories: enterotoxic, including the enterotoxemias and diarrheas, produced by *Clostridium perfringens*, *Clostridium colinum, Clostridioides difficile*, *Clostridium piliforme, Clostridium septicum*, *Clostridium spiroforme*, and *Paeniclostridium sordellii*; histotoxic, produced by *C. perfringens*, *Clostridium chauvoei*, *Clostridium haemolyticum*, *Clostridium novyi*, *C. septicum*, and *P. sordellii*; and neurotoxic, produced by *Clostridium botulinum* and *Clostridium tetani*.

Clostridial infections are serious infections because of the powerful toxins produced by these organisms. Many have been controlled successfully by immunization almost since the dawn of bacteriology. However, other ever more common and important clostridial infections are emerging, perhaps partly because their ability to form resistant spores gives them a selective advantage in growing in the intestinal tract whenever antibiotics are administered. Much remains to be discovered.

Descriptive Features of the Genus *Clostridium*

Morphology and Staining

Members of the genus *Clostridium* are gram-positive rods measuring 0.2–4 μm by 2–20 μm. The pathogenic clostridia all can produce spores. The location and shape of endospores are consistent within a species. Their ability to form spores is crucial to persistence in the intestine and the environment, and contributes to difficulties in their control (Figure 34.1).

Structure and Composition

Little of medical relevance is known of the ultrastructure and composition of clostridia. A surface-associated structure characterized by orderly paracrystalline protein arrays (S layer) in the cell wall of *Clostridioides difficile* may contribute to its ability to resist antimicrobial peptides in the intestine. Some clostridia produce pili (*C. perfringens, C. difficile*), and others produce adhesive structures, presumably cell wall proteins, but their role in disease is not well defined. Considerable cellular intraspecific antigenic diversity and interspecific cross-reactivity exists, but are of less interest than the antigenic properties of toxins because these latter are crucial in immunity. Those that are motile have peritrichous flagella or pilus-mediated "twitching motility." Of the pathogenic species, *C. perfringens* and *C. difficile* may form capsules.

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Table 34.1 Selected members of the genus *Clostridium* and their usual source or associated condition.

Clostridium species	Usual source or associated condition
C. botulinum	Botulism
C. chauvoei	Blackleg in ruminants; iatrogenic myositis of horses
C. colinum	Ulcerative enteritis and hepatitis in birds
C. difficile	Antibiotic-associated and spontaneous typhlocolitis in guinea pigs, hamsters, horses, humans, pigs, rabbits, and other species; pseudomembranous colitis in humans
C. haemolyticum	Bacillary hemoglobinuria in ruminants
C. novyi	Gas gangrene and pseudo-blackleg, big head in rams, black disease in ruminants; iatrogenic myositis of horses
C. perfringens	Enteric disease and enterotoxemias including necrotic enteritis of chickens, lamb dysentery, hemorrhagic and necrotizing gastroenteritis in neonatal and sometimes older farm animals and in other species, "pulpy kidney" disease of ruminants, food poisoning in humans and possibly dogs; gas gangrene; gangrenous mastitis of cows
C. piliforme	Tyzzer's disease
C. septicum	Gas gangrene in ruminants and pigs; pseudo-blackleg; gangrenous dermatitis of poultry; severe abomasitis (Braxy) of lambs; iatrogenic myositis of horses
P. sordellii	Gas gangrene and pseudo-blackleg; severe abomasitis of lambs; equine atypical myositis; possibly enteritis in other species
C. spiroforme	Antibiotic-induced and spontaneous typhlocolitis of rabbits
C. tetani	Tetanus

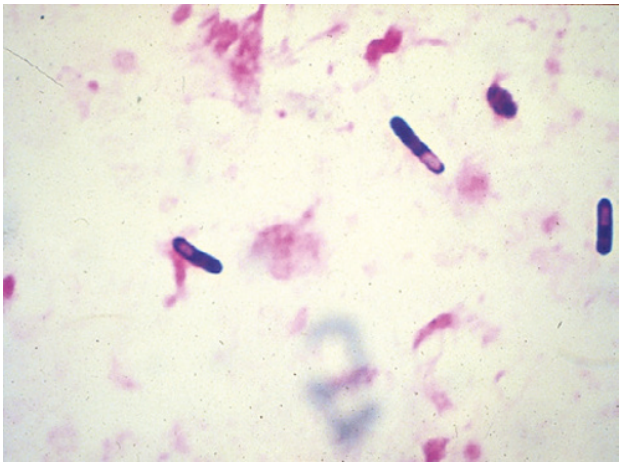


Figure 34.1 Gram stain of *Clostridium* species, showing the spores that are characteristic of the genus.

Growth Characteristics

Clostridia are anaerobes, but the strictness of anaerobic requirements varies among species. For example, *C. difficile* is far more readily killed by exposure to air than is *C. perfringens*. In general clostridia are characterized by simplicity of their growth requirements, though some require relatively rich and complex media; blood is beneficial. A temperature of 37 °C is optimal. Growth is visible within one or two days. Colonies are often irregular in shape and contour. Several clostridia swarm across moist agar media without forming colonies. Most clostridia produce hemolysis on blood agar. In liquid media, clostridia often grow in air provided a reducing agent is present (cooked meat pieces, thioglycolate), though growth occurs only in the anaerobic portions of the medium.

Biochemical Activities

Most clostridia are metabolically highly active and masters at attacking carbohydrates, proteins, lipids, and nucleic acids. Clostridial cultures typically emit putrid odors due to production of volatile fatty acids and hydrogen sulfide during the fermentative degradation of carbohydrates and proteins. Biochemical reactions and their end products furnish a basis for species identification.

Resistance

The vegetative form is as susceptible to environmental stresses and disinfectants as other bacteria. Endospores impart resistance to drying, heat, irradiation, and disinfectants.

Enterotoxic Clostridia

Clostridium perfringens

Descriptive Features

C. perfringens is a gram-positive, usually spore-forming, nonmotile, encapsulated anaerobic rod that produces a variety of toxins. The pathogenesis of *C. perfringens* diseases is directly associated with its prolific toxin-producing ability.

C. perfringens is associated with enterotoxic and other enteric disease including diarrhea in a variety of species, as well as histotoxic infections such as wound infections (gas gangrene) and serious mastitis. Our understanding of the role of *C. perfringens* in enteric disease in animals, particularly in serious hemorrhagic or necrotizing enteric disease, resembles our understanding of *Escherichia coli* in enteric disease 50 years ago, when it was thought to be exclusively

normal microflora. *C. perfringens* is as dynamic a bacterium as *E. coli,* increasingly recognized as having, like *E. coli*, the ability to adapt to cause disease in different host species. One important base of its adaptability is its possession of different conjugative plasmids that can move readily between *C. perfringens* in the intestine and that can also acquire mobile genetic elements carrying virulence determinants, and likely to change these through DNA recombination.

Cellular Products of Medical Interest

Adhesins

C. perfringens possesses genes encoding fibronectin-binding and collagen-adhesion proteins. These proteins are thought to be involved in binding to the extracellular matrix during infection.

Capsule

The capsule probably acts as a deterrent to phagocytosis. Encapsulation is an important virulence determinant in wounds (e.g. gas gangrene), but probably not in the intestinal tract.

Toxins

C. perfringens produces a large variety of protein toxins and tissue-degradative enzymes consistent with its adaptation to break down tissue quickly and efficiently. It is auxotrophic for 15 amino acids, which it needs to obtain intact as it is unable to synthesize them. Under optimal conditions, it divides every 10 minutes, the fastest known multiplication rate of any bacterium. It has been aptly described as an "anaerobic flesh-eater." Most toxins are regulated by a global regulatory system ("VirR/VirS") that is itself under the control of a "quorum sensing" system (discussed in Section Regulation of Toxin Genes). The *C. perfringens* toxins/enzymes are historically organized into two categories, "major" (mouse lethal) and "minor" (non-lethal) toxins. The major toxins are termed α (alpha), β (beta), ε (epsilon), and ι (iota) toxins. Traditionally, the major toxins have provided the basis for classification of individual strains into five toxinotypes, designated A through E. However, other toxins might have major roles in specific diseases. Therefore, the toxinotyping system of *C. perfringens* has been recently modified, and two additional types, type F (enterotoxin (CPE)-producing strains; no production of β-, ε-, and ι-toxins) and type G (NetB-producing strains), were included (Table 34.2). As new epidemiological and experimental findings emerge, the current *C. perfringens* typing scheme will almost certainly be further expanded to encompass the pathogenic diversity of this species, for example, to include NetF-producing strains as a novel future toxinotype. Some *C. perfringens* toxins are described below.

α (alpha)-Toxin α-toxin (CPA), also sometimes called PLC (for phospholipase C), is produced by all *C. perfringens*. The α-toxin gene (*cpa*) is located in a stable region within the bacterial chromosome. CPA is a zinc-dependent metalloenzyme (a lecithinase) that hydrolyzes phospholipids and sphingomyelin, the basic components of the host cell membrane, as part of its action in breaking down tissues to extract nutrients.

Table 34.2 *Clostridium perfringens* major toxinotypes in animal disease.

	Major toxins for classification						
Type	**α**	**β**	**ε**	**ι**	**CPE**	**NetB**	**Diseases produced**
A	+	−	−	−	−	−	Gas gangrene; gangrenous mastitis cows; sporadic hemorrhagic and necrotizing gastroenteritis in numerous species; hemorrhagic abomasitis calves. The full range of enteric disease associated with type A infections, and their toxin-associated basis, remain to be characterized
B	+	+	+	−	−	−	Lamb dysentery
C	+	+	−	−	±	−	Neonatal hemorrhagic and necrotizing enteritis of farm animals (calves, foals, lambs, piglets) "Struck" adult sheep
D	+	−	+	−	±	−	Ovine enterotoxemia (rarely calves); enterotoxemia and enterocolitis adult goats
E	+	−	−	+	±	−	Bovine hemorrhagic gastroenteritis
F	+	−	−	−	+	−	Foodborne infection (humans; other animal species?); antibiotic-associated enteritis in humans
G	+	−	−	−	−	+	Avian necrotic enteritis

β (beta)-Toxin The genes encoding β-toxin (CPB) are located on a conjugative plasmid. β-toxin is a mouse-lethal pore-forming toxin that damages host target cells (intestinal epithelial cells, endothelial cells). In addition, β-toxin affects nervous tissue by influencing the distribution of calcium ions across their membranes, thereby disrupting normal nerve conduction. It is susceptible to the proteolytic activity of trypsin.

ε (epsilon)-Toxin The gene encoding the mouse-lethal ε-toxin (ETX) is also located on a conjugative plasmid. Based on the mouse lethality assay, ETX is the most potent toxin of *C. perfringens* and the third most lethal clostridial toxin (after botulinum and tetanus toxins). ε-toxin targets lipid (cholesterol and sphingolipids) rafts found in eukaryotic cell membranes, though the toxin concentrates in the brain and kidney. It is a permease that acts by affecting the cellular cytoskeleton, increasing the permeability of epithelial and endothelial cells (both in the intestine but especially in the microvasculature of the brain, leading to leakage of toxin into that organ). The toxin seems to target neuronal granule cells in the cerebellum and elsewhere in the brain to release the neurotransmitter glutamate. ETX is secreted as a protoxin that is activated in the intestine by proteolytic enzymes. The toxin may delay intestinal transit time in affected animals.

ι (iota)-Toxin ι-toxin (ITX) is a binary mouse-lethal toxin composed of a binding portion (Ib) that binds the toxin to target epithelial cells, and an enzymatically active portion (Ia). Both components are encoded by two separate genes, *iap* and *ibp*, which are located on conjugative plasmids. Like ETX, ITX is produced as an inactive prototoxin in the intestinal tract, and proteolytic activation is an essential step for its toxicity. Once activated, the toxin binds to specific receptors on the cell surface, and the Ia enzymatic component gains entry into the cytoplasm. Although it is not clear precisely how entry occurs, it seems that a pore (composed of Ib) is formed in the cell membrane through which Ia traverses. Ia is an adenosine diphosphate (ADP)-ribosylating toxin that ribosylates actin within the host cell, resulting in disorganization of the cellular cytoskeleton and death of the affected cell.

Enterotoxin The genes encoding *C. perfringens* enterotoxin (CPE) are either chromosomal (isolates from cases of human food-related gastrointestinal disease), or conjugative plasmid-based (isolates from dogs or horses with diarrhea, or from human patients with non-food-related and sometimes antibiotic-associated diarrhea). Enterotoxin is produced during sporulation of *cpe*-containing *C. perfringens*. When the endospore is released, enterotoxin is also released into the surrounding milieu. Enterotoxin is a bifunctional toxin, first forming a pore in the apical portion of small intestinal epithelial cells resulting in fluid and electrolyte abnormalities, as well as providing access to tight junction proteins (specifically claudins and occludins). Interactions of CPE with tight junction proteins result in further losses in control of fluid and electrolytes.

Necrotic Enteritis Toxin B Necrotic enteritis toxin B (NetB) is a VirR/VirS-regulated pore-forming toxin related to the *C. perfringens* CPB toxin that is essential for the production of necrotic enteritis in chickens. Like almost all the major toxins (CPB, ETX, ITX) of *C. perfringens*, it is found on a conjugative plasmid.

NetF Toxin NetF toxin is a recently described toxin that is a member of the pore-forming toxin family, such as NetB and CPB. *C. perfringens* strains producing NetF also produce another putative pore-forming toxin called NetE; both are positioned on the same conjugative plasmid. In addition, all *netF*-positive strains carry a separate *cpe*-encoding plasmid.

Examples of Minor Toxins κ-toxin (Col for collagenase) is a collagenase. Col is thought to aid spread of clostridial cells through the tissue. μ-toxin (Nag for N-acetylgalactosaminidase) is a hyaluronidase. Nag aids spread of clostridial cells through the tissue. Perfringolysin O (PFO, θ-toxin) is a cholesterol-binding cytolysin (like novyilysin and chauveolysin, described later; and streptolysin O, Chapter 26; and listerial listeriolysin O, Chapter 31). Perfringolysin binds to cholesterol-containing rafts in the eukaryotic cell membrane and forms a pore, which kills the cell. Sialidase (neuraminidase, or Nan) removes sialic acid residues from glycoconjugates on the cell walls of eukaryotic cells, resulting in disruptions of the intercellular matrix. The TpeL toxin is a member of the clostridial glucosylating toxin family, and is currently the largest *C. perfringens* toxin. The gene encoding the TpeL toxin is localized on a large conjugative plasmid. As is the case for the CPE toxin, TpeL is highly expressed during sporulation, whereas its production is very low during vegetative growth. Isolation of *tpeL*-positive *C. perfringens* strains from chicken with necrotic enteritis has led to the suggestion, which is not yet proved, that it has an important accessory role in necrotic enteritis. The β-2 toxin (CPB2) is a weakly mouse-lethal pore-forming toxin that occurs in two variants, but its role in disease is thought to be minor. The gene encoding this toxin is also on a plasmid, and is controlled by the VirR/VirS system. The binary enterotoxin of *C. perfringens* (BEC) is another newly described toxin that shares some identity with ITX. This toxin is a member

of the ADP-ribosylating binary toxin family, and it consists of two components, BecA and BecB. Both genes are located on the same plasmid. BEC has been recently linked to several large outbreaks of human foodborne gastroenteritis in Japan.

Regulation of Toxin Genes

C. perfringens globally synchronously regulates its toxin production and production of major metabolic enzymes by the two-component regulatory system, VirR/VirS. VirS is a histidine kinase that acts as a "sensor" of environmental cues resulting in autophosphorylation of one of its histidine residues. This phosphate is then transferred to an aspartate residue, then to another histidine before being used to phosphorylate VirR, the "regulator." Phosphorylated VirR is a transcriptional activator of the genes encoding the proteins mentioned above. The environmental cues sensed by *C. perfringens* include microcolony formation ("quorum sensing") *in vivo*.

Cell–cell communication or quorum sensing is a procedure used by bacteria to communicate or "talk" with each other. In this system, bacteria are able to sense the cell population density not only of themselves but also of other species and thus to coordinate their activities by producing and detecting small diffusible signaling substances called "autoinducers" (AIs). When the bacterial cell density or autoinducer concentrations reaches a threshold, AIs bind to specific receptor proteins and trigger gene regulation. Therefore, quorum sensing systems are an important strategy used by bacteria to regulate the expression of virulence factors as well as cellular process such as sporulation and mating. Several quorum sensing systems have been identified in many bacteria. *C. perfringens* at least has two quorum sensing systems. The first system is called the "LuxS" system, which is common to both gram-negative and gram-positive bacteria. It has been speculated that this system can be used for interspecies cell–cell communication. The autoinducer used for LuxS system is called autoinducer 2 (AI-2), which is a small furanosyl borate diester compound. The second system is called the "Agr" (accessory gene regulator) system, which is only found in gram-positive bacteria. This system uses small autoinducing peptides (AIPs) as signaling substances. The receptor for AIPs is the histidine kinase part of a two-component regulatory system such as VirR/VirS. Like other bacteria, quorum sensing plays an important role in regulation of virulence-associated genes in *C. perfringens*. For example, it has been shown that the LuxS system positively regulates *cpa* and *pfo* genes at transcriptional levels, but *netB* is not regulated by this system. However, several toxins including CPA, CPB, CPE, CPB2, ETX, NetB, and PFO are under the control of the Agr system.

Reservoir and Transmission

Type A *C. perfringens* occurs in the intestinal tracts of humans and other animals and in most soils. Types B–G are found mostly in the intestinal tracts of animals, and their survival in soil is variable. Transmission is by ingestion and wound infection.

Pathogenesis and Disease Patterns

C. perfringens causes both serious enteric diseases, including important enterotoxemias, as well as histotoxic infections.

Enterotoxemias and Enteric Disease

Most animal diseases due to *C. perfringens* are intestinal and involve all toxinotypes.

Type A Type A C. *perfringens* is the most widespread toxinotype found in the environment, and by definition CPA is the only major toxin produced by type A. Although CPA plays a critical role in the pathogenesis of gas gangrene in humans and animals, its role in most enteric syndromes, notably hemorrhagic or necrotizing forms, in numerous species is still unproven. Because type A strains can also be found in the intestinal tracts of most clinically healthy animals, optimal microbiological diagnosis of these infections is challenging. For example, type A *C. perfringens* is an important cause of necrotizing and emphysematous abomasitis in young calves (Figure 34.2), but the basis of this disease is still unclear. Tissue destruction is usually attributed to the virulence determinants important in gas gangrene, but this assumption is probably incorrect. CPB2-producing *C. perfringens* may be associated with fatal typhlocolitis in adult horses following gentamicin treatment. Extraordinarily, the *cpb2* toxin gene is usually out of frame and therefore unread in horses isolates, but treatment with gentamicin may cause ribosomal distortion such that the mRNA

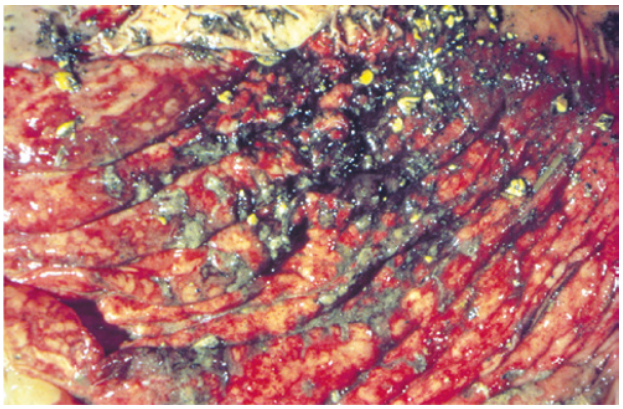


Figure 34.2 Necrotizing and emphysematous abomasitis in a young calf caused by type A *C. perfringens*. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

transcript is read in frame, and the toxin produced. Type A *C. perfringens* has been also linked to yellow lamb disease, a rare disease of sheep, but again this is not proved.

Type B Type B *C. perfringens* is an "Old World" infection usually causing "lamb dysentery" in newborn lambs. β-toxin is the principal factor producing hemorrhagic enteritis affecting the entire small intestine. Its trypsin susceptibility explains in part the predilection of the disease for the newborn, because colostrum contains antitrypsin substances. The signs are depression, anorexia, abdominal pain, and diarrhea. The course is rapid, with mortality rates approaching 100%. A chronic form occurs in older animals. Extraintestinal lesions include congestion, edema, serosal effusions, and hemorrhages in various organs. The signs and pathology associated with this disease are due to the action of the membrane-active toxins (β and ε). ε-toxin, being a permease, increases intestinal permeability, ensuring its absorption into the circulation, where it affects vascular endothelium, leading to fluid loss and edema as well as damage to kidney function. β- and ε-toxins also affect the nervous system, and the severe depression, lack of response to corrective therapy, and high mortality may be due in part to this activity. Because ε-toxin requires activation by proteolytic enzymes, its role in disease caused by type B strains is less important than that played by β-toxin.

Type C Type C *C. perfringens* causes hemorrhagic enteritis in neonatal calves, foals, piglets, and lambs worldwide (Figure 34.3). In some other species, including humans and rarely chickens, the organism can also produce a necrotic enteritis. An often rapidly fatal intestinal toxemia of older sheep is called "struck" (as in "struck dead," because the associated sudden death might suggest a lightning strike). β-toxin is the principal factor producing hemorrhagic enteritis affecting the small intestine. Its trypsin susceptibility explains in part the predilection of the disease for the newborn, because colostrum contains antitrypsin substances. The signs are depression, anorexia, abdominal pain, and diarrhea. The course is rapid, with mortality rates near 100%. The signs and pathology associated with this disease are due to the action of the membrane-active β-toxin. In humans in New Guinea, type C enteritis ("pig-bel") has been associated with feasting on undercooked and contaminated pork as well as on cassava; the trypsin-inhibiting properties of cassava prevent the breakdown of beta toxin produced by type C organisms in the small intestine.

Type D Type D *C. perfringens* produces an enterotoxemia ("overeating disease" or "pulpy kidney disease") in older lambs (<1 year), in goats of all ages, and occasionally in calves. ε-toxin is secreted as protoxin activated by intestinal proteases, explaining the predilection of this disease for older animals because colostrum contains antitrypsin substances. ε-toxin increases intestinal permeability, ensuring its absorption into the circulation, where it damages the vascular endothelium, leading to fluid loss and edema. When toxin levels are high, affected capillary endothelial cells in the brain are damaged, and the resultant edema greatly increases the intracranial pressure. When the amount of toxin is lower, however, as might be the case in a partially immune animal or when the amount of toxin produced in the intestinal tract is less, it damages the capillary endothelial cells in the brain so that toxin levels are increased in that organ. This results in a focal symmetrical encephalomalacia. In addition to these changes (which are toxin-dose related), ε-toxin triggers catecholamine release, resulting in adenylyl cyclase activation, cAMP-related hyperglycemia, and glycosuria, a frequent finding in enterotoxemia.

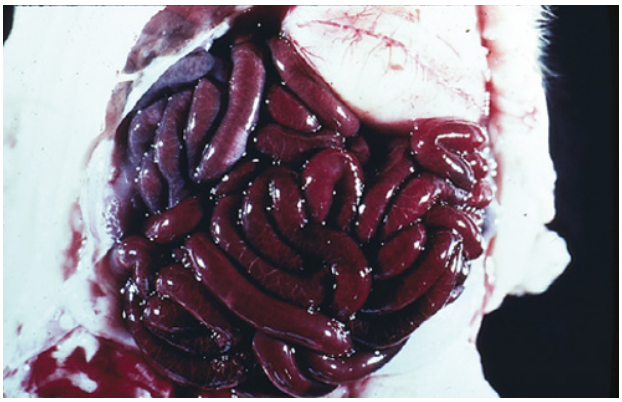


Figure 34.3 Necrohemorrhagic enteritis in a piglet caused by type C *C. perfringens*.

Gross lesions may be absent and death rates may be high in lambs. Postmortem autolysis is rapid because of vascular endothelial damage (Figure 34.4). Subserous and subendocardial hemorrhages and excess fluid in the body cavities are sometimes seen in lambs. Cerebral hemorrhage and degenerative lesions are common in less acute cases. Histopathology may reveal enteritis. Lambs may die without premonitory signs, but convulsions may occur in agonal stages and diarrhea in protracted cases. Cattle and older sheep show neurological manifestations. In adult goats, local necrotizing enteritis with diarrhea is common. In calves and goats, nonfatal subacute and chronic cases occur.

Type E Type E *C. perfringens* produces a relatively uncommon form of hemorrhagic enteritis in calves, and sometimes of lambs. The membrane-active ι-toxin produces this disease. Hemorrhagic enteritis and ulcerative abomasitis are the pathologic lesions. Type E disease was erroneously

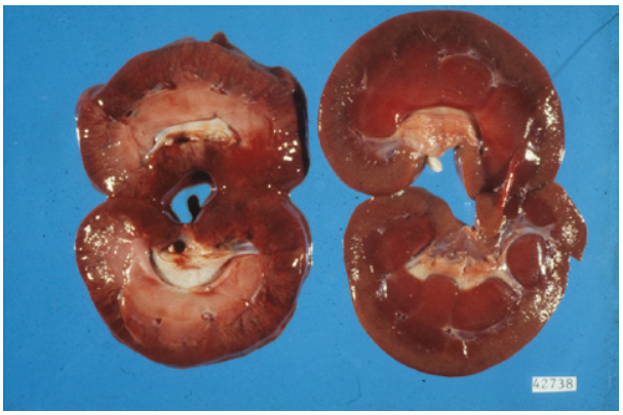


Figure 34.4 "Pulpy kidney" changes in kidneys at necropsy associated with autolysis of ε-toxin damaged kidney tissue. Kidney on the left is affected, and on the right is a normal kidney. *Source:* Courtesy of Department of Pathobiology, University of Guelph.



Figure 34.5 Necrotic enteritis caused by type G *C. perfringens* in a chicken. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

described in rabbits, because the *C. spiroforme* toxin is neutralized by ι-toxin antiserum.

Type F Type F *C. perfringens* (formerly known as type A), which produces CPE as the major toxin based on the new toxinotyping scheme, is involved in non-enterotoxemic food poisoning in humans (strains of which possess the chromosomally encoded *cpe* as well as being more resistant to heat) and antibiotic-associated diarrhea in humans (associated with plasmid-borne carriage of the *cpe* and *cpb2* genes). Type F food poisoning is the second most prevalent bacterial foodborne disease after *Salmonella* in the United States, where approximately one million cases occur per annum. Type F food poisoning symptoms include abdominal cramps and diarrhea that usually resolve within 24 hours. However, in people with preexisting fecal impaction, type F infection can progress to lethal enterotoxemia, where CPE is absorbed from the intestines and thus damages organs such as the liver. Antibiotic-associated diarrhea is another major public health problem caused by type F strains, developing in 5–25% of people after administration of broad-spectrum antibiotics. Antibiotic-associated diarrhea is typically more sever and of longer duration than type F food poisoning.

Type G Type G *C. perfringens* comprises strains that produce only two major toxins, CPA and NetB, and do not produce any other known major toxins such as CPB, ETX, ITX, and CPE. However, TpeL toxin may be produced in some type G strains and increase their virulence. Type G *C. perfringens* causes necrotic enteritis of chickens and other birds (Figure 34.5). The most common clinical symptoms of necrotic enteritis of chickens including anorexia, depression, diarrhea, dehydration, and ruffled feathers. Gross pathology such as gaseous lesion and mucosa necrosis are typically found in the small intestine. For many years, CPA has been considered the main virulence factor associated with chicken necrotic enteritis. It has only recently been recognized that strains that cause this infection are characterized by production of the NetB toxin, the gene for which is on one of the three pathogenicity loci (PAL) that are characteristic of chicken necrotic enteritis strains. The largest PAL is carried on a conjugative plasmid typical of host-adapted pathovars. This unexpected discovery illustrates the largely unrecognized likely ability of this bacterial pathogen to adapt to cause distinct diseases in different animal species.

NetF-Producing Strains NetF-producing type A strains are involved in two important gastrointestinal diseases in animals, foal necrotizing enteritis and canine acute hemorrhagic diarrhea syndrome (AHDS). These unique strains have not yet been included in the updated toxinotyping scheme, but are candidates for possible future inclusion. Foal necrotizing enterocolitis is an acute and severe disease with high mortality in neonatal foals less than four days of age, with no gender or breed predisposition. The most common and general clinical signs of this syndrome include anorexia, fever, dehydration, colic, and diarrhea. Diarrhea, when present, is often bloody. Signs of enterotoxemia and sudden death are usually common. Although type C *C. perfringens* is a well-known but uncommon cause of necrotizing enteritis in foals, the role of other *C. perfringens* is not well understood. Recently, a novel pore-forming toxin designated NetF and a highly significant association between the presence of NetF-producing strains and fatal foal necrotizing enteritis was discovered (Figure 34.6). These strains also produce a related pore-forming toxin designated NetE, and in some cases a conjugative plasmid encoded pore-forming



Figure 34.6 Severe necrohemorrhagic enteritis due to NetF-producing *C. perfringens* in a three-day-old foal, showing diffuse hemorrhage and necrotic ulcers on the serosal surface of small intestine. *Source:* Reproduced with permission from Mehdizadeh Gohari et al. (2020).

toxin designated NetG. Because of the consistent presence of *cpe* gene in *netF*-positive strains, a possible synergistic effect between these toxins has been proposed in the pathogenesis of necrotizing enteritis of foals.

AHDS is a relatively poorly documented disorder, but is well known to veterinarians. Clinical signs of AHDS vary from mild and usually self-limiting enteritis with accompanying vomiting and diarrhea to a severe disease characterized by passage of frank blood, with severe necrotizing inflammation of the intestine and sudden death. Small breed dogs, such as miniature and toy breeds, aged between two and four years are more affected, but no gender predisposition has been reported. No documented evidence regarding the morbidity and mortality rate exists, which may be because of the lack of clear diagnostic criteria. The classic hallmarks of *C. perfringens* enteric infections found in this syndrome, including rapid onset and development, characteristic intestinal necrosis lesions, and adhesion of a large number of *C. perfringens* to necrotic surfaces, suggest a major pathogenic role for *C. perfringens* in canine AHDS. Although *C. perfringens*–associated canine AHDS has been frequently reported, the infection is not yet well understood. Recently, a strong association between canine AHDS and NetF-producing *C. perfringens* has been identified. According to these findings, a crucial role for NetF-producing *C. perfringens* in canine AHDS is proposed. The reason that small breed dogs are at particular risk is possibly because of their recognized genetic predisposition to development of pancreatitis, which might reduce trypsin in their small intestine, is an intriguing suggestion that should be explored. The recognition of the role of *netF*-positive *C. perfringens* in canine AHDS and foal necrotizing enteritis has been a critical advance in understanding the basis of these infections, although more work is required to understand its epidemiology and the factors predisposing dogs and neonatal foals to diseases.

Non-enterotoxemic Diarrhea The role of *C. perfringens* in non-enterotoxemic and mild diarrhea in animals remains to be confirmed. For example, neonatal piglets may have a syndrome of weight loss, failure to thrive, and diarrhea associated with CPB2-producing type A strains. However, the very common colonization of the intestine by *C. perfringens* shortly after the birth of neonatal animals has likely led to the erroneous assumption that these are a cause of this syndrome. It is also possible that non-enterotoxemic diarrhea occurs in other species subsequent to the interaction of the CPE enterotoxin (with epithelial cells of the small intestine following sporulation of the microorganism in that environment), but this is not well described. In addition to altering the fluid and electrolyte flow of the epithelium, CPE damages epithelial cells and tight junctions, leading to sloughing with accompanying inflammatory changes.

Histotoxic Infections

C. perfringens type A, alone or with other bacteria, causes wound infections (gas gangrene, sometimes called malignant edema in animals), a gangrenous anaerobic cellulitis following inoculation into a normally sterile site. *C. perfringens* is also an occasional cause of severe and fatal necrotizing mastitis in newly calved cows.

The membrane-active toxins (α-toxin and perfringolysin O) account for the major tissue destruction. Spread of the infection is aided by collagenases, sialidases, and hyaluronidases. The numerous powerful degradative proteases, glycosidases, lipases, and other enzymes upregulated by the VirR/VirS system assist in the tissue destruction and nutrient assimilation, while encapsulation helps the organism resist phagocytosis. The disease is a necrotizing cellulitis and myonecrosis with edema, hemorrhage, gas production, and an often fatal toxemia. This type of *C. perfringens* infection in animals is rare, and follows introduction of clostridia from the environment into usually severely traumatized tissue, where trauma assists in production of an anoxic environment suitable for the establishment of clostridia.

Epidemiology

Many healthy animals commonly carry *C. perfringens* in their intestinal tracts, probably as frequently as they carry *E. coli*. During outbreaks of diarrheal disease, pathogenic strains survive in soil long enough to infect other animals.

The determinant of enterotoxemic (type D) disease is the intestinal environment, which is influenced by diet and age. Overeating, especially on protein and energy-rich food (milk, legume forage, grain) is almost a prerequisite. In young animals, the excess feed is often passed, inadequately digested, into the intestine, where it provides a rich medium for proliferation and toxinogenesis by ingested or resident bacteria. Overloading slows intestinal motility, thereby favoring retention of bacteria and absorption of their toxins. In addition, there are likely to be bacterial factors promoting intestinal colonization, but these are not well described. In broiler chickens, necrotic enteritis is predisposed by concurrent coccidiosis, as well as by wheat-based diets, and diets containing trypsin inhibitors (such as unheated soybean meal).

The age predilection of many of these enteric these diseases is due to the diet and the infantile digestive tract, which often lacks enzymes to inactivate the toxins. In particular, colostral antitrypsin activity exacerbates this aspect. Type D proliferation, for which expression of the ε-toxin requires trypsin activation, in older lambs appears to be favored by high-carbohydrate intake.

Seasonal prevalence relates to the seasonal abundance of susceptible populations and rich forage, with warm temperatures favoring proliferation of bacteria in the environment.

Type A–associated conditions occur worldwide. Type B lamb dysentery occurs in Europe and South Africa, while type B enteritis of sheep or goats is reported from Iran. Type C occurs worldwide, and type D is prevalent wherever sheep are raised. Type E is found in Britain, the United States, and Australia. A toxin similar to its ι-toxin is also produced by *C. difficile* and *C. spiroforme*. Type F-associated infections have been reported in every continent around the world. Chicken necrotic enteritis caused by type G C. *perfringens* has been reported in most countries.

Immunologic Aspects

Immunity is antibody-mediated and generally correlates with antitoxin levels. Immunizing preparations often include bacterial components as well. Active immunization is important in the control of the diseases (see Section Treatment and Control).

Laboratory Diagnosis

C. perfringens is relatively aerotolerant, and is easy to isolate and to identify in the diagnostic laboratory. Spores are rarely demonstrable in exudates obtained from normally sterile sites.

Isolation follows inoculation of blood containing agar media, and incubation in an anaerobic environment. If *C. perfringens* is to be isolated from a contaminated environment (e.g. intestinal contents), the sample can first be heated to 80 °C for 15 minutes because endospores will resist this treatment whereas vegetative forms will not, and then placed into or onto isolation media. Diagnostic features include the characteristic double zone of hemolysis on blood agar (CPA, PFO toxins) and the clotting of milk followed by gaseous disruption ("stormy fermentation"). In cases of enterotoxemia, stained (e.g. Gram's, Wright's, Giemsa) contents of the small intestine often contain large numbers of fat gram-positive rods typical of *C. perfringens*. However, this test is of limited value due to rapid postmortem bacterial overgrowth in all parts of the gut. DNA primers specific for the various genes encoding the toxins have been developed for detection in feces or cultures by the polymerase chain reaction (multiplex PCR).

Demonstration of toxin in the contents of the small intestine is definitive and involves injecting small amounts of clarified intestinal contents into the tail vein of mice. Death after more than a few minutes post injection constitutes presumptive evidence of enterotoxemia, and the toxin can be neutralized using specific antitoxins. Such a procedure, or guinea-pig intradermal toxin testing, is now generally regarded as barbaric, so that most diagnostic laboratories rely on PCR testing. Lambs with type D enterotoxemia usually test positively for glycosuria.

CPE is detected immunologically in feces of affected dogs or cats by ELISA. Although sporulation and CPE production are co-regulated, there is disagreement regarding the usefulness of determining the presence of spores in stained smears of feces as a method of diagnosis.

Treatment and Control

Most cases of enterotoxemia are too acute for successful treatment. The best method of preventing enterotoxaemia is active immunization of dams with two injections of bacterin-toxoid combinations prior to parturition. Commercial immunizing products usually cover types C and D. This ensures nurslings passive protection for the first weeks of life. During outbreaks, antitoxin and toxoid are sometimes given, and a second dose of toxoid is administered some weeks later. Protection of lambs against type D enterotoxemia requires two vaccinations at a monthly interval. The course should be completed two weeks before the lambs are placed on full feed. Milking goats respond poorly to immunization with pulpy kidney (type D) vaccines, so that vaccination is often repeated several times a year.

Antitoxin of the appropriate type may be given to sick animals and to those at risk. Protection lasts two to three weeks. Prophylactic dosages, given subcutaneously, can be doubled and given intravenously for therapy. However, antibiotics now usually replace antitoxins because of the cost and unavailability of antiserum.

Taking precautions against overeating is a worthwhile preventive measure where practicable. Feeding broad-spectrum antibiotics reduces the prevalence of enterotoxemia of lambs, but creates other problems. Feeding antibiotics to poultry prevents mortality and illness in chickens due to necrotic enteritis caused by type G *C. perfringens*. Diarrhea in dogs associated with NetF-producing *C. perfringens* responds to metronidazole, macrolides (tylosin), or ampicillin, although antibiotics are usually not required because of the generally rapid recovery from the infection. An autogenous vaccine has been developed in Kentucky, the United States, for prevention of *C. perfringens* enterocolitis in foals, which contains antibodies to NetF. In addition, hyperimmune plasma is available from horses immunized with types A, C, D, and NetF toxin *C. perfringens* supernatants for prevention/treatment of foal necrotizing enteritis. However, more research is required to support its value.

Clostridium colinum

C. colinum causes quail disease, an ulcerative enteritis and necrotizing hepatitis of several species of poultry (Figure 34.7). The agent is fastidious nutritionally and forms spores sparingly. Its life cycle is unknown, and a toxin has not been identified. The untreated disease is usually fatal.



Figure 34.7 Colitis in a parrot caused by *C. colinum*. *Source:* Courtesy of F.A. Uzal, University of California, Davis.

Clostridioides difficile

C. difficile is a gram-positive, motile, encapsulated, spore-forming anaerobic rod. *C. difficile* is a strict anaerobe, and the vegetative forms of this bacterium do not survive exposure to air for long. This species produces adhesins (pili or fimbria), and its cell wall contains paracrystalline arrays (S layer) visible with the electron microscope.

C. difficile is an important cause of serious diarrheal disease in humans, commonly associated with antibiotic use, that may progress to fatal pseudomembranous colitis. It has become a scourge in human hospitals, particularly in older patients (>65 years of age), and is characterized by the difficulty of controlling spores by disinfection in the hospital environment and by other features that include use of certain antibiotics and of gastric antacids. It may also cause non-antibiotic-associated diarrheal disease in people. *C. difficile* is increasingly recognized as an important cause of fatal diarrheal disease in animals, notably horses, hamsters, guinea pigs, and piglets, but its significance in other animals is only now being established. Other species in which *C. difficile* diarrheal disease has been described include dogs, cats, rabbits, and ratites. The microorganism has been isolated from symptomatic diarrheic as well as asymptomatic dogs and cats. Disease commonly follows broad-spectrum antibiotic use (e.g. third-generation cephalosporin, clindamycin). Pathological findings in horses, hamsters, and guinea pigs include hemorrhagic necrotizing enterocolitis, typhlocolitis, and pseudomembranous colitis, and in piglets necrotizing typhlocolitis and mesocolonic edema. There is usually a history of association with antimicrobial agents, though *C. difficile*–associated disease has been reported in previously normal, unmedicated foals.

Descriptive Features

Cellular Products of Medical Interest

Adhesins *C. difficile* produces pilus adhesins that probably play a role in adhesion to target cells in the large intestine. It also produces other adhesins likely play a role in adherence and gut colonization, including a cell wall protein, Cwp66, a heat shock protein, GroEL, the flagellin FliC, the flagellar cap protein FliD, fibronectin-binding proteins, and a surface layer protein, SlpA.

Capsule *C. difficile* produces a carbohydrate capsule that protects it from phagocytic cells.

Toxins *C. difficile* produces three toxins that are responsible for enteritis observed with this microorganism: toxin A ("enterotoxin"), toxin B ("cytolysin"), and the ADP-ribosyltransferase binary toxin, CDT. Most *C. difficile*

strains produce toxin A and toxin B or all three toxins. Strains that produce none of these toxins are non-toxigenic and clinically irrelevant. Therefore, strains that produce all of these toxins or some (e.g. A-/B+) are pathogenic and can cause disease.

C. difficile Toxin A (TcdA) is a glycosyltransferase that glucosylates Rho GTPases, rendering them ineffectual in interacting with their substrates (i.e. they become biologically inactive). In addition, glycosylation blocks interaction of Rho GDP with guanine exchange factor, and the interaction of Rho GTP with GTPase activating factor, thereby preventing membrane cycling. Several signaling pathways are disrupted, resulting in a breakdown of the cytoskeletal components of the affected cell including disruption of the tight junctions between intestinal epithelial cells. These changes result in death of the cell. The enterotoxic property of TcdA, in addition to the cytotoxic effects just described, is due to its ability to stimulate influx of PMNs by way of the enteric nervous system (through the release of substance P and mast cell degranulation). Prostaglandin synthesis by the recruited PMNs (and perhaps by affected host cells), as well as activation of various inositol-signaling pathways within affected host cells, results in the secretion of chloride ions and water (diarrhea).

C. difficile Toxin B (TcdB), like TcdA, is a glycosyltransferase that glucosylates Rho GTPases. However, TcdB has little enterotoxic activity but far more cytotoxic activity than TcdA. The genes encoding toxins A and B are located within a large pathogenicity locus in the chromosome.

The *C. difficile* ADP-ribosyltransferase CDT can be found in a small percentage of *C. difficile* strains, but its role in the pathogenesis of *C. difficile* infections has not been clearly demonstrated. CDT is a binary toxin (see *C. perfringens* ι-toxin, described earlier, and *C. spiroforme*, described below), composed of a binding portion (CDTb) that binds to the target intestinal epithelial cells and an enzymatically active portion (CDTa). After the toxin binds to specific receptors on the cell surface, CDTa gains entry into the cytoplasm. Although it is not clear precisely how entry occurs, it seems that a pore (composed of CDTb) is formed in the cell membrane through which CDTa traverses. CDTa is an ADP-ribosylating toxin that ribosylates actin within the host cell, resulting in disorganization of the cellular cytoskeleton and death of the affected cell.

Reservoir and Transmission

C. difficile is found in the large intestine of normal as well as clinically affected animals. The spores, being resistant to most environmental stresses, are widely distributed in locations where animals are housed. There is some overlap between those strains that produce disease in human patients and those that are associated with animals.

Pathogenesis

C. difficile adheres to mucus or the epithelial cells of the large intestine by pili as well as the surface protein Cwp66 and other adhesins mentioned earlier. Disease most often follows a "trigger" event (e.g. antibiotics, nonsteroidal drugs, chemotherapeutic agents) that results in relaxation of the control the normal intestinal flora has on the numbers of *C. difficile*. In some cases, these antibiotics may temporarily control *C. difficile* but once they are removed, the organism, which has survived the antibiotic as spores, can rapidly proliferate to produce disease. In addition, the reduced activity or quantity of antimicrobial peptides found as part of the innate defenses of the large intestine of elderly human patients also predisposes to infection. Toxins are produced (TcdA, TcdB, and CDT), resulting in death of the epithelial cells, which follows disruption of their actin cytoskeleton and tight junctions. Prostaglandin along with the products of the inositol pathway stemming from the intense inflammatory response (TcdA) results in fluid and electrolyte secretion (Figures 34.8 and 34.9). Diarrhea, with or without blood, results.

Epidemiology

C. difficile–associated diarrhea is often linked to administration of antibiotics, stress, chemotherapeutic agents, or nonsteroidal anti-inflammatory drugs. For this reason, disease appears to be more frequent and worse in those species with expanded large intestines (colon, cecum) such as horses, rabbits, and swine. Newborn foals have been shown to develop disease without a recognizable "trigger" event, but the diagnosis is complicated by the presence of toxin in the intestine of some healthy neonatal foals, presumably protected against disease by maternal antibodies. Spread



Figure 34.8 Fatal necrotizing colitis in a horse caused by *Clostridioides difficile*. The large colon and cecum of the horse can result in severe and often fatal disease caused by this bacterium. *Source:* Department of Pathobiology, University of Guelph.

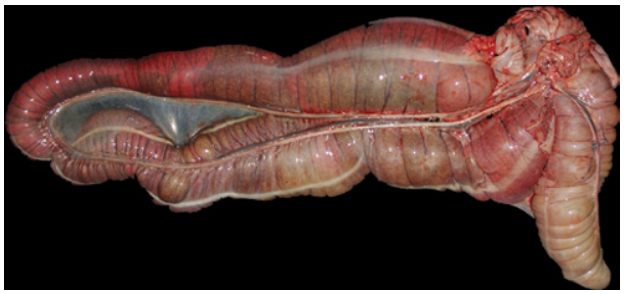


Figure 34.9 Horse with *C. difficile*–associated colitis, showing marked diffuse reddening of large intestine serosal surface as the result of mucosal and submucosal congestion and/or hemorrhage. *Source:* Courtesy of F.A. Uzal, University of California, Davis.

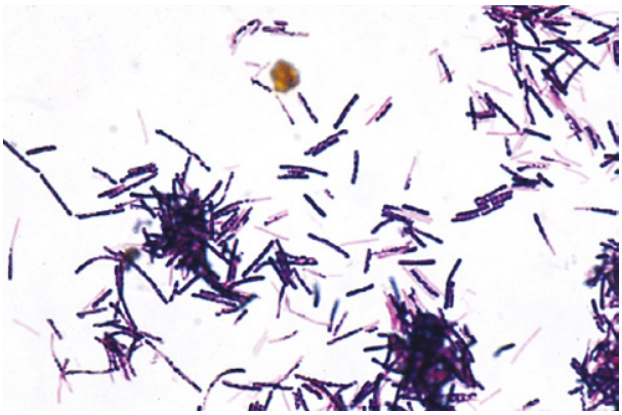


Figure 34.10 Gram stain of *C. difficile*. Like many clostridia, some organisms appear gram negative; spores are not readily visible in this smear.

occurs through contamination of the environment by spores; the numbers shed in the feces of animals treated with antibiotics can be massive, so that spores can commonly be found in hospital environments. The spore form is resistant to usual disinfection and cleaning procedures.

C. difficile isolated from dogs and cats with diarrhea do not commonly contain the genes encoding CDT. *C. difficile*–associated diarrhea in calves has been reported. However, the disease could not be reproduced after experimental infections in neonatal calves. The role of this bacterium in disease in adult cattle is still unproved. *C. difficile* has been isolated from different wildlife species, including insectivores, herbivores, omnivores, and wild carnivores. Whether this bacterium is a pathogen in wildlife species is not proved.

Immunologic Aspects

Immunity is probably antitoxic, although the role of antibodies to *C. difficile* is unknown. Orally administered antitoxin (made in bovines) is protective for human patients.

Laboratory Diagnosis

The genes encoding the toxin(s) can be detected in feces by assays based on PCR. Immunologically based tests are available for the detection of the toxin (TcdA, TcdB) in fecal specimens. *C. difficile* can also be isolated from feces by using a selective medium, CCFA (cycloserine, cefoxitin, and fructose agar), and the presence of toxigenic types confirmed by PCR (Figure 34.10). Perhaps surprisingly, an optimal approach to laboratory diagnosis is still being developed in human medicine, because enzyme immunoassays (EIA) for Toxin A and Toxin B are not as sensitive as direct toxicity assays and in addition are not always specific. The current "algorithm" for diagnosis in human medicine involves EIA for glutamate dehydrogenase (GDH) and toxins. If these are both negative, the diagnosis is reported as negative; and if both are positive, the diagnosis is regarded as positive. However, if the more sensitive GDH assay is positive while the toxin assay is negative, a cell cytotoxicity assay or real-time PCR for toxin genes is recommended as the second stage of testing. Enzyme assays are currently preferred over alternatives because of the speed of diagnosis, although real-time PCR is increasingly used because it is fast though expensive. Ideal algorithms for diagnosis of disease in animals have not been developed, although toxin testing remains a method of choice. Culture is slow and sometimes also hampered by the death of the organism in air during transport to the laboratory.

Treatment and Control

Diarrhea-associated *C. difficile* responds rapidly to metronidazole. Unfortunately, metronidazole-resistant strains exist. The alternative antibiotics used in humans are vancomycin and the new macrolide drug fidaxomicin. Recently, fecal microbiota transplantation (FMT; transfer of stool from a healthy donor to an infected patient) is recommended for the highly effective treatment of patients who have not responded to antibiotic treatment. There are no vaccines available. The use of orally administered yeast, *Saccharomyces boulardii*, has been shown to be useful in preventing the disease in human patients. In pigs, colonization with a non-toxigenic strain has prevented infection by toxigenic bacteria. Probiotics administered at the time of antibiotic use appear to reduce the incidence of *C. difficile* infection. In human hospitals, hand washing by health care personnel is an efficient mechanism for curtailing spread. Disinfectants are not effective against the spores.

Clostridium piliforme (*Bacillus piliformis*)

An acute fatal diarrheal disease of laboratory mice with focal liver necrosis, Tyzzer's disease is associated with a spore-forming organism, *C. piliforme*, which occurs in bundles

within hepatocytes. It is unable to grow on cell-free media. *C. piliforme* is linked to identical diseases in rabbits, hares, gerbils, rats, hamsters, muskrats, dogs, cats, snow leopards, foals, and rhesus monkeys. Tyzzer's disease has been reported in a human patient infected with the human immunodeficiency virus, but not in immunocompetent persons.

Descriptive Features

C. piliforme is a large, gram-variable, obligate intracellular, spore-forming rod that is motile by peritrichous flagella. Giemsa and silver stains are preferable to hematoxylin–eosin and Gram stains. Growth has been obtained in embryonated hen's eggs and on cultured mouse hepatocytes.

Vegetative cells die even when deep-frozen or freeze-dried. Spores survive moderate heating, freezing, and thawing. Litter remains infective for months. Strains are pathogenically and morphologically uniform.

Reservoir and Transmission

The source of the agent is the infected animal. The agent spreads by the fecal-oral route and transplacentally. Many infections are believed to be endogenous and stress triggered.

Pathogenesis

Lesions suggest hepatic invasion from the intestine via lymphatics and blood vessels. The most significant finding is hepatomegaly (Figure 34.11). Foci of coagulation necrosis are periportal (Figure 34.12). There may be dissemination to the myocardium. Parasitized cells include hepatocytes, myocardial cells, smooth muscle, and epithelial cells of the intestine, in which a dysentery-like condition may develop. Lymphadenitis, especially of hepatic nodes, is seen in foals. The course of disease is usually under three days.

Epidemiology

Outbreaks are often stress related (crowding, irradiation, steroid administration). Morbidity is high in laboratory animal colonies. Case fatality rates reach 50–100%, especially among young stock. In many colonies, subclinically infected individuals are evidently present; these are often identifiable serologically.

Laboratory Diagnosis

Laboratory diagnosis rests on the demonstration of typical bundles of intracellular bacilli (0.5 µm by 8.0–10 µm), especially in hepatocytes surrounding lesions (Figure 34.13). Fluorescent antibody aids diagnosis.

A complement fixation test that utilizes an infected mouse liver extract as antigen has been used to determine the degree on infection in mouse colonies. 16S rRNA PCR and immunohistochemistry can be also used for diagnosis of Tyzzer's disease.

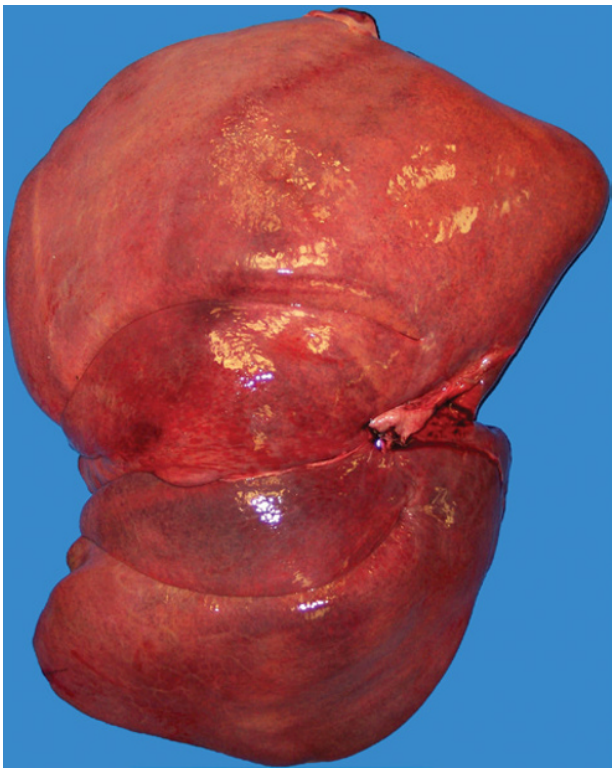


Figure 34.11 Tyzzer's disease in a foal, showing markedly enlarged liver with multifocal to coalescing white foci. *Source:* Reproduced with permission from Fresneda and Carvallo Chaigneau (2016).

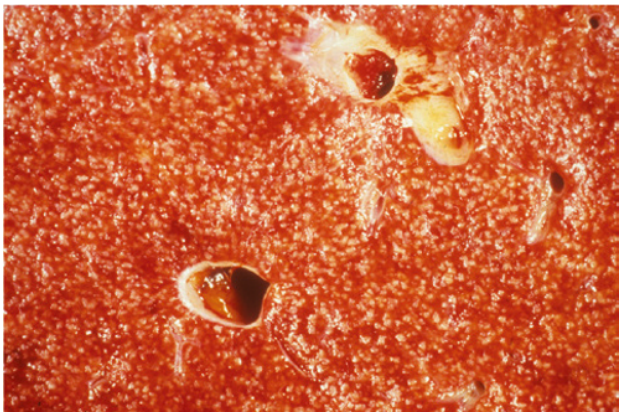


Figure 34.12 Tyzzer's disease in a foal, showing extensive focal hepatic necrosis. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

Treatment and Control

Treatment of clinical cases is usually unsuccessful. Prophylactically effective antimicrobial drugs include erythromycin and tetracycline.

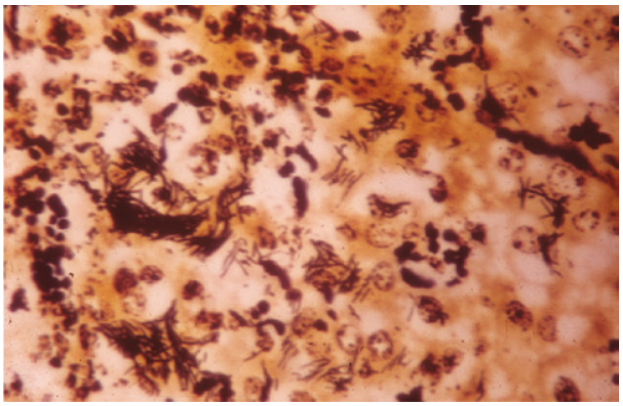


Figure 34.13 Tissue Gram stain of Tyzzer's disease, showing accumulations of *C. piliforme*. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

Clostridium spiroforme

C. spiroforme is isolated frequently from rabbits with typhlocolitis. Like *C. difficile* infection, it may also be predisposed by antibiotic use. There is some evidence that this bacterium can also cause enteritis in hares. *C. spiroforme* toxin (CST) is the main virulence factor produced by this microorganism and is identical to the ι-toxin of *C. perfringens* type E and the ADP-ribosyltransferase of *C. difficile*. It acts by ADP-ribosylating cellular actin.

Other Clostridia Causing Enteric Disease

Enteric disease caused by *C. septicum* and *P. sordellii* is described in the following section.

Histotoxic Clostridia

The histotoxic clostridia are characterized by their ability to cause serious soft-tissue, including muscle, infections associated with the presence of anaerobic environments, including those cause by severe traumatic events. Histotoxicity associated with *C. perfringens* has been briefly described earlier. Some of the histotoxic clostridia also cause enterotoxic infections.

Clostridium chauvoei

C. chauvoei is a gram-positive, motile, obligately anaerobic rod that produces subterminal or subcentral spores. *C. chauvoei* produces an endogenous emphysematous necrotizing myositis ("blackleg" or "blackquarter") in cattle. The classical blackleg disease is rarely reported in other animal species. However, *C. chauvoei*–associated gas gangrene has been reported in small ruminants.

Descriptive Features

Cellular Products of Medical Interest

Like other clostridia, *C. chauvoei* produces a number of powerful protein exotoxins that are responsible for the diseases it causes. The *C. chauvoei* cytotoxin A (CctA) is a pore-forming toxin similar to *C. perfringens* NetB, *C. perfringens* CPB, and *Staphylococcus aureus* α-toxin. It has been shown that the main cytotoxic and hemolytic activity of *C. chauvoei* is related to this toxin. Other extracellular products thought to be important in the pathogenesis of the infection include a DNAase ("β-toxin") and a neuraminidase (sialidase) that removes sialic acid residues from glycoconjugates on the cell walls of eukaryotic cells, resulting in disruption of the intercellular matrix. *C. chauvoei* also synthesizes hyaluronidase enzyme, which is able to break down hyaluronic acid. It is postulated that this enzyme is responsible for destruction of tissue in infected hosts. Chauveolysin ("δ-toxin") is a cholesterol-binding cytolysin similar to perfringolysin O, described earlier, which binds to cholesterol-containing rafts in the eukaryotic cell membrane to form a pore that results in the death of the cell. In addition, *C. chauvoei* produces flagella that contribute to bacterial invasion.

Reservoir and Transmission

C. chauvoei inhabits the intestine, liver, and other tissues of susceptible and resistant species. Blackleg occurs in endemic regions of the world, where the infection is thought to be acquired from soil, such as soil contaminated with the feces of carrier animals. Infection of the intestinal tract is followed by seeding of the organism into tissues via the liver, with survival of the bacterium in the form of spores in muscle throughout the body. The organism can also be introduced into traumatic wounds from soil, in which case it may cause gas gangrene.

Pathogenesis

Seeding of tissues, especially skeletal muscle, with spores from the intestine precedes disease in cattle. Conditions favoring spore germination, with subsequent bacterial growth, and toxin production, causes formation of local lesions marked by edema, hemorrhage, and myofibrillar necrosis, and toxemia systemically. It is usually not clear what factor triggers the spores to germinate, but any local anoxic condition including bruising or injection with irritating products will cause germination. It is suspected that the flight ("gadding") of groups of well-fed and probably physically slightly unfit young cattle to escape the vicious biting flies common in the summer is the major stimulus that causes local muscle anoxia and activates the spores. The necrotizing toxin CctA, together the other exotoxins mentioned, is responsible for the initial lesions. Bacterial



Figure 34.14 Blackleg in a cow, showing the characteristic blackened color of the affected muscle, with emphysema due to gas production by *C. chauvoei* in the center of the necrotic muscle. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

metabolism producing gas from fermentation may be contributory. The centers of lesions become strikingly dry, dark, and emphysematous because of the necrotizing infection, while the periphery is edematous and hemorrhagic (Figure 34.14). A rancid-butter odor is typical. Microscopically, there are degenerative changes in muscle fibers disrupted by edema, emphysema, and hemorrhage. Leukocytic infiltration is minor.

Clinically, there is high fever, anorexia, and depression. Rapidly developing lameness is common. Superficial lesions cause visible swellings, which crepitate on being handled. Although blackleg characteristically affects the focus in one of the major limb muscles, the site of infection sometimes involves more minor muscles such as those of the diaphragm, myocardium, or tongue. Some animals die suddenly, others within one or two days.

Activation of endogenous spores by injection of irritant chemicals has produced sometimes iatrogenic fatal myositis in horses.

Epidemiology

Blackleg occurs worldwide at rates that differ between and within geographic areas, which suggests a soil reservoir or climatic or seasonal factors yet to be defined. Young, well-fed cattle (<3 years) are preferentially attacked. As noted, exertion or bruising are suspected triggering events.

In sheep and some other species, *C. chauvoei* typically causes wound infections resembling malignant edema or gas gangrene. Other clostridia (*C. novyi*, *C. septicum*, and *P. sordellii*) may be present.

Immunologic Aspects

Circulating antibody to toxins and cellular components apparently determines resistance to *C. chauvoei*. Commercial formalinized adjuvant vaccines include components from up to six other clostridial species (*C. haemolyticum*, *C. novyi*, type C and type D *C. perfringens*, *C. septicum*, and *P. sordellii*).

Laboratory Diagnosis

Sporulated gram-positive rods can be demonstrated in smears of infected tissues and identified with immunofluorescent reagents.

C. chauvoei requires strict anaerobic conditions and media rich in cysteine and water-soluble vitamins. The agent resembles *C. septicum* and is frequently recovered with it. Unlike *C. septicum*, it ferments sucrose but not salicin and will not grow at 44 °C. The use of DNA primers to amplify the 16S–23S DNA spacer regions (by PCR) will differentiate *C. septicum* from *C. chauvoei*. This assay as well as fluorescent antibody test (FAT) can be used to detect the microorganism in tissue.

Detection in tissue or identification in culture can also be accomplished by using DNA primers designed to amplify species-specific portions of the gene encoding the flagellar protein flagellin by PCR, and have been used successfully to differentiate the histotoxic clostridia. Recently, MALDI TOF mass spectrometry has also been used to identify the isolated strains.

Treatment and Control

Treatment is often disappointing. Penicillin should be given intravenously at first, followed by repository forms intramuscularly.

Cattle are vaccinated against blackleg in endemic regions at three to six months of age and annually thereafter. Vaccination should precede exposure by at least two weeks. During an outbreak, all cattle are vaccinated and given long-acting penicillin. Pregnant ewes are vaccinated three weeks prior to parturition, when infection often occurs. Lambs may require vaccination during their first year. Because of the infectious nature of the disease, a change of pasture is often advisable when cases are first observed.

Clostridium haemolyticum

C. haemolyticum is a nonencapsulated, motile, strict anaerobe, producing large, oval, highly heat-resistant spores. *C. haemolyticum* (previously known as type D *C. novyi*) resembles type B *C. novyi* in practically all phenotypic traits. *C. haemolyticum* produces three exotoxins. Its main virulence factor, a phospholipase C, is identical to that of the type B *C. novyi* β-toxin, but is produced in much larger amounts. This toxin can cause hemolysis and necrosis of hepatocytes. The other two toxins of this microorganism are a lipase and a tropomyosinase. The role of these minor

toxins in pathogenesis of *C. haemolyticum* infection is not fully elucidated. Serological and toxigenic variants of *C. haemolyticum* have been noted.

C. haemolyticum produces bacillary hemoglobinuria ("red water") disease of ruminants (mainly cattle). This disease has been rarely reported in other animal species such as horses and pigs.

Reservoir and Transmission

C. haemolyticum exists in the ruminant digestive tract, in the liver, and in soil. The appearance of the disease in new, widely separated regions suggests that movement of cattle plays a part in its dissemination. Transmission is by ingestion.

Pathogenesis

The pathogenesis of bacillary hemoglobinuria involves ingestion of spores, colonization of the liver, liver injury, spore germination, and toxinogenesis (see *C. novyi* type B "black disease" in Section Infectious Necrotic Hepatitis). The exotoxin is a powerful phospholipase C that produces a hemolytic crisis and death within hours or days. Other pathological effects include serosal effusions and widespread hemorrhages. The diagnostic lesions are circumscribed areas of liver necrosis (misnamed "infarcts"), which are the effects of beta toxin (Figure 34.15). Clinical signs are fever, pale, icteric mucous membranes, anorexia, agalactia, abdominal pain, hemoglobinuria ("red water"), and hyperpnea. Pregnant cows may abort.

Epidemiology

Bacillary hemoglobinuria occurs in North America in the Rocky Mountain and Pacific Coast states and along the Gulf of Mexico, and in Latin America, parts of Europe, and New Zealand. Although swampy lowlands are associated with endemic disease and flooding with the spread of infection, little is known of the agent's persistence in soil. Shedder animals may play a role in dissemination.

Cases are clustered in the summer and fall, typically among well-nourished animals one year (or more) of age. Correlation with fluke infection is less consistent than in black disease (see *C. novyi* type B in Section Infectious Necrotic Hepatitis).

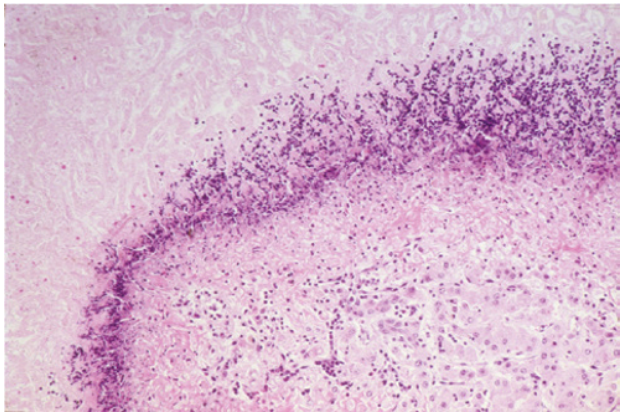


Figure 34.15 Histological section of liver of a cow with bacillary hemoglobinuria, showing coagulation necrosis at the top of the figure, bordered by a zone of intense inflammation. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

Immunologic Aspects

Immunity is antitoxic. Animals in endemic areas develop some immunity. Whole culture bacterin-toxoids are effective prophylactically.

Laboratory Diagnosis

Liver lesions are the best source of positive smears for Gram stains and immunofluorescence tests. Cultivation requires freshly poured blood agar and strict anaerobic conditions.

Detection in tissue or identification in culture can be accomplished by using PCR as noted for other histotoxic clostridia. MALDI TOF mass spectrometry is also used to identify this bacterium.

Treatment and Control

Early treatment of sick animals with an antibiotic (e.g. tetracycline or procaine penicillin G), antitoxin (if available), and blood transfusion produces good results. Animals in endemic areas are vaccinated at least every six months and preferably three to four weeks before anticipated exposure.

Clostridium novyi

Descriptive Features

C. novyi is a nonencapsulated, motile, gram-positive, obligately anaerobic rod producing large, oval, highly heat-resistant spores. *C. novyi* strains can be classified into four toxinotypes (A-D) based upon production of toxins (Table 34.3). There are two pathogenic types (A, B), which differ biochemically, epidemiologically, and pathogenically. Type C *C. novyi* is non-toxigenic. A different species, *C. haemolyticum*, discussed above, used to be known as type D *C. novyi*. *C. novyi* produces gas gangrene, "big head," and "black disease" of ruminants, and, rarely, blackleg of cattle.

Cellular Products of Medical Interest

Toxins

C. novyi produces a number of protein extracellular toxins that are responsible for the diseases associated with it. Of these, the α- and β-toxins, and the cholesterol-binding cytolysin novyilysin (γ-toxin), have proven roles in the diseases produced by this species.

Table 34.3 *Clostridium novyi* toxins and its associated diseases.

C. novyi type	Toxin	Disease	Species affected
A	α, γ	Gas gangrene, pseudo-blackleg, big head	Animals, humans
B	α, β	Gas gangrene, Necrotizing hepatitis	Animals
C	γ (?)	–	–
D (*C. haemolyticum*)	β	Bacillary hemoglobinuria	Animals

α-Toxin The α-toxin (TcnA) is produced by *C. novyi* types A and B. Genome sequencing of *C. novyi* indicated that α-toxin is expressed from a phage-encoded gene. The α-toxin is a member of large clostridial cytotoxin family similar to *C. perfringens* TpeL and *C. difficile* toxins A/B. This cytotoxin is a glycosyltransferase that glucosylates Rho GTPases, rendering them ineffectual in interacting with their substrates and therefore as cell signaling systems. In addition, glycosylation blocks interaction of Rho GDP with guanine exchange factor, and the interaction of Rho GTP with GTPase activating factor, thereby preventing membrane cycling. As a consequence, several signaling pathways are disrupted, resulting in a breakdown of the cytoskeletal components of the affected cell, followed by its death.

β-Toxin The β-toxin is produced by *C. novyi* types B and D. It is a phospholipase C (a lecithinase) that hydrolyzes the phosphatidylcholine and sphingomyelin constituents of host cell membranes, resulting in death of the cell. This toxin is also hemolytic.

γ-Toxin γ-Toxin or novyilysin is produced *by C. novyi* type A, and is a cholesterol-binding cytolysin (see also perfringolysin O, described earlier). Novyilysin binds to cholesterol-containing rafts in the eukaryotic cell membrane. Once bound, it forms a pore, resulting in the death of the cell.

Miscellaneous Toxins *C. novyi* produces a number of other protein extracellular enzymes or minor toxins with uncertain roles in the pathogenesis of disease. These include lecithinases, lipases, and myosinases.

Reservoir and Transmission

Type A is common in soil. Types A and B occur in the healthy intestine and liver of herbivores, and may also be found as spores persisting in muscle. All enter their hosts by ingestion or by wound infection.

Pathogenesis

Of several toxins produced by *C. novyi*, α-, β-, and novyilysin are lethal and necrotizing, and are of established pathogenic significance. The pathogenic roles of the minor non-lethal toxins are uncertain.

Gas Gangrene and Blackleg

C. novyi type A is implicated in gas gangrene ("clostridial wound infection") of humans and animals (Figure 34.16). One unique form of these, "big head of rams," starts as a fight injury at the top of the head of rams, and may result from the activation of spores endogenously present in muscles of the head. Toxic endothelial damage produces edema involving the head, neck, and cranial thorax. Death occurs in two days. The yellow tinge of the edema fluid, which is clear and gelatinous with little hemorrhage, is a postmortem change. *C. novyi* may also rarely cause a blackleg-like disease of cattle, sometimes known as "pseudo-blackleg" to distinguish it from the identical disease caused by *C. chauvoei*. Activation of endogenous spores by injection of irritant chemicals has produced sometimes iatrogenic fatal myositis in horses.

Infectious Necrotic Hepatitis

C. novyi type B causes infectious necrotic hepatitis ("black disease") of sheep and cattle, and rarely horses, swine, and other species. Spores originating in the intestine reach the liver and remain there dormant within Kupffer cells. When liver cells are injured, as commonly follows fluke migration, the resulting anaerobic conditions cause the spores to germinate. Vegetative growth results in toxin production and



Figure 34.16 Gas gangrene caused by *C. novyi* in a calf following castration, showing emphysema and subcutaneous edema. *Source:* Reproduced with permission from Silva et al. (2016).

dissemination. Death may be sudden or within two days of clinical onset. Signs include depression, anorexia, and hypothermia. Necropsy reveals edema, serosal effusion, and one or more areas of liver necrosis, containing bacteria. Subcutaneous venous congestion secondary to pericardial edema darkens the underside of the skin, hence the name "black disease." The occasional outbreak in swine might be associated with ascarids migrating through the liver.

Epidemiology

The agent occurs worldwide. Big head is recognized in Australia, South Africa, and North America. Distribution of "black disease" largely coincides with that of the liver fluke *Fasciola hepatica*. Both diseases occur mostly in adult sheep, during summer and fall. "Black disease" affects notably well-nourished animals.

Immunologic Aspects

Circulating antitoxins (antibodies to α-, β-, and novyilysin toxins) and antibodies to cellular components of the organism presumably are the basis of immunity to *C. novyi* infections. Whole culture bacterins and toxoids have prophylactic value.

Laboratory Diagnosis

Liver lesions contain large gram-positive to gram-variable rods with oval subterminal, large spores, which are identifiable by fluorescent anti-*C. novyi* conjugates.

Isolation of the bacteria requires the strictest anaerobic conditions, especially for type B, which is also nutritionally fastidious. *C. novyi* may be demonstrable in normal livers of herbivores within hours after death, so that postmortem invaders need to be differentiated from genuine pathogens.

Increasingly, detection in tissue or identification in culture is accomplished by using PCR, including real-time PCR, which allows quantitation of the bacterial load. DNA primers designed to amplify species-specific portions of the gene encoding the flagellar protein flagellin have been used successfully to differentiate histotoxic clostridia.

Treatment and Control

There is no effective treatment. Control is directed at eliminating flukes and other hepatopathic agents. Prophylactic vaccination with a bacterin-toxoid combination is generally effective.

Clostridium septicum

Descriptive Features

C. septicum is a short, stout, and pleomorphic gram-positive, motile, spore-forming obligately anaerobic rod. In some exudates, long filaments are found.

C. septicum is the leading cause of wound infections (gas gangrene = malignant edema) of farm animals and gangrenous dermatitis in poultry, but may also cause a fatal abomasitis in sheep and, unusually, blackleg ("pseudo-blackleg") in ruminants.

Cellular Products of Medical Interest

C. septicum produces a number of protein exotoxins that are purportedly responsible for the diseases associated with it. However, only α-toxin has been definitively shown to be a virulence factor. The α-toxin, like *C. perfringens* CPE enterotoxin, is a member of the aerolysin family of pore-forming toxins. Following secretion, it is bound to glycosylphosphatidylinositol (GPI)-anchored proteins on the eukaryotic cell surface (mainly endothelial cells). There, the cell-bound proteolytic enzyme, furin, cleaves it, resulting in fragments that insert into the membrane forming pores leading to death of the cell. *C. septicum* also produces a DNAase ("β-toxin") with leukocytotoxic activity, a hyaluronidase ("γ-toxin"), a cholesterol-binding cytolysin (septicolysin O) (with activity similar to that of perfringolysin O and novyilysin, etc.), and miscellaneous extracellular products (chitinases, neuraminidases, lipases, sialidases, hemagglutinins) with undefined roles in the pathogenesis of disease.

Reservoir and Transmission

C. septicum occurs in soils worldwide and in the animal and human intestine. It is acquired by wound infection and ingestion.

Pathogenesis

Wound infection produced by *C. septicum* is often called "malignant edema," but the term *gas gangrene* is probably better understood and less old fashioned. All of the toxic products outlined above probably play a role in this serious necrotizing infection, but only the α-toxin has been shown to be definitively involved. Systemic effects are thought to be the result of endothelial damage throughout the body, leading to severe fluid and electrolyte imbalances as well as for the edema seen locally. Membrane-active toxins, dissolution of connective tissue components, along with destruction of polymorphonuclear leukocytes and release of their digestive enzymes may account for the tissue destruction observed. The infectious process radiates from the point of inoculation within hours to days of exposure. A hemorrhagic, edematous, necrotizing process frequently follows fascial planes as adjacent muscle is darkened (Figure 34.17). With muscular involvement and emphysema, there may be close resemblance to "blackleg" (see Section *Clostridium chauvoei*). Crepitant swellings change from painful and hot to painless and cold. Signs include



Figure 34.17 Gas gangrene in a young piglet, likely associated with injection or castration. The necrotizing process has followed fascial planes. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

fever, tachycardia, anorexia, and depression. The course may be rapid and fatal within a day. Wounds may include traumatic wounds but may include the post-parturient uterus of animals.

C. septicum sometimes causes a blackleg-like disease of cattle, sometimes called pseudo-blackleg to distinguish it from the identical disease caused by *C. chauvoei*. Activation of endogenous spores by injection of irritant chemicals has produced iatrogenic fatal myositis in horses.

C. septicum also causes gangrenous dermatitis, a fatal necrotizing cellulitis, and myonecrosis of poultry including turkeys and broiler chickens. *C. perfringens* may be concurrently involved. The factors precipitating the sometimes devastating infection are unclear, but include poor hygiene, overcrowding, and likely fecal contamination of pressure sores.

Severe and fatal abomasitis (Braxy in Scotland or Bradsot in Denmark) is a fatal *C. septicum* cold-weather disease of sheep in which *C. septicum* produces a necrotizing lesion in the abomasal wall comparable to the subcutaneous one described above. Clinical signs are mostly toxemia and gastrointestinal distress. Most aspects of the pathogenesis of this unusual, dramatic, and generally fatal disease are poorly understood. The association of the infection with ingestion of frozen food including hay might suggest traumatic damage of the abomasal mucosa (Figure 34.18).

Human wound infections due to *C. septicum* may develop into serious cellulitis and gas gangrene, and are associated with intravenous illegal drug injection, abortion (producing septic metritis) under unhygienic conditions, malignancies, skin infections, peripheral vasculopathy associated with diabetes, and severe trauma. The organism also causes a "spontaneous" non-wound-associated myonecrosis ("gas gangrene") in humans with pathogenesis likely similar to that of blackleg in cattle.



Figure 34.18 Braxy in the abomasum of a lamb, showing extensive hemorrhage, edema, gas formation, and areas of focal necrosis. *Source:* Courtesy of Department of Pathobiology, University of Guelph.

Epidemiology

Gas gangrene may follow such procedures as castration, docking, shearing, tagging, and injections. Post-parturient genital infections are sometimes linked to dystocia and unskilled obstetrical assistance. Braxy or Bradsot occurs mostly in colder countries such as Canada, Scotland, and the Scandinavian countries.

Immunologic Aspects

Immunity is probably antitoxin-dependent.

Laboratory Diagnosis

Sporulated rods may be demonstrable in exudates by Gram stain or immunofluorescence.

C. septicum grows readily on blood agar under anaerobic conditions, producing hemolytic colonies up to 5 mm in diameter within 48 hours, with rhizoid contours and a frequent tendency to swarm. Although recovery and identification of this agent by culture are not difficult, positive results should be interpreted with caution as *C. septicum* is an aggressive postmortem invader. Its presence may therefore be unrelated to the problem at hand and may obscure that of more significant pathogens, such as *C. haemolyticum* and *C. chauvoei*.

Demonstration in tissue or identification of isolates is possible by fluorescent antibody staining or by PCR amplification of the α-toxin. PCR amplification of species-specific portions of flagellin gene has been to differentiate the organism from other clostridia causing severe tissue necrosis.

Treatment and Control

Prognosis is guarded. Possible therapy includes penicillin or tetracycline given systemically, incision and drainage of the wounds, and their irrigation with antiseptics.

Calves are vaccinated at three to four months of age, sheep and goats at weaning. Hygienic precautions at times of likely exposure are helpful.

Paeniclostridium sordellii

P. sordellii is a gram-positive, anaerobic, spore-forming rod that is motile by peritrichous flagella. *P. sordellii* grows on blood agar under anaerobic conditions, producing translucent to opaque colonies with small β-hemolysis. The agent is commonly found in soil and in the gastrointestinal tract of animals. The role of *P. sordellii* in serious infections of animals and humans may have been underestimated, or infections may be increasing. This organism produces numerous toxins. These toxins include lethal toxin (LT or TcsL), hemorrhagic toxin (HT or TcsH), sordellilysin, phospholipase, collagenase, and neuraminidase. However, the role of most of these toxins in the pathogenesis of *P. sordellii* infections is not well understood. The most extensively studied of these toxins are TcsL and TcsH. Both toxins belong to the large clostridial glucosylating toxins family. The amino acid sequence and biological activity of TcsL and TcsH are similar to *C. difficile* toxins A and B, respectively. It has been shown that TcsL is an essential virulence factor in *P. sordellii*.

P. sordellii is associated with a fatal myositis and hepatic disease in ruminants and horses, although the precise pathogenic process is not known. "Acute pasture myopathy" ("equine atypical myopathy," "seasonal pasture myopathy") associated with acute and usually fatal rhabdomyolysis has become an increasing problem in recent years in European countries. It occurs especially in young adult grazing horses under frosty and stormy, fall-like, conditions. The disease is associated with sudden onset of muscle weakness, the result of generalized myodegeneration although the focus of infection is unclear. It may be the intestine. The presence of the lethal toxin of *P. sordellii* (TcsL) has been demonstrated in myofibers of affected horses by immunohistochemistry.

P. sordellii has caused fatal disease in perinatal foals following infection of the umbilical remnants as well as fatal uterine infections in post-parturient sheep. It is also emerging as a pathogen of humans, associated with very serious and often fatal infections following abortion and "spontaneous" non-wound-associated myonecrosis ("gas gangrene") similar to blackleg.

There is increasing evidence that the organism can cause severe enteric disease in animals including a disease identical to *C. septicum* abomasitis ("braxy") in lambs. The organism has been isolated from fatal enteric disease in lions.

Mixed clostridial bacterin-toxoids for blackleg and gas gangrene usually contain *P. sordellii*.

Neurotoxic Clostridia

C. botulinum (the cause of botulism) and *C. tetani* (the cause of tetanus) produce disease strictly through the action of neurotoxins, botulinum and tetanus toxins, respectively. Though botulinum and tetanus toxins have the same mechanism of action, they produce different diseases with remarkably different manifestations because they affect different sites in the nervous system.

Botulinum toxin and tetanus toxin block neurotransmitter release. Both toxins are zinc endopeptidases that interfere with the fusion of vesicles containing neurotransmitter with the membrane lining the presynaptic cleft. Interference is due to hydrolysis of the proteins involved with "docking," which precedes fusion of neurotransmitter-containing vesicles with the membrane lining the cleft. Hydrolysis of these proteins leads to degeneration of the synapse and to the blockage of neurotransmission. Regeneration of the synapse takes several weeks to months.

Clostridium botulinum

C. botulinum is a gram-positive, spore-forming, obligately anaerobic rod that produces the disease botulism, a neuroparalytic intoxication characterized by flaccid paralysis.

Botulism is seen mainly in ruminants, horses, mink, and birds, particularly waterfowl, though the infection is increasingly diagnosed in commercial poultry. Swine, carnivores, and fish are rarely affected.

Descriptive Features

Morphology

C. botulinum is a gram-positive, spore-forming obligately anaerobic rod. At a pH near and above neutrality, it produces subterminal oval spores.

Cellular Products of Medical Interest

Toxin *C. botulinum* produces several proteins with "toxic" activity (botulinum toxin [BoNT for botulinum neurotoxin], C2 toxin, and C3 exoenzyme), but only botulinum toxin has a central role in the production of botulism.

BoNTs are traditionally classified into seven types (A to G) based upon recognition or neutralization with a type-specific antitoxin. Letters A through G depict the types (Table 34.4). These seven neurotoxins are identical in action but differ in potency, antigenic properties, and distribution. The type of neurotoxin characterizes the strain of *C. botulinum* producing it. Thus, a strain of *C. botulinum* producing type A BoNT would be described as *C. botulinum* type A. *bont* gene sequence comparisons have shown the presence of a large number of "mosaic" botulinum neurotoxins. Therefore, BoNT types can further be divided

Table 34.4 Classic botulinum toxin types: general distribution, origin, and pathogenicity.

Type (Toxin present)	Geographic distribution	Source	Species affected
A	Western N. America; Former Soviet Union; China; Taiwan	Soil, vegetables, fruits (meat, fish)[a]	Humans (chicken, mink)
B	Eastern N. America; Europe; Former Soviet Union; China; Taiwan	Soil, meat, pork products (vegetables, fish)	Humans (horses, cattle)
C	New Zealand; Australia; N. America; Africa; Japan; Europe	Vegetation, invertebrates, carrion, spoiled feed, carrion	Waterfowl, horse, cattle, mink (humans)
D	Africa; Australia; Former Soviet Union; SW US; Europe	Carrion, chicken manure	Cattle, sheep, chickens (horses, humans)
E	N. America; N. Europe; Japan; Former Soviet Union	Raw fish, marine mammals	Humans, fish, fish-eating birds
F	US; N. Europe; Former Soviet Union	Meat, fish	Humans
G	Argentina	Soil	Humans

[a] Parentheses indicate infrequency or variability.

Table 34.5 Groups and characteristics of clostridia capable of producing botulinum neurotoxin.

	Group I	Group II	Group III	Group IV	Group V	Group VI
Botulinum toxin type	A, proteolytic B, F	E, non-proteolytic B, F	C, D	G	F	E
Subtypes	A1-A8; B1-B2, B3, B5, B6, B7, B8; F1, F2, F3, F4, F5, F8	E1, E2, E3, E6, E7, E8, E9, E10, E11, E12; B4; F6	C/D, D/C	G	F7	E4, E5
Optimum growth temperature	35–40 °C	18–25 °C	40 °C	37 °C	30–37 °C	35–40 °C
Minimum growth temperature	12 °C	3.3 °C	15 °C	ND	12 °C	10 °C
Minimum pH for growth	4.6	5	5.1	ND	4.8	3.7
Neurotoxin-producing bacteria	*C. botulinum* (newly proposed *C. parabotulinum*)	*C. botulinum*	*C. botulinum* (newly proposed *C. novyi* sensu *lato*)	*C. argentinense*	*C. baratii*	*C. butyricum*

ND: no data available.

into subtypes. Currently, more than 40 subtypes have been described for BoNT type A, B, E, and F. For example, BoNT type A is subdivided into A1 to A8. A toxigenic strain usually synthesizes only one BoNT type and subtype. However, bivalent strains have been rarely described. These strains produce two BoNT types such as Ab, Af, and Bf (major toxins are written as uppercase letters and minor toxins as lowercase letters). Recently, a putative new BoNT type (proposed as BoNT H) was identified in a bivalent *C. botulinum* Bh strain isolated from a case of human infant botulism. However, the designation was not globally accepted because this strain can be neutralized by a combination of the type A and B antitoxins. Moreover, genome sequencing of this specific strain revealed that the putative neurotoxin is a BoNT/F5A1 hybrid.

The seven types of botulinum toxin are produced by a heterogeneous group of clostridia. All botulinum neurotoxin-producing bacteria share the same phenotype and have been traditionally designated *C. botulinum* without consideration of their genetic or physiological heterogeneity. However, the recent genome sequencing comparisons indicated that the clostridia-producing BoNT belongs to six different groups (I–VI), which can be considered different species (Table 34.5).

Analysis of genome sequencing of *C. botulinum* strains revealed that *bont* genes are typically located in a locus (named the botulinum locus) with their accessory genes. This locus is often flanked by insertion sequences and is found in different DNA structures, including chromosome, plasmid, transposon, and phage. This genetic feature

facilitates the movement of *bont* genes between various clostridial strains and possibly from clostridia to non-clostridia species.

All seven types of BoNT are zinc endopeptidases with identical activity, that is, hydrolysis of the docking proteins required by neurotransmitter-containing vesicles to fuse with the presynaptic membrane. Although the end result is the same (blockage of the release of neurotransmitter), the various types of BoNT hydrolyze different docking proteins. Types A and E hydrolyze SNAP (synaptosomal-associated protein), types B, D, F, and G hydrolyze VAMP (vesicle-associated membrane protein, also known as synaptobrevin), and type C hydrolyzes SNAP and syntaxin. Once hydrolyzed, the synapse degenerates, taking weeks to months to regenerate.

BoNT is a "di-chain" molecule consisting of a light chain (with zinc endopeptidase activity), a heavy chain composed of a translocation domain (responsible for forming a pore through which the light chain passes), and a binding domain (responsible for binding to nerve cells). Several "accessory" proteins thought to aid the survival of the toxin in the gastrointestinal tract are secreted with BoNT.

BoNT binds to cholinergic nerve cells, each type of BoNT binding to a different receptor. After binding, the toxin is internalized by way of receptor-mediated endocytosis. Vesicles containing BoNT remain at the neuromuscular junction. Following a cleaving event, the light chain (the zinc endopeptidase) translocates across the vesicle membrane into the cytosol of the nerve cell, where it hydrolyzes the docking proteins.

C. botulinum strains may also produce non-neurotoxins, including C2 toxin and C3 exoenzyme. C2 toxin is a binary toxin, consisting of an enzymatic effector subunit (C2I) and a cell-binding subunit (C2II). C2 toxin is produced by *C. botulinum* types C and D. *C. botulinum* producing C2 toxin occasionally induces hemorrhagic enteritis in animals, notably in birds. Both C2 toxin and C3 exoenzyme are ADP-ribosyltransferases. C2 toxin and C3 exoenzyme ribosylates G-actin and Rho, respectively, resulting in disruptions in the cytoskeleton. Neither enzyme appears to play a role in the botulism disease process.

Resistance

Although the heat resistance of spores varies between culture groups, toxin types, and strains, moist heat at 120 °C for five minutes is generally lethal. Exceptions exist. Low pH and high salinity enhance heat sterilization. Salt, nitrates, and nitrites suppress germination of spores in foods. Heating to 80 °C for 20 minutes inactivates the toxin. The seven types of toxins differ in antigenicity, heat resistance, and lethality for different animal species (probably related to receptor density on the surface of the motor neuron).

Reservoir and Transmission

Reservoirs of *C. botulinum* are soil and aquatic sediments. Vehicles of intoxication are animal and plant material contaminated from these sources. When animals die, *C. botulinum* spores, which are common in gut and tissues, germinate and generate toxin. This may contaminate the immediate local environment. An example of this is an animal killed during hay making and rolled into a "big bale" hay bale. In rotting vegetation, a similar process occurs. Apart from toxin ingestion, spore ingestion and wound contamination may lead to botulism. Spore ingestion is important in human infant botulism. Wound-infection botulism is seen rarely in humans and horses.

Pathogenesis

Botulinum toxin is the most toxic protein known; 1 µg can kill a person. Ingested BoNT is absorbed from the glandular stomach and anterior small intestine and distributed via the bloodstream. It binds to receptors and enters the nerve cell after receptor-mediated endocytosis. How BoNT gets from the bloodstream to the surface of nerve cells is not fully understood because these toxins are unable to cross the blood–brain barrier. However, the results of some studies have suggested that type A and E neurotoxins may reach the nervous system by axonal retrograde transport. Toxin-containing vesicles remain at the myoneural junction. A fragment of the toxin (light chain) translocates across the vesicle membrane into the cytosol of the nerve cell and subsequently hydrolyzes a "docking" protein (which protein depends upon which botulinum toxin). The synapse degenerates and flaccid paralysis results due to the lack of neurotransmitter (acetylcholine). When this affects muscles of respiration, death due to respiratory failure occurs. No primary lesions are produced.

Clinical signs include muscular incoordination leading to recumbency, extrusion of the tongue, and disturbances in food prehension, chewing, and swallowing (Figure 34.19). No changes in consciousness occur. The temperature remains normal unless secondary infections such as aspiration pneumonia supervene. In nonfatal cases, recovery is slow, and residual signs may persist for months. There is a still unproven but plausible suggestion that equine grass sickness, a commonly fatal dysautonomia affecting the enteric nervous system, is associated with the ingestion of BoNT secreted by *C. botulinum* type C living in association with blades of grass (as a biofilm).

In birds such as ducks the disease has been named "limberneck" after the characteristic drooping head posture, which often causes waterfowl to drown.



Figure 34.19 Nuchal ligament edema in horse with botulism. *Source:* Courtesy of Dr. F.A. Uzal, University of California, Davis.

Epidemiology

Types A and B are found in all soils, including virgin soils; C, D, E, and F are linked with wet environments – that is, muddy soils or aquatic sediment. In animals, types C and D predominate. Type C living in biofilms on the surface of blades of grass may be the source of BoNT seen in equine grass sickness, although whether equine grass sickness is truly the result of botulism remains to be definitively demonstrated. Type E is associated with aquatic environments, and so is characteristically associated with eating affected or contaminated fish.

Dead animals (birds, rodents, cats) in feed can be sources of outbreaks, as can chicken litter (which may contain dead chickens) when used as a cattle and sheep feed supplement, as bedding, or is spread as a fertilizer onto pastures to which ruminants have access. The increasing use of "big bale silage" and round hay baling in recent years, in which grass is ensiled without acidification in large plastic airtight bags or wrapped in tight round bales, has been associated with increased botulism in horses and cattle. It is likely that this is a combination of the cutting of grass very close to the ground, so that baby grassland nestling birds or rodents are collected, as well as the anaerobic conditions of ensilage or of tightly wrapped hay. Outbreaks on mink ranches are usually due to tainted meat, and those in fish hatcheries to fish food containing *C. botulinum* type E spores that germinate in bottom sludge. Decaying vegetation triggers outbreaks in waterfowl. As lakes recede in summer leaving muddy shores or shallow pools, rotting plant material becomes accessible. *Clostridium botulinum* and its toxin are ingested and after death permeate the carcass, which is fed upon by blowfly larvae, which absorb the toxin. Waterfowl ingesting these larvae become intoxicated. Type C and type D botulism is increasing in commercial laying and broiler chickens, apparently as a result of production of toxin within the intestine (toxicoinfectious botulism), although the reason for this increase is unclear. This intestinal infection may be related to the increased botulism observed in ruminants exposed to chicken litter.

Type B botulism has been seen in cattle and mules. In toxicoinfectious botulism of foals (shaker foal syndrome) and adult horses, no toxin has been demonstrated, but the agent has been isolated consistently from tissue.

Type D botulism is classically linked to phosphorus-deficient ranges, where grazing animals feed on carcasses and bones that often contain botulinum toxin. In South African cattle, the condition is called lamziekte ("lame disease"), which describes the lameness associated with phosphorus deficiency. Type D botulism is increasingly identified in ruminants in the United Kingdom exposed to broiler chicken litter.

Type E botulism has emerged in fish-eating birds in the Great Lakes of North America, the result of complex ecological changes induced in the aquatic environment by various invasive species. Migrating fish-eating birds catch fish with botulism, which makes them easier to catch, and succumb to intoxication.

Human botulism is usually traced to improperly processed meat, seafood, or canned vegetables. Infant botulism involves clostridial growth and toxinogenesis in the intestinal tract, producing the "floppy baby syndrome," and may be associated with ingestion of honey, because this frequently contains large numbers of *C. botulinum* spores. Wound-infection botulism results from contaminated external injuries.

Immunologic Aspects

Resistance to botulism depends on circulating antitoxin. Some carrion-eating animals, such as vultures, apparently acquire immunity through repeated sublethal exposure.

Laboratory Diagnosis

Diagnosis of botulism requires demonstration of toxin in plasma or tissue before death or from a fresh carcass. Isolation of the organism, especially from intestinal contents, or postmortem demonstration of toxin is not definitive. Demonstration of toxin in feedstuffs, fresh stomach contents, or vomitus supports a diagnosis of botulism.

Toxin is extracted from suspect material (unless fluids) overnight with saline. The mixture is centrifuged, and the clear portion filter-sterilized and trypsinized (1% at 37 °C for 45 minutes). Guinea pigs or mice are injected intraperitoneally with the extract, mixtures of extract and antitoxins, and extract heated to 100 °C for 10 minutes. Death due to botulism occurs within 10 hours to three weeks (average is four days) preceded by muscular weakness, limb paralysis, and respiratory difficulties. To confirm that animals die of intoxication rather than peritonitis, any toxin must be

neutralizable by one of the *C. botulinum* antitoxins. This method is still considered as the gold standard method for detection and identification of BoNTs, but it is very expensive, time consuming, and inhumane if the affected animals are not immediately euthanized once disease signs develop. In recent years, immunoassays such as ELISA have been developed to detect BoNTs.

Isolation of *C. botulinum* from suspect feeds or tissue begins with heating suspect material for 30 minutes at 65–80 °C to induce germination. Type E spores require, in addition, treatment with lysozyme (5 mg/ml of medium). Culture is anaerobically on blood agar plates. Identification is by biochemical reactions and toxin production. Immunofluorescence is used to identify some cultural groups. Primers designed to amplify the genes encoding the various toxins (by PCR) can be used to support the demonstration that *C. botulinum* has been isolated.

Treatment and Control

If recent ingestion is suspected, evacuation of the stomach and purging are helpful. Antitoxin treatment following onset of signs is sometimes beneficial, especially for mink and ducks, but prevention by vaccination is definitely preferred. Mink and other animals at risk should be vaccinated with toxoid (Types A, B, C, D). Clinical trials to prevent equine grass sickness with a botulinum toxoid vaccine are underway. Removal of affected waterfowl to dry land, if feasible, saves many from exposure and drowning. Placing food on dry ground may lure birds from contaminated areas. It is mandatory that farmers in the United Kingdom remove carcasses from chicken litter before selling it as fertilizer.

Guanidine and aminopyridine stimulate acetylcholine release, and germine intensifies neural impulses. Clinical reports on their use are few and mixed.

Clostridium tetani

C. tetani is a gram-positive, spore-forming, obligately anaerobic rod, which produces the disease tetanus, a neuroparalytic intoxication characterized by tetanic spasm of muscle and tonic–clonic convulsions. The intoxication is due to a protein neurotoxin.

All mammals are susceptible to varying degrees to tetanus, with horses, ruminants, and swine being more susceptible than carnivores, and birds are resistant. In all animals, the mortality rate is high. Humans and horses are the most susceptible species.

Descriptive Features

Morphology

C. tetani is a gram-positive, spore-forming, obligately anaerobic rod that is motile by peritrichous flagella. A distinguishing morphological feature of *C. tetani* is the spherical shape and terminal position of its spores ("drumstick" or "tennis racket"; Figure 34.1).

Cellular Products of Medical Interest *C. tetani* produces two toxins (tetanolysin and tetanus toxin), though only tetanus toxin is of clinical significance.

The genes encoding tetanus toxin (TeNT for tetanus neurotoxin, also called tetanospasmin) are located on a large plasmid. TeNT is released upon lysis of the clostridial cell. There is only one type of TeNT, a zinc endopeptidase, which hydrolyzes the docking proteins required by neurotransmitter-containing vesicles to fuse with the presynaptic membrane. TeNT hydrolyzes the docking protein VAMP (vesicle-associated membrane protein, also known as synaptobrevin). Once the docking proteins are hydrolyzed, the synapse degenerates, taking weeks to months to regenerate.

TeNT is a "di-chain" molecule consisting of a light chain (with zinc endopeptidase activity), a heavy chain composed of a translocation domain (responsible for forming a pore through which the light chain passes), and a binding domain (responsible for binding to nerve cells).

TeNT binds to cholinergic nerve cells by virtue of receptors that are different from those recognized by BoNT. These receptors are composed of lipid containing rafts and glycosylphosphatidylinositol (GPI)-anchored proteins. After binding, the toxin is internalized by way of receptor-mediated endocytosis. Vesicles containing TeNT travel by retrograde axonal transport to the inhibitory interneurons in the ventral horn of the spinal cord. Following a cleaving event, the light chain (the zinc endopeptidase) translocates across the vesicle membrane into the cytosol of the nerve cell, where it hydrolyzes the "docking" proteins involved with vesicles containing the neurotransmitters gamma aminobutyric acid (GABA) and glycine.

The other toxin, tetanolysin, is a cholesterol-binding cytolysin but has no known pathogenic significance in the production of tetanus.

Growth Characteristics *C. tetani* grows on blood agar under routine anaerobic conditions. There may be swarming. Its differential reactions (carbohydrate fermentation, proteolysis, indole production) vary with the medium used. Spores resist boiling up to 1.5 hours, but not autoclaving (121 °C for 10 minutes). Disinfection by some halogen compounds (3% iodine) can be effective within several hours, but phenol, lysol, and formalin in the usual concentrations are ineffective.

Reservoir and Transmission

C. tetani is widely distributed in soil and is often a transient in the intestine. Spores are introduced into wounds.

Pathogenesis

Spore germination requires an anaerobic environment as is found in tissues devitalized by crushing, burning, laceration, breakdown of blood supply (e.g. umbilical stump or placental remnants), or bacterial infection. Under these circumstances, *C. tetani* proliferates, and its toxin diffuses via vascular channels or peripheral nerve trunks. The toxin attaches to receptors on the nearest cholinergic nerve and is internalized within a vesicle, which travels retrograde inside the axons to the cell bodies in the ventral horns of the spinal cord. The light chain of the toxin translocates across the vesicle membrane into the cytosol, where it hydrolyzes "docking" proteins and suppresses the release of afferent inhibitory messenger substances (glycine, GABA), causing the innervated muscles to remain in sustained clonic or tonic spasms. The toxin also travels within the cord to other levels affecting additional muscle groups. Synapses degenerate following hydrolysis of the vesicular docking proteins, taking several weeks to regenerate.

The process described ("ascending tetanus") is typical of animals not highly susceptible to tetanus toxin (e.g. dogs and cats). Only nerve trunks near the toxigenic site absorb sufficient toxin to produce overt signs.

"Descending tetanus" (a generalized toxemia) is typical of highly susceptible species (horses, humans) in which effective toxin quantities are disseminated via vascular channels to nerve endings in areas remote from the toxigenic site. Toxin enters the central nervous system at many levels producing generalized tetanus, frequently beginning cranially because the cranial nerves have short distances into the central nervous system. The sequence reflects the susceptibility of various neurons.

Disease Patterns

Early signs, following an incubation period of a few days to several weeks, are stiffness, muscular tremor, and increased responsiveness to stimuli.

In horses, ruminants, and swine, which usually develop descending tetanus, retraction of the third eyelid (because of spasm of ophthalmic muscles), erectness of ears, grinding of teeth, and stiffness of the tail are observed. Bloat is common in ruminants. Feeding becomes impossible ("lockjaw"). Rigidity of extremities causes "sawhorse" attitudes and, eventually, recumbency. Tetanic spasms occur first in response to stimuli, but later become permanent. There is fecal and urinary retention, sweating, and high fever. Consciousness persists. Death, due to respiratory arrest, occurs in lambs and piglets within the first week, in adult animals in one to two weeks. Full recovery requires weeks to months. Mortality is at least 50% and highest in the young.

In carnivores, the incubation period tends to be longer, and local (ascending) tetanus (stiffness, tremors) is frequently seen near the original wound. Progression may be slower than in ungulates, but the signs and course are comparable.

Epidemiology

Occurrence of tetanus is linked to the introduction of *C. tetani* spores into traumatized tissue (see Section Pathogenesis). Penetrating nail wounds of the foot, barnyard surgery, the use of rubber bands for castrating and docking sheep, ear tagging, injections, shearing wounds, postpartum uterine infections, perinatal umbilical infections, and small animal fights and leghold traps all predispose to the type of injury and the soil or fecal contamination associated with tetanus.

Immunologic Aspects

TeNT is antigenically uniform. Acquired resistance to tetanus depends on circulating antitoxin. Small amounts have been demonstrated in normal ruminants. Surprisingly, but because of its great toxicity, survivors of tetanus with the possible exception of dogs and cats are susceptible to reinfection. The amount of toxin needed to produce tetanus in dogs and cats is sometimes large enough to elicit an antitoxin response. Passive and active protection is provided by administration of antitoxin or immunization with toxoid, respectively (see Section Treatment and Control).

Laboratory Diagnosis

A gram-stained smear from a suspect wound may reveal the typical "drumstick" type of bacteria (see Figure 34.1). Their absence does not exclude tetanus, and their presence is merely suggestive as the morphology is not unique. Wound exudate is plated on blood agar for anaerobic culture. Increased agar content (up to 4%) inhibits swarming, and a drop of antitoxin will inhibit hemolysis on that portion of the plate.

Traditionally, replicate cooked meat broth cultures were incubated, and some were heated at 80 °C for varying periods (up to 20 minutes) to kill bacteria other than spores. All were incubated at 37 °C for four days and subcultured to blood agar periodically during that time. Previously unheated ones were heated before subculture. Suspect isolates were identified by differential tests and confirmed as tetanus toxin producers by intramuscular injection of a 48-hour broth culture into two mice, one of which had received antitoxin. It is actually now rare for this to be done because the disease is so characteristic that laboratory diagnosis is not required.

Primers designed to amplify the gene encoding tetanus toxin (by PCR) can also be used to support the demonstration that *C. tetani* has been isolated.

Treatment and Control

Therapy aims at neutralization of circulating toxin, suppression of toxin production, and life support and

symptomatic relief to the patient. The first objective is pursued by injection of adequate doses of antitoxin: 10 000–300 000 units for horses. Some suggest intrathecal administration because this can be beneficial

Wound care and parenteral penicillin or metronidazole are aimed at stopping toxin production. Supportive treatment includes use of sedatives and muscle relaxants and exclusion of external stimuli. Artificial feeding by stomach tube or intravenously may be necessary after the hyperesthetic phase. Nursing care is most important.

Wounds should be properly cleaned and dressed. During surgical procedures, especially on a mass scale under farm conditions, appropriate hygienic precautions should be observed. Horses, unless actively immunized, are given antitoxin after injury or surgery, and depot penicillin.

Active immunization employs formalinized toxoid, given twice at 1-to-2-month intervals and often annually thereafter, although it is increasingly common to immunize less frequently. All highly susceptible species, especially horses, humans, and sheep, should be vaccinated against tetanus. Package insert directions should be followed. Passive immunity passes from immunized mare to nursing foals and provides protection for about 10 weeks, when toxoid can be given.

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35

Filamentous Bacteria: *Actinomyces, Nocardia, Dermatophilus*, and *Streptobacillus*

Megan E. Jacob

Members of the genera *Actinomyces*, *Nocardia*, *Dermatophilus*, and *Streptobacillus* are grouped together because of their filamentous morphological appearance. Apart from this characteristic, and a common clinical manifestation as pyogranulomatous inflammation, these organisms have little genetic relatedness. Table 35.1 summarizes their differential characteristics.

Actinomyces Species

Actinomyces species are anaerobic or facultative anaerobic, gram-positive, branching rods. These organisms are considered normal microbial members of the oral, gastrointestinal, and urogenital tracts of humans and animals. They are most commonly associated with colonization in the mouth and the nasal cavity near the throat, where they can subsequently result in pyogranulomatous inflammation after disruption of the mucus membrane. The genus comprises multiple species, and clinical manifestations may resemble other morphologically similar organisms such as *Nocardia* species.

Descriptive Features

Morphology and Staining

Members of the genus *Actinomyces* are gram-positive, non-acid-fast, diphtheroid or filamentous rods. They do not form spores; however, microscopically the branching filaments often appear beaded due to uneven staining. This is most commonly seen in pathological specimens. In culture, diphtheroid forms predominate.

Structure and Composition

Actinomyces species have distinct cell wall constituents, which allows for differentiation of morphologically similar species and *Nocardia*. Surface fibrils in *Actinomyces viscosus* may be adhesins for host cells or other bacteria. Surface antigens are related to chemotactic and mitogenic activities.

Growth Characteristics

Actinomyces species are slow-growing facultative or strict anaerobes that require a rich media, preferably containing serum or blood for growth *in vitro*. The organisms tend to be capnophilic, so growth is enhanced with carbon dioxide. Development of macroscopic colonies may require several days (two to three) of incubation at 37 °C. Colony morphology varies between and within species, and hemolysis is rare. Colonies often appear irregularly shaped.

Resistance

Actinomyces species are readily killed by heat and disinfectants, and require frequent passage to survive in culture. The antimicrobial susceptibility patterns of *Actinomyces* are predictable, particularly with regard to penicillin, and acquired antimicrobial resistance is not widely reported. Resistance to fluoroquinolones has been noted.

Ecology

Reservoir

Members of the genus *Actinomyces* are normal members of the host microbiome, most often present on the oral mucous membranes or tooth surfaces; however, they can colonize

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Table 35.1 Differential characteristics of *Actinomyces*, *Nocardia*, *Dermatophilus*, and *Streptobacillus* Genera.

	Actinomyces	***Nocardia***	***Dermatophilus***	***Streptobacillus***
Gram reaction	+	+	+	–
Acid-fast reaction	–	Partial +	–	–
Motility	–	–	Motile zoospores	–
Atmospheric preference	Strict or facultative anaerobe	Aerobe	Facultative anaerobe	Facultative anaerobe
Primary reservoir	Oral mucosa	Soil	Skin	Pharynx of rodents

the mucous membranes of the urogenital tract and be found secondarily in the gastrointestinal tract of many animal species. There are reports of *Actinomyces* species in soil and other environmental sources; however, they are most commonly considered an endogenous infection.

Transmission

Most *Actinomyces* infections are endogenous; that is, they are caused by introduction of a commensal organism into susceptible tissue of its host. Although rare, bites are another means of transmission.

Pathogenesis

Members of the genus *Actinomyces* typically evoke pyogranulomatous inflammatory reactions by largely unknown mechanisms. Bacterial colonies form in tissue, triggering suppurative responses in the immediate vicinity. Peripheral to this, granulation, mononuclear infiltration, and fibrosis furnish the granulomatous elements. Sinus tracts carry exudate to the outside; the exudate often contains characteristic yellow "sulfur granules." These sulfur granules can be greater than 5 mm in size and have also been referred to as rosettes or club colonies. *Actinomyces* species are often part of a polymicrobial infection.

Disease Patterns

Ruminants

In cattle, *Actinomyces bovis*, or rarely, *Actinomyces israelii*, typically present as "lumpy jaw" when they are introduced from an oral reservoir by a traumatic event (e.g. poor-quality feed or hardware) into the alveolar or paralveolar region of the jaw, initiating a chronic rarefying osteomyelitis (Figure 35.1). The physical appearance is often a visible large, hard lump. This rarefying osteomyelitis eventually leads to replacement of normal bone by porous bone, which is laid down irregularly and honeycombed with sinus tracts containing pus. There may be dislodgement of teeth, inability to chew, and mandibular fractures. The lesion expands but has little tendency for vascular dissemination. Similar infections occur in humans and marsupials, although not often due to *A. bovis*. Occasionally, *A. bovis* also has been associated with pulmonary infection in cattle.

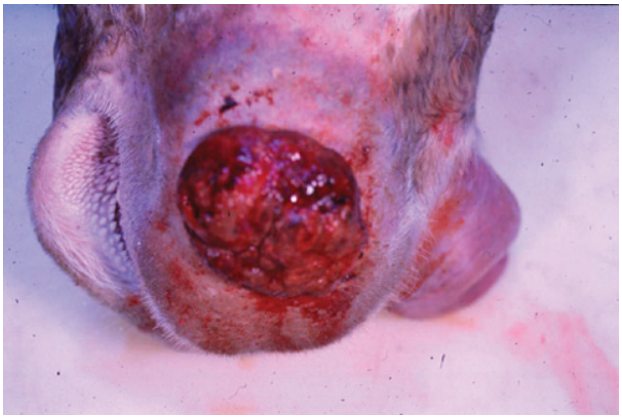


Figure 35.1 A lesion on a bovine mandible associated with *Actinomyces* species infection. *Source:* Courtesy of Noah's Archive. Original contribution of Dr. Crowell, University of Georgia.

Horses

Reports of *Actinomyces* isolated from abscesses, corneal swabs, and skin pustules have all been described in horses. Organisms have been associated with fistulous withers, often accompanying a *Brucella* species. In addition, because horses can develop a lymphadenitis, *Actinomyces* infection may mimic *Streptococcus equi* subsp. *equi* or "strangles" infections.

Dogs and Cats

Actinomyces have been associated with abscesses, mouth wounds, and thoracic and abdominal infections in companion animals. In dogs, actinomycosis may be associated with foreign bodies (e.g. by licking), particularly migrating

grass awns. If these are lodged near vertebrae, they can cause an actinomycotic discospondylitis. Cutaneous actinomycosis in dogs is a rare noduloulcerative lymphangitis.

Swine

Clinically, *Actinomyces* infection in swine, usually *Actinobacillus suis*, is associated with mastitis, likely initiated from suckling piglets. *Actinomyces* has also been recovered from lung lesions and aborted fetuses.

Epidemiology

Actinomycosis are not considered communicable, except rarely via bites. Contamination of bursae or body cavities may be hematogenous or result from perforations of the alimentary tract, or body wall. Actinomycosis associated with plant awns have been reported in outdoor dogs in semiarid areas. *Actinomyces*-associated disease has been reported in goats, sheep, wild ruminants, monkeys, rabbits, squirrels, hamsters, marsupials, and birds throughout the world.

Immunologic Factors

Infected humans show cell-mediated and humoral immune responses. Circulating antibody, produced during infection, confers no protection. Specific resistance, if any, is probably cell mediated. Phagocytes kill *Actinomyces*. No commercial vaccine is available.

Laboratory Diagnosis

Aspirates from unopened lesions or tissues, preferably including granules, are optimal for laboratory diagnosis. Suspected exudates are examined for "sulfur granules": yellowish particles, varying in firmness, up to several millimeters in diameter. Granules are washed, crushed, and observed under the microscope. Rosettes are suggestive of actinomycosis, especially if bacterial-sized filaments extend into the clubbed fringe. Although these are not unique to *Actinomyces* species, their presence should suggest the possibility of the organism.

Crushed granules, exudate, or tissue impressions can be stained with Gram and acid-fast stains. Branching, gram-positive, beaded, non-acid-fast filaments suggest *Actinomyces* species. Most *Actinomyces* strains obtained from animals do not require anaerobic incubation but benefit from increased carbon dioxide. Granules offer the best chance of isolation from the usually mixed flora. Colonies can develop within 48–72 hours after incubation at 35–37 °C. Organisms of suggestive morphology and staining characteristics can be evaluated biochemically or by MALDI-TOF to aid in identification. Different species can react differentially on most common biochemical tests. Colonies are typically non-hemolytic and white, and can appear either smooth or rough; colonies tend to adhere to the surface of the media. Meaningful species identification is improved with molecular techniques. DNA primers based upon the sequence of genes encoding the 16S ribosomal RNA have been designed for speciation of *Actinomyces* members.

Treatment and Control

In bovine actinomycosis, iodine compounds have been used for treatment, which, must be stopped when signs of toxicity appear. Accessible soft tissue lesions can be drained or excised. Antimicrobials including penicillin and aminoglycoside combinations are often administered concurrently. Treatment of lumpy jaw will not restore normal bone structure, but the process can be arrested by systemic medication aided by drainage, lavage (iodine), and debridement of lesions.

In other species, surgery (drainage, lavage, excision, removal of foreign bodies) is controversial but may be used and supplemented by long-term antimicrobial therapy. A penicillin derivative (e.g. ampicillin) is the drug of choice. Alternatives are erythromycin, clindamycin, doxycycline, or amoxicillin-clavulanic acid. Relapses are common, especially with short-term therapy or thoracic involvement. It is important to note that many *Actinomyces* infections are polymicrobial and may require modified treatment strategies.

Nocardia Species

Nocardia species are aerobic, saprophytic, gram-positive, filamentous bacteria that are ubiquitous in the environment including the soil and water. The list of recognized species and clinical associations is expanding and currently consists of more than 80 members, with approximately half of veterinary or human medical importance. Infections attributed to *Nocardia* species have been reported in mammals, fish, mollusks, and birds. Because of the opportunistic nature of these organisms, they are frequently found in immunocompromised individuals. *Nocardia* infections often begin as suppurative or pyogranulomatous inflammatory processes that can become chronic; however, species can differ in their ability to cause disease. *Nocardia asteroides*, historically the species

most often associated with diseases, is now designated as the *N. asteroides* complex with several member species and types. For this reason, in the discussion that follows, a species designation will not be given to the nocardial agents associated with the various diseases ascribed to members of this genus, unless isolates were identified by techniques (mostly DNA-based analyses) currently accepted for identification of this group of bacteria.

Descriptive Features

Morphology and Staining

Nocardia species are nonmotile, gram-positive bacteria that when gram-stained are difficult to distinguish from *Actinomyces* species. They often appear as branching, beaded filaments that can fragment into pleomorphic rods or cocci. They are partially acid fast due to the presence of mycolic acids in their cell wall and their relatedness to mycobacteria, which can distinguish them from morphologically similar genera (Figure 35.2).

Structure and Composition

The cell wall is typical of gram-positive bacteria, with the addition of meso-diaminopimelic acid (DAP), arabinogalactan, and mycolic acids. The cell wall contains a high concentration of lipids.

Growth Characteristics

Pathogenic *Nocardia* species are obligate aerobes, capable of growing on simple (e.g. Sabouraud's) or enriched (blood agar) media over a wide temperature range (10–50 °C). Small, indiscriminate colonies can appear after 48 hours. Older colonies can vary phenotypically, appearing opaque or variously pigmented. The colony surface has been described as waxy, powdery, and velvety depending on the abundance of aerial growth. The formation of aerial hyphae helps differentiate *Nocardia* species from related genera. Colonies may develop a wrinkled appearance with age. In broth, a surface pellicle or sediment is produced, but little turbidity.

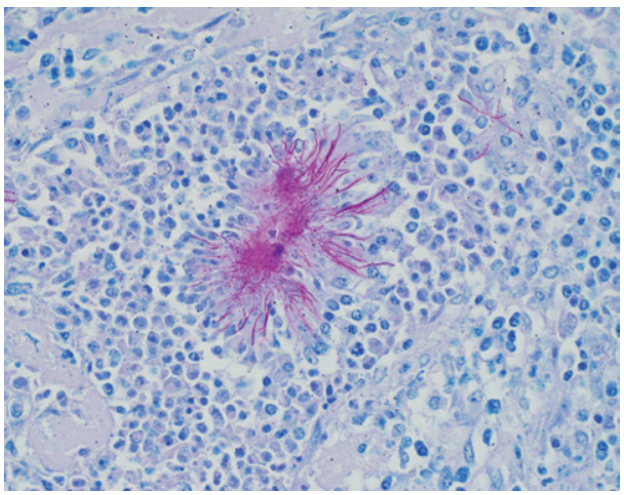


Figure 35.2 A modified acid-fast (Fite's) stain (600× magnification) of *Nocardia* species in an alpaca kidney. *Source:* Courtesy of Dr. Brandon Plattner, Veterinary Diagnostic Laboratory, Kansas State University.

Resistance

Nocardiae thrive in the environment, yet are susceptible to disinfectants. *Nocardia* species exhibit varying resistance patterns to antimicrobials, which have traditionally been used in the differentiation of species. *Nocardia farcinica* has been reported to be particularly resistant to antimicrobials. Resistance to trimethoprim-sulfamethoxazole has been reported in a large proportion of human *Nocardia* isolates, where it is considered the drug of choice.

Ecology

Reservoir

Nocardia species are common inhabitants of the environment including soil, water, and decaying vegetation, and it can also be shed in the feces of animals. The organisms have a worldwide distribution; however, species can cluster geographically. Because of the nature of these organisms, they can be environmental contaminants or rarely asymptomatically colonize humans or animals; when isolated from a clinical specimen – especially those with granulomas or draining tracts – they are generally considered pathogens.

Transmission

The three main routes of infection are inhalation, direct contact through trauma, and ingestion. Dust, soil, and plant material can serve as vehicles. *Nocardia* associated with bovine mastitis can be introduced and disseminated by equipment and personnel. The route of transmission is associated with the clinical presentation of disease.

Pathogenesis

Pathogenic *Nocardia* strains are facultative intracellular organisms that survive within phagocytic vacuoles, initially by preventing phagolysosome formation. The cell wall content and mycolic acids exhibited during the growth phase of the bacteria facilitate intracellular survival.

Superoxide dismutase and lysosomal enzyme inhibition protect against phagocytic killing. Other cell wall lipids may trigger granulomatous reactions. Variations between strains and growth phases in cell envelope constituents are paralleled by changes in virulence and infectivity.

Nocardiosis is a predominantly suppurative process with variable granulomatous features. Lymph nodes are consistently involved, particularly in wound cases. Lesions, particularly in the lung, can erode into blood vessels and result in dissemination and widespread abscess formation. Central nervous involvement is rare in animals, though fairly common in human patients. In dogs, the common form is thoracic empyema with granulomatous serositis. Exudates are sanguinopurulent and sometimes contain small (<1 mm diameter), soft granules consisting of bacteria, neutrophils, and debris ("sulfur granule-like"). They usually lack the microstructure of sulfur granules sometimes seen with *Actinomyces* infections (see Section 35.1).

Disease Patterns

Nocardiosis is frequently overlooked because of its ability to coexist with or masquerade as other familiar diseases. Infection due to a *Nocardia* species may be difficult to distinguish from mycobacterial and *Actinomyces* infections without laboratory diagnosis. Infections can be regional or disseminated. Human and animal cases of nocardiosis are almost always associated with the immune status of the individual.

Ruminants

Bovine mastitis is the most common manifestation of *Nocardia* infection in cattle. This infection is generally associated with the hygiene of the herd. Onset is sudden with fever, anorexia, and abnormal milk secretion. The affected gland is swollen, hot, and painful. Discharging fistulous tracts may develop. Lymphadenopathy is common, and there is occasional dissemination. The affected gland usually becomes nonfunctional. Rarely, pneumonia, abortion, and lymphadenitis associated with *Nocardia* species have been reported in ruminants.

Horses

In horses, local or disseminated infections with *Nocardia* have been reported, including *Nocardia*-induced mycetomas, pulmonary infection, and abortion. Nocardioform placentitis is a relatively uncommon condition of mares that can result in late-term abortion, still birth, or premature birth. Although morphologically similar, gram-positive, branching, and filamentous, the causative agent, *Crossiella equi*, is unrelated to *Nocardia*.

Dogs and Cats

Dogs and cats often develop debilitating, febrile illness with depression and anorexia. Pneumonia and suppurative pleuritis with empyema is the common finding in dogs. Dissemination occurs to liver, kidneys, bones, joints, and, rarely, the central nervous system. Coinfection with canine distemper virus is common. Cutaneous subcutaneous abscess formation is the more common clinical manifestation in cats, although pulmonary or systemic disease can also occur. There is a high mortality rate in these animals, although that may be related to predisposing factors such as being immunosuppressed or coinfection status.

Swine

Pneumonia, abortion, and lymphadenitis associated with *Nocardia* have rarely been reported.

Other Species

Nocardiosis observed in birds (rare), whales, and dolphins are mostly respiratory in nature with signs of dissemination. In fish, granulomas within the muscle or associated with internal organs have been reported.

Epidemiology

The distribution of *Nocardia* species in the environment is worldwide; however, some species cluster geographically. In animals and humans, disease is associated largely with immunodeficiency. Bovine nocardial mastitis is most often traceable to unsatisfactory hygienic practices. Typically, infection is introduced during the "dry period" with intramammary mastitis therapy.

Immunologic Aspects

Antibody and cell-mediated immune responses, including hypersensitivity, commonly develop during nocardial infections. Yet severe nocardiosis is commonly associated with immunosuppression, particularly of cell-mediated responses. Antibody apparently confers little protection. Specific resistance is largely cell mediated. No practical immunization method is presently available.

Laboratory Diagnosis

Branching, gram-positive, filaments and or shorter, coccobacillary forms are found in smears made from *Nocardia*-infected samples, impressions, or sections. The presence of

these organisms is not definitive for *Nocardia* diagnosis because of the morphological similarities with other genera. For confirmation, samples should be submitted for culture or molecular identification.

Nocardial colonies on blood agar, incubated at 37 °C, will initially appear small and indistinct; older cultures will vary phenotypically, ranging from dull opaque to orange or yellowish. The colony surface may appear velvety or powdery depending on the abundance of aerial growth. Colonies may be hard to dislodge by loop or needle, adhering to the surface of the medium. Stains confirm nocardial morphology that may be pleomorphic rods, coccobacilli, or branching filaments. Acid fastness may be lost on culture. Biochemical activities include catalase production, nitrate reduction, and acidification of various carbohydrates. Biochemical properties and susceptibility to various antimicrobial agents have traditionally been used in identification of specific *Nocardia* species; however, they are now considered insufficient to differentiate members of the *N. asteroides* complex.

Serological tests are not widely available for *Nocardia* detection, and their reliability is uncertain. Molecular methods are gaining popularity for identification of *Nocardia* species. Determination of restriction enzyme patterns from a heat shock protein gene, *hsp*65, had been used to differentiate species; however, the discriminative ability of this test has been challenged. Sequencing of the DNA encoding the 16S rRNA or *hsp*65 gene has been proposed as useful identification tools for *Nocardia* species. The usefulness of these techniques in clinical settings is not established.

Treatment and Control

Antimicrobial therapy of nocardial mastitis may produce temporary clinical relief and cessation of shedding, but no permanent cures. Control involves removal of infected animals, thorough disinfection of premises and equipment, and scrupulous stabling and milking hygiene.

In other forms of nocardiosis, trimethoprim–sulfonamide combinations have been considered the therapy of choice. Amikacin, linezolid, and some beta-lactam compounds (imipenem, cefotaxime) have also been used effectively in humans and animals. Fluoroquinolones are reported to have limited therapeutic value. Therapy of nocardial infections is typically prolonged (months), with relapses common in short-term courses. Abscesses or empyema are often treated surgically and/or drained, in combination with administration of antimicrobials. Antimicrobial resistance has been observed during the course of treatment, and there are species-associated susceptibility profiles.

Dermatophilus

Dermatophilus species are generally considered true pathogens associated with exudative dermatitis or skin lesions in animals and occasionally in humans. The most predominant and type species of the genus is *Dermatophilus congolensis*, which is responsible for dermatophilosis or streptothricosis (cattle and other species), rain scald or rain rot (horses), grease heal (horses), lumpy wool (sheep), and strawberry foot rot (sheep). *Dermatophilus chelonae* is also rarely isolated from skin lesions of more exotic animals, although the taxonomy of this organism has recently been challenged. *Dermatophilus* species are gram-positive, branching, filamentous rods that exhibit a unique bacterial life cycle. In temperate climates, dermatophilosis is often a cosmetic problem controllable by management practices. In sheep and cattle of tropical areas, it can severely affect productivity and may be a disposing factor for other problems including myiasis. *Dermatophilus* organisms are rarely found at sites other than the skin.

Descriptive Features

Morphology and Composition

D. congolensis are gram-positive bacteria that exhibit a unique life cycle that includes the germination of a motile coccoid "zoospore," forming a germ tube. Next, transverse and longitudinal septa formation occurs, forming a strand several layers thick. Binary fission of constituent cells become coccoid in shape and appears in parallel rows. Mature, motile (flagellated) zoospores are released, completing the life cycle. The cell wall of *Dermatophilus* contains meso-DAP, but lacks galactose and arabinose.

Growth Characteristics

D. congolensis grows well on rich blood agar media, but not on Sabouraud dextrose agar. It is a facultative anaerobe and capnophilic. Colonies, which develop in 48 hours, vary from mucoid to viscous and waxy, whitish-gray to yellow, and smooth to wrinkled (Figure 35.3). There is large phenotypic variation, including hemolytic and enzymatic activity between *D. congolensis* isolates. Generally, *D. congolensis* is highly hemolytic on blood agar plates. In addition, antigenic variation has been reported in field isolates. Genotyping work suggests that the variation between isolates is correlated to host species and not to geographical location.

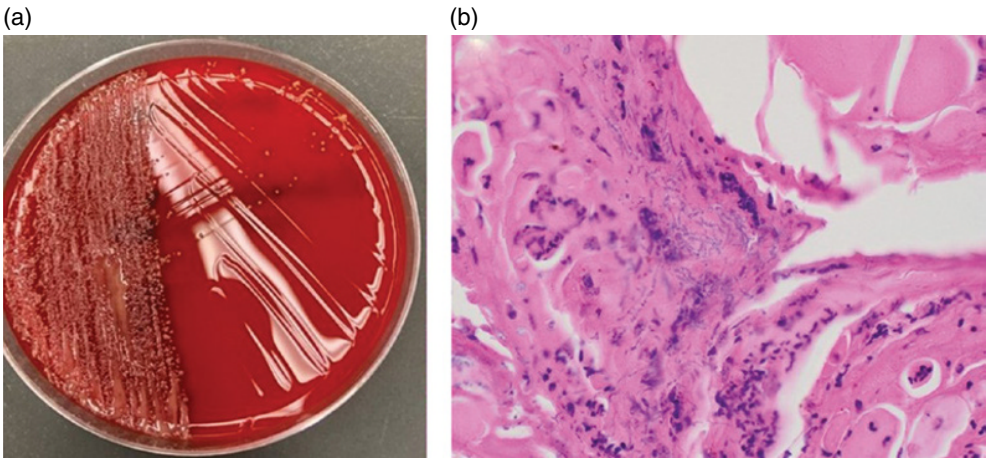


Figure 35.3 (a) *Dermatophilus congolensis* rough colonies grown in culture on a blood agar plate and (b) characteristic *D. congolensis* gram-positive organisms observed in a clinical specimen. *Source:* Courtesy of Dr. Brandon Plattner, Veterinary Diagnostic Laboratory, Kansas State University.

Ecology

Reservoir

D. congolensis does not multiply saprophytically; it is an obligate parasite. Cattle, sheep, and horses are common hosts; however, it has been diagnosed in goats, swine, dogs and cats, turkeys, primates (including humans), and wild mammals, including marine mammals. The distribution of *D. congolensis* is worldwide, but its greatest economic significance is in tropical regions in Africa. Younger animals appear to be more susceptible than older animals, possibly related to an immature immune system.

Transmission

Spread of *D. congolensis* is by direct physical contact of animals or indirect contact via arthropods. Injury by thorny range plants and shearing cuts may create portals of infection or inoculate the agent. Transmission is exacerbated in moist conditions.

Pathogenesis

D. congolensis results in an exudative epidermitis (Figure 35.4). Its primary activity is confined to the living epidermis. Infection results after soaking or trauma occurs in this area. Deposited zoospores respond to a CO_2 gradient and penetrate deeper cell layers. Upon germination, germ tubes and filaments arborize within the epidermis and colonize hair follicles. A layer of inflammatory cells (largely neutrophils) forms under the infected epidermis, which keratinizes. Beneath the neutrophils new epidermis forms, which can be invaded. The eventual result is a scab consisting of layers of neutrophilic exudate and infected keratinizing epidermis. The scabs are easily lifted by the hair, which protrudes from both surfaces. Virulence factors of *D. congolensis* including phospholipase and proteolytic enzymes increase the permeability of the epidermis and interact with the inflammatory response.



Figure 35.4 A *Dermatophilus congolensis* infection from the leg of an unknown species. *Source:* Courtesy of Noah's Archive, https://davisthompsonfoundation.org/noahs-arkive.Original contribution of Dr. John King, Cornell University.

The primary lesions from *D. congolensis* are painless and nonpruritic. Wetting favors their expansion. Biting arthropods can infect areas protected from soaking rain. Soaking provides the preparatory step for lesions on the back (equine "rain scald"), feet, and legs (ovine "strawberry footrot," equine "grease heel"). The extent varies from a few scabby areas of roughened hair or "lumpy wool" to widespread loss of epidermis, causing secondary parasitisms or infections and eventual loss of the animal. More severe disease is generally associated with infestation with the *Amblyomma variegatum* tick that promotes immunosuppression. The genetic makeup of the animal may also contribute to the severity of disease. Dermatophilosis in nonepidermal tissue including the tongue and lymph nodes has only been rarely reported in cats.

Epidemiology

Infection with *D. congolensis* is strongly correlated to environmental conditions, and as such, prevalence can vary significantly. The prevalence of dermatophilosis depends on (i) infected animals; (ii) dissemination, for example, by arthropods or thorny plants; and (iii) access to the susceptible hosts' epidermis, enabled by trauma or wetting.

Immunologic Aspects

Antibody is widespread among cattle in endemic areas. Its protective role is not well understood.

Laboratory Diagnosis

Gram or Giemsa stains of crushed scab or scab flakes usually show typical phases of the *D. congolensis* life cycle and provide a strong presumptive diagnosis. In subacute and chronic cases, bacterial elements may be rare and lack, for example, multicellular branching filaments. Zoospores resemble large cocci. They and other *Dermatophilus* fragments in skin debris may be identified by fluorescent antibody. Impression smears from the base of recently removed scabs may also be useful in identification.

D. congolensis is cultured from the undersurface of fresh, uncontaminated scabs on blood agar under 5–10% carbon dioxide at 35–37 °C. Small, grayish yellow hemolytic colonies typically appear within 24–48 hours. Colonies typically adhere to the media and after an extended incubation (three to four days) appear larger (3 mm), rough, and wrinkled (Figure 35.3). When colonies are gram-stained, branching filaments or coccoid forms are seen. A wet mount may reveal motile zoospores. The organisms are not acid fast; they are catalase and urease positive, and indole and nitrate negative.

Treatment and Control

Acute cases of *D. congolensis* infection are often self-limited. Mild cases respond to grooming and removal of the animal from the moist environment. Severe cases can be treated with antimicrobials including penicillin and streptomycin, tetracycline, and chloramphenicol. Control should be aimed at minimizing skin trauma and exposure to rain and arthropods. Multiple live and killed vaccines have been investigated for *D. congolensis* control in sheep and cattle; however, due to their ineffectiveness, none has been widely implemented.

Streptobacillus

Streptobacillus moniliformis is the best-known pathogenic species in a genus renamed numerous times, and now taxonomically placed in the family *Fusobacteriaceae*. The organism is the more common of two zoonotic agents (the other is *Spirillum minus*) associated with rat-bite fever. Rat-bite fever has also been described in a case of *Streptobacillus notomytis* infection in an elderly adult. At least five *Streptobacillus* species have now been described, including *Staphylococcus felis* isolated from a cat lung.

Descriptive Features

Streptobacillus are nonmotile, non-spore-forming, gram-negative, pleomorphic rods. There is no capsule. The organisms often grow in culture as long, beaded filaments bearing knobby irregularities. Cellular proteins vary with geographic and pathological sources. Cell-wall-deficient L-forms, which can be more difficult to grow, can form readily. Very little is known about specific virulence factors or cellular products; however, antigens expressed by the two forms may not be identical.

Ecology

The primary reservoir for *S. moniliformis,* the best-described *Streptobacillus* species, is the pharynx of rodents, particularly rats. Wild mice are not considered a primary

reservoir, and *S. moniliformis* has been associated with severe clinical disease in these animals. Other carnivores that feed on or have rats in their mouth may also be a source of the organism. Infection is generally attributed to being bitten by an infected animal reservoir; however, oral ingestion of contaminated material can also result in infection. Oral-related infection in humans, usually from rat excrement contamination of water, milk, or food, is referred to as Haverhill fever.

Bite lesions are inflammatory and often purulent or necrotic. Reports of clinical disease in rodents range from swelling of the joints and lymph nodes to bronchopneumonia, abscess formation in the liver, and septicemia. In turkeys and mice, septicemic infections lead to polyarthritis or synovitis and often death. Clinical infection of *S. moniliformis* in dogs has resulted in vomiting, diarrhea, and arthritis in the hind legs. Other animals associated with the rat reservoir may become infected and develop a range of clinical signs.

In humans, an abrupt fever, accompanied by a headache, vomiting, and a rash on the extremities precede localization in joints in 50–70% of *S. moniliformis* patients. Other complications including endocarditis, pneumonia, abscess formation on organs, and meningitis may occur. Mortality rates may approximate 10% in untreated cases. Treatment may induce the L-form of *S. moniliformis*, which may persist in the body and cause relapses, including recurrent fever, after therapy is completed.

Immunologic Aspects

Although antibodies against *S. moniliformis* are produced during infection, the role of immunity is not understood.

Laboratory Diagnosis

Streptobacillus species are fastidious and slow growing, yet can be cultured in the laboratory using blood- or serum-enriched media. Proper diagnostic submissions include blood or joint fluid, abscess aspirates, synovial fluid, and wound cultures. Some anticoagulants from blood culture systems may impede growth. The organisms are facultative anaerobes that often appear after 48 hours as small, translucent, or gray and smooth colonies. In enriched liquid media, *S. moniliformis* forms a characteristic "puff-ball" appearance. Subsequent identification is by morphological and biochemical characteristics. MALDI-TOF has not been highly successful in identifying *Streptobacillus* species, likely due to database limitations. Although *S. moniliformis* is relatively unreactive – it is catalase, oxidase, indole, urease, and nitrate negative – it can produce acid from select carbohydrates including glucose. In culture, the production of L-form colonies may be favored by special media, forming mycoplasma-like colonies that are recognizable among the conventional colonies.

Direct fluorescent antibody tests and molecular methods including PCR and sequencing have been used more recently for diagnostic identification of this organism, and in rodents, ELISA and immunoblot tests have been used.

Treatment and Control

Penicillin is the antibiotic of choice; however, tetracycline is also effective in treating *S. moniliformis* infections in penicillin-allergic individuals. Although antimicrobial resistance is not thought to be a large problem, resistance to cephalosporins and aminoglycosides have been reported. The most obvious preventive measure is avoidance of direct and indirect contact with likely carriers.

Acknowledgments

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36

Mycobacteria

Raul G. Barletta and David J. Steffen

Mycobacteria (a general name used for the members of the *Mycobacteriaceae* family) are aerobic, acid-fast rods with high guanine-cytosine (GC) content (57–71%) in their genomes. There are 188 species with valid published names. Most of their members are saprophytic organisms that live in the environment. However, the family *Mycobacteriaceae* also includes some of the most dreaded pathogens such as the agents of bovine and human tuberculosis, leprosy, and various types of granulomatous diseases in normal or compromised mammals, birds, reptiles, and fish.

Based on growth rate, mycobacteria can be classified in fast (colonies develop in agar plates in less than seven days (e.g. *Mycolicibacterium smegmatis* and *Mycolicibacterium fortuitum*) and slow growers (colonies take more than seven days to develop on agar, e.g. *Mycobacterium tuberculosis* and *Mycobacterium avium*). Most of the pathogenic species are slow growers with generation times of 12 hours and upward. It is noted that even the fast growers have a significantly longer generation time as compared to typical *Escherichia coli* strains that may develop colonies in agar plates upon overnight incubation. Another property useful in the classification of mycobacteria is the presence of carotenoid pigments in some species ("chromogens" vs. "nonchromogens") and their light dependence ("photochromogens" vs. "scotochromogens").

Amended Taxonomical Classification

In the intervening years since the previous edition of the book, the relationships among the mycobacterial species have been thoroughly determined by several analyses including 16SrRNA, 16S-23S spacer regions, identification of molecular markers, and multilocus and whole genome sequencing. These studies led to the division of the former genus *Mycobacterium* into the new restricted genus *Mycobacterium* and four new genera (gen. nov): *Mycolicibacter* gen. nov., *Mycolicibacillus* gen. nov., *Mycolicibacterium* gen. nov., and *Mycobacteroides* gen. nov. A fifth additional group (*Hoyosella*) of organisms originally classified in the suborder *Corynebacterineae* was also added to the *Mycobacteriaceae* family. The main features of the corresponding genera are described in Table 36.1. Although there is no official nomenclature for prokaryotes, the classification outlined herein comprises the entries associated with the NCBI taxonomy browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) and is consistent with species names published in the *International Journal of Systematic and Evolutionary Microbiology* and the *Rules of International Code of Nomenclature of Bacteria*. In this context, most of the fast-growing mycobacteria are now classified in the genus *Mycolicibacterium* gen. nov. Thus, it is likely that in the coming years, this new nomenclature will be firmly established in the field, and medical and veterinary professional and researchers would need to refer to this nomenclature to obtain reference microorganisms from culture collections. In the transition period, many scholars and scientists may continue using the older names (basonyms). Thus, we include a description of main basonyms for more common species (Table 36.2).

Morphology and Staining

Mycobacteria are nonflagellated, predominantly rod shaped, about 0.5 μm wide, and variable in length. Cytochemically, these microorganisms are gram positive, but often resist staining with the Gram stain. However, mycobacteria can be stained with carbolfuchsin (Ziehl–Neelsen stain; Figure 36.1) or fluorescent dyes (e.g. auramine-rhodamine). Their most noted staining property

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Table 36.1 Amended classification of the family *Mycobacteriaceae*.

Genus	Representative species	Properties
Hoyosella	*Hoyosella altamirensis*	Usually isolated from biofilms in caves, fast-growing, tolerant to 6% sodium chloride, optimum growth at 28 °C. Genomes sizes are about 4.8 Mbp with GC content of 61.8%.
Mycobacterium	*Mycobacterium avium* (subspecies: *avium*, *hominissuis*, *paratuberculosis*, *silvaticum*) *Mycobacterium intracellulare* *Mycobacterium leprae* *Mycobacterium marinum* *Mycobacterium canettii* *Mycobacterium mungi* *Mycobacterium tuberculosis* (type species) *M. tuberculosis* (variants: *africanum*, *bovis*, *caprae*, *microti*, *pinnipedii)* *Mycobacterium ulcerans* *Mycobacterium xenopi*	Slow-growing, mostly pathogenic or opportunistic mycobacteria. Includes major pathogens associated with human and veterinary diseases. Genomes of 3.2–7.3 Mbp with GC contents of 57.8–69.3%. Presence of the Type VII secretion system.
Mycobacteroides gen. nov.	*Mycobacteroides abscessus* (type species) *Mycobacteroides chelonae* *Mycobacteroides salmoniphilum*	Better growth at 30 °C. Genomes of 4.5–5.6 Mbp with 63.9–64.8% GC content. Slow-growing opportunistic pathogens causing lung, skin, and soft tissue infections. Resistant to multiple antibiotics.
Mycolicibacillus gen. nov.	*Mycolicibacillus trivialis* (type species)	Slow-growing bacteria. Opportunistic pathogen isolated from human and environmental sources. Small genomes of 3.89–4.08 Mbp with 69.4 GC content.
Mycolicibacter gen. nov.	*Mycolicibacter terrae* (type species)	Intermediate and slow-growing environmental mycobacteria. Most members are non-pathogenic, but some have been isolated from human and animal hosts. Genomes are 3.87–5.11 Mbp with 66.3–70.3 GC content.
Mycolicibacterium	*Mycolicibacterium aurum* *Mycolicibacterium fortuitum* (type species) *Mycolicibacterium phlei* *Mycolicibacterium smegmatis* *Mycolicibacterium vaccae* *Mycolicibacterium vanbaalenii*	Main group of fast-growing environmental mycobacteria. Most species are saprophytic. Some are opportunistic pathogens. Extensively used as model systems for pathogenic mycobacteria. Genomes range from 3.95 to 8.0 Mbp with 65.4–70.3 GC content.

is their acid fastness (i.e. once stained, they resist discoloration with 3% hydrochloric acid in ethanol). Growth in agar media leads to colonies of variable morphology, but typically they are dry and crumbly. Colonies of *M. avium* subsp. *hominissuis* are usually dome shaped with most virulent strains assuming a smooth, transparent appearance, gradually changing to rough and opaque as virulence may be lost upon repeated culturing in vitro.

The presence of an outer layer (capsule) of loosely bound carbohydrates and proteins has been demonstrated by cryoelectron microscopy. This material is shed during growth in liquid medium but is readily visible *in vivo*. Some experiments indicate that mycobacteria may form spores. These findings, highly dependent on microscopic examination of aged cultures, remain highly controversial.

Structure and Composition

Mycobacteria are characterized by lipid-rich cell walls. Lipids account for acid fastness and some pathogenic and immunologic properties. The surface mycosides (mostly glycolipids and peptidoglycolipids) determine colonial characteristics, serological specificities, and bacteriophage susceptibilities. Subsurface layers of long-chain branched mycolic acids and their esters make up the bulk of cell wall lipids. Mycolic acids are linked to the innermost peptidoglycan layer by way of arabinogalactans. Determination of mycolic acid chain length has diagnostic relevance for genus and species identification as mycobacteria have longer chains of 60–90 carbon atoms. Some mycolic acids are loosely bound to the disaccharide trehalose in the form of

Table 36.2 Important Widely Used Basonyms.

New Name	Basonym
Mycobacterium avium subsp. *avium*	*Mycobacterium avium*
Mycobacterium avium subsp. *hominissuis*	*Mycobacterium avium*
Mycobacterium avium subsp. *paratuberculosis*	*Mycobacterium paratuberculosis*
Mycobacterium avium subsp. *silvaticum*	*Mycobacterium silvaticum*
Mycobacterium tuberculosis variant *africanum*	*Mycobacterium africanum*
Mycobacterium tuberculosis variant *bovis*	*Mycobacterium bovis*
Mycobacterium tuberculosis variant *caprae*	*Mycobacterium caprae*
Mycobacterium tuberculosis variant *microti*	*Mycobacterium microti*
Mycobacterium tuberculosis variant *pinnipedii*	*Mycobacterium pinnipedii*
Mycobacteroides abscessus	*Mycobacterium abscessus*
Mycobacteroides chelonae	*Mycobacterium chelonae*
Mycobacteroides salmoniphilum	*Mycobacterium salmoniphilum*
Mycolicibacillus trivialis	*Mycobacterium trivialis*
Mycolicibacter terrae	*Mycobactterium terrae*
Mycolicibacterium aurum	*Mycobacterium aurum*
Mycolicibacterium fortuitum	*Mycobacterium fortuitum*
Mycolicibacterium phlei	*Mycobacterium phlei*
Mycolicibacterium smegmatis	*Mycobacterium smegmatis*
Mycolicibacterium vaccae	*Mycobacterium vaccae*
Mycolicibacterium vanbaalenii	*Mycobacterium vanbaalenii*

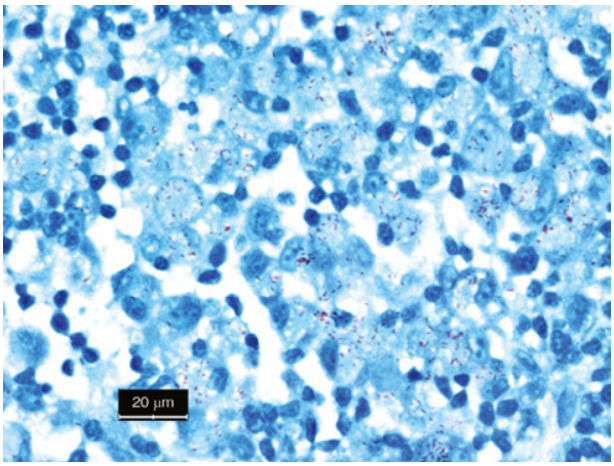


Figure 36.1 Large epithelioid macrophages are present among the crypts in the jejunum of a bovine with Johne's disease. Nuclei are eccentric and cytoplasm slightly vacuolated and sometimes granular (hematoxylin and eosin staining, 40×). (Specimen from the University of Nebraska Veterinary Diagnostic Center). *Source:* Reproduced from McVey et al. (2013).

trehalose monomycolates (TMMs) and dimycolates (TDMs). One of the capsular antigens (antigen 85) carries the enzyme activity responsible for the formation of TMM and TDM. TDM has recently been identified as "cord factor," the mycobacterial component that determines the characteristic serpentine-like appearance observed upon culture in certain liquid media. TDM is associated with multiple activities in the mechanisms of pathogenesis, both protecting mycobacteria from phagocytic killing and contributing to disease pathology of host cells within the granuloma.

The mycobacterial peptidoglycan has also an unusual structure, as the muramyl dipeptide subunit component has a 50% mixture of *N*-acetylated and *N*-glycosylated moieties that departs from the typical 100% *N*-acetylated moiety of most eubacteria. This composition seems directly linked to the ability of this component to potentiate humoral and cellular immune responses (adjuvanticity) and is the basis for its inclusion as the active component of Freund's complete adjuvant.

Mycobacteria possess typical cell membranes composed of lipid bilayers, but they have the salient characteristic that one major cell wall glycolipid (lipoarabinomannan, LAM) is covalently attached to this membrane. LAM is also involved in mycobacterial pathogenesis (phagocytosis and inhibition of phagolysosome fusions; see Section 36.6), especially in *M. tuberculosis* as LAM is capped with mannose residues (ManLAM). Other mycobacterial lipids (glycolipids, phospholipids, sulfolipids, and waxes) may also play a role in these processes.

The mycobacterial cell wall architecture is completed by the presence of porins and other proteins such as transporters involved in nutrient acquisition. Some of the proteins are antigenic and can be readily isolated in large amounts from broth culture supernatant fluids. In addition to the already mentioned antigen 85, mycobacteria "secrete" superoxide dismutase, L-alanine dehydrogenase, and glutamate synthetase.

Overview of Pathogenic Species, Epidemiology, and Microbial Evolution

Host-Adapted Strains

The group of closely related mycobacteria causing tuberculosis in humans and animals is denominated the *M. tuberculosis* complex. These host-adapted species still cause significant morbidity and mortality worldwide. In medical terms, a complex is a group of microorganisms producing similar disease, but the term does not have taxonomic implications. Moreover, the new suggested classification refers to these microorganisms as variants (Table 36.1). The microorganisms *M. tuberculosis* (var.) *bovis* and *M. tuberculosis* were described as agents of tuberculosis in animals

and humans by Robert Koch in 1882. These studies provided the basis for developing Koch's postulates. Rodents, especially mice and guinea pigs, are especially susceptible to experimental infection with the *bovis* variant and *M. tuberculosis*. *M. tuberculosis* is a human-adapted pathogen and rarely infects other animals and has limited environmental survival. Transmission occurs from one individual to another by infected aerosols. The *bovis* variant is a zoonotic microorganism that can be transmitted by aerosols bidirectionally from (to) animals to (from) humans. Unpasteurized milk from infected animals (cattle and other ruminants) serves as the main source of infection to humans. The work of Koch involved samples from both humans and animals. Thus, Koch worked with both *M. tuberculosis* and the *bovis* variant, at a time when the subtle distinctions between these microorganisms were not defined. The variants *pinnipedii* (seals) and *caprae* (goats) represent two additional host-adapted mycobacterial strains. Some citations still refer the *caprae* variant as a subspecies of the original *Mycobacterium bovis*. The antituberculosis vaccine BCG (bacillus Calmette–Guerin) is an attenuated strain of the *bovis* variant.

Another related human-restricted pathogen is the African version of the tubercle bacillus designated *M. tuberculosis* variant *africanum*. Another microorganism that has a taxonomical status in the new classification is *Mycobacterium canettii*, which has also been described in North Africa. The zoonotic variant *M. tuberculosis* variant *mungi* was identified as pathogens in banded mongooses. The host range of *M. canettii* remains unknown. *M. tuberculosis* variant *microti* is another member of the complex identified as the agent of tuberculosis in voles and more recently isolated from some human cases. Molecular and genomic studies established that mycobacterial genomes were generated by reductive evolution involving both single nucleotide polymorphisms and serial deletions of genetic materials. The deletions gave rise to regions of difference in the corresponding genomes and defined major speciation events. This analysis leads to the conclusion that an ancestral strain of *M. canettii* is the precursor of *M. tuberculosis* and their variants. Thus, the old paradigm that the human pathogen *M. tuberculosis* evolved from the bovis variant (e.g. human tuberculosis has its origin in bovine tuberculosis) is no longer tenable. Interestingly, the *bovis*, the species with the smaller genome, has a greater host range than *M. tuberculosis*. This has been attributed to deletions of "avirulence" islands (e.g. encoding regulatory elements that modulate the expression of virulence determinants) from the genome, allowing the variant *bovis* to have a greater host range (Figure 36.2).

Mycobacterium leprae is a peculiar human pathogen that is considered an obligate intracellular pathogen as it has never been grown *in vitro*. The nine-banded armadillo is one of the few experimental hosts used to grow the microorganism under laboratory conditions. *M. leprae* has a favorite niche in bodily areas of lower temperature, such as the extremities (feet and hands) followed by spreading to the face. Transmission of leprosy occurs by contact and is mediated by aerosols. Very few cases of zoonotic transmission are reported. *M. leprae* attacks the peripheral nervous system. These features result in mentally normal patients with characteristic disfigurements due to peripheral lesions. This

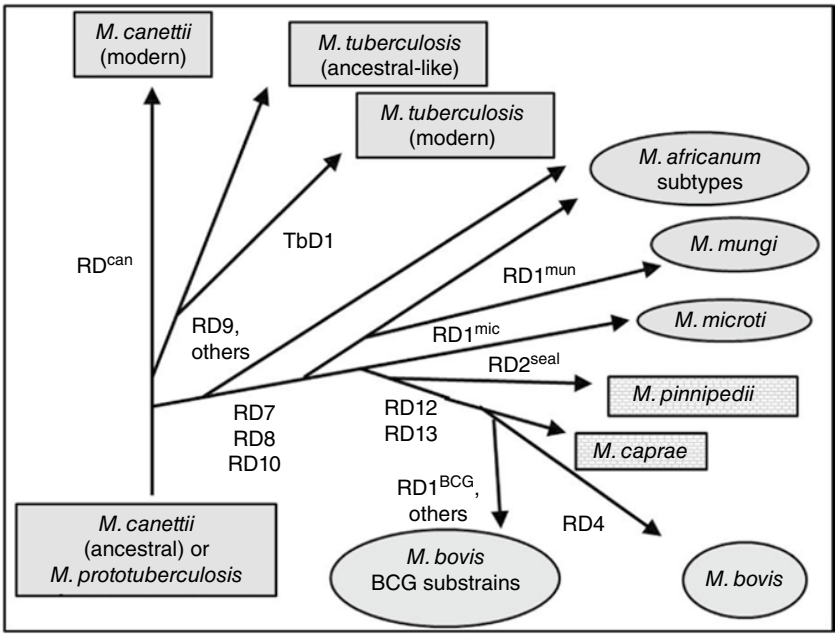


Figure 36.2 Proposed evolution of mycobacterial pathogens from a common ancestral *M. canettii* strain by single nucleotide polymorphisms and sequential genomic deletions. Names of microorganisms enclosed in rectangular grey boxes are from exclusive human pathogens, names (basonyms) enclosed in rectangular patterned boxes are host-adapted nonhuman animal pathogens, and zoonotic agents, including BCG per extension, are enclosed by oval boxes. RD, region of difference (deletion). *Source:* Reproduced from Thoen and Barletta (2014).

disease was well documented in biblical times. This disease was popularized in theaters in the epic 1959 movie "Ben-Hur." From the ancient times until the early years of the twentieth century, leprosy patients were isolated. The disease is now readily treatable with dapsone and other medications.

Environmentally Adapted Strains

Microorganisms of the *M. avium* complex (MAC) produce common infections in birds and are found in surface water of ponds inhabited by birds. The ability of the bacteria to replicate and maintain itself in surface water is not clear. Similarly, *M. avium* ssp. *hominissuis* is found in household dust and showerheads, but the ability to replicate and survive is not clear in those environments. These non-host-adapted species occur in animals as opportunistic pathogens. MAC infections were once a common infection of AIDS patients. Advances in AIDS therapy have resulted in patients maintaining a higher, yet less than normal, level of T cell CD4 counts, and thus in better control of MAC infections. *M. tuberculosis*, the most virulent mycobacterial species, has now overtaken MAC as the main cause of complicating coinfections in AIDS patients. The MAC complex includes related but distinct species: *Mycobacterium intracellulare* and *M. avium* (these two usually grouped as the MAI complex, a term without taxonomical relevance), and the fast growers *Mycobacteroides chelonae* and *M. fortuitum*. As a result of biochemical and genetic analysis, already firmly established in the literature, the species *M. avium* is now divided in four subspecies: *M. avium* subsp. *avium*, the agent of a tuberculosis-like disease in birds; *M. avium* subsp. *hominissuis*, the classical *M. avium* in the medical literature that affects humans and swine, and the main example of "environmental mycobacteria"; *M. avium* subsp. *paratuberculosis* (MAP), the agent of Johne's disease in ruminants and associated with Crohn's disease in humans; and *M. avium* subsp. *silvaticum* found colonizing wildlife such as wood pigeons, whose status as a pathogen has not been well defined. Both aerosols and contaminated water have been associated with infection, especially with the subsp. *hominissuis*. MAP is unique in that it represents a nontuberculoid strain that is host adapted, in this case to ruminants. The main route of infection in ruminants is fecal-oral transmission. Calves are infected by drinking contaminated milk from their dams or by the ingestion of fecal material from objects contaminated by manure. An environmental exposure of susceptible individuals or consumption of unpasteurized or incompletely pasteurized (adulterated) milk is a potential source of human exposure. It is suggested that an ancestral strain of the MAC led to two subspecies, *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*, and the species *M. intracellulare*. The subsp. *paratuberculosis* seems to have originated from the subsp. *hominissuis*.

Another microorganism of relevance is *Mycobacterium kansasii*, a species related to the MAC complex that may cause disseminated infections in AIDS patients and pulmonary diseases in patients with noninfectious lung disease. *Mycobacterium marinum*, a pathogen of fish, also causes human skin granulomas. *Mycobacterium ulcerans* is associated with severe skin ulcerations prevalent in tropical regions. Other mycobacteria such *as Mycolicibacterim aurum, M. smegmatis*, and many others are usually saprophytic environmental species, but from time to time may cause opportunistic infections in human and animal hosts. Identification usually requires molecular sequencing or restriction fragment length polymorphism analysis of DNA generated from infected tissue.

Primary Interaction with Phagocytic Cells and Disease Onset

Upon entry via the mucosa, or by penetration of skin, resident alveolar, intestinal, or dermal macrophages engulf the pathogenic mycobacteria. This phagocytic process is receptor mediated and antibody independent with the involvement of complement receptors, the mannose receptor, and/or integrins. Pathogenic mycobacteria avoid antibody-dependent receptors that normally trigger macrophage activation and a bactericidal response. By avoiding macrophage activation, bacilli remain in phagocytic vacuoles, preventing phagosome activation and the release of bactericidal reactive intermediates. Phagosome acidification and fusion with lysosomes are also inhibited. Blocking of phagosome responses allow mycobacterial bacilli to survive and multiply within phagocytic cells. There are reports that highly virulent strains of *M. tuberculosis* are capable to escape from the phagosome into the cytosol, where accelerated replication occurs (Figure 36.3).

Monocytes (blood phagocytic cells) recruited to sites of infection will evolve into epithelioid macrophages and form a granuloma or tubercle. Focal round granulomas appear grossly as yellow nodulations near the entry/exit of blood vessels from affected organs (e.g. the Ghon complex in the lungs). The granuloma contains infected macrophages at its center encircled by enlarged phagocytic cells (epithelioid cells), lymphocytes, and fibroblasts intermixed with collagen. Caseous necrosis develops in the center of tuberculous lesions. Depending upon disease outcome, this lesion may centrally calcify if infection is contained, or liquefy as infection advances. Tubercles may enlarge, coalesce, and eventually efface large areas of

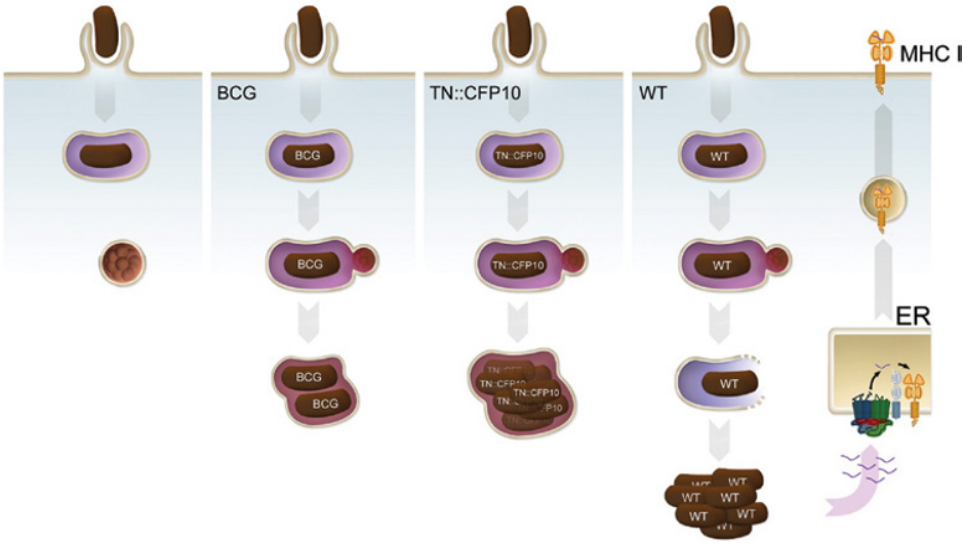


Figure 36.3 Subcellular pathways in myeloid cells for different mycobacteria. Left panel represents the current view in which mycobacteria reside in an "early" phagosome. The two middle panels show traffic of *M. bovis* BCG and *M. tuberculosis* attenuated transposon mutant Tn::cfp-10 (a transposon mutant that does not express CFP10 and ESAT-6) after uptake, both residing and multiplying in a lysosomal-associated membrane protein-containing membrane-enclosed compartment that fuses with lysosomes. Right panel shows virulent *M. tuberculosis* or *M. leprae* present in phagolysosomes and the subsequent translocation to the cytosol. Here possible replication, degradation, and peptide delivery to the MHC I pathway occurs. ER, endoplasmic reticulum. *Source:* Reproduced from van der Wel et al. (2007), figure 7B, with permission from Elsevier (Cell Press) with grateful acknowledgment.

tissue. Mature large tubercles consist of abundant caseous material.

A favorable immune response may prevent disease altogether or keep bacilli in remission with bacteria entrapped in calcified granulomas. The bacilli remain in a nonreplicating, latent state in mature tubercles. Weak or waning host responses can allow centers of tubercles to become necrotic and release infectious bacilli, sometime after long periods of remission, allowing reactivation and spread by cough. This clear sequence of latency and reactivation is not well documented in nonhuman hosts. Certainly, granulomas form in animal hosts, but it is unclear whether a true process of latency and reactivation occurs. A shorter life span and weaker host response, in many animal hosts, allows more frequent progression of mycobacterial infection from subclinical to overt disease. Opportunistic dermal infection in small animals and paratuberculosis in ruminants are similar: inflammation is granulomatous and more diffuse, lacking the typical encapsulation that results in tubercles.

Immune Response to Mycobacterial Infections

Mycobacteria possess numerous antigenic macromolecules including proteins, lipids, glucan, and glycolipids that induce humoral immunity. However, as intracellular pathogens, the humoral response is ineffective in controlling the infection. The humoral response appears later in disease progression. The delayed humoral response has diagnostic significance as a marker of disease progression and can serve as evidence of exposure but usually late in the course of infection. Recent research has identified a role for humoral immunity in the host defense as antibodies against surface virulence determinants can prevent entry of mycobacteria into phagocytic cells and could have a protective effect if developed or administered before infection advances.

The major host defense is an acquired cellular immune response that results in macrophage activation and the production of cytotoxic T cells. Mycobacteria may activate macrophages to a first level of responsiveness via an innate immune process mediated by the interaction of mycobacterial products with Toll-like receptors present on the phagocytic cells. This process results in dendritic cells and macrophages producing the polypeptide messenger interleukin-12 (IL-12, a type of cytokine) and presentation of mycobacterial antigens to helper CD4+ T cells. This antigen presentation process requires antigen degradation in the phagosome and association of degraded peptides with class II major histocompatibility complex (MHC) molecules. In the presence of IL-12, and possibly other cytokines such as IL-17, these cells undergo a differentiation process and become T_{H1} cells with the property of producing γ-interferon that further activates macrophages undergoing a respiratory burst. This process leads to the increased production of highly bactericidal products known as reactive oxygen

(superoxide anion and hydrogen peroxide) and nitrogen (nitric oxide [NO] and nitrite) intermediates. It seems that NO and a combined product of nitrogen and oxygen intermediates (peroxynitrite) exert the most important bactericidal actions. Still, mycobacteria partially resist these activities by producing catalase, superoxide dismutase, and alkyl hydroperoxidase, enzymes that detoxify these reactive intermediates by a redox mechanism. NO has also an immunostimulatory role that results in further cytokine secretion and the activation of macrophage apoptosis that further reduces the number of host cells that can be used for mycobacteria to replicate. Activated macrophages also undergo maturation with phagosome acidification and phagolysosome fusion events, thus complementing the bactericidal action of NO and other reactive intermediates.

IL-12 (IL-17)-activated T_{H1} cells also secrete IL-2, which triggers the differentiation of CD8+ T cells into cytotoxic T cells. Their role is to lyse infected macrophages that then release the intracellular bacilli. These microorganisms can be further phagocytosed and cleared by activated macrophages. Thus, the effective control of mycobacterial infections requires both branches of the acquired cellular response: macrophage and cytotoxic T cell activations upon antigen presentation. Activation of CD8+ cells by antigen presentation requires the assistance of class I MHC molecules. This process involves secretion of mycobacterial antigens from the phagosome into the cytosol, escape of mycobacteria into the cytosol, and/or an effective crosstalk of the antigen presentation pathways.

Depending upon the immune status of the host, differentiation of CD4+ cells into the T_{H1} pathway does not occur. Infected macrophages secrete IL-10 and IL-4 instead of IL-12. This process leads to the maturation of CD4+ cells via the T_{H2} pathway. T_{H2} cells secrete more IL-4 that stimulates B cells to mature into antibody-secreting cells. Unfortunately, this delayed humoral response dependent on T_{H2} cells is completely nonprotective, and it has devastating consequences for the host (Figure 36.4). For example, in tuberculosis infections, it can lead to miliary tuberculosis, a form of disseminated infection with large numbers of bacilli with many organs compromised in addition to the lung. In leprosy, cases of disfigurement occur upon activation of the T_{H2} response.

Tuberculous Infections, an Overview of Disease Patterns in Veterinary Medicine

Birds

Birds are naturally susceptible primarily to *M. avium* subsp. *avium*. Most poultry infections occur via the alimentary canal and disseminate to the liver and spleen. Bone marrow, lung, and peritoneum are often affected. Although the agent has been isolated from eggs, transovarian infection of chicks is rare. Although *M. avium* subsp. *avium* affects many species of birds, psittacines are resistant but are susceptible to *M. tuberculosis*. Canaries are also more susceptible to mammalian than to avian bacilli.

Cattle

Cattle infected with *M. tuberculosis* variant *bovis* have pathology centered on the respiratory tract, adjacent lymph nodes, and serous cavities. The disease is commonly progressive via airways. Hematogenous dissemination to the liver and kidney can occur. The uterus may serve as a portal for fetal infection, a pattern virtually unknown in other domestic animals. Surviving calves commonly develop liver and spleen lesions. Udder infection is rare (<2% of cases) but has obvious public health implications. Infection with *M. avium* subsp. *hominissuis* is generally subclinical. Abortions resulting apparently from localization of bacilli in the uterine wall can also occur. In experimental infections, human-adapted *M. tuberculosis* strains causes minor, non-progressive lesions in cattle.

Cervidae

M. tuberculosis variant *bovis* readily infects free-ranging and domesticated cervidae; it is problematic in captive herds and is maintained in some wild populations. Disease progression and lesions are similar to those in cattle, although species variations exist among cervidae, particularly at the histological level. Lesions are, in some species, more suppurative, varied in numbers of acid-fast bacteria, giant cells, and in degree of encapsulation.

Dogs and Cats

Dogs and cats are readily infected with *M. tuberculosis* variant *bovis* but rarely with *M. avium* subspecies. Dogs are also susceptible to *M. tuberculosis* human strains. Intestinal and abdominal localization of infection is more common in cats than in dogs, reflecting a likely alimentary route of exposure. Ulcerative skin lesions are more common in cats than in other hosts, as is eye involvement, with tuberculous choroiditis leading to blindness. Lesions, especially in dogs, often more closely resemble a foreign body reaction than tubercles, since they may lack typical epithelioid and giant cells, and neither caseate and calcify, nor liquefy. The course is usually progressive. *M. tuberculosis* variant *microti* (vole origin) occasionally is associated with tuberculous lesions in cats, which are normally quite resistant to tuberculous lesions.

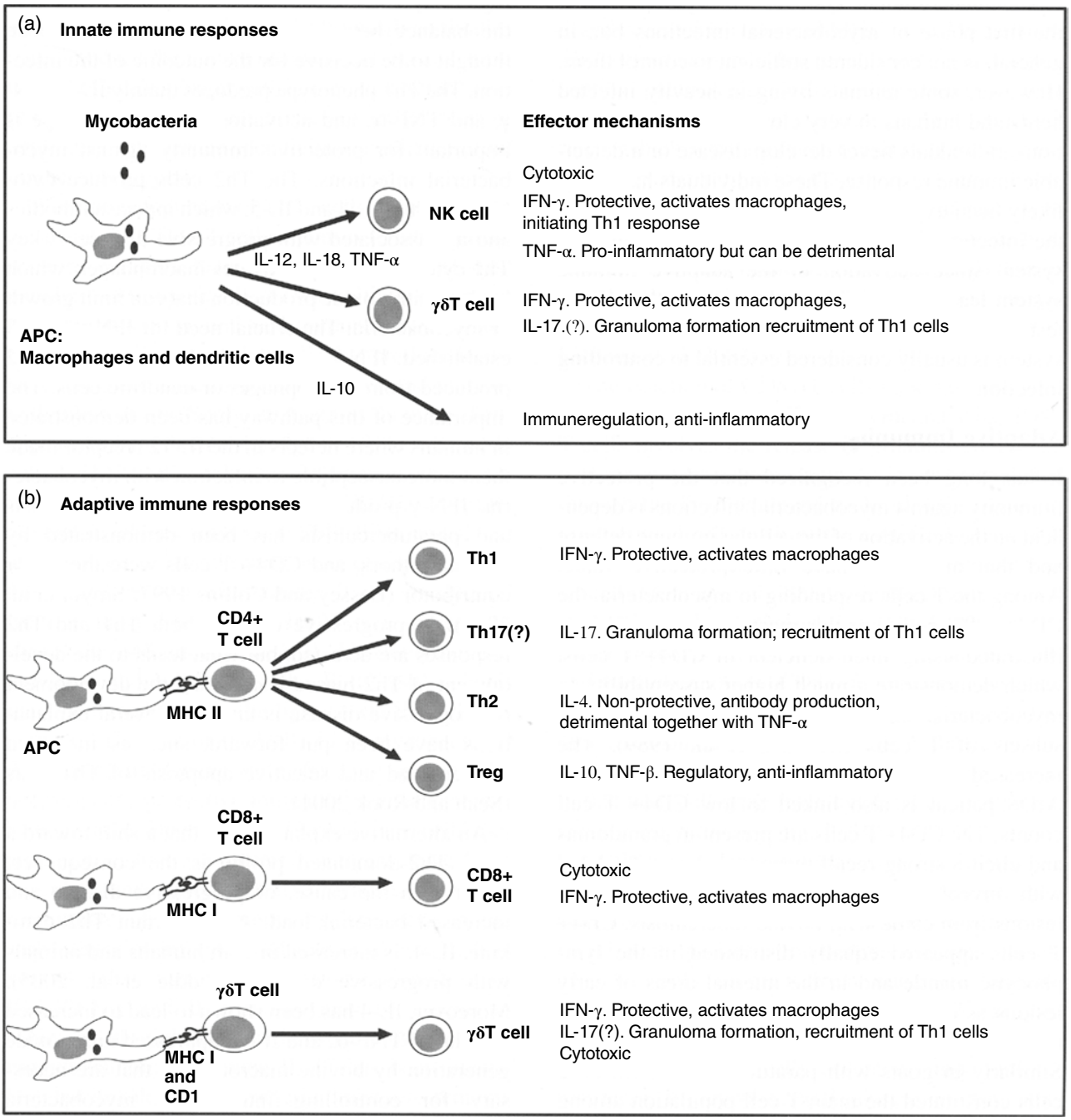


Figure 36.4 Immune responses to mycobacterial infections. The figure shows an overview of important (a) innate and (b) adaptive immune responses in mycobacterial infections. CD4+ T cells can develop into different subtypes designated Th1, Th2, Th17, and Treg. More recently, the roles of Th17 and IL17 have been demonstrated in mycobacterial infections in humans and laboratory animals. *Source:* Reproduced from Olsen et al. (2010).

Horses

Horses are rarely infected, but relatively more often with *M. avium* subsp. *hominissuis* than with *M. tuberculosis* variant *bovis*. Infection enters usually by the alimentary tract, with primary complexes related to the pharynx and intestine. Secondary lesions may be in the lung, liver, spleen, and serous membranes. Lesions in cervical vertebrae may be due to a secondary, nonspecific hypertrophic periostitis. Gross lesions are tumor-like. They lack caseation and gross calcification and contain few lymphocytes. There is fibroblast proliferation but usually no firm encapsulation.

Primates

Nonhuman primates are susceptible to *M. tuberculosis* and the *bovis* variant, but resistant to *M. avium* subsp. *hominissuis* unless severely compromised, as by simian immunodeficiency virus infection or preexisting bronchopulmonary disease.

Sheep and Goats

Sheep and goats are susceptible to *M. tuberculosis* variant *bovis* and *Mycobacterium avium* subsp. *paratuberculosis*, but slightly less so to *M. avium* subsp. *hominissuis*, and they are resistant to progressive infection by *M. tuberculosis*. Disease patterns resemble those described in cattle.

Swine

Swine are infected by tubercle bacilli, usually via the alimentary route, but only *M. tuberculosis* variant *bovis* causes progressive disease with classical lesions. Human-adapted *M. tuberculosis* infections do not advance past regional lymph nodes. *M. avium* subsp. *hominissuis* infections, the predominant form in many countries, may disseminate to viscera, bone, and meninges. The lesions lack the organization of tubercles but contain granulomatous elements. Caseation, calcification, or liquefaction is negligible. Bacteria may be abundant.

Wildlife and Infection Reservoirs

With near eradication of bovine tuberculosis in North America and Europe, the traditional reservoir in domestic mammals might be of diminished concern. However, resurgence is a problem where efforts toward eradication are losing support and consolidation of livestock enterprises makes elimination of large, infected herds cost prohibitive. Game farms, animal parks, zoos, and isolated wildlife pockets of infection can serve as reservoirs of *M. tuberculosis* variant *bovis*. Tuberculosis is typically a disease of captivity and domestication. Limited spread in free-living animals and clinical improvement contrast with high communicability in confined domestic groups. *M. tuberculosis* variant *bovis*, probably originating from cattle, is endemic in badgers of southern England and in brush-tailed opossums of New Zealand, both of which are sources of infection for livestock. Infections in raccoons, opossums, foxes, and coyotes can also be important as transmission to and from cattle in shared environments may frustrate infection control measures. Coyote–deer interaction is also common, and coyote sampling is used to monitor infection spread in some wild deer populations. Farming of exotic animals comingling with ruminants has also significantly increase risks of bovine tuberculosis transmission, as captive cervidae are highly susceptible to this variant. Sporadic cases of canine tuberculosis often prove to be *M. tuberculosis* infections traceable to human contacts, a case of reverse zoonoses. These events also occur in nonhuman primates in laboratory colonies and zoos.

In commercial poultry establishments, rapid population turnover (<1 year) and elimination of transgenerational transmission has eradicated *M. avium* subsp. *avium* from that population. Mycobacteria remain a problem in barnyard flocks, aviaries, and zoological parks, particularly because the agent can survive in soil for several years. It has been hypothesized that amoeba may be involved in the transmission of *M. avium* subsp. Support for this hypothesis has been provided by experiments indicating that bacilli grown in amoeba have increased infectivity and pathogenicity than microorganisms grown in broth or agar.

Effects of Age, Breed, and Sex

In general, across all animal species, immature individuals often develop more severe lesions than older ones to a given mycobacterial infection. Breed susceptibilities differ, with zebu cattle are more resistant than European breeds, and fox terriers and Irish setters are more often infected than dachshunds and Doberman Pinscher dogs. The higher prevalence in dairy than beef cattle may reflect closer confinement and greater productivity stress among dairy cows. Pregnancy and lactation may explain the higher disease prevalence in cows than in bulls, although in dogs the reverse sex ratio is reported.

Nontuberculous Mycobacterial Infections

In areas with a strong history of tuberculosis control in domestic species, nontuberculous mycobacterial infections predominate. Johne's disease caused by MAP, a host-adapted nontuberculous species, is problematic in ruminants. Infections with this microorganism are widespread, and frustration of early diagnosis related to the biological limitations of mycobacteria make elimination from herds difficult. *M. avium* subsp. *avium* can cause nontuberculous reactors in cattle. Schnauzer dogs with CARD9 mutations are predisposed to mycobacterial disease. There are numerous saprophytic mycobacteria, including both slow- (*M. avium* subsp. *hominissuis*, *M. intracellulare, Mycobacterium malmoense*) and fast-growing (*M. fortuitum, M. smegmatis*) strains, that cause granulomatous lesions in cats and dogs. *Mycobacterium genavense* and *M. avium* subsp. *avium* cause systemic disease in aviary birds along with other less frequent

opportunist pathogens (*M. fortuitum, M. intracellulare*). There are myriad species of non-tuberculosis mycobacteria opportunist that frustrate simple inexpensive diagnostic approaches.

Laboratory Diagnosis

Sample Collection

Collection of sputum and serum samples is common in human cases. In animals, especially ruminants, samples include aspirates or biopsies of active lesions, tracheobronchial and gastric lavages, lymph nodes (thoracic and abdominal), urine, or feces. Lung and lymph nodes associated with the respiratory tract including the pharynx and lymph nodes of the intestinal tracts and liver are common sites for lesions and sampled at necropsy. Freezing samples during transit for molecular diagnostics or preserving tissue in sodium borate is effective, preserving mycobacterial viability for culture and diminishing the growth of microbial contaminants. It is best to obtain multiple or split biopsy samples from domestic pet skin lesions. Section distribution includes formalin fixed (pathology), chilled or in sodium borate (culture), and frozen for molecular diagnostics.

Direct Examination

Fluid samples are concentrated by centrifugation in tightly capped containers. Smears of sediment or tissue were traditionally processed using the acid-fast staining procedure, or with auramine-rhodamine when fluorescence microscopy is available. Histological sections stained with hematoxylin–eosin and acid-fast stains demonstrate bacteria. Light emitting diode fluorescence microscopy using Auramine O staining, as recommended by the World Health Organization, is a sensitive and affordable method of direct detection of bacteria from sputum. Culture or molecular (nucleic acid detection) validation of direct staining results is required for confirmation and speciation of acid-fast bacteria.

Culture, Molecular Detection, and Identification

Digestion and selective decontamination are advisable, especially with specimens likely to contain a mixture of microorganisms. Identification is based upon growth characteristics (fast vs. slow), pigmentation (in the presence or absence of light), and biochemical reactions. DNA probes, specific for the main groups, are commercially available. Tests for the amplification of mycobacteria-specific DNA fragments with the polymerase chain reaction (PCR) are methods of choice to demonstrate or identify members of this genus. Improved methods for nucleic acid detection are more sensitive and faster than culture, and allows speciation. Whole genome sequencing is also affordable for absolute identification.

Immunodiagnosis

Tuberculin Test

The Mantoux tuberculin skin test consists of the intradermal application of tuberculin (tuberculin-purified protein derivative, tuberculin PPD), which is a cell-free extract of proteins and peptides released by *M. tuberculosis* into the culture medium. This inoculation, in infected individuals, results in swelling characterized by an induration area 48–72 hours post application (delayed-type hypersensitivity reaction). In cattle, a 0.2–0.3 mg/dose of bovine PPD (PPD from the variant *bovis*) is injected intradermally in the caudal, vulvar, or anal skin or, in some situations, the neck region. In positive cases, a swelling (>5 mm) develops within 72 hours. Although tuberculin cannot induce the hypersensitive state, it may desensitize animals for weeks or months. A positive test implies past or present infection, requiring the reacting animal to be slaughtered and necropsied. False-positive reactions occur and are explained by hypersensitivity to nontuberculous mycobacteria (e.g. *M. avium*) and related agents such as *Nocardiae*. Simultaneous use of avian "tuberculin" (*M. avium* PPD) often helps to decide, by comparative size assessment of the two reactions, whether sensitivity is due to *M. tuberculosis* variant *bovis* or other mycobacteria. False-negative results occur in animals too recently infected and in advanced cases in which anergy (lack of reactivity) develops due to antigen excess or immunosuppression. Nonspecific factors, such as malnutrition, stress, and impending or recent parturition, are alternative causes of anergy. "Tuberculins" of appropriate specificity are used on swine and poultry. In swine, the ears are injected, in poultry the wattles. The reliability of tuberculin tests on horses, sheep, goats, dogs, and cats is not established. The ease and the sensitive and inexpensive nature of the skin test establish it as the most effective antemortem screening test in ruminants.

γ -Interferon Assays

These tests are based on the release of γ -interferon from whole blood of infected animals when mixed with mycobacterial antigens (e.g. PPD) as the result of the activation of the cell-mediated immune response. Recent studies have demonstrated increases in specificity by altering the antigens used to stimulate the interferon

response. Other studies suggest that adding cytokines to the analysis such as interleukin 1β may increase test sensitivity as well.

Serology

Serological tests have been moderately useful. These assays are sensitive but are produced late in the disease process. The greatest success has been in the detection of animals infected with MAP.

Treatment and Control

First-line drugs for tuberculosis therapy in humans are isoniazid (INH), rifampin, pyrazinamide, streptomycin, and ethambutol. Second-line drugs are para-aminosalicylic acid, kanamycin, D-cycloserine, capreomycin, and ethionamide. Because resistance often develops under a single-drug regimen, a combination is commonly used; a favored one in human medicine is INH-ethambutol rifampin given for at least nine months. The standard short-term course involves initial daily therapy with isoniazid, rifampin, and pyrazinamide, plus either streptomycin or ethambutol for two months. This regimen is then followed by another four-month phase of daily therapy with isoniazid and one other primary (first-line) drug. The current increase in human infections due to MDR (multiple drug-resistant; *in vitro* resistance to isoniazid and rifampin) and XDR (extensively resistant; resistance to at least isoniazid and rifampin plus resistance to fluoroquinolones and one second-line injectable drugs such as amikacin, kanamycin, or capreomycin) strains is especially concerning.

Because of the public health hazards inherent in the retention of tuberculous animals, antituberculous chemotherapy for animals is discouraged. Prophylactic treatment with INH is sometimes used for pets recently exposed to tuberculosis. INH for prophylaxis and treatment of calves is sometimes successful. Treatment of animal tuberculosis is generally discouraged or illegal in countries with control programs.

Bovine tuberculosis control is by identification and elimination of infected animals. This approach has resulted in the near eradication of the infection in many countries. Continued surveillance is required to prevent resurgence. These efforts have changed tuberculosis from a clinical disease in cattle and a frequent source of zoonotic infection to a subclinical disease and rare source of human infection in many Western countries.

Vaccination of humans with BCG produces temporary immunity and hypersensitivity. The benefits of vaccination have been greatest where exposure is high and negligible where prevalence is low. The use of BCG in calves is inappropriate in countries attempting to eradicate tuberculosis because it interferes with the interpretation of the tuberculin test. Vaccines of moderate efficacy are available against MAP, but they interfere with diagnostic tests. Development of more effective vaccines that allow the differentiation of infected from vaccinated animals are needed (data-interpolating variational analysis, DIVA formulation).

M. avium subsp. *paratuberculosis*, the Agent of Johne's Disease

MAP is the causative agent of a chronic, irreversible wasting disease of ruminants called Johne's disease. Young animals are the most susceptible to infection. Older animals can be infected with larger inocula. Economic losses are traced to reduced milk production and breeding problems (e.g. longer calving intervals), and an increased incidence of mastitis (not caused by MAP), and to general ill thrift and early culling (elimination).

Like the rest of the genus *Mycobacterium*, MAP is aerobic but grows extremely slowly (8–12 weeks of incubation at 37 °C) in Herrold's egg yolk medium. Primary clinical isolates require the addition of the siderophore mycobactin (an iron-binding compound derived from hydroxamic acid). This growth dependency on mycobactin is of diagnostic value to identify and confirm MAP in clinical samples. Culture is used infrequently for diagnosis as molecular approaches are faster and now highly sensitive.

The reservoir for MAP is the intestinal tract of infected animals, both the clinically affected and asymptomatic shedders. Infected, asymptomatic fecal shedders may be 20 times more numerous than those showing clinical signs. An infected animal may shed MAP into colostrum and milk with potential for passing the microorganism to the fetus in utero. MAP has been isolated from semen, seminiferous tubules, and the prostate glands of infected bulls. Infection is most often acquired through the ingestion of fecal contaminated feeds or ingestion from contaminated surfaces.

MAP enters the intestinal mucosa through M cells and is later found within macrophages in the submucosa of the ileocecal area and the adjacent lymph nodes (ileocecal) (Figures 36.5–36.7). The establishment of the primary focus of infection is the intestine. Initially, the animal shows no signs of disease. The incubation period before overt clinical disease is 12 months or longer. Disease progression follows the course of other mycobacterial infection with the formation of slowly progressing granulomatous lesions.

The disease in affected animals progresses to malabsorption, protein-losing enteropathy, and overt clinical disease,

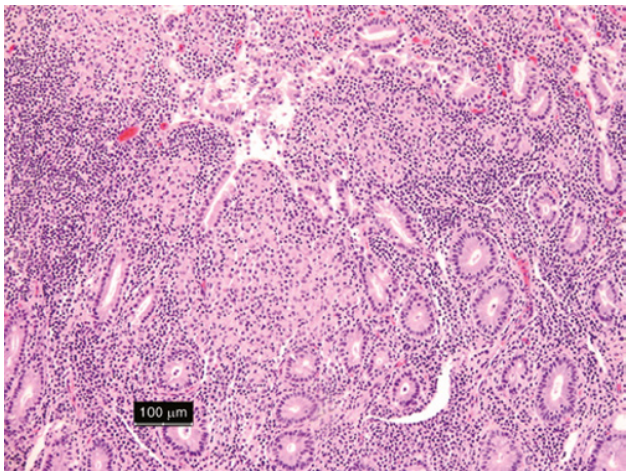


Figure 36.5 Typical histopathology of the jejunum of a bovine infected with Johne's disease. Abundant epithelioid macrophages expand the lamina propria; crypts are rarified and contain debris (hematoxylin and eosin staining, 10×). (Specimen from the University of Nebraska Veterinary Diagnostic Center). *Source:* Reproduced from McVey et al. (2013).

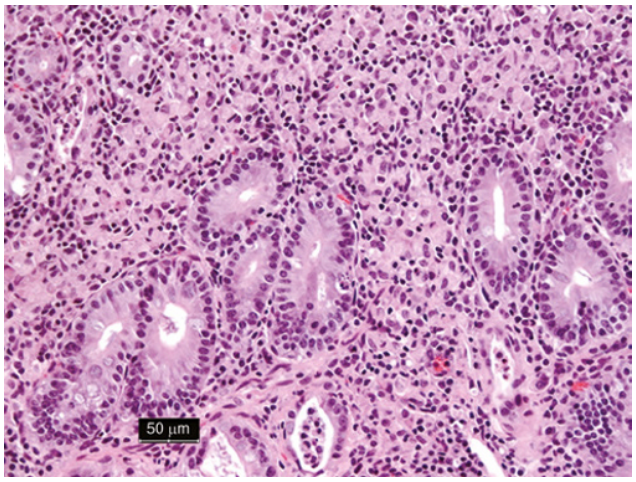


Figure 36.6 Large epithelioid macrophages. Epithelioid macrophages dominate the superficial lamina propria and separate crypts in the jejunum of a bovine with Johne's disease. A few degenerate cells are evident in crypts (hematoxylin and eosin staining, 20×). (Specimen from the University of Nebraska Veterinary Diagnostic Center). *Source:* Reproduced from McVey et al. (2013).

with only 3–5% of the animals in an infected herd progressing to this terminal clinical disease. The mucosal epithelium displays a characteristic gross appearance (corrugated mucosa) that underlies the malabsorption syndrome. Diarrhea is not a common clinical sign of the disease in sheep and goats. Poor growth efficiency and loss of condition should prompt examination for MAP in sheep or goats. Intestinal lesions in sheep and goats are less grossly obvious than in cattle. Weight loss is a consistent finding in affected goats.

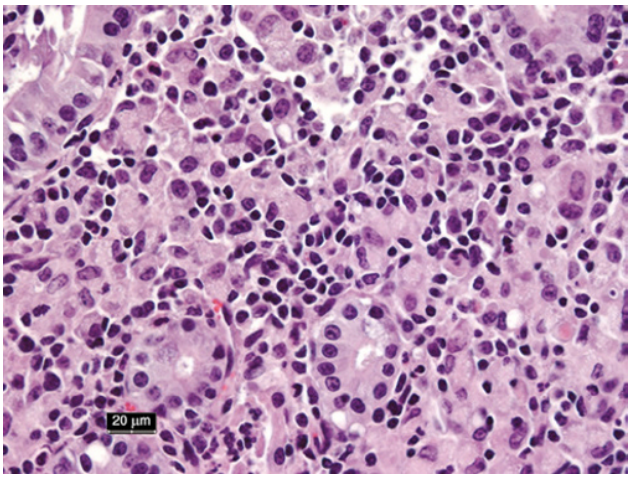


Figure 36.7 Large epithelioid macrophages are present among the crypts in the jejunum of a bovine with Johne's disease. Nuclei are eccentric and cytoplasm slightly vacuolated and sometimes granular (hematoxylin and eosin staining, 40×). (Specimen from the University of Nebraska Veterinary Diagnostic Center). *Source:* Reproduced from McVey et al. (2013).

Several DNA tests were developed to demonstrate the presence of MAP. These assays utilize a specific sequence for the development of probes or for the design of primers needed to amplify DNA by using the PCR. Specific sequences found in the gene encoding the 16S ribosomal RNA and the IS*900* characteristic multi-copy insertion sequence are usually targeted. Phage-based methods for detection of viable MAP have been recently developed as well.

At present, there are no economically useful antimicrobial agents effective against MAP *in vivo*. The newer macrolides, such as clarithromycin, and some experimental fluoroquinolones are effective *in vitro*, but are too expensive to use clinically. Live attenuated and killed vaccines are not universally available as they are not highly effective.

Eliminating infected animals and preventing possible spread within the herd is an effective means of disease control. Husbandry procedures include segregating the neonate from the dam and other adult infected animals, ensuring that parturition takes place in non-contaminated areas, and taking precautions against feeding neonates infected unpasteurized colostrum or milk are critical in high-prevalence herds. Serology and fecal molecular tests help to identify animals that are exposed and those that are shedding. Early identification and culling of shedders is critical. Careful hygiene and cleaning manure-handling equipment is important to prevent feedstuff contamination. http://Johnes.org (University of Wisconsin) is a great resource for developing herd-specific control programs.

Pasteurization is an important step in control in dairies, ensuring that calves receive milk or colostrum that is free

of MAP. Given the potential relationship between MAP and Crohn's disease, this process has also significance from a public health perspective. High-temperature, short-time pasteurization is not 100% effective in killing MAP in naturally infected milk when the numbers of microorganisms in the milk are large. This may have minimal public health consequences when large quantities of milk pooled in processing dilute bacterial counts. Fecal contamination of water can be a source of MAP infection in Crohn's disease cases.

Feline Leprosy Syndrome

There is no true leprosy in domestic animals. In human leprosy, the disease picture ranges between two extremes. In lepromatous leprosy, bacterial proliferation is profuse. Poorly circumscribed nondestructive lesions develop, dominated by a monocytic response, but little other inflammatory reactivity. Cell-mediated immunity is suppressed, and circulating immunoglobulins are high. In tuberculoid leprosy, bacteria are scarce, lesions are granulomatous, and cell-mediated inflammatory responses are well developed. Neural damage leading to anesthesia, paralysis, dystrophy, disfigurement, and mutilation can occur.

Feline leprosy is a distinct chronic syndrome and is a nodular and ulcerative mycobacterial infection of the skin. It exists in two histomorphologic forms, lepromatous (organism rich) and tuberculoid (paucibacillary). *Mycobacterium lepraemurium*, the rodent leprosy bacillus, is identified in tuberculoid and occasionally lepromatous lesions, particularly in younger cats. Several mycobacterial species are described in case reports and reviews associated with cutaneous lesions. Sources, mode of spread, and pathogenic mechanisms are not well defined. The common location of lesions on the head, neck, and forelimbs suggests transmission by rodent bite or arthropods. Nodules occur in cutis or subcutis in many sites and are freely movable and painless. Ulceration and lymph node involvement are frequent. Disease is more common in older cats and is slowly progressive. The form of disease does not consistently predict rate of progression. The general health of the cat may be unaffected. The tendency is toward local spread of lesions and post-surgical recurrence without antimicrobial treatment. Untreated disease can result in widespread lesions. Nonulcerating, overlooked, early lesions can advance and present as disseminated disease. Internal lesions can occur with advanced disease. Microscopically, the lesions are granulomas largely monocytic with variable neutrophils (pyogranulomatous), lymphocytes, plasma cells, and giant cells. Neurotropism is not a feature. Acid-fast bacteria abound within histiocytes. The bacilli can stain as negative shadows on routine cytology. Culture for rapidly growing mycobacteria is typically negative. PCR technology and sequencing are usually necessary to make a definitive etiologic diagnosis.

Treatment includes surgical excision of affected sites coupled with antibiotics. Clofazimine, fluoroquinolones, rifampicin, doxycycline, and clarithromycin individually or more often as a combination of two or more exhibited varied success. Treatment for two to three months is required to prevent relapse.

Canine Leproid Granuloma Syndrome

Canine leproid granuloma syndrome is caused by an uncultured, saprophytic species of mycobacteria (based on sequence comparisons of DNA obtained from infected tissue with other members of this genus as regards the gene encoding the 16S ribosomal RNA). Diagnosis is based on demonstration of numerous acid-fast organisms in stained (Ziehl–Neelsen) biopsies or smears of affected tissue or sites. The condition affects the subcutis and skin of the outer pinnae, face, and forelimbs. Lesions can be single, but multiple lesions are common. Large lesions may ulcerate. Histologic features are of pyogranulomatous inflammation with necrosis and limited numbers of giant cells. Nerve bundles are not involved, and thus leproid refers only to the mycobacterial etiology rather than to pathological similarity to human disease. The disease can be self-limiting and cured by surgical excision or antibiotic therapy. A combination of rifampicin and clarithromycin has been effective, as has enrofloxacin. The disease was described first in Africa, has been well characterized in Australia and Brazil, and is present in North America. The disease is most common in large breed, short-haired, outdoor dogs during summer months. This summer occurrence and a strong predilection for lesions on the pinnae suggest that arthropod injury may contribute to transmission. Cases in the United States, Brazil, and Australia all share common mycobacterial 16S ribosomal sequence identity.

Ulcerative Dermatitis of Cats and Dogs due to Rapidly Growing Mycobacteria

The most common cases of ulcerative dermatitis are chronic (months to years), nonhealing skin lesions. There is a tendency for lesions to occur on the ventral and inguinal regions and for disease to be progressive and requiring protracted treatment. Histopathologic evaluation of biopsies of affected tissue reveals pyogranulomatous inflammation. Microorganisms may stain poorly ("ghosts" or

"speckled" structures, thought to represent nonstaining or poorly staining mycobacteria, respectively) when stained with Gram's or a Romanowsky-type stain (e.g. Wright's and Giemsa). When samples are stained by the acid-fast method (Ziehl–Neelsen), microorganisms are usually not observed. Cavitated nonstaining areas may contain clusters of bacteria.

Culture on standard blood agar medium will result in the isolation of rapidly growing (48–72 hours incubation) colonies. Isolates are identified by biochemical traits and DNA tests. The most isolated in western North America are *M. smegmatis* and *M. fortuitum*. Prevalent bacterial species tends to vary based on geography. The distribution of opportunistic mycobacteria in the environment is the critical factor in anticipating what agent might be infecting cats in a particular location. A wide array of reported opportunistic mycobacteria complicates etiologic diagnosis. Other species identified in cases of dermatitis and panniculitis include *M. chelonae*, *Mycobacteroides abscessus, Mycobacteroides abscessus* subsp. *massiliense, M. tuberculosis* variant *microti, Mycolibacterium flavescens*, *M. ulcerans*, and a more recently characterized *Mycobacterium* sp. strain Tarwin, a microorganism related to the *Mycobacterium simiae* complex isolated from the New South Wales near the town of Tarwin. *M. tuberculosis* variant *bovis* and *M. avium* are identified in individual cases of feline dermatitis.

Percutaneous wound infection is the likely portal of entry. Mycobacteria that can be isolated from soil and water are likely to lead to opportunistic infections of skin wounds. Treatment includes surgical excision of affected sites coupled with antibiotic treatment. Clofazimine, fluoroquinolones, doxycycline, and clarithromycin, individually or in combination, are used with varied success. Antimicrobial resistance patterns vary between bacterial species in the studies published, and thus, etiologic diagnosis can be critical for proper medical management. Treatment as long as two to three months is required to prevent relapse.

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37

Chlamydiaceae: *Chlamydia*

Roman R. Ganta

Descriptive Features

The family Chlamydiaceae includes obligate, intracellular pathogenic bacteria that can survive and multiply only inside a host cell. They also have a cell wall like most other gram-negative organisms, respond to antibiotics (tetracyclines and chloramphenicol), and possess ribosomes, DNA, and RNA. Members of the order Chlamydiales are transmitted primarily by inhalation of dust particles and droplets (all of which contain chlamydial organisms, known as the elementary bodies) and through interpersonal contact. These microorganisms infect the host epithelial cells and mucous membranes. The cell tropism of Chlamydiales to epithelium is similar to *Mycoplasma* species. Thus, infections in target tissue and the diseases caused by *Chlamydia* and *Mycoplasma* species are very similar. Based on the clinical symptoms, sometimes it can be difficult to distinguish a Chlamydial infection from a *Mycoplasma* species infection. Taxonomically, members of Chlamydiales are classified in the order Chlamydiales, which has only one family, Chlamydiaceae, which has only one genus, *Chlamydia*. Five major pathogens are *Chlamydia psittaci*, *Chlamydia abortus, Chlamydia pecorum*, *Chlamydia trachomatis*, and *Chlamydia pneumoniae. C. psittaci*, *C. trachomatis*, and *C. pneumoniae* cause diseases in people. Veterinarians and poultry and slaughterhouse workers are at the highest risk of acquiring chlamydial infections from birds and farm animals.

Morphology and Staining

Chlamydiales are short cocci bacteria, with the size of the organisms ranging from 0.2 to 1.0 μm, similar to Rickettsiales. Giemsa or other polychromatic stains are used to stain these organisms.

Life Cycle and Growth Characteristics

Infectious organisms called elementary bodies, 0.2–0.4 μm in size, enter susceptible cells by receptor-mediated endocytosis. The phagosome containing the organism is not fused with lysosomes, and the elementary body changes into a noninfectious and replicating form that is metabolically more active than elementary bodies. The replicating form is called the reticulate body, which is larger in size (0.6–1.0 μm) compared to elementary bodies. The reticulate bodies replicate by binary fission, which later transform to elementary bodies, and are released upon complete lysis of the infected host cell or by exocytosis.

Chlamydiales do not grow in a standard bacteriological medium or on a media plate. They require eukaryotic cells or yolk sacs of chicken embryos for their growth under *in vitro* conditions. Chlamydiales undergo replication inside a phagosome of a host cell by binary fission, very similar to Anaplasmataceae pathogens of the genera *Ehrlichia*, *Anaplasma*, and *Neorickettsia*. Chlamydiales also have a broad host preference that is similar to Rickettsiales.

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Diagnosis, Immunity, and Control

Diagnosis is typically based on serological methods to detect chlamydial infection-induced antibody response, although several molecular tests are reported and are in use as well. Diagnostic parameters also include clinical signs and the demonstration of the organism by Giemsa or other polychromatic-stained impression smears (Figure 37.1) of an infected animal tissue. Tests also include an enzyme-linked immunoadsorbent assay (ELISA) to detect chlamydial antigens from a sample. Similarly, complement fixation and ELISA to detect antibodies in the serum of an infected animal are also employed. Many different and sensitive molecular methods have been developed to amplify a nucleic acid target of chlamydial pathogens. Molecular methods allow the most sensitive means of rapidly detecting an infection in a tissue sample from clinically ill animals or people or from a sample collected from a persistently infected patient.

As in Rickettsiales, an infected host induces a cell-mediated and humoral immune response, resulting in the rise of antibody titers, but the immune response may not necessarily eliminate the infectious agents. Chlamydial pathogens establish persistent infections in a host. Tetracycline is the drug of choice for treating chlamydial infections. It is also offered as part of the feed additive. The dose used in feed additives for controlling infections is moderate, whereas a higher dose is used to treat active infections. Vaccination against the pathogen infections is sometimes successful.

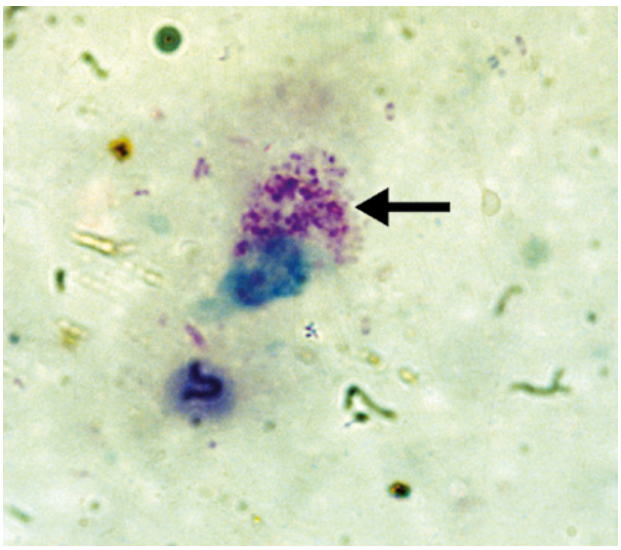


Figure 37.1 *C. abortus* inclusions identified from an impression smear sample prepared from an aborted placenta collected from an infected sheep. The smear is stained with Diff-Quick stain (magnification, 1000×). *Source:* The slide prepared by Dr. Jerome C. Nietfeld, Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University.

Pathogenicity and Toxins

Chlamydial pathogens infect epithelial cells and mucous membranes. These bacteria possess hemagglutinin that facilitates attachment to cells. The cell-mediated immune response is largely responsible for tissue damage during inflammation. Cell wall lipopolysaccharides and low-density lipoprotein oxidation by the bacterial heat shock proteins are implicated in the pathogenicity.

Diseases Caused by Chlamydiales

Because chlamydial infections are primarily in the epithelium, they infect the respiratory tract, eyes, urogenital tracts, and joints, thus causing respiratory disease, conjunctivitis, urogenital infections, and polyarthritis, respectively. With respect to the host cell preference, the mode of transmission, and the diseases they cause, chlamydial infections resemble many *Mycoplasma* infections.

C. psittaci/C. abortus Infections

C. psittaci is reclassified into *C. psittaci* and *C. abortus*. *C. abortus* is the causative agent of abortions in sheep and goats in many countries. It is also responsible for abortions in cattle, pigs, and women. *C. psittaci* is responsible for the majority of infections in various vertebrates, including birds, goats, sheep, pigs, cattle, and humans.

Birds. *C. psittaci* infections in birds are referred to as avian chlamydiosis (AC), psittacosis, and parrot fever in parrots, and ornithosis in all other bird species. It is an important cause of systemic illness in companion birds and poultry and can cause large economic losses to the poultry industry throughout the world. A great variation in pathogenicity exists for this species, likely resulting from strain variations. AC could be an asymptomatic, acute, subacute, or chronic clinical disease. Clinical signs and mortality from AC depend on the species of birds, virulence of a strain, infective dose, stress factors, age, and extent of treatment or prophylaxis. Birds infected with *C. psittaci* typically exhibit clinical signs such as lethargy, anorexia, and ruffled feathers. Other signs may include

ocular or nasal discharge, diarrhea, and excretion of green to yellow-green urates. Infected birds may die soon after the onset of the illness or may become emaciated and dehydrated before death.

Cattle. *C. psittaci* is a well-recognized and important bovine pathogen in many countries. Its infection in the neuroepithelium has been implicated in the neurological disease sporadic bovine encephalomyelitis (SBE), which is also known as Buss disease. SBE is more common in cattle less than three years of age and is characterized by encephalitis, fibrinous pleuritis, and peritonitis. The disease manifestation typically includes profound depression usually associated with fever that persists until recovery or death. A staggering gait is often seen, and some infected animals tend to walk or stagger in circles. The disease can be transmitted to calves through the milk of infected dams. In addition to SBE, *C. psittaci* infections can lead to polyarthritis, pneumonitis, conjunctivitis, abortion, and infertility. *C. psittaci* infections can cause great economic losses. *C. pecorum* is also isolated from cattle and sheep. It is responsible for various diseases, including sporadic encephalitis, polyarthritis, pneumonia, and diarrhea.

Sheep. *C. psittaci* infections in sheep cause pneumonitis, polyarthritis, and conjunctivitis. *C. abortus* causes abortion.

Goats. Pneumonitis, abortion, enteritis/diarrhea, and arthritis in goats are caused by *C. psittaci*/*C. abortus*.

Horses. Chlamydial infections by *C. psittaci* also cause pneumonitis, arthritis, abortion, and infertility in horses.

Cats. *C. psittaci* infections in cats may begin with conjunctivitis and nasal discharge, followed by interstitial bronchopneumonia.

Swine. Infections in swine are associated with pneumonitis, conjunctivitis, and polyarthritis.

Other animal species. *C. psittaci* also infects guinea pigs, rabbits, and mice, causing conjunctivitis and fertility problems.

C. pecorum Infections

C. pecorum infects cattle, sheep, and pigs. Clinical signs of *C. pecorum* infection vary from no symptoms to a severe disease involving the central nervous, respiratory, and digestive systems, joints, and conjunctiva. After recovery from a clinical disease, many animals remain as the carriers of infection and excrete elementary bodies for a long time.

Chlamydiosis: A Zoonotic Disease

C. psittaci

Infections in people with *C. psittaci* are reported worldwide. The disease is referred to as psittacosis, which can cause fatal pneumonia. Most cases in people are acquired by aerosol from the organisms shed from parrots, pigeons, turkeys, sheep, and goats. The disease agent can also be transmitted from person to person. Infected birds and animals shed elementary bodies in their feces, urine, saliva, ocular and nasal secretions, and feather dust. These infectious particles are inhaled or ingested by other birds, animals, and people. Humans are usually infected by the inhalation of infective particles from the air. The incubation period is one to two weeks. Symptoms are generally similar to those of the flu, such as fever, diarrhea, chills, conjunctivitis, and sore throat. *C. psittaci* infections in both people and birds are treated with doxycycline or tetracycline. People are treated for three weeks, while birds are treated for 45 days.

C. trachomatis

C. trachomatis is the most common sexually transmitted pathogen of humans in the United States. It is responsible for approximately 3–4 million cases each year. Pelvic inflammatory disease (PID), a serious complication of chlamydial infection, has emerged as the leading cause of infertility among childbearing-age women. Infection may be passed on from a pregnant mother to her newborns during delivery, which can also cause neonatal conjunctivitis and/or pneumonia. *C. trachomatis* cases are five times more in females compared to males. Clinical symptoms are also often milder in males, although some infected females can be asymptomatic. Chlamydiosis in both males and females can lead to a serious disease and can cause sterility due to the scarring of the tubes in women. *C. trachomatis* infections can also cause blindness in humans. *C. trachomatis* infections are also reported in pigs, monkeys, and laboratory mice.

C. pneumoniae

C. pneumoniae is responsible for causing a respiratory disease in humans (Figure 37.2). This is very similar to pneumonia caused by *Mycoplasma*. The mode of transmission of the organism is by inhalation. *C. pneumoniae* infections have also been implicated in atherosclerosis.

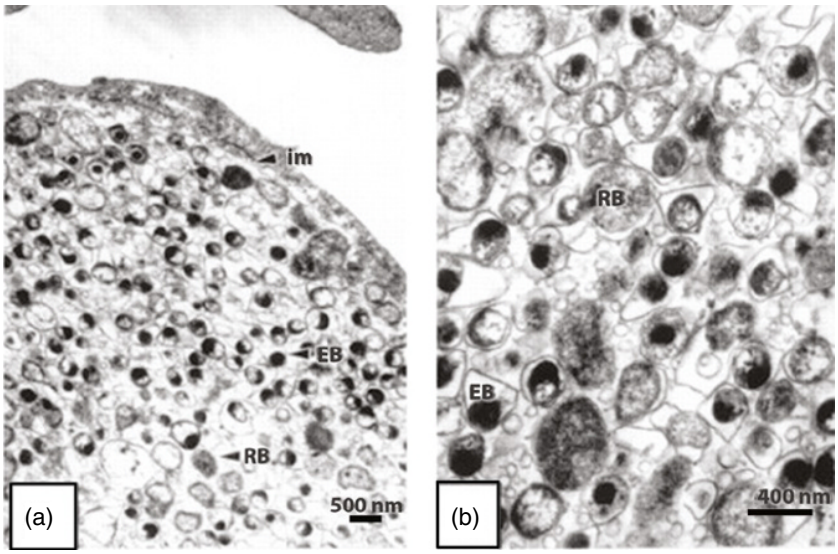


Figure 37.2 *C. pneumoniae* infection in HEp-2 cells (a and b) containing typical inclusions inside phagosomes of the infected cells. EB, elementary bodies; RB, reticulate bodies; im, inclusion membrane. *Source:* Reproduced from Kutlin et al. (2001) with permission from the American Society for Microbiology.

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38

Mollicutes

Bonto Faburay and D. Scott McVey

Introduction

The mollicutes taxonomically belong to the class Mollicutes (Latin: *mollis*, soft; *cutis*, skin). This includes the orders Mycoplasmatales, Entomoplasmatales, Acheloplasmatales, Haloplasmatales, and Anaeroplasmatales. Mollicutes are characterized by the lack of a cell wall and a small genome size of 0.58–1.4 Mbp. Whereas formerly the trivial name "mycoplasma" commonly denoted any member of the class Mollicutes, it now refers exclusively to a member of the genus *Mycoplasma*. Members of the genus *Acholeplasma* are sometimes encountered, but usually as contaminants. Mollicutes are ubiquitous in nature and have been found in mammals, reptiles, fish, arthropods, insects, and plants living on and in host cells. They are also common tissue-culture contaminants. It is likely that only a small fraction of the total number of mollicutes have been identified, as it seems that wherever one searches, a new species can be found. Only the genera *Mycoplasma* and *Ureaplasma* within the family Mycoplasmataceae have pathogenic species of veterinary importance. Over 113 animal mycoplasmas and 5 ureaplasmas have been identified, with many more at the "candidatus" stage, and of these more than 45 complete genomes have been sequenced. In addition, hemotrophic rickettsial species of the genera *Haemobartonella* and *Eperythrozoon* were reclassified to the genus *Mycoplasma* in 2001, based on 16S ribosomal RNA gene sequence analysis. They have collectively been given the common name "hemoplasmas."

Infections with mollicutes are parasitic in nature. They are most often subclinical to mild but severely debilitating, and sometimes fatal diseases can also be observed. Most pathogenic mycoplasmas and ureaplasmas infect the mucosa of the respiratory and/or urogenital tract, but are also found in other body sites such as the conjunctiva, synovial surfaces, and mammary glands. The hemoplasmas infect red blood cells and most commonly cause hemolytic anemia.

Descriptive Features

Morphology and Staining

The cell morphology of the mollicutes is extremely pleomorphic. Cell shapes include spherical, ring-shaped, pear-shaped, spiral-shaped, and filamentous forms. Cells sometime appear as chains of beads, the result of asynchronized genomic replication and cell division. Ring forms found in red blood cells infected with hemoplasmas have also been observed. The diameter of the spherical form ranges from 0.3 to 0.8 μm. Due to the size and shape of these organisms, they are able to pass through 0.22 and 0.45 μm membrane filters commonly used to filter sterilize liquid media; therefore, it is difficult to decontaminate tissue-culture cell lines from these organisms.

Mollicutes stain poorly by the Gram method due to their lack of a cell wall. Giemsa, Castaneda, Dienes, cresyl-fast violet, orcein, and acridine orange stains are preferred.

Structure and Composition

The mollicutes lack the genetic capacity to produce a cell wall. Instead, they are bound by a single trilaminar membrane composed of proteins, glycoproteins, lipoproteins, phospholipids, and sterols. Cholesterol in the membrane provides osmotic stability. Carbohydrate capsules have been described for some species.

The mollicutes have a small genome (ranging from 540 to 1380 kb) relative to other bacteria and are the smallest self-replicating organisms that have yet been described. Their base composition is low in guanine and cytosine content, with their G + C content ranging from 24% to 40%. They are related most closely to the *Clostridium–Streptococcus–Lactobacillus* group, from which it is

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presumed the mollicutes evolved through a process of reductive or degenerative evolution. Transposons, plasmids, and bacteriophages have been demonstrated in some species.

Nonhemotrophic Mollicutes

The nonhemotrophic mollicutes include members of the genus *Ureaplasma* and those of the genus *Mycoplasma* that do not colonize red blood cells. Unlike the hemotrophic mollicutes (see Section 38.16), they can be grown *in vitro* on axenic (lifeless) media. Clinical manifestations include respiratory and urogenital tract infections, conjunctivitis, arthritis, mastitis, septicemia, and otitis media. Most species exhibit a high degree of host specificity, but infections across host species are also observed, most commonly in immunocompromised individuals (Table 38.1).

Growth Characteristics

The nonhemotrophic mollicutes are generally slow growing and often require 3–10 days (or more) of incubation before colonies are apparent on agar. *In vitro* growth is generally optimal at 33–38 °C in an atmosphere of increased CO_2. Mollicutes lack many biosynthetic pathways and are unable

Table 38.1 Pathogenic *Mycoplasma* species and their primary host(s).

Animal species	Agent	Disease
Feline (cats)	*Mycoplasma felis*	Conjunctivitis
	Mycoplasma feliminutum	Respiratory disease
	Mycoplasma gatae	Arthritis
Bovine	*Mycoplasma alkalescens*	Arthritis, mastitis
	Mycoplasma bovigentialium	Infertility, mastitis, seminal vesiculitis
	Mycoplasma bovis	Abscesses, arthritis, mastitis, otitis, pneumonia
	Moraxella bovoculi	Keratoconjunctivitis
	Mycoplasma californicum	Arthritis, mastitis
	Mycoplasma canadense	Arthritis, mastitis
	Mycoplasma dispar	Alveolitis, bronchiolitis
	Mogibacterium diversum	Infertility, pneumonia, vulvovaginitis
	Mycoplasma mycoides ssp. mycoides (SC)	Contagious bovine pleuropneumonia (CBPP)
	Mycoplasma wenyonii	Anemia
Avian (chickens)	*Mycoplasma gallisepticum*	Respiratory disease
	Mycoplasma synoviae	Airsacculitis, sternal bursitis, synovitis
Canine	*Mycoplasma canis*	Urogenital tract disease
	Mycoplasma cynos	Pneumonia
	Mycoplasma spumans	Arthritis
Felids (wild cats)	*Mycoplasma felifaucium*	Respiratory disease
	Mycoplasma leocaptivus	Respiratory disease
	Mycoplasma leopharyngis	Respiratory disease
	Mycoplasma simbae	Respiratory disease
Caprine	*Mycoplasma agalactiae*	Agalactiae, arthritis, conjunctivitis
	Mycoplasma capricolum ssp. capricolum	Arthritis, mastitis, pneumonia, septicemia
	M. capricolum ssp. capripneumoniae	Contagious caprine pleuropneumonia (CCPP)
	Mycoplasma conjunctivae	Keratoconjunctivitis
	M. mycoides ssp. mycoides (LC)	Abscesses, arthritis, mastitis, septicemia
	M. mycoides ssp. capri	Pneumonia
	Mycoplasma putrefaciens	Arthritis, mastitis

(*Continued*)

Table 38.1 (Continued)

Animal species	Agent	Disease
Equine	*Mycoplasma equigenitalium*	Infertility
	Mycoplasma equirhinis	Respiratory disease (suspected)
	Mycoplasma fastidiosum	Respiratory disease (suspected)
	M. felis	Pleuritis
	Mycoplasma subdolum	Infertility
Murine (Mice)	*M. neurolyitcum*	Conjunctivitis, neurological disease
	Mycoplasma pulmonis	Respiratory disease
Murine (Rats)	*Mycoplasma arthritidis*	Arthritis
	M. pulmonis	Respiratory disease, genital tract disease
Ovine	*M. agalactiae*	Contagious agalactiae
	M. conjunctivae	Keratoconjunctivitis
	Mycoplasma ovipneumoniae	Pneumonia
Swine	*Mycoplasma hyopneumoniae*	Enzootic pneumonia
	Mycoplasma hyorhinis	Arthritis, pneumonia, polyserositis
	Mycoplasma hyosynoviae	Arthritis
Avian (Turkeys)	*M. gallisepticum*	Sinusitis, respiratory disease
	Mycoplasma iowae	Embryo mortality, leg deformities
	Mycoplasma meleagridis	Airsacculitis, decreased egg hatchability, perosis
	M. synoviae	Sternal bursitis, synovitis

to synthesize amino acids, and have either a complete or partial inability to synthesize fatty acids. Exogenous sterols are required by mycoplasmas that they acquire from serum. Because of the high nutritional demands of the mollicutes, media supporting growth of the various mycoplasmas and ureaplasmas are often highly enriched and complex. Most are based on beef heart infusion, peptone, yeast extract, and serum along with various other supplements. Optimal pH for growth ranges from 6.0 to 8.0 depending on the normal host environment in which they are found. Mollicute colonies are small and difficult to visualize with the unaided eye. Colony sizes vary from 0.01 to 1.0 mm. When observed with a dissecting microscope, many mycoplasmas exhibit a "fried-egg" morphology (Figure 38.1). This umbonate appearance is the result of the central portion of the colony embedding into the agar with a peripheral zone of surface growth. Some species produce film spots, which are composed of cholesterol and phospholipids and appear as a wrinkled film on the media surface. Differences in colony morphology and size can be used to distinguish some of the mollicutes. For example, the colony size of *Mycoplasma mycoides* ssp. *mycoides* isolates from goats is consistently larger than those isolated from cattle. This size difference is used to distinguish between the two variants. *Ureaplasma* species produce substantially smaller colonies than other mollicutes and often lack the fried-egg colony morphology.

Ecology

Reservoir and Environmental Stability

The major reservoir for the nonhemotrophic mollicutes is the host they infect. Subclinically infected animals carry organisms on mucosal surfaces, including the upper and lower respiratory tracts, the urogenital tract, conjunctiva, alimentary canal, mammary glands, and/or joints. In general, mollicutes can survive outside the host for substantial lengths of time in moist, cool environments. They are

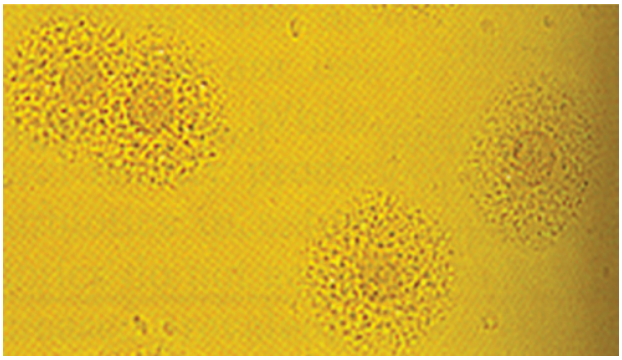


Figure 38.1 Fried-egg colony morphology that is typical of many *Mycoplasma* species. *Source:* Courtesy of Dr. M. M. Chengappa, Kansas State University.

very susceptible to heat and most detergents (tween) and disinfectants (quaternary ammonium, iodine, and phenol-based compounds).

Transmission and Epidemiology

Although there are several clinically and economically important species of mollicutes, primary pathogens such as *M. mycoides* ssp. *mycoides*, small colony (SC), variants in cattle are the exception rather than the rule. Mollicutes most often cause mild to moderate disease that can be exacerbated by cofactors including age, genetic predisposition, environmental conditions, crowding, stress, and concurrent infections. Minimizing predisposing stress factors will minimize disease. Virulence varies not only among species but also among strains within species. This also accounts for some of the observed variations in disease manifestation.

Transmission occurs predominately by spread from animal to animal through direct contact and is mediated by aerosolization of respiratory secretions or venereal transmission. Introduction of an infected animal into an uninfected population is a common source for dissemination and spread of infection. Asymptomatic carriers, usually colonized on mucosal surfaces, serve as the source for maintaining organisms within a population. Long-distance spread of infectious *Mycoplasma* can occur through wind under optimal conditions. For instance, viable *Mycoplasma hyopneumoniae* has been recovered >9 km from an infected swine herd source. Mechanical transmission through milking machinery is a means for spreading bovine and caprine *Mycoplasma* mastitis, and contaminated milk can be a source of infection for calves and goat kids. In poultry, vertical transmission through hatching eggs is an important means of spread for many of the pathogenic avian species. Little is known regarding the role of ectoparasites and/or flying insects in transmission; however, pathogenic caprine species have been isolated from ear mites of goats.

Pathogenesis and Immunity

Mollicutes employ a variety of strategies in order to occupy such a wide range of host niches and to maintain themselves as chronic, parasitic infections. These organisms have a complex relationship with their host(s) especially with regard to immunity, making it difficult to separate the pathogenesis of the organism from the host's own immune response against them. In fact, the immune response of the host is intimately involved in the pathogenesis of disease (immunopathology).

The chronic course of mollicute infections suggests that the immune response is not effective at eliminating infection once it is established, and latent infections are common. In the face of an intense inflammatory response in the acute stage of infection, mycoplasmas can generally avoid elimination and at least a number of hosts maintain long-term infections. Antigenic variation is a mechanism employed by some mollicutes to evade host defenses. The pMGA gene family, which accounts for 16% of the entire genome of *Mycoplasma gallisepticum*, generates antigenic variants of a major surface protein. Incorporation of host antigens by mycoplasmas, a condition referred to as capping, further helps some mollicutes escape detection by the immune system. Phase shift in *Mycoplasma hyorhinis* helps evade the host immune response by varying the length of surface proteins so that they are less accessible to immune cells. Alternatively, antigens shared between *Mycoplasma* species and the host tissues may result in a biological mimicry, whereby the host recognizes the *Mycoplasma* as self, leading to persistent infections. Similarly, shared host and *Mycoplasma* antigens, such as the galactans found in the lungs of cattle and in *M. mycoides* ssp. *mycoides*, can also result in autoimmune disease. Another mechanism to achieve carrier state in a host is to form biofilms, which are a community of individual organisms living in a matrix of extracellular polymeric substance as a means of avoiding the host immune response and antibiotic therapies. Several mycoplasmas readily form biofilms (Figure 38.2a). Underlying factors such as age, overcrowding, concurrent infections, and transportation stresses lead to overt disease.

Attachment to host cells is the first step in establishing infection and is facilitated by the anionic surface layer on most mycoplasmas (Figure 38.2b). Host receptors for attachment are surface proteins, especially glycoconjugates that allow colonization of mucosal surfaces. In some species such as *M. gallisepticum*, attachment is mediated through a specialized tip structure. A polar bleb has been demonstrated in some species, including *M. gallisepticum*, and also has a role in adherence to host cell surfaces. These blebs aid in the motility of mycoplasmas that possess them, which is referred to as gliding motility. There is evidence that some *Mycoplasma* and *Ureaplasma* species can penetrate and exist inside nonphagocytic cells, protecting them from the immune system and facilitating their dissemination within the host. Some mycoplasmas that cause pneumonia are able to induce ciliostasis, which damages and impedes the effectiveness of the mucociliary escalator, aiding colonization.

Downregulation of the innate immune response, such as inhibition of macrophage phagocytic activity, has been associated with some *Mycoplasma* infections. Alternatively, lipoproteins abundant in the plasma membrane of mollicutes activate the innate immune system through Toll-like

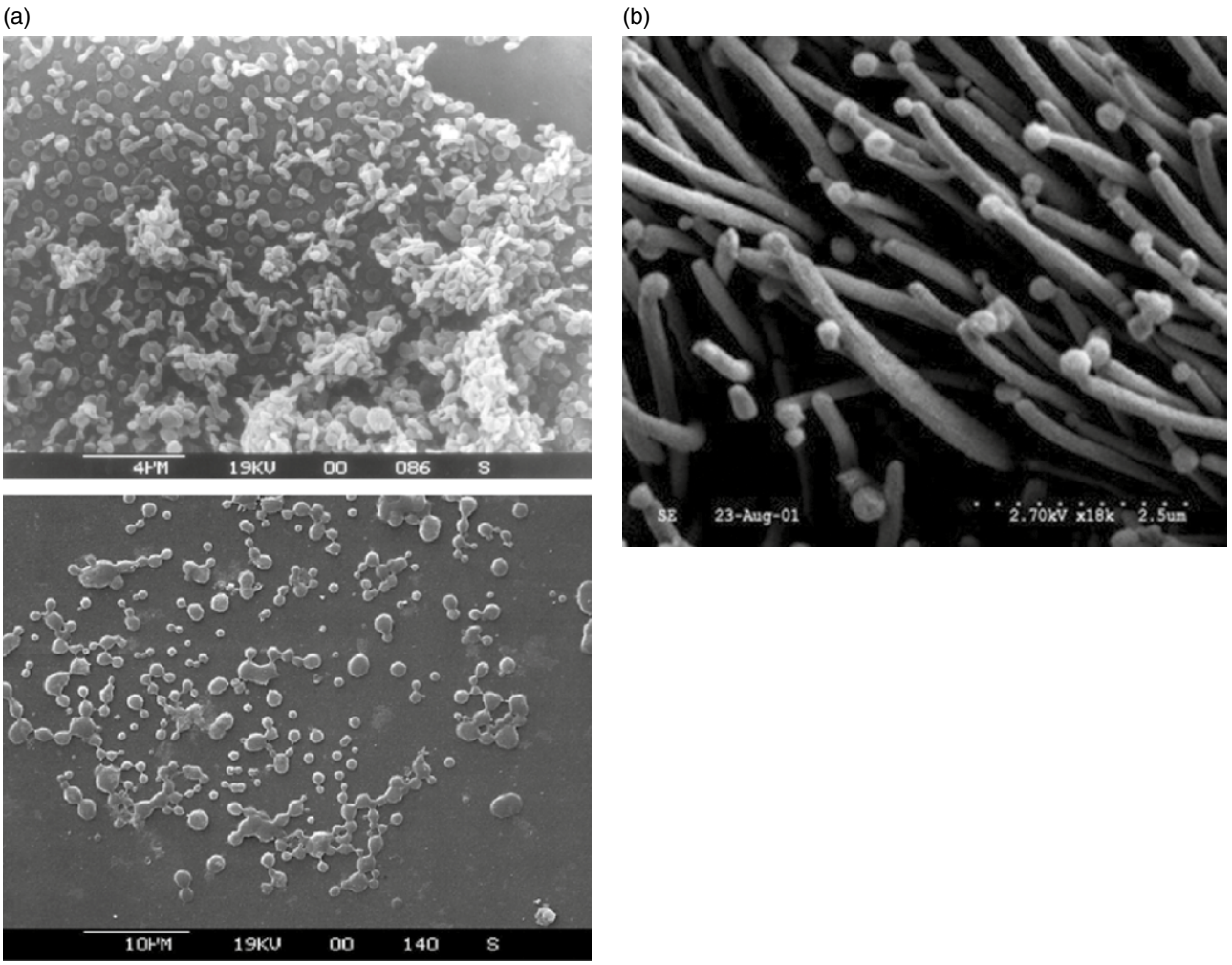


Figure 38.2 (a) Scanning electron micrograph analysis of biofilm formation by *Mycoplasma bovis* (left panel) and *Mycoplasma mycoides* subsp. mycoides SC (right panel) cultures grown on glass coverslips. *Source:* Courtesy of Dr. L. McAuliffe. (b) Electron micrograph of *Mycoplasma hyopneumoniae* attached to swine ciliated respiratory tract epithelium. *Source:* Courtesy of Dr. Carlos Pijoan.

receptors 2 and 6 and stimulate a pro-inflammatory response mediated by the production of cytokines and various other factors. In addition, mycoplasmas activate the complement cascade by the classical pathway, which contributes to the inflammatory response. The inflammatory response results in damage to bystander host cells that may benefit the mycoplasmas by freeing nutrients from host cells that these parasitic organisms require for growth while the ensuing immune response does not effectively clear the organisms.

Injury to host tissue may also be induced through other mechanisms. Products of metabolism such as hydrogen peroxide and superoxide radicals in mycoplasmas result in local host cell damage. The ureaplasmas have potent enzymes as well, including phospholipases and urease. Persistence of antigen in selected sites, such as joints, allows for further damage due to development of an immune-complex-mediated inflammatory response. Induction of interleukin 1 (IL-1), IL-6, IL-12, IL-18, and tumor necrosis factor from activated macrophages leads to activation of cytotoxic T-lymphocytes and results in an endotoxin-like effect in the host. Acute, septicemic forms of disease can result in a coagulopathy and widespread vascular thrombosis, which resembles a gram-negative septicemia and is, at least in part, mediated through induction of cytokines.

Both the cellular and humoral immune systems respond to infection by mollicutes, and each has been demonstrated to play a role in controlling the number of *Mycoplasma* organisms present in the host. Yet, similar to the innate immune response, mollicutes are able to subvert these host response mechanisms as well. For example, *M. bovis* has been shown to suppress lymphocyte responses; conversely, many *Mycoplasma* species have been shown to possess unique mitogenic factors that induce nonspecific polyclonal B- and/or T-cell stimulation. *Mycoplasma*

arthritidis possesses a small peptide, MAM, which acts as a superantigen and stimulates a broad population of T-lymphocytes. Although the mechanism of action has not been elucidated for all suppressive and mitogenic factors utilized by mollicutes, it is clear that they are important in the pathogenesis of these organisms.

Disease Patterns and Pathology

Infections can manifest in a variety of ways including septicemias, disseminated infections involving multiple sites, or localized infections. Diseases and common clinical manifestations caused by the different pathogenic species in major animal species are listed in Table 38.1. In addition to specific effects related to *Mycoplasma*, generalized effects on the immune system may increase susceptibility to secondary infections with other bacterial and viral pathogens.

The lesions associated with *Mycoplasma* infections vary from acute to chronic and are dependent on the agent involved and the site affected. In acute infections, there is an inflammatory reaction with an infiltration of neutrophils, macrophages, and lymphocytes and an accumulation of fibrin. An infiltration of lymphocytes and plasma cells is often observed in *Mycoplasma* infections, particularly around vessels, airways, and in the submucosa. Peribronchial, peribronchiolar, and perivascular lymphoplasmacytic cuffing is a characteristic finding in respiratory tract infection. The profound perivascular and peribronchiolar lymphoid hyperplasia observed in many infections is due to nonspecific mitogenic effects as well as to a specific anti-mycoplasmal immune response.

Generalized infections lead to a fibrinopurulent exudate on serosal surfaces and synovial membranes. In persistent localized infections, tissue destruction can be substantial. Abscesses may develop at pressure sites in calves and are characterized by an eosinophilic coagulative necrosis with peripheral fibrosis. In cases of *Mycoplasma* mastitis, pockets of purulent exudate may develop in affected mammary tissue. Eventually the affected gland becomes fibrosed. In the acute stage of *Mycoplasma* infections of the joint, the joint becomes distended with fluid containing fibrin. As infections become chronic, there is villus hypertrophy of the synovia and a proliferative and erosive arthritis develops.

Mycoplasma Infections in Animals

Avian

Mycoplasmosis in poultry has important economic consequences. *M. gallisepticum* causes a chronic respiratory disease in chickens and infectious sinusitis in turkeys, and infects a number of other domestic avian species. Clinical signs include coughing, nasal discharge, and tracheal rales. Turkeys can develop sinusitis with production of a thick, mucoid exudate that results in severe swelling of the infraorbital sinuses (Figure 38.3). Natural outbreaks of conjunctivitis in house finches due to *M. gallisepticum* have caused substantial reductions in the finch population on the eastern coast of the United States. Occasionally, clinical signs related to brain and joint involvements are recognized. Decrease in egg production also occurs. *Mycoplasma synoviae* also infects a wide range of avian species. Synovitis resulting in lameness, swelling of joints and tendon sheaths, and retarded growth are common presentations. Sternal bursitis is also frequently observed in turkeys. Airsacculitis, which is usually subclinical, is another manifestation. *Mycoplasma meleagridis* and *Mycoplasma iowae* infections are mostly limited to turkeys. *M. meleagridis* causes respiratory disease, predominately an airsacculitis, which is often clinically mild or inapparent. Skeletal deformities, including bowing or twisting of the tarsometatarsal bone and cervical vertebrae, are occasionally detected. Decreased egg hatchability is a serious consequence of *M. meleagridis* infections. Airsacculitis, leg deformities, and stunting in poults have been demonstrated with *M. iowae*. Decreased egg hatchability has also been noted with *M. iowae* infections.

Bovine

M. mycoides ssp. *mycoides* SC variant was isolated in 1898 as the first *Mycoplasma* species and considered the most

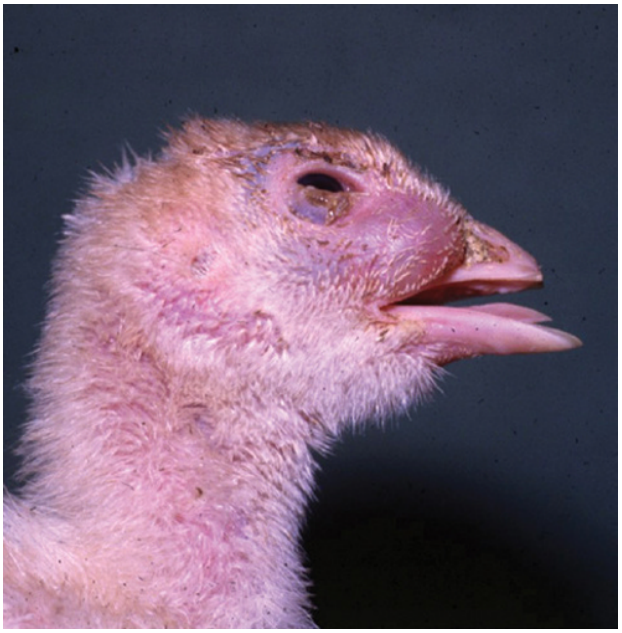


Figure 38.3 *Infraorbital sinusitis in a turkey caused by* M. gallisepticum. *(Courtesy of Dr D. Trampel.)*

virulent of the bovine mycoplasmas. It is the causative agent of a respiratory disease, contagious bovine pleuropneumonia (CBPP), in cattle. Susceptible cattle become infected by inhaling droplets disseminated by coughing in affected cattle, and the disease manifests in a variety of symptoms ranging from a persistent, subclinical infection to an acute, sometimes fatal disease. Mortality can reach 50% in herds experiencing the disease for the first time. The organism can also be found in saliva, urine, fetal membranes, and uterine discharges. Transplacental infection can also occur. Viability of the organism is poor in the environment in ambient temperature. After reaching near global spread in the nineteenth century, the disease was eradicated from most continents through stamping-out policies. However, it has remained in many countries in Africa.

Clinical signs include respiratory distress, coughing, nasal discharge, and reluctance to move. Most infections are limited to the respiratory tract, although arthritis occurs in calves. The incubation period varies, but most cases occur three to eight weeks. after exposure. In some localities, susceptible herds may show up to 70% morbidity, but much lower infection rates (~10%) associated with clinical signs are more common. Mortality is likely to be ~50% in herds experiencing the disease for the first time. In acute cases, the animal suffers fever with temperature up to 107 °F (41.5 °C), anorexia, and painful difficult breathing. In a hot tropical climate, the animal often stands by itself in the shade, its head lowered and extended, its back slightly arched, and its elbows turned out (Online Figure 1). At necropsy, the affected portion is enlarged and solid. On section of the lung, the typical marbled appearance of pleuropneumonia is evident because of the thickened interlobular septa and subpleural tissue that encloses gray, yellow, or red consolidated lung lobules (Online Figure 2), Percussion of the chest is painful with rapid respiration, shallow and abdominal. The disease progresses rapidly, and the animal loses condition, with breathing becoming very labored and distressed often accompanied by a grunt expiration. Subsequently, the animal becomes recumbent and dies after one to three weeks. Cattle that are chronically infected usually exhibit signs of varying intensity for three to four weeks, after which the lesions gradually resolve, and the animals appear to recover. Subclinically, affected animals serve as a source for maintaining and spreading infection in the herd. Infected calves may present with polyarthritis that exhibits as swelling of joints and lameness.

Mycoplasmal Mastitis

M. bovis is currently recognized as one the most important and frequently isolated *Mycoplasma* species associated with disease in cattle worldwide. The pathogen was first isolated in the United States in 1961 from a severe case of mastitis in a dairy herd experiencing an outbreak affecting more than 30% of the animals. In general, *Mycoplasma* mastitis is caused by a number of species, although *M. bovis* is the most common cause and results in the most severe disease. *Mycoplasma californicum* and *Mycoplasma canadense* are also frequently involved. *Mycoplasma alkalescens* and *Mycoplasma bovigenitalium* have also been implicated as etiologic agents on occasion. Typically, there is a drop in milk production. The milk becomes thick and intermixed with a watery secretion and may progress to a purulent exudate. The udder is often swollen, although not painful. Sometimes all four quarters are involved. It is a destructive mastitis and often refractory to treatment. Most infections are limited to the mammary gland; however, arthritis subsequent to bacteremia occurs. In some cases, disseminated infection results in periarticular involvement and fasciitis. Spread from cow to cow is directly related to inadequate management and sanitation practices.

Respiratory Tract Infections

Mycoplasma respiratory tract infections in calves often present as pneumonia in association with other bovine respiratory pathogens (Figure 38.4). *M. bovis* is usually the predominant species recovered from respiratory cases. Other mycoplasmas of clinical significance include *Mycoplasma dispar*, which causes a mild respiratory disease characterized by bronchiolitis and alveolitis and is usually precipitated by environmental stresses or a primary viral infection. Both *M. bovis* and *M. dispar* can be recovered as commensals from the upper respiratory tract.

Urogenital Tract Infections

Urogenital tract infections are caused by *M. bovigenitalium* and *Ureaplasma diversum*. Seminal vesiculitis in bulls and



Figure 38.4 Pneumonia in a cow caused by *M. bovis*. *Source:* Courtesy of Dr. R. Rosenbusch.

granular vulvitis, vaginitis, endometritis, infertility, and abortion in cows are associated with both of these organisms. Both are found as normal commensals in the lower urogenital tract.

Arthritis in calves occurs sporadically. Although a number of different species can cause arthritis, *M. bovis* is most frequently recovered. Other less common presentations include otitis media and decubital abscesses. Otitis media usually occurs in conjunction with respiratory disease. Decubital abscesses have been associated with confined housing conditions. *M. bovis*, again, is the usual agent.

Canine

A number of *Mycoplasma* species have been isolated from dogs; however, little is known regarding their role in disease. Experimental and clinical evidence suggests that *Mycoplasma canis* can cause urogenital tract disease including prostatitis, cystitis, endometritis, orchitis, and epididymitis. The role of *Mycoplasma* in reproductive disorders of the bitch is uncertain although infertility has been associated with mycoplasmal infections, especially in kennels with poor hygiene. Canine infectious respiratory disease (CIRD) is a common respiratory infection found mostly in kenneled animals. *Mycoplasma cynos* is the primary *Mycoplasma* associated with the disease and is found to exacerbate the pathogenesis of CIRD. *Mycoplasma spumans* has been reported to cause arthritis.

Mycoplasma Infections in Small Ruminants

Mycoplasma infections are quite common in small ruminants. Although various *Mycoplasma* species have been isolated from both sheep and goats, it appears that caprine species account for majority of pathogenic infections described in literature.

Caprine

Mycoplasma infections in goats are economically important and can result in disease of epizootic proportion. Some of the most important and recognized ones include the following. Contagious caprine pleuropneumonia (CCPP) is a highly contagious and rapidly spreading mycoplasmal disease of goats, occasionally sheep and wild ruminants, caused by *Mycoplasma capricolum* subspecies *capripneumoniae (Mccp),* originally known as the F38 biotype. The disease affects a vast majority of goat populations in Africa, the Middle East, and Asia. CCPP has been identified as an emerging transboundary infectious disease that poses epidemic threat to non-endemic countries due to regular trade with affected countries. The disease has been detected in Turkey and poses a constant threat to the European Union, Armenia, Georgia, and Russia. The disease has not been reported in North and South America.

Clinical Signs

CCPP is characterized by severe serofibrinous pleuropneumonia, very high morbidity up to 100% and 80–100% mortality. The disease manifests in peracute, acute, and chronic forms. In the peracute form, goats may die within one to three days with minimal clinical signs. In acute form, clinical signs include fever (41–43 °C), lethargy, and anorexia, followed within two to three days by coughing and labored breathing. The cough is frequent, violent, and productive. In the final stages, the goat stands with its front legs wide apart and its neck stiff and extended (Online Figure 3). Often saliva can drip continuously from the mouth, and the animal may bleat in pain. Frothy nasal discharge may be seen. Pregnant goats may abort. Death usually ensues within 7–10 days. Chronic cases manifest as chronic cough, nasal discharge, and debilitation.

M. mycoides ssp. *mycoides* (large colony variant) infections present as a mastitis, pneumonia, bursitis, or arthritis in adult animals. Some develop a generalized toxic disease that can be fatal. A rapidly fatal septicemia is common in kids. Those that survive develop a chronic, destructive arthritis and/or bursitis. *M. mycoides* ssp. *capri* causes a pleuropneumonia similar to that of goat strains of *M. mycoides* ssp. *mycoides*. It has been proposed that *M. mycoides* ssp. *mycoides* (large colony variant) be combined with *M. mycoides* ssp. *capri* as a single subspecies *capri* based on the high DNA homology they share. Septicemia, arthritis, and mastitis occur with *M. capricolum* ssp. *capricolum* infections. Both *Mycoplasma agalactiae* and *Mycoplasma putrefaciens* cause mastitis. The mastitis due to *M. putrefaciens* is purulent in nature, whereas infections with *M. agalactiae* result in a decrease or total cessation in milk production. Both species can cause arthritis.

Mycoplasma conjunctivae causes a keratoconjunctivitis that presents with lacrimation, conjunctival hyperemia, and keratitis. Pannus is sometimes evident

Ovine

M. agalactia is the cause of contagious agalactia in small ruminants, primarily sheep, which is endemic in most Mediterranean countries and parts of Africa. The disease has been reported in Brazil and the Canary Islands but rarely in the United States. Upon introduction into a flock, the infection spreads rapidly. In addition to primary mastitis, *M. agalactia* may also cause arthritis, septicemia, keratoconjunctivitis, and pneumonia, most notably in naïve populations. Pregnant animals typically exhibit more severe clinical signs and have

a higher morbidity rate than intact male animals, which is thought to be associated with immunosuppressive effects of progesterone. Mortality rates may reach 25%. Affected animals commonly manifest signs of depression and severe mastitis several days after parturition.

Mycoplasma ovipneumoniae is the causative agent of ovine nonprogressive pneumonia. With the agent first isolated in New Zealand in 1974, the atypical pneumonia caused by the pathogen has been described as such to be different from the more severe and progressive enzootic pneumonia caused by *Pasteurella* spp. The atypical pneumonia is most common in lambs 2–12 months of age and presents as paroxysmal coughing, which predisposes lambs to rectal prolapse and reduced weight gain. Naïve animals are more susceptible and may exhibit more severe clinical signs. It should be mentioned that agalactic mastitis caused by *M. agalactiae* is similar to that observed in goats. Sheep can also be infected with many of the other species that affect goats.

M. conjunctivae has been implicated in outbreaks of keratoconjunctivitis in sheep.

Equine

Mycoplasma felis is the only species that has been conclusively associated with disease in the horse. It is recovered from the upper respiratory tract as a commensal, but can cause a pleuritis, usually related to some exertional activity. The pleuritis is self-limiting and frequently resolves spontaneously. *Mycoplasma equirhinis* and *Mycoplasma fastidiosum* have been suspected of causing respiratory disease, but this has not been confirmed. *Mycoplasma equigenitalium* and *Mycoplasma subdolum* have been identified in the genital tract of both stallions and mares and are implicated as a cause of infertility in mares.

Feline

A variety of commensal mycoplasmas have been recovered from mucosal surfaces of cats. Relatively few are associated with disease. *Mycoplasma gatae* has been recovered from cats with arthritis. *M. felis* has been associated with a serous to mucoid conjunctivitis. Typically, the conjunctiva is edematous; however, the cornea is not involved. A *Mycoplasma*-like organism has been associated with subcutaneous abscesses, but neither the disease nor the organism has been well characterized. *Mycoplasma* spp. have been isolated as part of the polymicrobial component from pyothorax exudates.

Murine

Mycoplasma pulmonis causes a low-grade respiratory disease in rats. Infections involve the nasal cavity, middle ear, larynx, trachea, and lungs. The most common clinical sign is a low-pitched wheezing or snuffling resulting from the purulent nasal exudate. In mice, clinical signs are often inapparent, although a chattering sound and continued rubbing of the eyes and nose may suggest infection in the colony. Mortality is low, and when it occurs is related to pneumonia. Genital tract infections with *M. pulmonis* are also recognized in rats. *M. arthritidis* causes a polyarthritis in rats and mice, although many infections are subclinical. Experimental infections in mice result in joint swelling and, in some cases, posterior paralysis. Natural infections with *Mycoplasma neurolyticum* generally do not cause disease, although conjunctivitis has been reported. Experimental inoculation with *M. neurolyticum* or cell-free filtrates causes a neurologic syndrome referred to as rolling disease.

Porcine

A number of clinical entities are associated with *Mycoplasma* infections in swine. *M. hyopneumoniae* causes a chronic respiratory disease referred to as "porcine enzootic pneumonia." There is high morbidity, but low mortality associated with this disease. The principal clinical sign is a chronic nonproductive cough, and pigs exhibit ill-thrift and impaired weight gain. *M. hyopneumoniae* is a significant contributor to the porcine respiratory disease complex (PRDC). The PRDC has a severe negative impact on swine production, and *M. hyopneumoniae* has been shown to potentiate the disease caused by other agents in this complex, such as the porcine reproductive and respiratory syndrome virus. *M. hyorhinis* causes a systemic infection in pigs between 3 and 12 weeks of age. Initial signs include fever, inappetence, and listlessness. Arthritis and lameness frequently follow. There is a characteristic polyserositis that involves pleural, peritoneal, and pericardial serosa. *Mycoplasma hyosynoviae* causes arthritis in growing pigs 12–24 weeks of age (Figure 38.5). Lameness and associated difficulty with mobility are the principal clinical signs.

Laboratory Diagnosis

Sample Collection

The appropriate sample for isolation attempt is determined by the clinical presentation and includes exudates, swabs from affected sites, affected tissues, and milk. Because of the fastidious nature of mollicutes, samples should be submitted to the laboratory as soon as possible after collection. During transportation, samples should be kept cool and moist. Various commercially available media (Stuart's and Amies' without charcoal) are suitable for transporting swabs. If a prolonged transport time

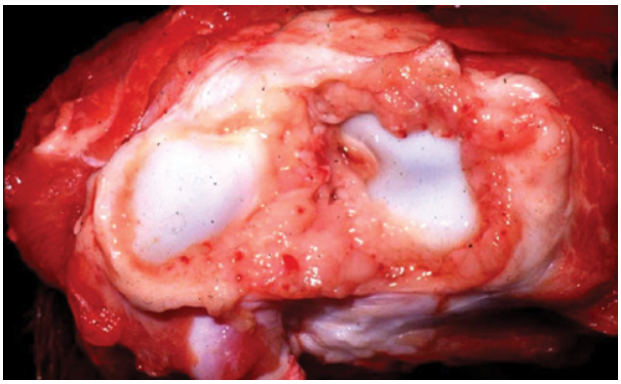


Figure 38.5 Arthritis in a pig with villous synovial tissue proliferation caused by *Mycoplasma hyosynoviae*. *Source:* Courtesy of Noah's Archive, https://davisthompsonfoundation.org/noahs-arkive/. Original contribution of Dr. E. Harrington, Purdue University.

(>24 hours) is expected, samples should be shipped frozen and preferably on dry ice or in liquid nitrogen.

Direct Examination

The variability in microscopic morphology and poor staining with the Gram method make direct examination for most mollicutes unrewarding. Direct fluorescent antibody tests and DNA fluorochrome staining have been described, particularly for diagnosing conjunctivitis and mastitis, but they are not widely used.

Immunoperoxidase, immunofluorescent, and immunohistochemistry staining of histopathologic sections has been used successfully for identification of some species in tissues, including *M. bovis* in cattle tissues, *M. hyopneumoniae* in pig lungs, and some of the poultry mycoplasmas.

Microbial Culture Isolation

The identification and detection of *Mycoplasma* species has traditionally been performed via microbial culture. However, due to their structural simplicity, mycoplasmas are unable to synthesize amino acids and have complete or partial inability to synthesize fatty acids. In order to meet this high nutritional demand, the media used to grow mycoplasmas is specific and often highly enriched with beef heart infusion, serum, yeast extract, peptone, and other supplements with buffering to a final pH of 7.3–7.8 required. No one media formulation is suitable for growth of all of the mollicutes. The media selected should be based on the specific species or group of species of interest. In general, a fairly complex and enriched medium is required. Serum is the usual source of sterols and is required by most species. Different species, however, grow better with different sources of serum. Yeast extract is included as a source of growth factors. Growth of some species is enhanced or requires incorporation of specific substances such as vaginal mucus (*M. agalactiae*) and nicotinamide adenine dinucleotide (*M. synoviae*). Some goat mycoplasmas grow on sheep blood agar as small colonies with an α-type ("greening") hemolysis. Mollicutes are resistant to a number of antibiotics and growth inhibitors such as nafcillin, bacitracin, cefobid, thallium acetate, and amphotericin B are commonly added to media to inhibit contaminating bacteria and fungi. Specific immune sera directed against commensal mycoplasmas can be incorporated in media to allow for selective isolation for pathogenic species. For optimal recovery, samples are inoculated into both a liquid and a solid media and incubated at 36–38 °C in 5–10% CO_2 for at least seven days. Some species require longer incubation times. Semen and joint fluids may contain inhibitory factors and should be diluted prior to culture to enhance recovery. Blind passages from broth to broth for up to three passages may enhance the recovery. *Ureaplasma* species are susceptible to pH changes as a result of hydrolysis of urea included in the media and must be subcultured frequently to maintain their viability when isolation attempts are made. None of the hemoplasmas are cultivable.

It is important to note that for *Mycoplasma* infection to be diagnosed by culture, the animal must be shedding viable organisms at the time of sample collection. Intermittent shedding is frequently reported with chronic and subclinical *Mycoplasma* mastitis cases, which can result in a diagnosis failure from a single sample.

Microbial Culture Identification

Agar cultures must be examined with the aid of a dissecting microscope. Colonies with the typical umbonate morphology can be stained directly on the agar to differentiate them from other bacteria or can be smeared on a glass slide. Mollicutes stain poorly by the Gram method due to their lack of a cell wall. Dienes is a commonly used stain. The mollicutes stain blue because of their inability to reduce methylene blue in the stain. Other bacteria reduce methylene blue by using it as a hydrogen acceptor in maltose oxidation, and therefore appear colorless with the Dienes stain. The exceptions are L-form bacteria, which exhibit a similar colony morphology and staining reaction as the mollicutes. L-forms must be differentiated from mollicutes by demonstrating reversion of the L-form bacteria back to a walled form. Other stains used to detect mollicutes include Giemsa, Castaneda, Wright's, cresyl-fast violet, orcein, and acridine orange.

Digitonin sensitivity can be used to distinguish *Mycoplasma* and *Ureaplasma* from *Acholeplasma*. A large zone of inhibition around paper disks saturated with 1.5% digitonin will be present with *Mycoplasma* and *Ureaplasma*, but only a small

or no zone is observed with *Acholeplasma*. Commonly used biochemical tests to further characterize isolates include detection of phosphatase activity, fermentation of glucose, and hydrolysis of arginine or urea.

Serological Detection and Identification

Although some antigens are shared among the mollicutes, antigenic differences are usually sufficiently specific to allow for species identification. A number of identification methods have been employed based on either the ability of specific antisera to inhibit growth or metabolism or the demonstration of reactivity with specific antisera using either a fluorescence or chromogen-based detection system. Growth inhibition tests employ antisera-impregnated disks or antisera placed in wells in media and demonstrating a zone of inhibition. Metabolic inhibition tests use growth inhibition in liquid media and a color change based on pH as an indicator system. Other test procedures that have been used to demonstrate specific reactivity are direct or indirect immunofluorescence on colony impressions, colony epifluorescence, and immunoperoxidase staining of colonies on agar plates.

A number of immunodiagnostic tests have been developed for many of the important *Mycoplasma* diseases, although problems with sensitivity, especially related to asymptomatic carriers, are common and can complicate interpretation of results. Lack of specificity as a result of cross-reacting antibodies is also a problem.

Enzyme-linked immunosorbent assays, plate agglutination, and hemagglutination inhibition tests are routinely used to detect flock infections with *M. gallisepticum*, *M. meleagridis*, and *M. synoviae* in poultry and are an important part of overall eradication programs used by commercial poultry operations. Because *M. bovis* is currently considered the most common and important species, much of the assay development and research available is specific to this species. This includes commercially available indirect ELISA kits for anti-*M. bovis* antibody detection for use on serum and milk samples.

Molecular Diagnostics

Fast replacing the traditional assays described earlier are molecular assays, primarily based on the polymerase chain reaction (PCR). Amplification of specific DNA sequences by PCR, or less frequently restriction endonuclease analysis of PCR products, has been used for identification and characterization of isolates. A number of PCR methods have been described for identification of pathogenic species directly from clinical material that bypasses the need for culture.

Conventional PCR

The use of PCR to detect *Mycoplasma* species from various sample types has demonstrated a higher efficiency, specificity, and sensitivity for laboratory diagnosis when compared with conventional culture-based diagnostic methodologies. The 16S rRNA gene is one of the most common genes targeted for bacterial identification due its presence in all bacteria and its unchanged function over time. Results are usually produced within 24 hours, making the PCR method more rapid and increasingly preferred to culture. However, although the specificity of the PCR assays targeting the 16S rRNA gene of *M. bovis* appeared to be adequate against most *Mycoplasma* species, less specific amplification was seen with *M. agalactiae*, a species affecting small ruminants. Further development of 16S rRNA gene PCR assays allowed the detection of other species such as *M. dispar* and *Mycoplasma bovirhinis*.

Real-Time PCR

The main approaches for real-time PCR utilized for diagnostics of *Mycoplasma* species utilizes SYBR green dye and fluorescent reporter probes. A real-time multiplex PCR assay allowing the simultaneous detection of *M. bovis*, *M. californicum*, and *M. bovigenitalium* in a single reaction has also been explored. Specificity testing showed specific amplification for all *M. bovis*, *M. californicum*, and *M. bovigenitalium* isolates, with no amplification against other Mollicutes and Eubacterial isolates tested.

Isolate Typing

Several molecular tools are used for identification and genetic characterization of *Mycoplasma* species. For instance, pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (RFLP), multiple locus variable number tandem repeat analysis (MLVA), and multilocus sequence typing (MLST) have been used to characterize *M. bovis* isolates and other mycoplasmal species. PFGE has been widely used for *Mycoplasma* strain typing and identification; the technique involves initial digestion of chromosomal DNA across a several numbers of sites using a

restriction enzyme, resulting in large DNA fragments being separated in an agarose gel producing banding patterns specific to different strains. PFG has been employed in several *Mycoplasma* outbreak investigations.

Whole Genome Sequencing

Whole genome sequencing using next generation sequencing (NGS) is increasingly used for investigating bacterial genome sequences as sequencing becomes faster, cheaper, and more readily available. The entire genome of several *Mycoplasma* species has been sequenced and is used to aid clinical diagnostics, disease outbreak investigation and antimicrobial resistance control. Complete genome sequences are available for *M. bovis* and *M. californicum* isolates, as well as a *Mycoplasma arginini*, *M. bovigenitalium*, *M. canadense*, *M. bovoculi*, and *M. leachii* isolates are available. The availability of these genome sequences will provide opportunities to compare the genetic diversity between many isolates.

Treatment, Control, and Prevention

Current understanding of the immunology related to *Mycoplasma* and *Ureaplasma* infection(s) is very incomplete, and the necessary components of a protective immune response to these organisms are not well defined. Both killed and attenuated vaccines have been shown to be at least partially protective against *Mycoplasma*-induced disease, but do not provide complete immunity. Vaccinated animals demonstrate fewer lesions and have a reduced number of organisms present relative to nonvaccinated animals, but disease is still observed in both groups. Some vaccine preparations have actually resulted in increased disease after subsequent infection. The mechanism by which this occurs is not known.

An attenuated vaccine is used to protect cattle in areas where CBPP is enzootic. Immunization in endemic countries in Africa is mostly carried out using the attenuated vaccine, T1/44 strain. Protection lasts for approximately 18 months. There are increasing concerns about the efficacy of this vaccine due to possible appearance of antigenically diverse strains. Attenuated and killed, adjuvanted vaccines have been used with variable success to control some caprine infections, specifically those caused by *M. agalactiae*, *M. mycoides* ssp. *capri*, and *M. capricolum* ssp. *capripneumoniae*. Killed vaccines for *M. hyopneumoniae* in swine reduce the severity of mycoplasmal pneumonia, but do not prevent disease or colonization. In poultry, live (attenuated strain) and inactivated vaccines are employed to control egg production losses and respiratory disease associated with *M. gallisepticum* infections. No vaccines for ureaplasmas or hemoplasmas are currently available.

Antibiotics are used to treat *Mycoplasma* and *Ureaplasma* infections. The lack of a cell wall renders mollicutes resistant to the action of antimicrobial agents that target the cell wall or its synthesis, such as the glycopeptides and β-lactams, but are sensitive to compounds that interfere with protein and nucleic acid synthesis. Mycoplasmas show sensitivity to the following antibiotics: tetracyclines, macrolides, lincosamides, ketolides, aminoglycosides, cephalosporins, pleuromutilins, and fluoroquinolones. Treatment success varies depending on the species involved, the affected site, and the time course of the disease. Isolates of mycoplasmas and ureaplasmas have been identified with resistance to many of these antibiotics, which is mediated through a variety of mechanisms. The incidence of antibiotic resistance is not well documented because of the difficulties in culturing these organisms that is necessary for most antibiotic susceptibility assays, and this testing is only performed by specialized laboratories.

Control measures depend on the disease status of the country, specific disease, and the animal species infected. Diseases such as CBPP and CCPP, which affect large populations of animals, are controlled by test and slaughter of affected herds in countries attempting to maintain a disease-free status. Vaccination, culling of infected animals, and management changes to prevent dissemination are employed in countries where the disease is enzootic. In general, because of the poor success in treating infected animals, culling of clinically ill animals is often employed as a control measure in infected populations where test and slaughter are not feasible. Routine culturing of bulk tanks is used to monitor for *Mycoplasma*-induced mastitis in cow and goat herds. Animals identified as shedding organisms in the milk are usually culled. Industry-driven efforts, particularly for poultry, have outlined measures to eliminate or prevent infection. Attempts to eradicate infections, particularly in breeding flocks, include serological testing and elimination of positive flocks and antibiotic treatment of hatching eggs to produce *Mycoplasma*-free chicks. Treatment of eggs involves immersing warmed eggs in a chilled antibiotic solution, which promotes antibiotic penetration into the egg.

Preventing infection should be based on following strict biosecurity practices to preclude introduction of infected animals into a *Mycoplasma*-free herd. New animals should be quarantined and tested before being mixed with the herd, but subclinically infected animals can be difficult to identify. Taking animals to shows and fairs and returning them to the herd may also serve to introduce infection. Good hygiene and management practices are important in preventing spread among animals where infections are enzootic. Because milk can be a source of infection, especially in goats and cattle, it should be pasteurized to prevent infecting young animals in the herd.

Table 38.2 Hemotrophic *Mycoplasma* species and their primary host.

Animal species	Agent	Disease
Cat	*Mycoplasma haemofelis*	Feline infectious anemia
Cattle	*Mycoplasma wenyonii*	Anemia
Dog	*Mycoplasma haemocanis*	Anemia
Mice	*Mycoplasma coccoides*	Anemia
	Mycoplasma haemomuris	Anemia
Sheep	*M. ovis*	Anemia
Swine	*Mycoplasma suis*	Anemia

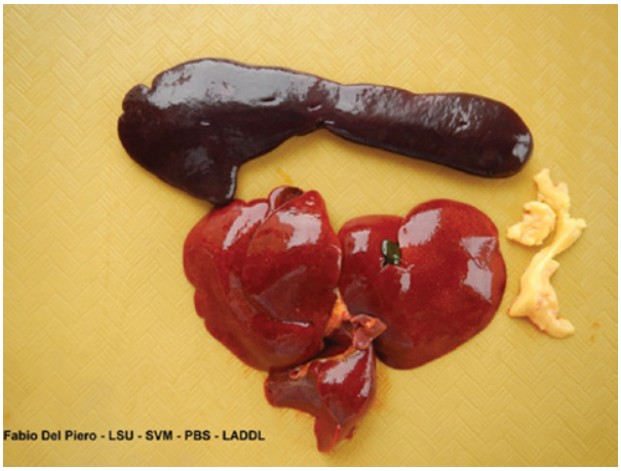


Figure 38.6 *Mycoplasma haemofelis* infected cat – hepatomegaly and splenomegaly with lipid icterus. *Source:* Courtesy of Noah's Archive, https://davisthompsonfoundation.org/noahs-arkive/. Original contribution of Dr. Fabio Del Piero, Louisiana State University.

Hemotrophic Mollicutes

The hemotrophic mollicutes include microorganisms previously included in the genera *Haemobartonella* and *Eperythrozoon* (Table 38.2). All are parasites of red blood cells and produce hemolytic anemia, which can be observed in animals of any age, but is often observed in immunocompromised or otherwise stressed individuals. Subclinically infected animals are the source of hemoplasmas, and they are spread by blood-to-blood contact. Several members of this group are transmitted by ectoparasites. Unlike the non-hemotrophic mollicutes, they have not been cultivated on axenic media.

Infections with the hemoplasmas may cause icterus, splenomegaly, and/or bone marrow hyperplasia (Figure 38.6). Diagnosis is based upon clinical signs, demonstration of the agent in stained (e.g. Wright's, Giemsa, or acridine orange) smears of peripheral blood, or detection by PCR. Treatment includes correction of the hemolytic anemia and antibiotic treatment, usually with tetracyclines.

Acknowledgment

Revised from the chapter *Mollicutes* by E. Strait and M. Madsen in the third edition.

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39

Rickettsiaceae and Coxiellaceae: *Rickettsia* and *Coxiella*

Roman R. Ganta

Descriptive Features

The order Rickettsiales includes small obligate intracellular gram-negative bacteria. Two families within this order are Anaplasmataceae and Rickettsiaceae, both of which include several important pathogens causing diseases in various vertebrate animals and people. The family Anaplasmataceae of the class Alphaproteobacteria includes intraphagosomal pathogens of the genera *Anaplasma, Ehrlichia*, and *Neorickettsia*. These organisms look very similar microscopically, as they reside within a phagosome of an infected host cell. The cell tropism is a major distinguishable feature for identifying the organisms in a vertebrate host. The family Rickettsiaceae includes pathogens of the genus *Rickettsia* that invade vascular endothelium. The family Coxiellaceae of the class Gammaproteobacteria includes one pathogen, *Coxiella burnetii*, which infects macrophages. *C. burnetii* is similar to Rickettsiales as it is primarily an obligate pathogen that replicates within a phagosome vacuole of the infected host cell cytoplasm.

Most of the Rickettsiales, except *Ehrlichia* and *Anaplasma* species, have a cell wall like most other gram-negative organisms. The pathogens respond to antibiotics (such as tetracycline derivatives and chloramphenicol), and possess ribosomes, DNA, and RNA. They are small coccid to short rod-shaped bacteria. The organism ranges from 0.2 to 0.5 μm in diameter and from 0.8 to 2 μm in length. Giemsa or other polychromatic stains are used to detect the organisms microscopically. These aerobic pathogenic bacteria are responsible for many fatal and chronic infections in animals and people. They do not grow in standard bacteriological media. *In vitro* growth requires the close association with eukaryotic cells such as yolk sacs of chicken embryos, macrophages, or epithelial cells of vertebrate or arthropod vectors. The only exception is *C. burnetii*, which can be cultivated in the absence of a host cell in a conditioned medium. The pathogens replicate by binary fission inside a phagosome or in the cytoplasm. They depend heavily on their host for energy (ATP) and other nutrients. Some pathogens (e.g. *Rickettsia* species) have an ATP/ADP translocase with which they obtain ATP molecules from the host in exchange for ADPs.

The pathogenicity of Rickettsiales is not well defined and may be complex because they represent multiple species having widely different host specificities and host cell associations. Virulence may be due to the release of endotoxins (lipopolysaccharides), production of immune complexes, and hypersensitivity reactions. The pathogens mostly damage vascular endothelium or hematopoietic cells and severely weaken the host defense mechanisms. Rickettsiales are highly pathogenic and are responsible for high mortality rates in humans and animals. Typical diagnosis is based on the clinical presentation, detection of organisms in specific host cell types and cell organelles, vector associations, serology, and molecular techniques. Culture isolation methods are available for some bacteria, but are time-consuming and are not practical for routine diagnosis. However, *in vitro* culture methods serve as excellent research tools for studies on these pathogens. Vertebrate hosts induce a host response, including humoral and cell-mediated immunity. Antibodies cross-react between species; thus, it is difficult to use an antibody test as a specific diagnostic tool. Inactivated or live attenuated vaccines are available for some pathogens, but appear to lose effectiveness rapidly, probably due to continuous changes in the expression of their outer membrane proteins. Control may be best achieved by controlling the vector burden for some of the pathogenic infections.

The pathogens of veterinary importance of the family Rickettsiaceae are discussed in this chapter. Anaplasmataceae family pathogens are discussed in Chapters 40 and 41.

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Two major pathogens of the genus *Rickettsia* are *Rickettsia rickettsii* (the disease-causing agent of Rocky Mountain spotted fever [RMSF] in dogs and humans in the Americas) and *Rickettsia prowazekii* (epidemic typhus disease agent in people). The parasitic bacteria of the genus *Rickettsia* infect the vascular endothelium. Transmission from arthropod vectors to vertebrate hosts is achieved when arthropods take a blood meal or through open wounds or skin abrasions. The capillary endothelium is damaged, thus producing hemorrhagic skin rashes. Hallmark symptoms of acute disease are fever, petechial rashes, and intractable headache. The organisms escape the phagosome and multiply freely in the cytoplasm or nucleus. The genus *Coxiella* includes one major pathogen, *C. burnetii*. This organism is transmitted from one host to another, primarily by means of aerosol transmission, although tick transmission is another means of acquiring infections. This organism differs from *Rickettsia* species in how it grows in the host cell cytoplasm. It replicates within the cytoplasmic phagocytic vacuoles of under acidic conditions.

Rickettsia rickettsii

Ecology

R. rickettsii is responsible for the century-old disease, RMSF, in dogs and humans. RMSF is primarily found in North, Central, and South America. *R. rickettsii* is transmitted by various tick species, including *Dermacento* species ticks. Other likely tick vectors are *Amblyomma americanum* and *Rhipicephalus sanguineus*. Cell-to-cell migration of *R. rickettsii* from within the cytoplasm of infected cells occurs because of polymerization of host cell actin induced by the pathogen (Figure 39.1). Infection may result in a spectrum of diseases ranging from subclinical to severe or fatal multiorgan collapse. The disease agent is maintained in nature in small mammals and ticks. Canines and humans acquire infections primarily during blood feeding by infected ticks.

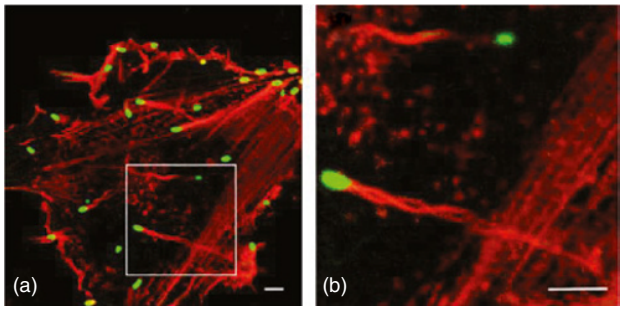


Figure 39.1 (a) *Rickettsia rickettsii* showing long actin tails that are frequently comprised multiple, twisting, distinct F-actin bundles. (b) High magnification of an *R. rickettsii* actin tail in panel A comprising two F-actin bundles. *Source:* Reproduced with permission from the American Society for Microbiology; Van Kirk et al. (2000).

Pathogenesis

Clinical signs in dogs with *R. rickettsii* infections may include decreased appetite and severe depression, as judged by the lack of interest in socializing. Infected dogs may also have petechial rashes on the ears, gums, buccal mucosa, and testes. The rashes and inflammation can be prominent on the testes as evidenced by the presence of swelling of scrotal sacs and darkened scrotal skin within a week post infection. Dogs may also develop edema in various organs, with the facial region and feet becoming edematous. Most severe cases of RMSF result in death within two weeks of acquiring the infection. A significant drop in hemoglobin, RBC numbers, and packed cell volume and elevated levels of monocytes and neutrophils are also observed in animals with severe disease. *R. rickettsii* organisms persist in blood, which can be detected by *in vitro* amplification molecular methods, such as polymerase chain reaction (PCR). Differentiation of RMSF cases from canine ehrlichiosis by clinical signs during the early stages of infection can be difficult (described in Chapter 41). Both tick-borne diseases are characterized by fever, depression, and lymphadenopathy. Distinctive red rashes, particularly in humans, are one of the important symptoms of RMSF. RMSF cases, like most other tick-borne illnesses, are recognized in summer months as they result from high tick activity. Younger dogs and children develop the more rapid and severe RMSF clinical disease.

R. rickettsii replicates within the cytoplasm of the epithelium of an infected tick and is transferred to the salivary glands and ovarian tissue. After an infected tick consumes a blood meal on a host, the pathogen enters the bloodstream of that animal and infects the vascular endothelium by endocytosis and subsequently escapes from the phagosome and multiplies within the cell cytoplasm and nucleus. Rickettsial phospholipase and proteases damage endothelial cell membranes, leading to necrosis, vasculitis, hemorrhage, edema, perfusion inadequacies, thrombosis, and dyspnea. Infected dogs present clinical signs that include high fever (40 °C), anorexia, vomiting, diarrhea, petechiated or ecchymotic mucous membranes, and tenderness over lymph nodes, joints, and muscles. Central nervous system disturbances may also occur. In some instances, heart and kidney involvement leads to fatal outcomes. Immune complexes are suspected in the pathogenesis of late vascular manifestations of RMSF. No vaccine is available for RMSF, although a recent study reports the potential for developing a bacterial whole cell inactivated-antigen-based vaccine.

Laboratory Diagnosis

Indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA) are most commonly performed to detect antibodies specific to rickettsial antigens, and the detectable antibody response may take at least two weeks following the rickettsial infection. However, by this point infected animals may die due to disease severity. The organisms may also be detected in the host endothelial cells by direct immunofluorescence assays. Molecular methods such as the PCR are used to detect *R. rickettsii* DNA in ticks and dogs.

Treatment and Control

Treatment with tetracycline, doxycycline, or chloramphenicol for 14–21 days is effective against *R. rickettsii* infections. Infected dogs with clinical signs must also receive aggressive supportive therapy. Control of RMSF is also better achieved by reducing the tick burden and exposure.

Epidemic Typhus Fever

Epidemic typhus, caused by *R. prowazekii*, is a highly fatal human disease. It caused more than 3 million deaths during the last century, with a considerable number of fatalities occurring during major wars such as the World Wars I and II. This louse-borne typhus spreads rapidly in crowded areas under cold weather and unhygienic conditions. The mortality rate with this agent is very high (>30%). The organism persists in wild animals such as wild squirrels.

Coxiella burnetii

Ecology

C. burnetii is responsible for the zoonotic disease Q fever in humans. This organism, which has a global distribution, differs from Rickettsiales as it is transmitted by aerosol as well as from infected ticks. Cattle, sheep, and goats are the primary reservoirs of *C. burnetii* infection. This pathogen does not usually cause clinical disease in these animals, although abortions in goats and sheep are implicated in *C. burnetii* infections. The organisms in infected animals are excreted into milk, urine, and feces. During birth, the organisms are shed in high numbers within amniotic fluids and the placenta. *C. burnetii* is resistant to heat, drying, and to many common disinfectants, which enables it to survive for long periods in the environment. Moreover, inhalation of a single organism can cause the disease in a person.

Human infections usually occur by inhalation airborne organisms from barnyard dust contaminated by dried placental material, birth fluids, and excreta of infected herd animals. Humans, particularly those in close contact with the animals shedding the organisms, are often very susceptible to this disease. Just one organism is sufficient to cause infection and disease. About one-half of all people infected with *C. burnetii* show signs of clinical illness. Most acute cases of Q fever begin with the sudden onset of one or more of the following: high fevers, severe headache, general malaise, myalgia, confusion, sore throat, chills, sweats, nonproductive cough, nausea, vomiting, diarrhea, abdominal pain, and chest pain. Fever usually lasts for one to two weeks. Weight loss can occur and persist for some time. *C. burnetii* is a highly infectious agent that is also resistant to heat and drying. It can become airborne and be inhaled by humans. Because a single *C. burnetii* organism may cause disease in susceptible humans, it could be developed for use in biological warfare and is considered a potential bioterrorism agent. However, as the fatality rate is low, it is not a serious cause of concern.

Pathogenesis

C. burnetii is acquired through inhalation, ingestion, or from a tick and gains access to lung macrophages. It replicates within a phagosome of a macrophage, similar to *Ehrlichia* and *Neorickettsia* species. About 50% of people acquiring the pathogen exhibit no clinical signs or show mild, self-limiting infection. Less than 10% may develop severe disease with signs including vasculitis, pneumonitis, splenomegaly, fever, and lymphocytosis. Mortality in human cases is primarily due to pneumonia, hepatic infection, or endocarditis.

Immunologic Aspects

A vaccine is available in some countries but is reported to have poor efficacy. A whole cell killed vaccine may be protective. There is considerable interest in the development of a Q fever vaccine, as it is classified as a select agent.

Laboratory Diagnosis

C. burnetii can be isolated following injection into guinea pigs and mice, cultured in chicken eggs, or grown in cell cultures. This is not a useful diagnostic tool. Indirect immunofluorescence, complement fixation, or ELISA tests are used to detect antibodies. In cell culture, eggs, and experimental animal tissues, direct immunofluorescent staining aids in identifying the organisms. PCR–based molecular

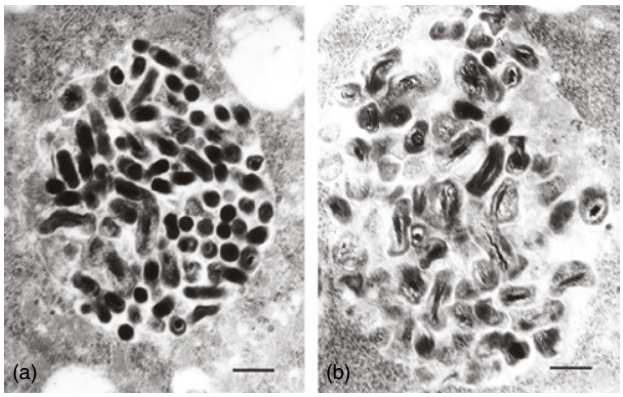


Figure 39.2 Transmission electron micrograph image showing a typical vacuole in J774A.1 cells infected with *C. burnetii* (100 nm). Image in panel a contains small variants, and the image in panel b contains mostly large variants. *Source:* Reproduced with permission from the American Society for Microbiology; Howe and Mallavia (2000).

assays are also reported for detecting the *Coxiella* DNA from a sample. In culture, *C. burnetii* replicates within a phagosome. The two forms of the organism are (i) the replicating large variant and (ii) the non-replicating infectious form, referred to as the small variant (Figure 39.2).

Treatment and Control

The major challenge for successful therapy is that the phagosome where *C. burnetii* persists is highly acidic, and most antimicrobial agents are not effective at low pH. Alkalinizing cells by using chloroquine has been shown to improve clinical efficacy of tetracycline. Drugs with moderate efficacy for *C. burnetii* infection include tetracycline, chloramphenicol, clarithromycin, enrofloxacin, and trimethoprim-sulfa. Long-term tetracycline feeding is considered an attempt to control shedding of *C. burnetii* organisms from animals. Pregnant sheep and goats may be treated with tetracycline to reduce the risk of abortions.

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40

Anaplasmataceae: *Anaplasma*

Roman R. Ganta

Descriptive Features

The order Rickettsiales includes small, pathogenic, obligate intracellular gram-negative bacteria. Two families within this order belonging to the class Alphaproteobacteria are Anaplasmataceae and Rickettsiaceae. Several bacteria from both families are responsible for causing diseases in various vertebrate animals and humans. The family Anaplasmataceae of includes intraphagosomal pathogens of the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia*. These organisms look very similar when examined microscopically as they reside within a phagosome of an infected host cell. The cell tropism is a major distinguishable feature for identifying the organisms in a vertebrate host. The family Rickettsiaceae includes pathogens of the genus *Rickettsia* that invade vascular endothelium.

Rickettsiales multiply only inside a host cell (intracellular). Most of the Rickettsiales, except *Ehrlichia* and *Anaplasma* species, have a cell wall like most other gram-negative organisms, respond to antibiotics (such as tetracycline derivatives and chloramphenicol), and possess ribosomes, DNA, and RNA. They are small coccoid to short rod-shaped bacteria. The organism ranges from 0.2 to 0.5 μm in diameter and from 0.8 to 2 μm in length. Giemsa or other polychromatic stains are used to stain the organisms. These aerobic organisms cause many fatal and chronic infections in animals and humans. They do not grow in standard bacteriological media. *In vitro* growth requires close association with eukaryotic cells such as macrophages or epithelial cells originating from a vertebrate host, tick cell cultures, yolk sacs of chicken embryos, or arthropod vectors. The pathogens replicate by binary fission inside a phagosome or within the cytoplasm. They depend heavily on their host for energy (ATP) and other nutrients.

The pathogenicity of Rickettsiales is not well defined and may be complex because they represent multiple genera having widely different host specificities and host cell associations. Virulence may be due to the release of endotoxins (lipopolysaccharides), production of immune complexes, and hypersensitivity reactions. The pathogens mostly damage vascular endothelium or hematopoietic cells and severely weaken the host defense mechanisms. Rickettsiales are highly pathogenic and are responsible for high mortality rates in humans and animals. Typical diagnosis is based on the clinical presentation, detection of organisms in specific host cell types and cell organelles, serology, and molecular techniques. Culture isolation methods are available for some bacteria, but are time-consuming and are not practical for routine diagnosis. However, *in vitro* culture methods serve as excellent research tools. Vertebrate hosts induce both humoral and cellular responses. Several species share extensive antigen similarity. Antibodies cross-react between species; thus, it is a challenge to use antibody-mediated tests as specific diagnostic tools. Inactivated or live attenuated vaccines are available for some pathogens, but appear to lose effectiveness rapidly, probably due to continuous changes in the expression of their outer membrane proteins. Protection may be best achieved by controlling the vector burden for some of the pathogen infections.

The pathogens of veterinary importance of the genus *Anaplasma* from the family Anaplasmataceae are discussed in this chapter. Pathogens of the genera *Ehrlichia* and *Neorickettsia* are discussed in Chapter 41, and the Rickettsiaceae family pathogens are discussed in Chapter 39. The genus *Anaplasma* includes the species *Anaplasma marginale* and *Anaplasma centrale* (both are bovine erythrocytic pathogens), *Anaplasma ovis* (the causal agent of ovine anaplasmosis, also infecting erythrocytes),

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Anaplasma platys (canine cyclic thrombocytopenia agent, which infects thrombocytes), and *Anaplasma phagocytophilum* (granulocytic pathogen that causes disease in a broad range of hosts, including cattle, horses, dogs, and humans).

A. marginale

Descriptive Features

In Giemsa- or polychromatic-stained blood smears, *A. marginale* organisms appear as purple inclusions within the cytoplasm of bovine erythrocytes and are typically located near the periphery of erythrocytes (Figure 40.1). These marginal (inclusion) bodies are membrane-bound phagocytic vacuoles containing few organisms. The organism can be serially propagated by needle inoculation of infected blood from one animal to the other. Recent studies also reported the establishment of *in vitro* cultivation and serial passaging of the organism *Ixodes scapularis* in tick cell culture under aerobic conditions.

Ecology

Reservoir and Transmission

Infected ruminants are the primary reservoirs of infection for *A. marginale*. Although many vertebrates can be infected, this organism is the major cause of illness in cattle, through tick transmission and mechanical transmission.

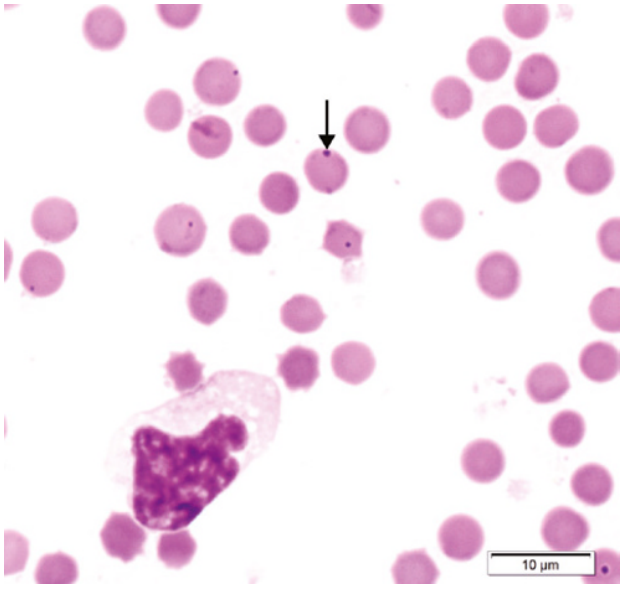


Figure 40.1 *A. marginale* in bovine erythrocytes (Wright–Giemsa stain, 1000×).

Bovine anaplasmosis is reported from all continents. Transmission from contaminated needles, dehorning procedures, or from the use of other surgical instruments is a major source of the disease spread from infected animals to naïve animals. In nature, most often transmission occurs from infected ticks, such as *Dermacentor variabilis*, when taking blood meals on naïve animals. Transmission by blood-sucking flying insects, such as horseflies, is another means of mechanical transmission.

Pathogenesis

Infectious organisms enter erythrocytes by endocytosis after adhering to the cell surface through its cell-surface-expressed proteins and replicate within a phagosome by binary fission. New infectious organisms are released from the infected erythrocytes by lysing the cells. Rapid loss of red blood cells occurs, and in most severe cases, the erythrocyte loss accounts for up to 50% drop. Clinical signs of bovine anaplasmosis vary from diarrhea, fever, anemia, increased heartbeat, anorexia, depression, constipation, abortion, muscle weakness, myocardial hypoxia, and finally to cardiac arrest.

The severity of the disease varies and depends on the age of the infected animal. Typically, infected calves less than six months old exhibit no clinical signs, whereas cattle from six months to three years may develop a serious illness. Cattle over three years old can experience high mortality rates up to 30–50%. However, animals that recover from the disease establish persistent, chronic infections with no apparent signs of illness. The chronically infected animals serve as the source of infection for spread to naïve animals. Bovine anaplasmosis is generally seen in cattle one year of age or more.

Immunologic Factors

A. marginale–infected animals induce both humoral and cell-mediated responses. Several major surface proteins are expressed by the pathogen during its growth in erythrocytes. During the organism's persistent growth in cattle, it varies the expression of some of its major antigenic surface proteins. The antigenic variation is likely to aid in the pathogen's escape from host immune clearance. Animals that recover from the early clinical stage of infection appear normal, but the infection persists, serving as reservoirs of infection for transmission to ticks and naïve animals. Thus, immunity developed against the clinical disease is not an indication that the pathogen has been cleared.

Infection with *A. centrale* causes a milder disease. Animals infected with *A. centrale* are also protected against infection with *A. marginale*. Infection followed by

treatment with tetracyclines is considered a good method of conferring protection against bovine anaplasmosis clinical disease, but this type of vaccine does not induce sterile immunity. Although whole-organism-derived inactivated antigens are used as a vaccine in the United States, its efficacy in protecting against bovine anaplasmosis is not defined. Currently, research is underway at Kansas State University on the development of a live attenuated vaccine with good protection against virulent infection challenge.

Laboratory Diagnosis

A. marginale is demonstrable in erythrocytes of a blood smear following the staining with polychromatic stain (Figure 40.1). Immunofluorescence assays can be performed to detect antibodies raised in animals infected with *A. marginale*. Blood-smear-staining-based detection of organisms may not be useful to monitor infection beyond the clinical phase of the disease, which occurs after several weeks. Molecular techniques such as the polymerase chain reaction (PCR), utilizing pathogen gene-specific primers, is the best technique for the sensitive detection of *A. marginale*.

Treatment and Control

Tetracycline is the most effective antibiotic against *A. marginale* infection in cattle.

Descriptive Features

In polychromatic-stained thick blood smears, *A. phagocytophilum* appears in neutrophils in phagosomes within the cytoplasm as morulae consisting of several organisms (Figure 40.2). The infection is common among ruminants, horses, dogs, and humans. *A. platys* infection occurs within canine platelets (Figures 40.3 and 40.4) and causes a cyclic thrombocytopenia. This organism may be visible in thick blood smears as well. Blood smear positives may be seen during the early clinical phages of infection for both organisms. Clinical diseases with these pathogens, as with most other tick-borne illnesses, are most likely reported during summer months. *A. phagocytophilum* is transmitted to vertebrate hosts from *Ixodes* species ticks, whereas the vector tick for A. platys remains to be established, but is most likely the brown dog tick, *Rhipicephalus sanguineus*.

Ecology, Reservoir, and Transmission

Rodents are considered the primary reservoirs of *A. phagocytophilum* infection. The reservoirs of infection may also be significantly different in different regions. In the eastern region of North America, the white-footed mouse (*Peromyscus leucopus*) and the *I. scapularis* tick are the primary reservoir host and vector, respectively. In the US West Coast, *Ixodes pacificus* is considered the major vector. In Europe, the primary transmitting vector is *Ixodes ricinus*, and various rodent species are implicated as reservoirs of infection. The reservoir host(s) and vector(s) for *A. platys* remain to be determined, although the pathogen transmission to dogs is likely caused by *R. sanguineus*.

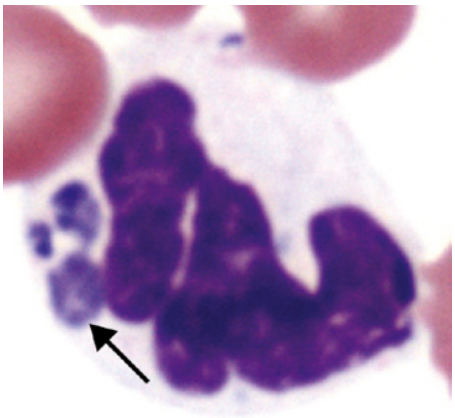


Figure 40.2 *A. phagocytophilum* infection (morula) in the cytoplasm of a neutrophil in blood from an infected dog (Wright–Giemsa stain). *Source:* Reproduced with permission from Harvey (2012).

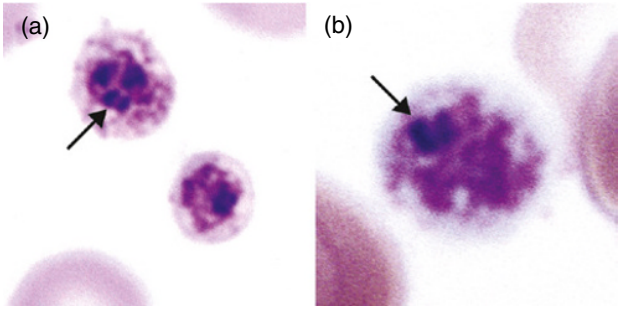


Figure 40.3 *A. platys* infection (morula) in the cytoplasm of platelets in blood from an infected dog (Wright–Giemsa stain). The panels a and b have two different images of A. platys in blood smears prepared from infected dogs. *Source:* Reproduced with permission from Harvey (2012).

Pathogenesis

Granulocytic infection with *A. phagocytophilum* is originally documented in ruminants in Europe, where it used to be referred to as tick-borne fever. Later, pathogenic infections were reported in North American horses and referred to as the equine ehrlichiosis agent, *Ehrlichia equi*. The pathogen also infects humans and dogs in North America.

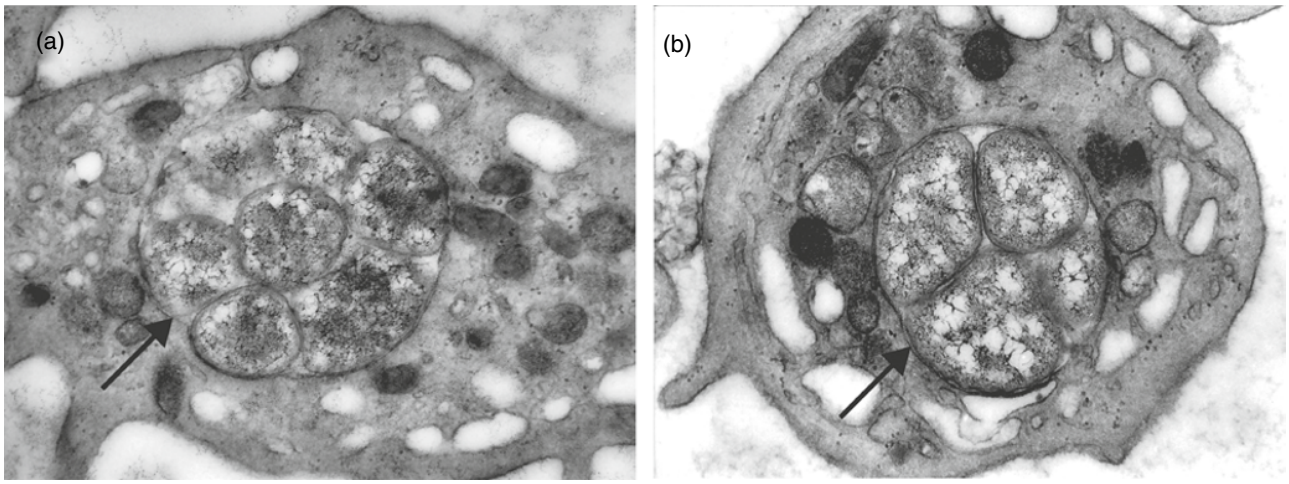


Figure 40.4 Transmission electron micrograph of *A. platys* infection (morula) in the cytoplasm of platelets in blood from an infected dog, with morulae containing (a) multiple and (b) four visible organisms. *Source:* Reproduced with permission from Harvey et al. (1978).

It is now commonly known as *A. phagocytophilum,* the human/equine/canine granulocytic anaplasmosis agent. All hosts develop vasculitis associated with thromboses, thrombocytopenia, edema, and hemorrhage. Vascular changes are more noticeable in the testes and ovaries of ruminants. Lesions from *A. phagocytophilum* infection may include splenomegaly and hemorrhages. Clinical symptoms may include fever, depression, accelerated breathing, lack of apatite, edema, anemia, icterus, and ataxia. Ataxia appears to be more common in horses. The infection in cows may also decrease the milk yield. The disease is milder in young animals. As a result of depressed neutrophil count, the infected animals are also more susceptible to a secondary infection. More serious complications of the disease can occur in some animals, which may include respiratory infections and/or laminitis. Canine *A. platys* infection results in cyclic thrombocytopenia, and the organism is globally distributed.

Immunologic Aspects

Host immune response against the pathogens includes cellular and humoral responses. Antigenic variation in *A. marginale, A. phagocytophilum*, and *A. platys* may contribute to their persistence and immune evasion in a host. Although considerable research has been carried out to understand the variation in some of the outer-surface-expressed antigens of *A. marginale* and *A. phagocytophilum*, little is known about the mechanism of antigenic variation for *A. platys*. However, cyclic thrombocytopenia in *A. platys* may be caused by cyclical changes in the bacteremia levels, leading to cyclical changes in platelet counts. Significant diversity in the expressed major surface proteins is also reported for *A. phagocytophilum* and *A. marginale* strains and over a period of time in infected cattle for *A. marginale*.

Laboratory Diagnosis

Inclusions in the cytoplasm (replicating organisms in phagosomes) of granulocytes may be detected during the early clinical phage of infection following polychromatic staining (such as the Giemsa stain) of a thick smear of blood or a buffy coat sample (Figures 40.1, 40.1 and 40.3). Indirect immunofluorescent antibody and enzyme-linked immunosorbent assay (ELISA) tests have been developed. The organisms can also be detected by transmission electron microscopy (Figure 40.4). Recombinant antigens are typically used for ELISA. Molecular methods, such as PCR, are used to detect the pathogens during the clinical phase of the disease. Similarly, *A. platys* organisms may be detected in polychromatic-stained platelets of infected dogs.

Treatment and Control

As with other Rickettsiales, tetracycline derivatives are the most effective antibiotics against *A. phagocytophilum* and *A. platys*. Vector control is also effective in reducing the disease in animals. Currently, there are no vaccines available for *A. phagocytophilum* or *A. platys* infections in animals.

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Anaplasmataceae: *Ehrlichia* and *Neorickettsia*

Roman R. Ganta

Descriptive Features

The order Rickettsiales includes several obligate intracellular gram-negative pathogenic bacteria. Two families within this order, Anaplasmataceae and Rickettsiaceae, belong to the class Alphaproteobacteria. Both families include several important disease-causing agents impacting various vertebrate animals and humans. The family Anaplasmataceae includes intraphagosomal pathogens of the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia*. These organisms have a very similar morphology as they reside within a phagosome of an infected host cell. The cell tropism is a major distinguishable feature for identifying the pathogens in a vertebrate host. The family Rickettsiaceae includes pathogens of the genera *Rickettsia* and *Orientia*. The Rickettsiaceae pathogens replicate within the cell cytoplasm of an infected host cell. They typically invade the vascular endothelium.

Most of the Rickettsiales, except *Ehrlichia* and *Anaplasma* species, have a cell wall like most other gram-negative organisms. Rickettsial infections respond to antibiotic treatments such as tetracycline derivatives and chloramphenicol. They possess ribosomes, DNA, and RNA. The organisms are small coccoid to short rod-shaped bacteria. The organism ranges from 0.2 to 0.5 μm in diameter and 0.8 to 2 μm in length. Giemsa or other polychromatic stains are used to stain the organisms to observe the infection under a microscope using a 100× oil immersion objective lens. These aerobic, and mostly pathogenic, bacteria are responsible for many fatal and chronic infections in animals and people. They do not grow in standard bacteriological media. *In vitro* growth requires close association with eukaryotic cells such as yolk sacs of chicken embryos, macrophages, or epithelial cells of vertebrate or arthropod vectors. The pathogens replicate by binary fission inside a phagosome or in the cytoplasm. They depend heavily on their host for energy sources (ATP), metabolites, and other nutrients. Some pathogens (e.g. *Rickettsia* species) have an ATP/ADP translocase with which they obtain ATP molecules from the host in exchange for ADPs.

The pathogenicity of Rickettsiales is not well defined and may be complex because they represent organisms belonging to multiple genera having widely different host specificities and host cell associations. Virulence may be due to the release of endotoxins (lipopolysaccharides), production of immune complexes, and hypersensitivity reactions. The pathogens mostly damage the vascular endothelium or hematopoietic cells and severely weaken the host defense mechanisms. Rickettsiales are highly pathogenic and are responsible for high mortality rates in humans and animals. Typical diagnosis is based on the clinical presentation, detection of organisms in specific host cell types and cell organelles, serology, and molecular techniques. Culture isolation methods are available for some bacteria, but are time-consuming and are not practical for routine diagnosis. However, *in vitro* culture methods serve as excellent research tools for studies on these pathogens. Vertebrate hosts produce antibodies, and several species share extensive antigen similarity. Antibodies cross-react between species; thus, it is difficult to define an infection based on an antibody-mediated test. Inactivated or live attenuated vaccines are available for some pathogens, but appear to lose effectiveness rapidly, probably due to continuous changes in the expression of their outer membrane proteins. Protection may be best achieved by controlling the vector burden for some of the pathogen infections.

The Anaplasmataceae family pathogens of the genera *Ehrlichia* and *Neorickettsia*, which are of veterinary importance, are discussed in this chapter. *Anaplasma* species pathogens are discussed in Chapter 40, and the Rickettsiaceae family pathogens are discussed in Chapter 39. *Ehrlichia* species are transmitted from infected ticks to

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vertebrate hosts, whereas *Neorickettsia* species are fluke transmitted. The major pathogens of the *Ehrlichia* genus causing diseases in animals include *Ehrlichia canis* (an important monocyte/macrophage tropic canine pathogen), *Ehrlichia ruminantium* (ruminant heartwater disease agent, which infects the vascular endothelium of a wide range of domestic and wild ruminants), *Ehrlichia ewingii* (mostly a canine granulocytic pathogen), and *Ehrlichia chaffeensis* (the human monocytic ehrlichiosis agent, which also infects dogs, goats, and coyotes). Infections in people with *E. canis* and *E. ewingii* are also reported, but the number of documented cases is low. Two species of the *Neorickettsia* causing diseases in animals are *Neorickettsia risticii* (Potomac horse fever agent) and *Neorickettsia helminthoeca* (canine salmon poisoning agent). Both the *Neorickettsia* species infect monocytes and macrophages.

Ecology

Reservoir and Transmission

E. canis is the major pathogen responsible for causing the disease canine monocytic ehrlichiosis. It is transmitted by the *Rhipicephalus sanguineus* tick, commonly referred to as the brown dog tick. The organism causes a clinical disease within the first two weeks following infection. The pathogen persists in recovered animals for the rest of their life with high titers of antibody response, but the organism is often undetectable in the blood. Infected and carrier status animals are also a major source of infection for ticks. Young German shepherd breed dogs are the most severely affected and exhibit clinical signs. *E. canis* infection is prevalent in the tropical and subtropical regions of the world and occurs on all continents except Australia. A few cases of human monocytic ehrlichiosis caused by *E. canis* infection are reported in Venezuela.

The vector for *E. ewingii*, the agent of canine granulocytic ehrlichiosis, is *Amblyomma americanum*. This vector is commonly referred as the lone star tick. *E. ewingii* infections are more common in central North America; however, as the tick is more widely distributed throughout the southeastern part of the United States, the disease occurrence is also reported from coastal areas. *E. ewingii* infections in people, also transmitted from the lone star tick, are reported from the mid-western region of the United States.

E. ruminantium (formerly *Cowdria ruminantium*) is an important tick-borne pathogen in sub-Saharan Africa, as it is responsible for causing heartwater disease in domestic and wild ruminants. This organism infects the vascular endothelium and causes a severe neurological disease in cattle, sheep, and goats. This pathogen is vectored by several exotic ticks of the genus *Amblyomma*. The importation of this pathogen together with the *Amblyomma* ticks into the Caribbean islands raised a serious concern that the pathogen might be introduced to the American mainland. In particular, if the pathogen is introduced into a nonendemic region, the mortality rates can reach up to 90% in domestic and wild ruminants.

E. chaffeensis was first identified as the pathogen causing the human monocytic ehrlichiosis. Subsequently, infections with this organism were reported in dogs, coyotes, and goats. All four *Ehrlichia* species share a close genetic similarity, and thus antibodies against one pathogen have extensive cross-reactivity against other members of *Ehrlichia*. The extensive genetic similarity, establishment of persistent infections, and serological cross-reactivity, together with the shared cell tropism for some pathogens, pose a challenge for the accurate diagnosis of clinical diseases and treatment initiation. The white-tailed deer has been identified as playing a major role in maintaining the pathogen in nature.

Pathogenesis

Acute onset of diseases is more common within two to three weeks following the tick transmission of *Ehrlichia* species pathogens to an animal, during which time the organisms replicate within a phagosome of an infected host cell by binary fission. In severe acute cases where the disease may last up to several weeks, there may be several clinical symptoms that may be undistinguishable for some of the pathogens. For example, clinical symptoms significantly overlap for *E. canis*, *E. chaffeensis*, and *E. ewingii*. They may include high fever, muscle aches, vomiting, anorexia, and lethargy. Leukopenia, thrombocytopenia, and anemia are common, as the blood cell count drops due to *Ehrlichia* species infections. Other clinical signs include malaise, depression, loss of appetite, weight loss, lymphadenopathy, and joint pain. Joint pain is more common in dogs and humans with *E. ewingii* infections. Canine monocytic ehrlichiosis is characterized by three stages: acute, subclinical, and chronic. Dogs with *E. canis* infection remain infected for their entire life, and the persistent presence of the pathogen occurs even after the animals are treated with the antibiotic doxycycline. Similarly, chronic infections in dogs are documented with *E. chaffeensis* infections. Chronic phases are also common in animals with *E. ruminantium* infections. The infections in chronic phages persist for several months to several years and may contribute to several clinical abnormalities and immune suppression.

Heartwater in ruminants caused by *E. ruminantium* is responsible for causing high economic losses, as it affects a wide range of ruminants including cattle, sheep, goats,

several species of antelope, and wild ruminants. Characteristic symptoms of heartwater range from fever, respiratory distress, and edema in lungs, heart, thorax, peritoneum, and liver. Clinical disease with *E. ruminantium* varies in animal species and is also impacted by the age and breed of animals and their geographic origin. In addition, *E. ruminantium* strains induce variable levels of pathogenesis. Animals with a rare peracute form of the disease exhibit sudden death following high fever. Animals with the acute form of the disease may present with a few days of high fever, depression, cough, heavy breathing, and neurological signs leading to high mortality rates. Subacute and subclinical forms of the disease cause moderate to high morbidity; clinical signs are a few days of fever, labored breathing, and cough, followed by recovery or death. Subclinical heartwater disease is characterized by transient fever and is reported to be less severe in some breeds of sheep, cattle, or antelope with natural resistance to the disease. *E. ruminantium* infects the vascular endothelium and induces vascular damage resulting in edema and hemorrhages within various organs.

Immunologic Aspects

Infections with *Ehrlichia* species induce strong humoral and cellular immune responses. Antibodies are generated by an infected host against several outer membrane proteins, and the antibody titers increase with time post infection. Despite the strong humoral and cellular response, and even after treating the animals with an antibiotic such as doxycycline, pathogens often remain in the host at very low levels. Immune responses against all tick-borne *Ehrlichia* species infections are also very similar.

Laboratory Diagnosis

Giemsa- or polychromatic-stained blood smears of buffy coats or total blood are typically evaluated for the presence of intracellular inclusions (commonly referred to as morulae). The inclusions, however, are most likely to be visible during the acute phase of infection. The morulae with all species of *Ehrlichia*, *Neorickettsia*, and *Anaplasma* are very similar in their appearance (Figures 41.1–41.5). Thus, the distinguishing features of infection with a specific pathogen may depend on the host cell tropism and vector and host associations with the pathogen. All *Ehrlichia* species, except *E. ewingii*, can be cultivated under *in vitro* conditions using a canine macrophage cell line or using tick cell cultures. Serodiagnosis by indirect immunofluorescence antibody or enzyme-linked

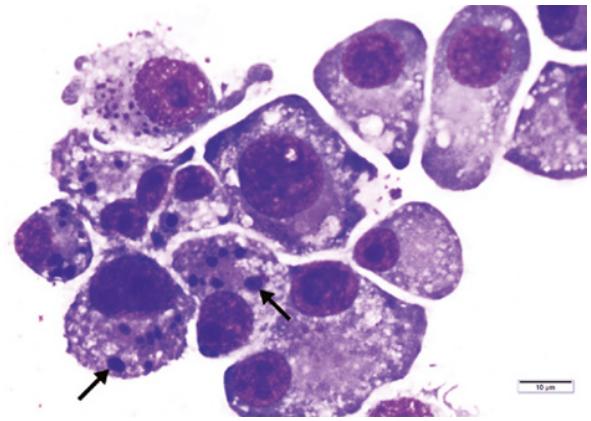


Figure 41.1 *Ehrlichia canis* cultured in the canine macrophage cell line, DH82. Inclusions within the cytoplasm contain many organisms. *In vitro* cultures transferred to a slide and stained with polychromatic stain.

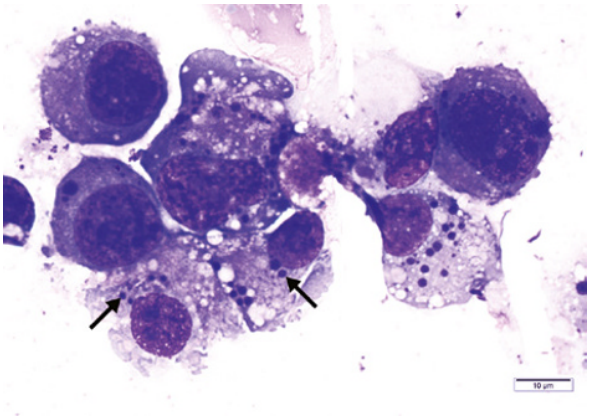


Figure 41.2 *Ehrlichia chaffeensis* cultured in the canine macrophage cell line, DH82. Inclusions within the cytoplasm contain many organisms. *In vitro* cultures transferred to a slide and stained with polychromatic stain.

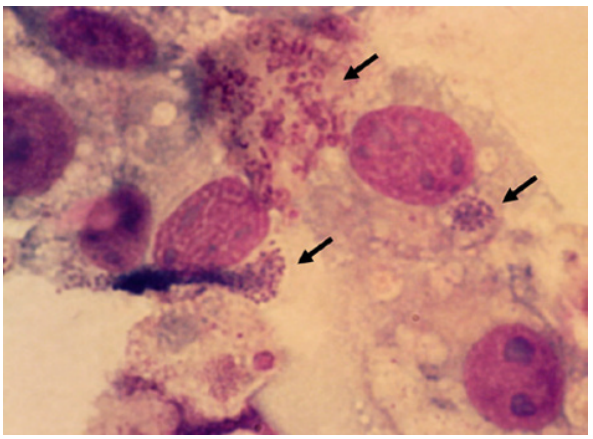


Figure 41.3 *Ehrlichia ruminantium* cultured in bovine primary endothelial cell culture. Phagosomes within the cytoplasm contain *E. ruminantium* organisms. *In vitro* cultures transferred to a slide and stained with polychromatic stain.

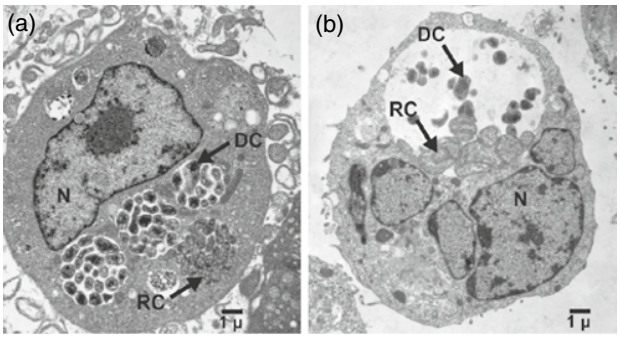


Figure 41.4 Transmission electron micrographs (TEM) of *Ehrlichia chaffeensis* infection (morula) in the cytoplasm of (a) cultured canine macrophages (DH82) and (b) tick cells. The TEM images with morulae containing multiple *Ehrlichia* organisms can be seen in the figure. *Source:* Reproduced with permission from Ganta et al. (2009).



Figure 41.5 *Ehrlichia ewingii* infection (morula) in the cytoplasm of a neutrophil in blood from an infected dog (Wright–Giemsa stain). *Source:* Reproduced with permission from Harvey (2012).

immunosorbent assay (ELISA) to detect antibodies against outer-membrane-expressed proteins is a commonly used diagnostic tool. This method, however, often results in serological cross-reactions among different *Ehrlichia* species antigens. Molecular methods such as polymerase chain reaction (PCR) assays are developed for the specific pathogen diagnosis, and this method is most useful for detecting active infection in blood during the early clinical phage of infection and prior to initiating the antibody treatment. Transmission electron microscopy of infected host cells of Anaplasmataceae

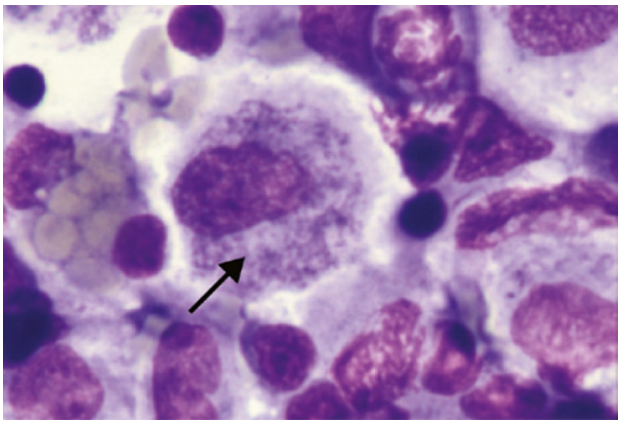


Figure 41.6 *Neorickettsia helminthoeca* inclusions in canine lymph node peripheral aspirate (Romanowsky; HP oil). *Source:* Reproduced with permission from Raskin and Meyer (2010).

pathogens possess two distinct forms: the infectious dense core (DC) cells and the replicating form referred to as reticulate cells (RC) (Figure 41.6).

Treatment and Control

Tetracycline derivatives, such as doxycycline, are the most effective in treating the early clinical phase of infections with *Ehrlichia* species, but less effective in clearing chronic infections. Controlling the tick burden and live infection vaccination in younger animals or infection and treatment vaccination protocols are some of the means of reducing *Ehrlichia* species infections, such as *E. ruminantium*, in sub-Saharan African countries. Although several inactivated whole-cell or subunit vaccines are under evaluation, currently no vaccines give complete protection against *Ehrlichia* infections.

N. risticii

In the late 1970s, Potomac horse fever in the Potomac River Valley of Maryland, United States, was recognized as an epidemic in horses. The pathogen *N. risticii* (previously known as *Ehrlichia risticii*) is the causative agent of this disease. Clinical signs in animals include depression, fever, poor appetite, and diarrhea, often accompanied by laminitis (founder). Diarrhea is common and can vary from being profuse and watery to scant and cow-like in consistency. Death occurs in 5–30% of untreated horses and can result in toxemia, colic, or euthanasia due to severe founder.

Ecology

Reservoir, Transmission, and Pathogenesis

The infection was initially recognized in North America. Later it was also reported from Canada and Europe. *N. risticii* infects equine monocytes, the intestinal epithelium, and colonic mast cells and is transmitted by cercaria, a free-swimming larval stage of a parasitic fluke, released from the infected snails into water, which are taken up by the horses when drinking the contaminated water. Clinically, Potomac Horse Fever resembles ehrlichiosis with fever, listlessness, anorexia, and variable leukopenia. Diarrhea and laminitis are the symptoms associated with the disease at the later stages and can lead to a mortality rate of up to 30%.

Immunologic Aspects

Immunologic factors are not fully understood, but the infected animals induce humoral and cell-mediated responses.

Laboratory Diagnosis

Microbiological diagnosis involves demonstration of the agent in monocytes in polychromatic-stained blood smears. Serological diagnosis is made by an indirect fluorescent antibody test or by ELISA. Nucleic acid assays, such as PCR, are described for the pathogen diagnosis.

Treatment and Control

Tetracycline, if given early, is considered important in reducing mortality. Supportive care is also necessary. A vaccine is commercially sold, but it appears to be less effective against the disease.

N. helminthoeca

Descriptive Features

N. helminthoeca causes salmon poisoning in dogs. This pathogen multiplies within the phagosomes of monocytes and macrophages of an infected dog. This infection is very similar to *E. canis* and *E. chaffeensis* in dogs, except that the organism is acquired from fish bearing *N. helminthoeca*–infected flukes. After staining a blood sample with a Giemsa or a polychromatic stain, the organism may be visible as cytoplasmic inclusions in the monocytes from a blood smear made from a clinically ill animal.

Ecology

Reservoir and Transmission

The reservoir of infection for *N. helminthoeca* is the fluke *Nanophyetus salmincola*. The life cycle of the fluke includes passage through a fish and a snail. Dogs eating fish infested with a fluke containing *N. helminthoeca* are likely to be infected. The geographical distribution of salmon poisoning is limited by the range of the snail, an intermediate host of the fluke. In particular, the distribution of the snail includes North American coastal regions, such as northern cost of California, Oregon, Washington, and British Columbia. If an *N. helminthoeca*–infected fluke gains entry into a dog that has eaten infected fish, the pathogen establishes itself in the dog. Fluke eggs are shed in canine feces and hatch miracidia, which can reach a snail in the environment. Cercaria emerges from the fluke-infected snail and invades a fish.

Pathogenesis

Exposure to an infected raw fish in an endemic area is the primary reason for *N. helminthoeca* infection in dogs; the fluke enters the dog from the fish. It then matures in the dog's gastrointestinal tract, where *N. helminthoeca* organisms are released, which enters the canine bloodstream and lymph nodes. In blood, the organism infects monocytes. The replication of the organism in the dog results in the clinical signs associated with the disease. Within one to two weeks of exposure, dogs develop fever, anorexia, depression, weight loss, swollen lymph nodes, and often hemorrhagic enteritis. Vomiting and diarrhea are also common clinical signs. Mortality can be up to 90% in untreated animals, which can occur within two weeks.

Immunologic Aspects

Dogs recovered from the clinical disease appear to be protected from the disease.

Laboratory Diagnosis

The presence of organisms within the cytoplasmic vacuoles of monocytes in a stained blood smear or in lymph node aspirates is evidence of the disease. The organisms may be cultured, but it is a time-consuming method. Molecular methods can also be used to detect the bacterial nucleic acids from a blood or lymph node sample.

Treatment and Control

Tetracycline, chloramphenicol, and sulfonamides are effective in treating *N. helminthoeca* infections. Supportive treatment is also necessary. The best preventive measure is the exclusion of infected salmon from the canine diet. Flukes and *N. helminthoeca* are killed by cooking at high temperatures or by freezing for 24 hours.

Further Reading

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42

Bartonellaceae

Kathryn E. Reif

Members of the family Bartonellaceae are small, thin, short and slightly curved, gram-negative rods. Until the early 1990s, the genus *Bartonella* consisted of only one species, *Bartonella bacilliformis*. The genus now contains all the species that were once included in the genera *Bartonella*, *Rochalimaea*, and *Grahamella*. The genus *Bartonella* has been placed into the family Bartonellaceae (which has also been removed from the order Rickettsiales). Members of the genus *Bartonella* belong to the α-2 subgroup of the Alphaproteobacteria. Most of these bacteria are hemotropic, erythrocyte-invasive bacilli.

The present family can be divided into the ancestral *Bartonella* (of which *B. bacilliformis* is the only member) and the modern *Bartonella* (which includes all other *Bartonella* species). Virtually ubiquitous among mammals, the *Bartonella* family consists of more than 50 species, subspecies, and *Candidatus* species, with new species regularly reported. Bartonellosis is considered a (re)emerging disease as an increasing number of *Bartonella* species are zoonotic and can cause disease in humans. At least 21 *Bartonella* species, subspecies, or *Candidatus* species are human pathogens.

1) Associated with disease in human patients:
   B. bacilliformis is the etiologic agent of Oroya fever, an acute bacteremic infection characterized by sepsis and hemolysis, and verruga peruana – mainly a cutaneous nodular vascular eruption representing chronic infection.

   Bartonella quintana, the agent of trench fever, has also been found to be one of the agents of bacillary angiomatosis (BA), a vascular proliferative lesion observed in immunocompromised individuals, mainly with acquired immunodeficiency syndrome (AIDS). BA caused by *B. quintana* usually occurs in homeless people infested with body lice. BA can also be caused by *Bartonella henselae*.

   B. henselae causes mainly cat scratch disease (CSD) in immunocompetent individuals.

   Bartonella elizabethae, *Bartonella koehlerae*, *Bartonella alsatica*, and *Candidatus B. mayotimonensis* have been associated with endocarditis in immunocompetent patients.

   Bartonella vinsonii ssp. *berkhoffii* and *B. washoensis* are associated with endocarditis or myocarditis. *Bartonella grahamii* has been associated with neuroretinitis.

   B. vinsonii ssp. *arupensis* was isolated from the blood of a rancher with fever and mild neurological symptoms and detected in a patient with endocarditis.

   B. melophagi has been associated with pericarditis and fatigue.

   Bartonella tamiae has been isolated from febrile people in Thailand.

   Bartonella clarridgeiae is suspected to also be a minor agent of CSD.

2) Associated with animal patients:

In their primary reservoir host species, *Bartonella* rarely cause overt clinical disease. In accidental host species, some of the *Bartonella* species that are pathogenic to humans (*B. vinsonii* ssp. *berkhoffii*, *B. clarridgeiae*, *B. henselae*, *B. elizabethae*, *B. quintana*, *B. washoensis*, *B. elizabethae*, *B. grahamii*, and *Bartonella taylorii*) have been associated with various clinical entities, including endocarditis, in domestic dogs. Several other species have been diagnosed in dogs without report of specific clinical entity, such as *B. vinsonii* ssp. *arupensis*, *B. volans*–like, *Bartonella bovis* or strain HMD (proposed *Candidatus B. merieuxii*). Cases of endocarditis caused by *B. henselae* in cats and by *B. bovis* in cattle have also been described.

Several other *Bartonella* species, such as *B. vinsonii* ssp. *vinsonii*, *Bartonella doshiae*, *B. taylorii*, *Bartonella peromysci*,

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Bartonella birtlesii, *Bartonella tribocorum*, *Bartonella talpae*, *B. bovis*, *Bartonella schoenbuchensis*, and *Bartonella capreoli*, have only been isolated from the blood of various animal species, including various wild rodents, squirrels, rabbits, felids, canids, bovids, and cervids. At present, these species are not known to induce any specific disease in the infected animal.

Descriptive Features

Morphology and Staining

Bartonellaceae are fastidious, slow-growing, aerobic, facultative intracellular bacteria that are catalase, oxidase, urease, and nitrate reductase negative. Morphologically, they are short, pleomorphic gram-negative coccobacillary or bacillary rods (0.6 µm by 1.0 µm). Depending on their lineage group, *Bartonella* species may also possess multiple unipolar flagella (Lineage 1, 2, and 3) or be decorated with hair-like filaments of variable lengths (40–240 nm) originally thought to be pili, but now known to be trimeric autotransporter adhesins.

Bartonella spp. can take from 5 to 15 days and up to 45 days on primary culture to form visible colonies on enriched blood-containing media, as they are highly hemin dependent (Figure 42.1). Culture success on enriched blood-containing media has the greatest chance of success when isolating *Bartonella* sp. from their respective reservoir host. Attempted culture from accidental hosts is more difficult and often requires the use of special enrichment media. In infected tissues, Warthin–Starry silver impregnation stain reveals small bacilli, which tend to appear as clumps of tightly compacted organisms. Similarly, small organisms can be identified in red blood cells by May-Grünwald Giemsa coloration. Bartonellae have a close evolutionary resemblance with members of the genera *Brucella*, *Agrobacterium*, and *Rhizobium*.

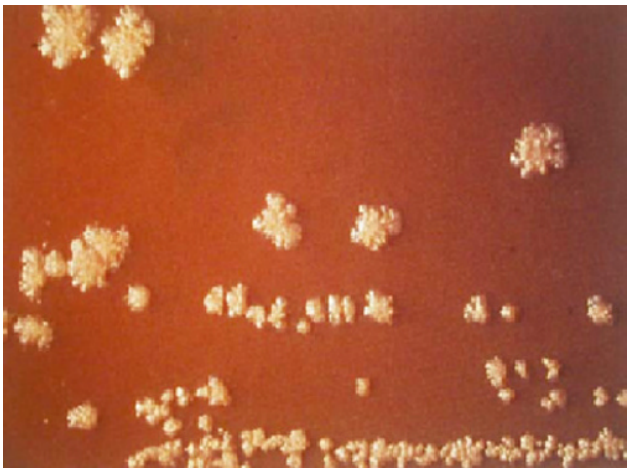


Figure 42.1 *B. henselae* colonies on blood culture on 5% rabbit blood agar.

Cellular Composition

Because of their slow growth, standard biochemical methods are not as useful for identification. The bartonellae are oxidase and catalase negative. Measurements of preformed enzymes and standard testing have revealed differences between species. Most species are biochemically inert except for the production of peptidases. The MicroScan Rapid Anaerobe Panel (Baxter Diagnostics, Deerfield, Illinois, United States) has been reported to provide species identification. Whole cell fatty acid (CFA) analysis for the genus has proved useful for identification because bartonellae have a unique and characteristic whole CFA composition. The bartonellae have gas–liquid chromatography fatty acid profiles consisting mainly of $C_{18:09}$, $C_{18:19}$, and $C_{16:0}$. Molecular genetic methods, such as restriction fragment length polymorphism (RFLP) of genes encoding citrate synthase, 16S ribosomal RNA (rRNA) or 16S-23S rRNA spacer region, and more recently analysis based on polymerase chain reaction (PCR) of random, repetitive extragenic palindromic sequences, have been used to distinguish strains and species of *Bartonella*. RFLP or sequence analysis of DNA encoding 16S rRNA and citrate synthase genes after PCR amplification both directly from specimens or pure cultures have been largely used for detecting and characterizing *Bartonella*. Identification is mainly performed with the amplification of the DNA encoding the 16S-23S rRNA intergenic spacer region (ITS) or protein-encoding genes. The genes most widely used are those encoding the citrate synthase (*gltA*), the heat shock protein (*groEL*), the riboflavine (*ribC*), a cell division protein (*ftsZ*), and a 17 kDa antigen.

Growth Characteristics

Traditionally, members of the genus *Bartonella* are cultivated in semisolid nutrient agar containing fresh rabbit blood (or sheep or horse blood) at 35 °C (except for *B. bacilliformis*, which grows best at 28 °C) in 5% CO_2. On primary isolation, some *Bartonella*, such as *B. henselae*, *B. clarridgeiae*, *B. vinsonii*, or *B. elizabethae*, have colonies with a white, rough, dry, raised appearance and pit the medium. They are hard to break up or transfer. Other bartonellae such as *B. quintana* have colonies that are usually smaller, gray, translucent, and somewhat gummy or slightly mucoid. A novel preenrichment liquid medium *Bartonella*/alpha-Proteobacteria growth medium (BAPGM) has been developed and used successfully to detect *Bartonella* in biological products.

Ecology

Reservoir, Transmission, and Geographic Distribution

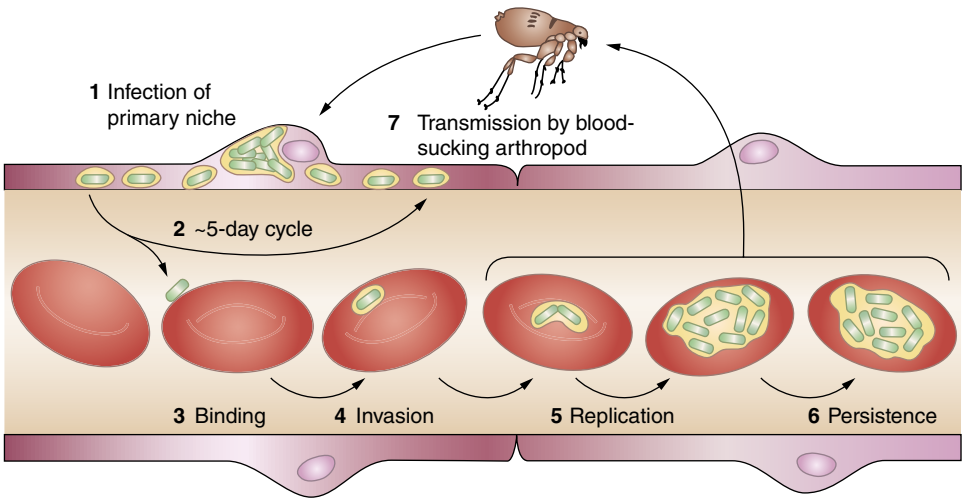
Bartonella species have been recovered from a broad spectrum of mammalian species, with individual species likely co-evolving with their primary reservoir host species. Most members of the genus *Bartonella* are vector-borne organisms, transmitted by a variety of fleas, ticks, lice, and other blood-feeding arthropods depending on the particular species. The arthropod vector acquires *Bartonella* by feeding on an infected (reservoir) host and ingesting *Bartonella*-infected red blood cells (Figure 42.2). There are two general routes of vector-borne *Bartonella* transmission: infected blood is ingested by the arthropod vector and transmitted to a naïve primary reservoir or accidental host through (i) *Bartonella*-infected arthropod feces that enters the host through self-inflicted skin abrasions due to host scratching or biting; or (ii) direct transmission through the bite of an infected vector (Figure 42.2). The efficiencies of these different transmission routes depend on the particular *Bartonella* species and arthropod vector. Other potential non-vector routes of transmission include blood transfusions and iatrogenic transmission via *Bartonella*-infected blood-contaminated needles or other instruments.

Distributed worldwide, more than 50 *Bartonella* species, subspecies, or Candidatus species have been isolated or detected from a combination of human, domestic animal, and wildlife hosts. Phylogenetically, *Bartonella* clusters into four lineages. The only member of the ancestral Lineage 1 is *B. bacilliformis*, for which humans serve as the primary reservoir. The other three lineages consist of species collectively referred to as "modern" *Bartonella* species. Lineage 2 consists of species that primarily utilize ruminant species as their primary reservoirs (e.g. *B. bovis*, *B. chomelii*). Lineage 3 and 4 contain the greatest diversity of *Bartonella* species, and this diversity is further reflected in their diverse preferences for different mammalian reservoir hosts (e.g. *B. henselae*, *B. vinsonii berkhoffii*).

The most common *Bartonella* species that cause clinical disease in humans are *B. bacilliformis*, *B. henselae*, and *B. quintana*. The most common *Bartonella* species that cause clinical disease in companion animals are *B. clarridgeiae*, *B. elizabethae*, *B. henselae*, *B. koehlerae*, *B. quintana*, *Bartonella rochalimae*, and *B. vinsonii berkhoffii*; importantly, all of these have zoonotic potential. *Bartonella* species are most commonly observed in their respective primary host and reservoir, however, incidental infection of other host species may occur, and infection of these accidental hosts may result in clinical disease. A comprehensive list of *Bartonella* species, their primary reservoirs, vectors, and accidental host(s) is provided in Table 42.1.

Pathogenesis

In their reservoir host, modern *Bartonella* species rarely cause overt clinical disease. In contrast, acute and/or chronic disease is observed in humans infected with the ancestral species, *B. bacilliformis*. Modern *Bartonella* are stealth pathogens that have evolved a variety of strategies to persist in their preferred mammalian reservoir species (e.g. hiding in



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Figure 42.2 Example of *Bartonella* transmission between the blood-feeding arthropod vector and the mammalian host.

Table 42.1 *Bartonella* species, subspecies, or *Candidatus* species presently described, their main reservoir, confirmed or possible vectors (confirmed or possible), and accidental host(s).

***Bartonella* spp.**	**Primary reservoir**	**Vectors (confirmed, *possible*)**	**Accidental host(s)**
B. acomydis	Mice (*Acomys russatus*)	Unknown	Unknown
B. aff. *schoenbuschensis*	Rusa deer (*Cervus timorensis russa*)	Deer keds?	Unknown
Bartonella alsatica[a]	Rabbits (*Oryctolagus cuniculus*)	Fleas (*Spilopsyllus cuniculi*), ticks?	Humans
Candidatus B. ancashi[a]	Unknown	Unknown	Humans
Candidatus B. antechini	Yellow-footed Antechinus (*Antechinus flavipes*)	Fleas (*Acanthopsylla jordani*) Ticks (*Ixodes antechini*)	Unknown
B. alsatica[a]	Rabbit	Fleas?	Humans
B. australis	Eastern gray kangaroos (*Macropus giganteus*)	Fleas?, ticks	Unknown
Bartonella bacilliformis	Humans, primates	Sand flies (*Lutzomyia verrucarum*), fleas	None
Candidatus B. bandicootii	Western barred bandicoot (*Perameles bougainville*)	Flea (*Pygiopsylla tunneyi*)	Unknown
Bartonella birtlesii	Wood mice (*Apodemus* spp.)	Fleas	Unknown
Bartonella bovis (weissi)[a]	Domestic cattle (*Bos taurus*)	Biting flies? Ticks?	Humans, dogs, cats
B. callosciuri	Squirrel (*Callosciurus notatus*)	Unknown	Unknown
Bartonella capreoli	Roe deer (*capreolus capreolus*)	Biting flies? Ticks?	Unknown
Bartonella chomelii	Domestic cattle (*B. taurus*),	Biting flies? Ticks?	Unknown
Bartonella clarridgeiae[a]	Cats (*Felis catus*)	Fleas (*Ctenocephalides felis*), ticks?	Humans, dogs
Bartonella coopersplainsensis	*Rattus leucopus*	Fleas?	Unknown
Bartonella doshiae[a]	Meadow voles (*Microtus agrestis*), rats (*Rattus* spp.)	Fleas	Humans
B. elizabethae[a]	Rodents, (e.g. *Rattus norvegicus*)	Fleas	Humans, dogs
B. florenciae	Shrew (*Crocidura russula*)	Unknown	Unknown
B. grahamii[a]	Several species of wild rodents (e.g. mice, voles)	Rodent fleas (e.g. *Ctenophthalmus nobilis*)	Humans, dogs
Bartonella henselae[a]	Cats (*Felis catus*), dogs (*Canis familiaris*)	Fleas (*Ctenocephalides felis*), ticks (*Ixodes* spp.), mites?	Humans, dogs, horses, marine mammals, sea turtles
B. japonica	Small Japanese field mouse (*Apodemus argenteus*)	Lice (*Hoplopleura affinis*)	Unknown
Bartonella koehlerae[a] *subsp. bothieri*	Bobcat (*Lynx rufus*), cheetah (*Acinonyx jubatus*)	Fleas?	Unknown
B. koehlerae[a] *subsp. boulouisii*	Mountain lion (*Puma concolor*)	Fleas?	Unknown
B. koehlerae[a] *subsp. koehlerae*	Cats (*Felis catus*), lion (*Panthera leo*), gerbils (*Meriones libycus*)	Fleas (*Ctenocephalides felis*)	Humans
B. massiliensis	Rodents	Soft tick (*Ornithodoros sonrai*)	Unknown
Candidatus B. mayotimonensis[a]	Daubenton's bat (*Myotis daubentonii*)	Bat flies, fleas?	Humans
B. melophagi[a]	Sheep (*Ovis aries*)	Sheep ked (*Melophagus ovinus*)	Humans
Candidatus B. merieuxii	Dogs (*Canis familiaris*), jackal (*Canis aureus*)	Fleas, ticks	Unknown
B. pachyuromydis	Mice (*Pachyuromys duprasi*)	Unknown	Unknown

(*Continued*)

Table 42.1 (Continued)

Bartonella spp.	Primary reservoir	Vectors (confirmed, *possible*)	Accidental host(s)
Bartonella peromysci	Field mice (*Peromyscus spp.*)	Fleas	Unknown
B. phoceensis	Rats (*Rattus norvegicus*)	Lice (*Hoplopleura pacifica*), fleas?	Unknown
Bartonella queenslandensis	Rats (*Rattus* spp.)	Fleas	Unknown
Bartonella quintana[a]	Humans, primates, gerbils (*Meriones libycus*)	Human body lice (*Pediculus humanus*), fleas, bed bugs, pigeon mites?	Cats, dogs, monkey
Bartonella rattaustraliani	Australian rats	Fleas	Unknown
B. rattimassiliensis	Rats (*Rattus norvegicus*)	Fleas	Unknown
Bartonella rochalimae[a]	Dogs (*Canis familiaris*), wild carnivores, Gray and red foxes, (*Urocyon cinereoargenteus, Vulpes vulpes*) Raccoons (*Procyon lotor*)	Fleas (*Pulex irritans, P. simulans*), ticks, sandflies	Humans
Bartonella schoenbuchensis[a]	Roe deer (*Capreolus capreolus*)	Deer keds (*Lipoptena cervi, Lipoptena mazamae*), biting flies, ticks	Humans
B. senegalensis[a]	Burrowing rodents	Soft tick (*Ornithodoros sonrai*)	Humans
B. silvatica	Large Japanese field mouse (*Apodemus speciosus*)	Fleas?	Unknown
Bartonella talpae	Moles (*Talpa europaea*)	Fleas	Unknown
B. tamiae[a]	Rat (*Rattus* spp.)	Fleas, chigger mites?, ticks?	Humans
Bartonella taylorii	Wood mice (*Apodemus spp.*), gerbils (*Meriones libycus*), voles (*Clethrionomys* spp.)	Fleas	Unknown
Candidatus B. thailandensis	Rodents (*Rattus* spp.)	Fleas?	Unknown
Bartonella tribocorum[a]	Rats (*Rattus spp.*), mice (*Apodemus* spp.)	Fleas	Unknown
Bartonella vinsonii ssp. arupensis[a]	White-footed mice (*Peromyscus leucopus*)	Ticks (*Ixodes scapularis*), fleas?	Humans, dogs
B. vinsonii ssp. berkhoffii[a]	Coyotes (*C. latrans*), dogs (*Canis familiaris*), foxes (*Urocyon* spp.)	Ticks, fleas (*Pulex* spp.)?	Humans
B. vinsonii ssp. vinsonii	Meadow voles (*Microtus pennsylvanicus*)	Ear mites (*Trombicula microti*)?	Unknown
B. volans[a]	Southern flying squirrel (*Glaucomys volans*)	Fleas	Humans, dogs, horses
B. washoeensis[a]	California ground squirrel (*Spermophilus beecheyi*), rabbits (*Oryctolagus cuniculus*)	Fleas (*Oropsylla montana*), ticks	Humans, dogs
Candidatus B. woyliei	Woylie (*Bettongia penicillata*)	Flea (*Pygiopsylla hilli*) Tick (*Ixodes australiensis*)	Unknown

? Indicates most likely vector species.
[a] *Bartonella* species or subspecies reported to be zoonotic.

immune-privileged sites, extensive immunomodulation of the host immune system) and facilitate their subsequent transmission by blood-feeding arthropod vectors (e.g. blood-feeding vectors ingest infected erythrocytes). The infection cycle of *Bartonella* begins with the inoculation of the organism into a mammalian host, followed by colonization of a primary niche (e.g. nucleated cells such as endothelial cells), followed by seeding of bartonellae into the bloodstream to infect erythrocytes. Colonization of this primary niche is critical as bartonellae require a period of host priming after inoculation by the arthropod before they are ready to infect host erythrocytes. Within the mammalian host, *Bartonella* spp. utilize both passive mechanisms (e.g. subcellular location, non-reactive LPS) and active mechanisms (e.g. trimeric

autotransporter adhesins, VirB/D4 T4SS, secreted GroEL, Trw T4SS, hemin binding proteins, flagellation, filamentous hemagglutinins) of immune evasion. Infected erythrocytes are ingested by blood-feeding arthropod vectors to start the cycle anew.

The severity of disease in accidental hosts or (less commonly) reservoir hosts is likely influenced by a multitude of host factors including species, immune status, concurrent infection or diseases, and nutritional status. In addition, persistent *Bartonella* spp. infection may also trigger autoimmune and immune-mediated disease manifestations, both of which may contribute to the increased susceptibility of the host to other infections or non-infectious disorders.

Mechanisms

B. quintana and *B. henselae* are clinically associated with proliferative neovascular lesions (Figure 42.3). The pathogenesis of BA involves injury and proliferation of the vascular endothelium through induction of vascular endothelial growth factor (VEGF) with *B. henselae, B. bacilliformis*, *B. quintana,* and *B. vinsonii berkhoffii* (in dogs), which can lead to vascular tumor formation and vasoproliferation (particularly in immunosuppressed patients). These organisms induce endothelial cell proliferation and migration *in vitro*, and a protein fraction was identified as the angiogenic factor. *Bartonella* infection (*in vitro*) stimulated endothelial cell proliferation and induced obvious morphological changes due to modifications of the cytoskeleton. *B. henselae* has been shown to induce infected cells to produce VEGF, which in turn stimulated the proliferation of endothelial cells and the growth of *B. henselae*.

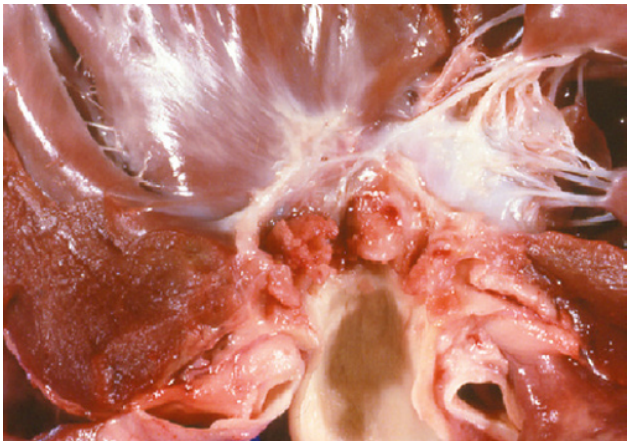


Figure 42.3 Canine endocarditis caused by *B. clarridgeiae*. *Source:* Used with permission from Chomel et al. (2001).

B. henselae seems to share with *B. bacilliformis* a common mechanism for mediating pathogenesis. A bacteriophage-like particle similar to the bacteriophage observed in *B. bacilliformis* has been found in culture supernatant from *B. henselae*. This particle has at least three associated proteins and contains 14 kbp linear DNA segments that are heterogeneous in sequence. It has been speculated that an ancestor of *B. henselae* and *B. bacilliformis* acquired the ability to mediate angioproliferation as a means of enhancing its dissemination or its acquisition of nutrients within the host. It is possible that a common transducing phage may be the mechanism of genetic exchange by which the two organisms acquired this pathogenic trait.

All *Bartonella* species multiply and persist in red blood cells; however, *in vitro* studies have demonstrated that *Bartonella* spp. can also infect dendritic cells, microglial cells, pericytes, monocytes, macrophages, and CD34+ bone marrow progenitor cells, all of which may contribute to dissemination of these organisms throughout the host's body. *B. bacilliformis* possess polar flagella that have been shown to mediate erythrocyte adhesion. For non-flagellated *Bartonella*, bundle-forming pili as well as surface proteins may play a role in erythrocyte adhesion. Until recently, mechanisms of persistence of *Bartonella* bacteremia in mammals were not well understood. Recent reports have revealed the intraerythrocytic localization of these bacteria, which is a unique strategy for bacterial persistence. Nonhemolytic intracellular colonization of erythrocytes would preserve the organisms for efficient vector transmission, protect *Bartonella* from the host immune response, and contribute to decreased antimicrobial efficacy. Persistence of infection was demonstrated in a rat model using *B. tribocorum*. After a five-day "hidden/silent" phase following experimental infection, the organism multiplied until there were an average of eight *Bartonella* per red blood cell. Thereafter, the organisms remained within the cell for the life of the erythrocyte. Nonhemolytic intracellular colonization of erythrocytes is likely a bacterial persistence strategy that preserves the *Bartonella* species for potential transmission by arthropods because the reservoir host would serve as a source of infection for blood-feeding arthropods, which could then subsequently infect a new host.

Bacterial type IV secretion systems, which are supramolecular transporters ancestrally related to bacterial conjugation systems, represent crucial pathogenicity factors that have contributed to a radial expansion of the *Bartonella* lineage in nature by facilitating adaptation to unique mammalian hosts. At the molecular level, the type IV secretion system VirB/VirD4 is known to translocate a cocktail of different effector proteins into host cells, which subvert multiple cellular functions to aid the infecting pathogen. Furthermore, bacterial adhesins mediate a critical, early step in the pathogenesis of the bartonellae by binding to

extracellular matrix components of host cells, which leads to firm bacterial adhesion to the cell surface as a prerequisite for the efficient translocation of type IV secretion effector proteins. The best-studied adhesins in bartonellae are the orthologous trimeric autotransporter adhesins, BadA in *B. henselae* and the Vomp family in *B. quintana*. Genetic diversity and strain variability also appear to enhance the ability of bartonellae to invade not only specific reservoir hosts but also accidental hosts, as shown for *B. henselae*.

Disease Patterns and Epidemiology

The ever-expanding list of *Bartonella* species represents a challenge to understanding their associated epidemiology and disease patterns as prevalence, infection characteristics, and disease manifestations vary based on a variety of factors such as host species (reservoir or accidental host), risk of exposure to arthropod vectors, host immune status (healthy or immunocompromised), and other co-infections. Often, *Bartonella* species do not cause disease in their primary reservoir host, where these bacteria live within erythrocytes or the vascular endothelium for extended periods of time (months to years). However, in immunosuppressed reservoir species or accidental hosts, clinical signs of disease may occur. Common severe clinical manifestations of *Bartonella* infections include verruga peruana, BA, bacillary peliosis, endocarditis, myocarditis, aneurysm, vasculitis and/or thrombosis, hemangiosarcoma, hemangiopericytoma, epithelioid hemangioendothelioma, and systemic reactive angioendotheliomatosis. These disease manifestations have been observed across several *Bartonella* species infecting a variety of mammalian hosts. Whether and when these more severe clinical manifestations occur is unclear and likely depends on numerous factors such as pathogen strain, host immune status, other concurrent infections or disorders, immunosuppression, and host nutritional status.

Examples of clinical signs of disease caused by *Bartonella* species in humans, cats, dogs, and rodents are presented below.

Human Patients

Cat Scratch Disease

CSD is caused by *B. henselae*, a pathogen spread by cats. Humans become infected with *B. henselae* when an infected cat licks an open wound or breaks the skin surface through bites or scratches of a naïve person, transferring *Bartonella* organisms present in their oral cavity or on their paws (contaminated with infected flea feces). About 40% of cats carry *B. henselae* at some point in their life, with kittens more likely to be infected and more likely to transmit infection due to their greater tendency to scratch and bite when playing. In CSD, one to three weeks elapse between the scratch or bite of a cat and the appearance of clinical signs. In 50% of the cases, a small skin lesion, often resembling an insect bite, appears at the inoculation site, usually the hand or forearm, and evolves from a papule to a vesicle and partially healed ulcers. These lesions resolve within a few days to a few weeks. Lymphadenitis develops approximately three weeks after exposure and is generally unilateral. It commonly appears in the epitrochlear, axillary, or cervical lymph nodes. Swelling of the lymph node is usually painful and persists for several weeks to several months. In 25% of the cases, suppuration occurs. The large majority of the cases show signs of systemic infection: fever, chills, malaise, anorexia, and headaches. In general, the disease is benign and heals spontaneously without sequelae. Atypical manifestations of CSD occur in 5–10% of the cases. The most common of these is Parinaud's oculoglandular syndrome (periauricular lymphadenopathy and palpebral conjunctivitis), but meningitis, encephalitis, osteolytic lesions, and thrombocytopenic purpura may also occur. Encephalopathy is one of the most serious complications of CSD, which usually occurs two to six weeks after the onset of lymphadenopathy. However, it usually resolves with complete recovery and few or no sequelae.

There were an estimated 22 000 human cases of CSD in the United States in 1992, some 2000 of whom were hospitalized. The estimated annual health cost of CSD was more than $12 million. Another study of insurance claim records from 2005 to 2013 identified 13 273 patients with CSD (12 735 outpatients and 538 inpatients; average annual incidence range 4–5.7 outpatient cases and 0.17–0.22 inpatient cases per 100 000 population), with the greatest case incidence reported in the West, South Central, East South Central, and South Atlantic regions (6.1–6.4 cases per 100 000 population), with 26% of cases occurring in the South Atlantic region. In this study, the estimated annual health cost of CSD was >$9.7 million in direct medical hosts. Both studies found that the majority of CSD cases were in children (<14 years old) and young adults (<20 years old), with >30% of all cases occurring children 14 years and younger in the more recent study. Both studies also found a seasonal pattern for CSD cases, with most cases seen in autumn and winter.

Additional rare clinical presentations affecting the brain, eyes, heart, and other internal organs have been associated with *B. henselae* infection in immunocompetent persons and children between 5 and 14 years of age. Neuroretinitis or bacteremia as a cause of chronic fatigue syndrome and cases

of *B. henselae* endocarditis in a cat owner have been described in immunocompromised persons. *B. henselae* was also determined as a frequent cause of prolonged fever and fever of unknown origin in children. Rheumatic manifestations of *Bartonella* infection have been described in children, including a case of myositis and a case of arthritis and skin nodules. Arthritis has also been described in a very limited number of cases. Other rheumatic manifestations related to *Bartonella* infection in humans include erythema nodosum, leukocytoclastic vasculitis, fever of unknown origin with myalgia, and arthralgia. Chronic infection and bacteremia have been reported in humans, especially in the United States.

Endocarditis

Several *Bartonella* spp. have also been recognized as causative agents of blood culture-negative endocarditis or myocarditis in humans, including *B. henselae*, *B. quintana*, *B. elizabethae*, *B. vinsonii* ssp. *berkhoffii* and *B. vinsonii* ssp. *arupensis*, *B. alsatica*, and *Candidatus B. mayotimonensis*. *Bartonella* spp. account for approximately 3% of all human cases of endocarditis, a percentage similar to endocarditis cases caused by *Coxiella burnetii*, the agent of Q fever (see Chapter 39).

Bacillary Angiomatosis

For BA in immunocompromised persons, the signs and symptoms are very different from CSD. BA, also called epithelioid angiomatosis, is a vascular proliferative disease of the skin characterized by multiple blood-filled, cystic tumors. It is usually characterized by violaceous or colorless papular and nodular skin lesions that clinically may suggest Kaposi's sarcoma, but histologically resemble epithelioid hemangiomas. When visceral parenchymal organs are involved, the condition is referred to as bacillary peliosis hepatis, splenic peliosis, or systemic BA. Fever, weight loss, malaise, and enlargement of affected organs may develop in people with disseminated BA. Endocarditis has also been reported in patients with BA.

Cats

Domestic cats are the primary host and reservoir for *B. henselae*, *B. clarridgeiae*, and *B. koehlerae*. No major clinical signs of CSD have been reported in cats under natural conditions, but infection is very common (~40% of cats carry *B. henselae* at some point in their life), especially in young kittens. It is estimated that about 10% of pet cats and up to 30–50% of stray cats are *Bartonella* bacteremic at a given time. In western North America (California), a 40% prevalence of bacteremic cats was found in the San Francisco–Sacramento area. Occasionally, *B. henselae* infection may cause self-limiting, transient, febrile illness that lasts 48–72 hours. Clinical symptoms of more serious infection, although rare, include fever, vomiting, lethargy, red eyes, swollen lymph nodes, and/or decreased appetite. Although even more rare, cases of uveitis, mild neurological symptoms, and a few cases of *B. henselae* endocarditis have been diagnosed in pet cats. Potential association between either seropositivity or bacteremia and presence of oral lesions (gingivitis, stomatitis) has been reported too. In addition, reproductive disorders (lack of pregnancy or pregnancy only after repeated breedings and stillbirths) have been observed in experimentally infected queens. Bacteremia usually lasts a few weeks to a few months; however, chronic relapsing bacteremia may occur, the long-term consequence of which is unknown. Previous studies have documented chronic relapsing *B. henselae* or *B. clarridgeiae* bacteremia for as long as 454 days.

Cats can yield more than 1 million colony-forming units (CFUs) of bartonellae per milliliter of blood. Direct transmission from cat to cat, as well as vertical transmission from bacteremic female cats to kittens, was unsuccessful in various experiments. Transmission from cat to cat was successfully achieved by depositing infected fleas collected from bacteremic cats onto noninfected kittens. It was shown that intradermal inoculation of flea feces from infected fleas through cat scratching or grooming are how cats become infected. This was later confirmed by direct intradermal inoculation of naïve cats with flea feces from *B. henselae*–infected fleas. In the flea, *B. henselae* replicates in the hindgut and is excreted within the feces, where it can remain environmentally stable and viable for at least nine days. The role of saliva as a possible source of infection still needs to be fully demonstrated. *B. henselae* DNA was found in infected fleas. Epidemiological studies clearly demonstrate that antibody prevalence and bacteremia prevalence is the highest in stray cat populations living in warm and humid areas, where flea infestation is usually higher.

Dogs

Dogs can be infected with *B. henselae* and are a primary reservoir for *B. vinsonii berkhoffii*. Dogs may also be infected with *B. clarridgeiae*, *B. elizabethae*, *B. koehlerae*, *B. quintana*, *B. rochalimae*, and *B. washoensis*. Dogs can present with a wide range of clinical and pathological abnormalities, such as fever, intermittent lameness, bone pain, BA, endocarditis and myocarditis, granulomatous lymphadenitis, granulomatous hepatitis, peliosis hepatis, cardiac arrhythmias, granulomatous rhinitis, and epistaxis. *B. vinsonii* ssp. *berkhoffii* has been identified as an important cause of canine endocarditis, especially in large breed dogs. In a two-year prospective study

of endocarditis cases, almost one third of the 18 cases were caused by *Bartonella* species. *B. clarridgeiae*, *B. washoensis*, *B. rochalimae*, *B. quintana*, *B. koehlerae*, and *B. henselae* have been associated with dog endocarditis cases, often with large vegetative lesions on the aortic valve. In some dogs, intermittent lameness, bone pain, or fever of unknown origin can precede the diagnosis of endocarditis by several months. *B. clarridgeiae* DNA was detected in a dog with lymphocytic hepatitis. *B. henselae* DNA was initially detected in a dog with peliosis hepatis, and in a dog with hepatopathy and in three dogs with various clinical entities. These three *B. henselae*–DNA-positive dogs presented nonspecific clinical abnormalities, such as severe weight loss, protracted lethargy, and anorexia. A fourth dog was diagnosed as being infected with *B. elizabethae* by PCR amplification and sequencing, increasing the number of *Bartonella* species identified in infected dogs. Serological studies in North America and Europe indicate that *Bartonella* infection in domestic dogs is quite rare (less than 5%), whereas high seroprevalence has been reported from dogs living in tropical countries (up to 65% of dogs tested from Sudan). In North America, especially in the Southeast, high seroprevalence has been reported in dogs also seropositive for various tick-borne pathogens (mainly *Ehrlichia*, *Babesia*, and *Anaplasma*). A high seroprevalence (35%) has been reported in coyotes from California, and in one specific California county, 28% of the coyotes tested were bacteremic.

Rodents

Experimental infection of pregnant laboratory mice with *B. birtlesii* showed pathogenic effects on the reproductive function of these mice. Bacteremia was significantly higher in virgin females than in males. In mice infected before pregnancy, fetal loss and resorption was higher in infected mice than in controls, and the weight of viable fetuses was significantly lower for infected than for uninfected mice. Transplacental transmission was also demonstrated, as 76% of the fetal resorptions were culture positive for *B. birtlesii*. The histopathological analysis of the placentas of infected mice showed vascular lesions in the maternal placenta, which could explain the reproductive disorders observed.

Immunologic Aspects

Infection by *Bartonella* organisms stimulates both the cellular and the humoral responses. *B. henselae* and *B. quintana* induce proliferation and migration of endothelial cells. These effects are due to a trypsin-sensitive factor that appears to be associated with the bacterial cell wall or membrane or intracellular molecules. *B. henselae* infects and activates endothelial cells. *B. henselae* outer membrane proteins are sufficient to induce NFκß activation and adhesion molecule expression, followed by enhanced rolling and adhesion of leukocytes.

In infected individuals, specific antibodies can be detected a few days to a few weeks after infection. Most of the clinical cases of CSD or BA are associated with elevated titers against *B. henselae* or *B. quintana*. Immunity is usually long-lasting in cases of CSD. Human cases of endocarditis are frequently associated with very high indirect fluorescent antibody (IFA) titers (>1:800). However, immunity is not complete against all strains, and cats become reinfected by different strains.

In a murine model, spleen cells from infected C57BL/6 mice proliferated specifically upon stimulation with heat-killed *Bartonella* antigen and CD4 T-lymphocytes mainly mediate proliferative responses. These responses increased during the course of infection and peaked at eight weeks post infection. Gamma interferon, but not interleukin-4, was produced *in vitro* by spleen cells from infected animals upon stimulation with *Bartonella* antigens. As described also in humans, cats, and dogs, *Bartonella*-specific IgG antibodies were detectable in the serum of the infected mice by the second week, and the antibody concentration peaked at 12 weeks post infection. IgG_{2b} was the prominent isotype among the *Bartonella*-specific serum IgG antibodies. Therefore, *B. henselae* induces cell-mediated immune responses with a T_{H1} phenotype in immunocompetent C57BL/6 mice.

In cats, *B. henselae* antibodies detected by IFA or enzyme-linked immunosorbent assay (ELISA) appear two to three weeks after experimental inoculation and usually persist for several months. Most infected cats are bacteremic for several weeks despite high antibody titers. Chronic bacteremia, despite a humoral immune response, is commonly observed among cats. There is no direct correlation between antibody titer and the magnitude of bacteremia; however, cats with IFA serologic titers of 512 or more are more likely to be bacteremic than cats with lower titers.

Dogs with endocarditis often show high antibody titers. In experimentally infected dogs, *B. vinsonii* ssp. *berkhoffii* establishes chronic infection, which may result in immune suppression, characterized by defects in monocytic phagocytosis, an impaired subset of CD8 T-lymphocytes, and impaired antigen presentation within the lymph node.

Laboratory Diagnosis

Accurate diagnosis of *Bartonella* infection or bartonellosis is a clinical, microbiological, and pathological challenge based on the broad spectrum of clinical abnormalities

caused by these pathogens. Further, no negative diagnostic test result can guarantee absence of infection. For years, the diagnosis of CSD was presumptive, based on clinical criteria, history of exposure to a cat, failure to isolate other bacteria, and/or histological examination of biopsies of lymph nodes. Currently, a combination of serological, direct culture, and molecular techniques are used to diagnose *Bartonella* infection or disease. Direct culture remains the gold standard for definitive diagnosis of *Bartonella* infection, but microbiological isolation can be very difficult and can require specialized culture techniques to improve chances of culture success – especially when trying to recover *Bartonella* from accidental hosts. Depending on the infecting *Bartonella* species, certain diagnostic techniques are more effective than others, and diagnosis of some *Bartonella* species remains challenging for any of the available diagnostic assays (e.g. *B. koehlerae* and *B. vinsonii berkhoffii*).

In the past, a skin test using antigen prepared from pasteurized exudate from lymph nodes of patients with CSD was also used in diagnosing CSD, but this test was not standardized and elicited concerns about the safety of the product.

Serological tests, such as IFA or ELISA, and techniques to isolate the organism from human, dog, and cat specimens have been developed since the mid-1990s. Because bartonellae are intraerythrocytic bacteria, cell lysis using a lysis-centrifugation technique greatly facilitates bacterial isolation from the blood. However, blood isolation is seldom obtained from human cases of CSD and from domestic dogs. On the contrary, isolation is more commonly successful from human BA cases, for which serology is often negative. Isolation from the blood of natural reservoirs is also quite common with bacteremia prevalence ranging from 10% to 20% (such as for wild felids or coyotes) to up to 95% (beef cattle and deer).

For blood culture from cats, 1.5 ml of blood is drawn into lysis-centrifugation tubes (ISOSTAT Microbial System, Wampole Laboratories, Princeton, United States); more commonly, cat blood collection in EDTA tubes kept frozen at 0 °C to −70 °C for a few days or weeks has been a preferred alternative because of easier handling and lower cost. For dogs or cattle, a larger volume of blood can be collected (3–5 ml). The tubes are centrifuged, and the pellet spread onto infusion agar plates containing 5% fresh rabbit blood, which are maintained at 35 °C in a high-humidity chamber with 5% CO_2 for three or four weeks. Colonies usually will develop in a few days from cat blood, although some strains may require a few weeks (Figure 42.1).

Diagnostic detection by molecular methods such as PCR using *Bartonella* genus and species-specific primers is often used/attempted, especially when serology results are negative, and isolation is not successful. However, low or intermittent circulation of *Bartonella* organisms can result in false negative results due to variable pathogen presence and assay sensitivity. PCR tests can be performed from a variety of patient sample types such as peripheral blood, cerebrospinal fluid, joint fluid, bacterial cultures, oral swabs, and lymph node or other tissue samples or aspirates.

Evidence of infection can be detected in humans or animals by detection of antibodies by IFA or ELISA. An IFA titer of at least 1:64 is considered positive. *B. henselae* antibodies can be detected despite concurrent bacteremia in cats and sometimes in humans. Detection of antibodies can indicate active or past infection, and these tests often cross-react against multiple *Bartonella* species that may or may not be the cause of the current clinical signs. Interestingly, seronegative animals with a history of chronic illness can test seropositive for *Bartonella* after a course of antimicrobial treatment, suggesting that the chronic disease signs were, at least in part, attributable to *Bartonella* spp. infection. Antibodies to *B. vinsonii* ssp. *berkhoffii* antigens are infrequently detected (<4%) in a sick referral or healthy (<1%) dog population. Detection of *B. vinsonii* ssp. *berkhoffii* antibodies in a sick dog provides strong clinical evidence for prior exposure to and potentially active infection with this organism.

Other means of isolation of *Bartonella* have been by using the BACTEC blood culture system or BACT/Alert blood culture system. Identification of isolates as *Bartonella* can be performed by using enzyme-based identification systems but is usually confirmed by DNA amplification using PCR-RFLP analysis. Several restriction endonucleases, such as TaqI and HhaI for citrate synthase gene, are used to digest the single product amplified by specific primers. PCR has also been used to identify *Bartonella* spp. in tissues, in the absence of culture. Diagnosis of *Bartonella* endocarditis relies heavily on this method in humans and dogs in conjunction with high antibody titers.

In 2005, Maggi et al. described a novel, chemically modified, insect-based liquid culture medium (BAPGM) that supports the growth of at least seven *Bartonella* spp. This medium also supported co-cultures consisting of different *Bartonella* spp. Subsequently, a unique diagnostic platform that utilizes pre-enrichment culture utilizing BAPGM, followed by a highly sensitive PCR assay (which has a sensitivity of 0.5 bacterial genome copies per microliter of sample DNA template) targeting the 16S-23S ITS region or Pap31, a bacteriophage-associated gene was developed. This approach has allowed characterization and quantification of *Bartonella* infection in blood, cerebrospinal fluid, joint fluid, pathological effusions, seroma fluids, transudates, and modified transudates from dogs with idiopathic cavitary effusions and tissue biopsy samples obtained at surgery.

Biochemical and laboratory findings may be otherwise normal in chronically infected reservoir hosts (e.g. cats infected with *B. henselae*). When laboratory test abnormalities are observed in sick cats or dogs, the most commonly reported abnormalities are anemia, eosinophilia (found in ~33% of infected dogs), hyperproteinemia, hyperglobulinemia, neutropenia, neutrophilic leukocytosis, monocytosis (frequently occurs in dogs or cats with endocarditis), and thrombocytopenia (found in ~50% of infected dogs). Importantly, some of these finding may be secondary immune mediated. Compared to dogs, these laboratory finding are much rarer in *Bartonella*-infected cats.

Treatment

In humans, antimicrobial treatment is not generally indicated for CSD as most cases resolve on their own. However, whether a course of antibiotics can effectively shorten CSD or prevent or reduce infection dissemination is debated. Macrolides such as azithromycin have been shown to decrease lymph node volume more rapidly compared to no treatment. The CDC-recommended dose of azithromycin for CSD for adults and children > 45.5 kg is 500 mg on day 1, followed by 250 mg for four days; and, for children ≤ 45.5 kg is 10 mg/kg on day 1, followed by 5 mg/kg for four days. Antimicrobial treatment is generally indicated for patients with BA, bacillary peliosis, or relapsing bacteremia. For treatment of *Bartonella* infections other than CSD, aminoglycosides are typically used as a first-line treatment because they are bactericidal. With serious infections, more than one antibiotic is often used. Other antibiotics that are effective against *Bartonella* infections include penicillins, tetracyclines, and cephalosporins. Treatment with azithromycin, erythromycin, rifampicin, or doxycycline for at least two to three months in immunocompromised people is recommended, but relapses can occur. In such cases, patients should receive lifelong treatment with one of these antibiotics. In cases of *Bartonella* endocarditis, patients receiving an aminoglycoside were more likely to fully recover, and those treated with aminoglycosides for at least 14 days were more likely to survive than those with shorter therapy duration.

For dogs and cats, there is no standardized antimicrobial protocol for treating *Bartonella* infection. Although multiple antimicrobials have been demonstrated effective, treatment efficacy can vary among individual patients. Current therapeutic strategies for dogs and cats with *Bartonella* infection are listed in Table 42.2.

Table 42.2 Current therapeutic strategies from for cats and dogs infected with *Bartonella* spp.

Host	Clinical manifestation/*Bartonella* spp.	Drug	Dose/Duration
Cat	Bacteremia and uveitis/*Bartonella* spp.	Doxycycline	50 mg/cat PO q12–24 h/14–28 days
		Doxycycline	10 mg/kg PO q12–24 h/4–6 weeks
		Doxycycline + Pradofloxacin	5 mg/kg PO q12/4–6 weeks +5 mg/kg PO q12/4–6 weeks
		Azithromycin	10 mg/kg PO q24 (or q48) h/7 days followed by every other day for 6–12 weeks or daily for 3 weeks
		Marbofloxacin	5 mg/kg PO q24 h/6 weeks
	Endocarditis/*Bartonella henselae*	Marbofloxacin + Azithromycin	5 mg/kg PO q24 h/6 weeks +10 mg/kg PO q24 h for 7 days and then q 48 h for 6 weeks
	Osteomyelitis and polyarthritis/*Bartonella vinsonii berkhoffii*	Amoxicillin-clavulanate (with azithromycin)	62.5 mg PO q12 h/2 months +10 mg/kg PO q48 h/3 months
Dog	Splenic vasculitis, thrombosis and infarction/*B. henselae*	Doxycycline + Trimethoprim-sulfamethoxazole	5–10 mg/kg PO q12 h/4 weeks +23 mg/kg PO q12 h/6 weeks
	Neurological and ocular disorders/*Bartonella* spp.	Doxycycline + Enrofloxacin	5–15 mg/kg PO q12 h/4–6 weeks +5 mg/kg PO q12 h/4–6 weeks
		Doxycycline + Rifampicin	5–10 mg/kg PO q12/4–6 weeks +5 mg/kg PO q24 h/4–6 weeks
	Endocarditis/*Bartonella koehlerae*	Ampicillin + Enrofloxacin	22 mg/kg PO q8 h/4–6 weeks +5 mg/kg PO q12–24 h/4–6 weeks
	Hemangiopericytoma/*B. vinsonii berkhoffii*	Enrofloxacin	5 mg/kg PO q12 h/4–6 weeks

Source: Adapted from Álvarez-Fernández et al. (2018) and Pennisi and Marsilio (2020).

An earlier standard treatment regimen using azithromycin (5–10 mg/kg daily for seven days followed by every other day administration for an additional five weeks) has proved effective for most, but not all cats and dogs; however, this treatment is no longer recommended as a first-line treatment option due to the rapid development of resistance among *B. henselae* strains. Fluoroquinolones alone, or in combination with amoxicillin, have also elicited a positive therapeutic response in dogs, which is accompanied by a progressive decrease in *B. vinsonii* antibody titers. Doxycycline may or may not be effective for treatment of *B. vinsonii* ssp. *berkhoffii*, but data from cats experimentally or naturally infected with *B. henselae* or *B. clarridgeiae* indicate that a high dose of doxycycline (10 mg/kg, q 12 h, for four to six weeks) may be necessary to eliminate *Bartonella* infection in cats, dogs, or other animal species.

Various antibiotics (doxycycline, erythromycin, enrofloxacin) have been shown to reduce the level of bacteremia in experimentally infected cats but do not eliminate infection, and the level of bacteremia may surpass the initial level a few weeks after the cessation of treatment. In animals with pre-treatment *Bartonella* antibody titers, antibody titers will typically decrease within three to six months and remain below detection in cases of successful treatment and possible clearance (the latter is difficult to confirm). Treatment of pets with no clinical signs is a source of debate given the duration of treatment, the uncertainty of infection elimination, and the growing concern of antimicrobial resistance; however, in households with immunocompromised individuals, attempts at treatment for infection clearance should be considered.

Prevention

Bartonella-infected companion animals pose the greatest potential risk for human infection because of frequent physical contact between companion animals and people. A large reservoir for *B. henselae* and possibly for *B. clarridgeiae* exists among the 86.4 million pet cats residing in one third of homes in North America. The most effective means of preventing *B. henselae* infection are common sense, hygiene, strict flea/tick control, and modification of cat owners' behaviors. To reduce transmission potential, hands should promptly be washed with soap and running water after playing with cats and especially after any bites or scratches. Avoid rough play that would encourage scratching and biting (Figure 42.4). Do not allow cats to lick open wounds. Avoid petting or touching stray or feral cats.



Figure 42.4 Rough play with cats that provokes biting or scratching can facilitate *B. henselae* transmission from infected cats to humans.

Seronegative cats are not likely to be bacteremic, but young kittens, especially impounded kittens and flea-infested kittens, are more likely to be bacteremic. Therefore, people who want to acquire a pet cat, especially if they are immunocompromised, should seek a cat raised in a cattery or an adult cat coming from a flea-controlled environment. Unfortunately, there is no correlation between seropositivity and bacteremia. Bacteremia can also be transient with relapses.

Keep cats' nails trimmed. Declawing cats has also been suggested but has a limited value, because fleas can transmit infection from cat to cat. Use flea/tick control products to reduce or prevent flea infestations and flea bites that can spread from cat to cat. Inspect cats for fleas or "flea dirt" (flea feces) using a flea comb. Control fleas in the home by vacuuming frequently and using a pest control agency when necessary. To prevent introduction of fleas into a home, keep cats indoors to reduce their contact with fleas carried by wildlife and to prevent them from fighting with other potentially infected animals. Although some evidence suggests CSD may be directly spread directly to people by an infected flea bite, this has not been definitively demonstrated. Further, *B. henselae* transmission between co-housed cats can be effectively managed if they live in a flea-free home as direct transmission between cats (in the absence of blood-to-blood contact) does not occur.

For dog bartonellosis, where tick infestation could be a risk factor for acquiring infection, tick and flea control measures should be used during the tick and flea season.

Systematic inspection of the dog for the presence of ticks after a walk in an infested area is highly recommended.

Several topical and oral flea and tick control products are available to control and prevent flea and tick infestation on dogs and cats. Common active ingredient(s) in these products include: isoxazoline class drugs, fipronil, methoprene, imidacloprid, or permethrin. Owners should consult with their veterinarian on the best product for their pet based on risk-based factors (e.g. exposure to vectors, outdoor/indoor lifestyle, geographic location) and their likelihood of complying with product administration instructions. Importantly, permethrin should never be used on cats.

As no vaccines are currently available to prevent *Bartonella* infections in people, cats, or dogs, prevention of associated disease relies on good vector control, good hygiene, and commonsense measures when interacting with potentially infected animals.

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Part III

Fungi

43

Yeasts

Cryptococcus, Malassezia, and Candida

Lisa M. Pohlman and M.M. Chengappa

Whether a fungus is categorized as a mold or a yeast is based upon its microscopic appearance in tissue or on routine culture media (the asexual stage).

Microscopically, if hyphal structures are observed, the fungus is termed a mold; if single-celled budding structures are observed, the fungus is termed a yeast. On routine culture media, molds will have a "fuzzy" or wooly appearance, and a yeast will be bacteria-like in its colonial morphology and consistency. Some pathogenic fungi will produce either hyphal structures or yeast-like structures, depending upon the conditions in which they are growing. Such fungi are called dimorphic fungi (Chapters 45 and 46).

In this chapter, three important representatives of yeast fungi are discussed: *Cryptococcus neoformans, Cryptococcus gattii*, *Malassezia pachydermatis*, and *Candida albicans*.

Cryptococcus neoformans and *Cryptococcus gattii*

There are at least 19 encapsulated yeast species in the genus *Cryptococcus*. The majority of infections are caused by *Cryptococcus neoformans* and *Cryptococcus gattii* (formerly *C. neoformans var. gattii*), which are now recognized as distinct, but related species, as part of the *Cryptococcus* pathogenic species complex. For that reason, only *C. neoformans* and *C. gattii* will be covered here.

Cryptococcosis is typically associated with ulcerative lesions affecting the mucous membranes of the upper respiratory tract (including nasal sinuses), the central nervous system (meninges), and eye (chorioretinitis) and has been reported in many veterinary species including felids (the domestic animal most commonly affected), canids, equids, camelids, ruminants, ferrets, birds, and marsupials, among others. It is often reported to be the most common systemic mycosis of cats, although in the authors' opinion that is geographically dependent. Even so, the incidence in cats is eightfold that of dogs. It is an uncommon cause of mastitis in cattle and has been, in rare instances, associated with intestinal disease, endometritis, and abortion in horses. However, this yeast has the potential to affect all animals, including humans. It is an opportunistic pathogen of human patients, especially immunocompromised ones, all over the world. In all species, there is the tendency for the central nervous system to become involved.

Descriptive Features

Morphology

C. neoformans and *C. gattii* are basidiomycetous, dimorphic fungi that grow as budding yeasts in the environment, in the infected host, and in culture media, but during reproduction (either mating between cells or unisexual) transition to a filamentous monokaryon or dikaryon. The spherical cells (2–20 μm diameter) produce single (usual) buds with slender stalks (narrow-based budding) and surrounded by polysaccharide capsules (Figure 43.1). The – often very thick – capsule is characteristic of this fungus.

Cellular Products of Medical Interest

Capsule

The polysaccharide capsule, composed primarily of a glucuronoxylomannan (GXM), is a major virulence factor in that it mediates many deleterious effects on the host immune response. These harmful effects include, but are not limited, to the following: prevention of effective antibody-mediated phagocytosis, stimulation of T-regulatory (formerly known as suppressor) lymphocytes, inhibition of leukocyte migration, alteration of cytokine production, blockage of

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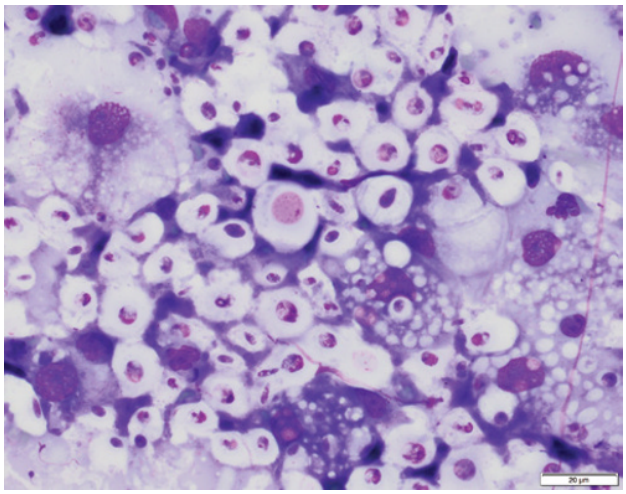


Figure 43.1 Fine-needle aspirate preparation of a nasal lesion from a cat with cryptococcosis. Note the characteristic unstained capsules surrounding the blue-stained yeast. *Source:* Modified Wright's stain, 1000×.

co-stimulatory molecules, and limitation of complement activation to the alternate pathway. Capsule size is significantly affected by the environment.

Melanin

Melanin (which is produced from phenols by phenoloxidase via the laccase pathway) is a powerful virulence factor and antioxidant (free radical scavenger) and thus reduces the toxicity of hydroxy radicals, superoxides, and singlet oxygen radicals found within the phagolysosome. Melanin also provides temperature tolerance and protects the organism against enzyme degradation, radiation, and heavy metals while allowing acquisition of nutrients; the packing of melanin granules within the cell wall helps inhibit the entry of antifungal compounds.

Phospholipase

Phospholipase is important for survival within macrophages, and is needed for the systemic spread of the yeast from the respiratory tract to the central nervous system. Phospholipase is believed to be involved in the membrane disruption process in the host.

Sialic Acids

Sialic acids found within the cell wall direct complement proteins toward the degradative pathway, rather than generating effective opsonizing fragments and anaphylotoxins.

Growth Characteristics

Cryptococcus spp. grow on common laboratory media at room temperatures or at 30 °C. Encapsulation is optimal on media such as chocolate agar incubated at 37 °C. Colonial growth may be apparent within two days. Colonies are convex, grayish white to white, and mucoid, and can reach diameters of several millimeters.

Biochemical Reactions

C. neoformans and *C. gattii* are significantly different in their utilization of nitrogen and carbon. These differences assist species differentiation. *C. neoformans* utilizes creatinine as a nitrogen source, and *C. gattii* more effectively utilizes D-glycine and other dicarboxylic acids as carbon sources. Both species produce melanin-pigmented, a virulence factor, on media containing diphenolic and polyphenolic compounds.

Resistance

Cycloheximide concentrations found in some fungal isolation media inhibit *C. neoformans*. Replication ceases above 40 °C. Highly alkaline environments kill the agent.

Variability

Five antigenic types (A, B, C, D, and AD) based on the antigenic makeup of the capsular polysaccharides have been described. Molecular typing methods have resulted in additional division into eight major molecular types: four each within *C. neoformans* and *C. gattii*. *C. neoformans* molecular-based serotypes include VNI, VNII (serotype A); VNIII (serotype AD); and VNIV (serotype D). *C. gattii* molecular-based serotypes include VGI, VGII, VGIII, and VGIV (serotypes B and C). Each species and molecular type exhibits differences in pathogenicity, clinical presentation, epidemiology, and drug susceptibility.

Ecology

Reservoir

C. neoformans lives in surface dust and dirt and decaying plant matter, especially in the hollows of certain trees. In soil, it does not compete well with resident microbiota. In dried bird (especially pigeon) droppings (rich in creatinine, which inhibits other microorganisms), the fungus reaches high concentrations and survives for more than a year at much reduced capsular and cell size.

C. gattii lives mainly in association with the decaying wood of the red river gum group of eucalyptus trees and in the tree bark of Garry oak, maple, cedar, and pine trees It has also been found in seawater, air, and soil.

Transmission

The route of infection is usually respiratory, rarely percutaneous. Cryptococcosis is noncontagious.

Pathogenesis

In an environment where moisture and nutrients are plentiful, *Cryptococcus* spp. make little if any capsular material. In arid conditions, the capsule collapses and protects the yeast from dehydration. In either case, the size (approximately 3 μm) is small enough for it to reach the lung alveoli. At physiological concentrations of bicarbonate, CO_2, and free iron, a capsule is produced. The cryptococcal capsule is a very efficient activator of the alternate complement pathway, resulting in the deposition of C3b on its surface. Although opsonized, even in the presence of anticapsular antibody, the yeast is poorly phagocytosed, as capsular components block the binding of IgG via interference of the interaction between the Fc portion of bound antibodies and receptors on host phagocytes. Capsular polysaccharide increases participation of regulatory T lymphocytes (formerly known as suppressor T lymphocytes) and decreases antigen processing, leading to a poor antibody response. Capsular polysaccharide also diminishes the chemoattractive effects of the anaphylotoxins C3a and C5a generated by activation of the alternate complement pathway. In the event phagocytosis occurs, melanin and mannitol produced by the yeast scavenge free radicals and reduce the hostile environment within the phagolysosome by inactivating superoxides, hydroxyl, and singlet oxygen radicals. In addition, phospholipase is produced, further diminishing the ability of phagocytic cells to eliminate the fungus. Thus, inflammatory responses are minimal, and cryptococci grow into large space-occupying "myxomatous" masses consisting of the characteristic capsular gelatinous slime (Figure 43.2), yeast cells, and a few inflammatory cells. Eventually these masses acquire histiocytes, including epithelioid, and multinucleated macrophages.

Development of pulmonary lesions is erratic. Infections often localize in the central nervous system (perhaps due to lower complement concentrations in the central nervous system (CNS) and to high concentrations of catechols, a substrate for phenoloxidase, the enzyme the yeast uses to produce melanin) following dissemination from the lungs and are manifested by neurological signs. Eye involvement, leading to chorioretinitis and blindness, is relatively common.

Disease Patterns

Cats and Dogs

Cats and dogs are most often clinically affected, with cats having an eightfold greater incidence of infection than dogs. Signs include chronic, ulcerative lesions of the mucous membranes in the nose, mouth, pharynx, and sinuses or myxomatous nasal masses (Figure 43.3).



Figure 43.2 Trapped community cat with large, progressive facial lesion due to cryptococcosis. Note the characteristic glistening, gelatinous appearance of the lesion. *Source:* This image is reprinted from Steen and Pohlman, Clinician's Brief (2020), with permission.

Central nervous system and ocular involvement are common. Disseminated disease lesions involving the skin and



Figure 43.3 Trapped community cat with large, progressive facial lesion due to cryptococcosis. *Source:* This image is reprinted from Steen and Pohlman, Clinician's Brief (2020), with permission.

subcutaneous tissue, lymph nodes, the kidney, spleen, liver, adrenal and thyroid glands, the gastrointestinal tract, and others have been reported. Infection in dogs is less common than in cats. Infection in cats is generally seen between three and seven years of age. In the United States, most dogs are infected with *C. neoformans*. Conversely, most cats, are infected with *C. gattii*, especially in California, where the disease is common.

Cattle

Cattle typically acquire cryptococcosis during administration of contaminated intramammary medication, resulting in bovine cryptococcal mastitis. There is gross swelling, hardening of the gland, and gradual changes in the secretions. Destruction of the lactiferous epithelium is extensive. Several glands may be irreversibly damaged. The disease rarely advances beyond the regional lymph nodes.

Sheep and Goats

Cryptococcosis is not common in sheep and goats.

Horses

C. neoformans causes meningitides, nasal granulomas, and occasionally rhinitis and granulomatous pneumonia in horses. In rare instances, it has been associated with intestinal disease, endometritis, and abortion.

Cryptococcosis has been reported in other many other species, and it is suspected that it can infect all mammals and some birds.

Epidemiology

Cryptococcus can probably affect any mammal. Its occurrence is sporadic and worldwide. Birds, particularly pigeons, often carry the agent in their intestinal contents and contribute to its reservoir (see above). They are rarely affected clinically, and then mostly on mucosal surfaces.

In human beings, *C. neoformans* infection is most often associated with immunosuppression (organ transplants, Hodgkin's disease, pregnancy, acquired immunodeficiency syndrome [AIDS], malignancy) or intensive exposure. Attempts to relate animal infections to similar circumstances have been speculative.

In contrast, *C. gattii* infections can infect immunocompetent hosts. In these human patients, the disease is often characterized by pulmonary or intracranial cryptococcomas (mass lesions).

Immunologic Aspects

Immunosuppression is a predisposing factor. However, *C. gattii* is known to infect immunocompetent hosts. The capsular polysaccharides produce immune paralysis, complement depletion, and antibody masking.

Humoral and cell-mediated phenomena (TH1 subset resulting in macrophage activation) evidently contribute to defense against cryptococcal infection. Macrophages participate in disposal of the agent. There is some evidence that T lymphocytes (CD4 and CD8) as well as natural killer cells kill or inhibit *C. neoformans* directly.

Results of experimental immunization have been equivocal. No vaccines are available.

Cryptococcosis is not more common in cats with retroviral infections (e.g. feline immunodeficiency virus [FIV] and feline leukemia virus [FeLV]), whereas it is much more common in human patients infected with human immunodeficiency virus (HIV). FIV- or FeLV-positive cats may or may not respond to appropriate antifungal therapy; human HIV-positive patients respond poorly if at all.

Laboratory Diagnosis

Direct Examination

In the clinical setting, cytological preparations stained with Romanowsky-type stains (Wright's, Giemsa) are frequently used to diagnose infection of *Cryptococcus* spp. via visualization of the pale blue/purple or clear capsule surrounding the blue-stained yeast (see Figure 43.1). Characteristic narrow-based budding may be identified but is not necessary to make a diagnosis of cryptococcosis. To enhance visualization, a small amount of sediment from exudates, tracheobronchial washes, and cerebrospinal fluids can also be mixed with an equal amount of India ink on a slide; a coverslip is placed on top and the sample is viewed under a microscope. However, india-ink-stainingis not usually required. The encapsulated organisms appear as bright circular lacunae in a dark field, with the yeast cells in their centers. Yeast cells with or without budding cells are visible. In sections processed by the usual histological methods, with hematoxylin and eosin (HE) stain the organism appears as a round to oval yeast body within a clear (unstained) capsule that separates the yeast cells from tissue constituents or from each other. Fungal stains – for example, periodic acid Schiff (PAS) and Gomori methenamine silver (GMS) – delineate the cell wall but not the capsule, which is stainable by mucicarmine.

Culture

Fungal isolation media such as Sabouraud's dextrose agar, incubated at 25 or 30 °C and at 37 °C, are recommended when the differential diagnoses include fungal agents. To

minimalize bacterial growth, antibiotics can be added. Characteristic white, creamy colonies may take 2–10 days to grow although 2–3 days is typical. *C. gattii* is reported to be more mucoid than *C. neoformans*, but this feature should not be used to differentiate the two species. *Cryptococcus* spp. are identified based on the microscopic encapsulated yeast morphology. Examination of an India ink wet mount may aid identification. Commercial test kits are also available.

Differentiation between *C. gattii* and *C. neoformans* is performed via culture on canavanine-glycine-bromothymol blue (CBG) agar. Growth of a brilliant blue colony is indicative of *C. gattii*. *C. neoformans* does not grow in this media. Selective media incorporating antibacterial and antifungal drugs, creatinine, and diphenyl are used for environmental sampling.

Some normal dogs and cats harbor small numbers of *Cryptococcus* spp. in their nasal cavities. Therefore, care should be taken when interpreting culture results from samples obtained from this site. Examination of direct smears (the numbers of yeast in smears from clinically normal animals are too low to see) and/or analysis of serum for capsular antigen are helpful adjuncts to culture. Capsular antigen is not detectable in the serum of normal dogs, regardless of whether they harbor *Cryptococcus* spp. in their nasal passages.

Serology and Molecular Diagnostics

Cryptococcal polysaccharide capsular antigen demonstration via latex agglutination is widely available and is performed using serum, urine or cerebrospinal fluid (CSF). Latex agglutination is sensitive and specific for detection of antigen for *C. neoformans* and *C. gattii*. However, a negative result does not completely rule out cryptococcosis as the sensitivity is not 100%. Enzyme-linked immunosorbent assay (ELISA) assays are also readily available. They also require serum or CSF.

Real-time polymerase chain reaction (PCR) is offered by some laboratories. However, the assays are highly sensitive, and a positive result may not reflect disease. A positive PCR should be supported by clinical disease consistent with cryptococcosis.

Antibody is irregularly demonstrable because of the "sponging" action of circulating capsular antigens. Its presence (demonstrated by indirect fluorescent antibody tests or by latex particles coated with capsular polysaccharide) is a favorable sign of decreasing antigen levels.

More recently a versatile mass spectrometry technique known as MALDI-TOF has begun to be used in the diagnostic laboratories for the identification of *Cryptococcus* spp. MALDI-TOF stands for Matrix-Assisted Laser Desorption Ionization – Time Of Flight.

Treatment and Control

Cats

The initial antifungal agent of choice is fluconazole. With failure of response, as often occurs with *C. gattii*, itraconazole or ketoconazole (although its use is controversial in cats) may achieve remission. Liver enzymes should be monitored when treating with azoles. Amphotericin B may also be used alone or in combination therapy in severe disseminated cases. It is used sometimes in combination with 5-flucytosine or fluconazole.

Dogs

Initial recommended treatment is a combination therapy of fluconazole or lipid-complexed amphotericin B in combination with deoxycholate. Long-term treatment with terbinafine has also been successful.

Horse

Multimodal therapy may be required. Successful multimodal therapy has included fluconazole, organic ethylenediamine dihydroiodide, amphotericin B, and formalin together with surgical debulking.

Therapy should be continued until clinical signs are resolved and antigen disappears from serum and CSF. This usually takes anywhere from take 2–12 months but has been reported to take longer.

Contaminated surfaces (pigeon lofts, attics) can be disinfected with lime solution (1 lb hydrated lime/3 gal water) prior to physical cleanup. The dirt removed is placed in containers and covered with hydrated lime powder, which can also be used on exposed floors and beams. Masks are worn during the operation.

Malassezia pachydermatis

Malassezia spp. are present in low numbers in the normal flora of skin in animals. However, they are opportunistic organisms and, with overgrowth, they are commonly associated with disease, having been isolated from the skin and external ear canals of normal and clinically affected dogs, cats, ruminants, and horses, among others. There are numerous species of *Malassezia*, most of which are lipid dependent, However, *M. pachydermatis* (which is non-lipid dependent in culture) comprises the vast majority of the isolates from dogs and cats, and so will be the focus of this discussion. That said, speciation of the organism is not routinely done in clinical practice.

Descriptive Features

Morphology and Composition

M. pachydermatis is an oval budding yeast (2 µm by 5 µm). In direct smears (and from colonies obtained from culture), a single bud attached to a broad base (0.9–1.1 µm) is seen (Figure 43.2). Filaments are not usually observed, regardless of culture conditions. The cell wall is composed of glycoproteins (75–80%), lipids (15–20%), and chitin (1–2%).

Growth Characteristics

Though not requiring lipids for growth, *M. pachydermatis* is lipophilic, and growth is improved when lipids are added to the medium. Most strains of *M. pachydermatis* grow on blood agar plates, though the colonies will be very small (<1 mm in diameter, and sometimes only a "greenish" tint will be seen on the surface of the plate) after several days of incubation (the optimum temperature is 37 °C, although it will grow at temperatures ranging from 25 to 41 °C). The yeast will grow in either an aerobic or microaerophilic atmosphere (it does not grow well anaerobically).

Biochemical Reactions

M. pachydermatis assimilates the carbon of glucose and D-mannitol, but does not ferment carbohydrates. Urea hydrolysis is strain dependent. However, *M. pachydermatis* strains produce enzymes such as proteinase, chondroitin sulfatase, hyaluronidase, and phospholipase, which are believed to contribute to the disease process. An immune-mediated hypersensitivity reaction is also believed to be a contributing factor of the disease.

Resistance

M. pachydermatis is resistant to cycloheximide.

Variability

There are a number of biotypes of *M. pachydermatis* as reflected in the variability in D-mannitol and sorbitol assimilation, the hydrolysis of urea, and cell wall fatty acid concentration.

Seven genetic types (a–g) have been described using the sequence of DNA encoding the large ribosomal subunit as the basis for comparison. Others have delineated four genetic types (A–D) after using the random amplification of polymorphic DNA method, together with sequence comparisons of the gene encoding chitin synthase.

Ecology

Reservoir

M. pachydermatis lives on the skin and external ear canal of healthy animals, including dogs, cats, ferrets, pigs, and rhinoceros (from which it gets its name). The surface of *M. pachydermatis* has mannose-containing glycoproteins that are responsible for binding to mannose receptors on the surface of corneocytes, thus allowing adherence in this niche. It is rarely isolated from human skin (*Malassezia furfur* is the opportunistic *Malassezia* species associated with the normal flora of humans) or the environment.

Transmission

M. pachydermatis is an opportunistic fungus that aggravates the disease processes already in progress (e.g. allergic dermatitis). The source of the yeast is endogenous (i.e. a member of the patient's normal flora). Studies show that although owners of dogs that have atopy are 11 times more likely to carry *M. pachydermatis*, carriage of this organism poses minimal risk to human health.

Pathogenesis

M. pachydermatis plays a secondary but significant role in otitis externa and dermatitis in a variety of animals, but most commonly in dogs, and to a lesser extent, in cats. The exact role played by *M. pachydermatis* remains unclear, and what causes the yeast to change from a harmless commensal to one that contributes to disease is unknown. However, it is believed that the disease may be associated with prolonged treatment with antibiotics, immunosuppression, glucocorticoid administration, as well as other predisposing conditions. If its presence is ignored when formulating a treatment regimen, however, resolution of the disease process is problematic.

Epidemiology

M. pachydermatis is a parasite of the skin (especially moist areas such as skin folds, interdigital regions, and the external ear canal) of nonhuman animals. *M. pachydermatis*–associated dermatitis is more commonly reported in Australian silky terriers, basset hounds, cocker spaniels, dachshunds, poodles, and West Highland white terriers. Cat breeds most commonly affected by *Malassezia* dermatitis are Devon Rex cats and Sphynx. The yeast has worldwide distribution.

Immunologic Aspects

M. pachydermatis is an opportunistic yeast that exploits preexisting compromises of the skin and external ear. It is unknown how *M. pachydermatis* contributes to disease. However, *M. pachydermatis* strains produce enzymes such as proteinase, chondroitin sulfatase, hyaluronidase, and phospholipase, which are believed to contribute to the disease process. An immune-mediated hypersensitivity reaction is also believed to be a contributing factor of the disease.

Laboratory Diagnosis

Direct Examination

The numbers of yeasts on normal skin or in the normal external ear canal are usually too low to visualize in samples taken from such sites. Thus, determining whether *M. pachydermatis* is a contributing factor to otitis externa or to a dermatological condition is relatively easy, because the numbers of yeasts will be high enough to be seen in samples taken from affected areas. However, some atopic dogs have been shown to have increased numbers of *M. pachydermatis* in normal as well as affected areas.

Samples taken with cotton-tipped swabs are the easiest to obtain from cases of otitis externa. Swabs are "rolled" over the surface of a microscope slide; the smear is air-dried, then stained with a Romanowsky-type stain (Wright's, Giemsa). Examination of smears will reveal yeasts with characteristic "bottle-shaped" or "shoe print" morphology (see Figure 43.4). If the swabs are also streaked onto the surface of a blood agar plate, small colonies (or a greenish coloration) will appear within 24–48 hours after incubation of the plate at 37 °C incubator. *M. pachydermatis* grows well on fungal selective media such as Mycosel or Sabouraud dextrose agar plates incubated at 37 °C.

Molecular Techniques

PCR amplification of DNA encoding either the large or the small ribosomal subunit together with the internal transcribed spacer region may be used to detect *Malassezia* spp. The same technology has been applied to speciation of the yeast.

Culture

To determine whether *M. pachydermatis* is a contributor in otitis externa, culture is not worth the effort, because most samples taken from such conditions contain bacteria (e.g. *Pseudomonas*) that quickly overgrow the slower-growing yeast. The involvement of *M. pachydermatis* can be confirmed more quickly by microscopic examination of samples.

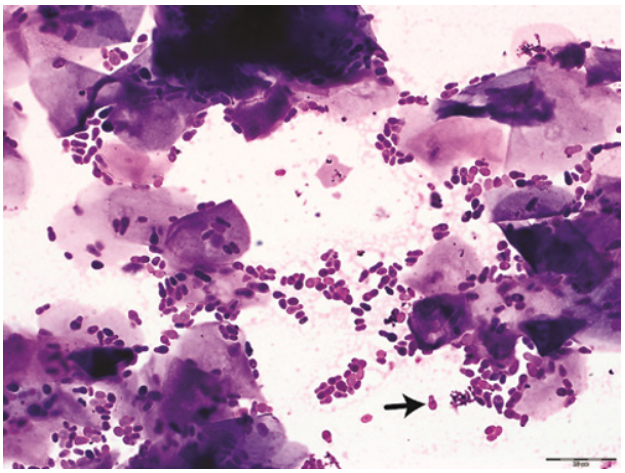


Figure 43.4 Exudate of canine otitis externa containing many *Malassezia pachydermatis* yeasts. Note characteristic "shoe print" pattern of budding yeasts (arrow). *Source:* Modified Wright's stain, 1000×.

Culture plays a role in assessing the microbiological makeup of dermatological conditions, as well as in formulating a treatment regimen. Media that have proved useful include Mycosel or Sabouraud dextrose agar. Plates are incubated at 37 °C.

Treatment and Control

Correction of the underlying condition is the most important aspect of treatment of *M. pachydermatis*–associated otitis externa or dermatitis. Almost all commercially available topical preparations that contain an antifungal agent (nystatin, clotrimazole, miconazole, etc.) are effective in treating the fungal component of otitis externa. Medicated shampoos (e.g. miconazole + chlorhexidine), along with systemic antifungal administration (ketoconazole, itraconazole, fluconazole, or terbinafine) are effective in reducing the influence of *M. pachydermatis* upon dermatological diseases. Griseofulvin is not effective.

Candida albicans

Candidiasis is usually due to the parasitic yeast *C. albicans*, which inhabits the mucous membranes of most mammals and birds. Of the more than 200 other species of *Candida*

that are associated with many diverse habitats, few are associated with animal disease, and *C. albicans* is the most important pathogen of animals and humans. Disease produced by *C. albicans* usually occurs in an immunocompromised host. Overuse of antibiotics and prolonged use of steroids and hormonal therapy could disrupt the normal cutaneous defense mechanisms, leading to candidiasis in animals and humans.

Descriptive Features

Cell Morphology and Composition

On routine laboratory media and mucous membranes, *C. albicans* typically grows as oval budding yeast cells (blastoconidia), 5–8 µm in size. Under certain conditions of temperature, pH, nutrition, and atmosphere, yeast cells sprout germ tubes (Figure 43.5) that develop into septate-branching mycelium. Pseudohyphae are produced by elongation of the blastoconidia and their failure to separate. *In vivo*, mycelial (a collection of hyphae) or pseudomycelial growth is associated with active proliferation and invasiveness.

The so-called chlamydospore (chlamydoconidium) is a thick-walled sphere of unknown function, attached by a suspensor cell to (pseudo)mycelium and essentially confined to *in vitro* growth (Figure 43.6) of *C. albicans* (rarely other *Candida* spp.).

The cell wall contains glycoproteins; the polysaccharide portions are glucans and especially mannans. Lipids and chitin are also present. Mannoproteins are found on the cell surface. Cellular products include peptidolytic enzymes, which may be virulence factors. Two major cross-reacting serogroups are recognized. These are termed A and B, and are identifiable with absorbed sera.

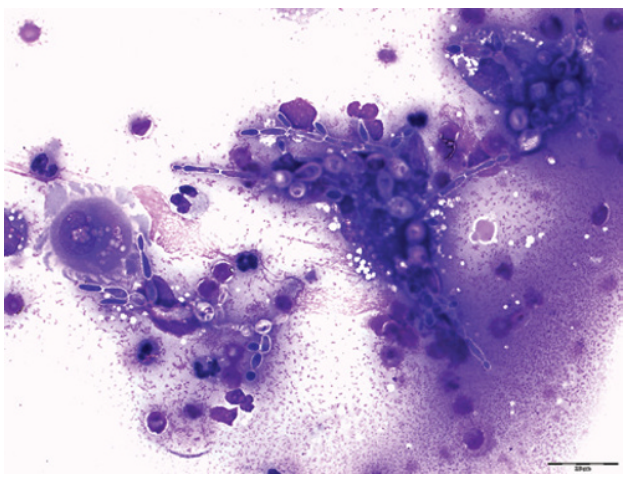


Figure 43.5 *Candida albicans* in peritoneal fluid from a dog. Note the yeast cells (blastoconidia and pseudohyphae). Modified Wright's stain, 1000×.

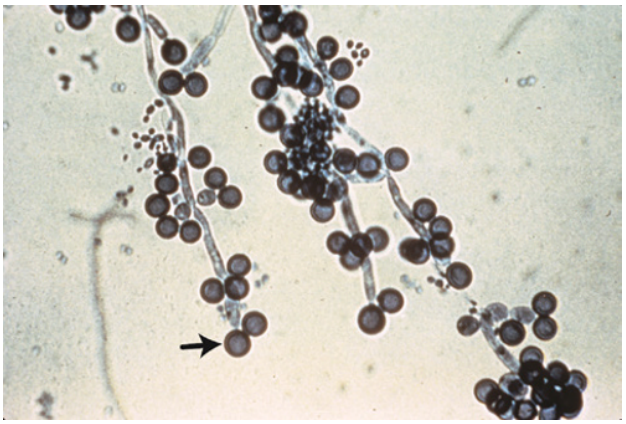


Figure 43.6 *Candida albicans* culture on Chlamydospore agar, showing blastoconidia (arrow).

Candida can be visualized under light microscopy when stained with Romanowsky-type stains (Wright's, Giemsa) (Figure 43.5), PAS, GMS, and other fungal stains, but is usually studied in culture unstained. With Gram stain, *Candida* cells are gram variable.

Cellular Products of Medical Interest

Adhesins

Various cell wall components (chitin, mannoproteins, and lipids) have been associated with adherence to extracellular matrix proteins.

Miscellaneous Products

Proteases and neuraminidases have been proposed to play a role in pathogenesis. Cell wall glycoproteins have endotoxin-like activity. Virulence factors such as phospholipases and proteases have been demonstrated in *C. albicans*. These enzymes seem to promote tissue invasion and adherence of yeasts to host cells, respectively.

Growth Characteristics

C. albicans, an obligate aerobe, grows on ordinary media over a wide range of pH and temperature. At 25–30 °C, creamy to pasty white colonies consisting predominantly of yeast cells appear in 24–48 hours. Incubation temperatures above 35 °C, a slightly alkaline pH, and a rich, carbohydrate-free fluid medium are often recommended. It grows well on a variety of non-selective fungal and bacterial media including Sabouraud dextrose or blood agar plates.

Differential ability to ferment or assimilate carbohydrates is the basis of species identification.

Candida albicans cells are killed by heat above 50 °C, ultraviolet light, chlorine, and quaternary ammonium-type disinfectants. They withstand freezing and survive well in the inanimate environment. They are susceptible to polyene antimycotics, and usually to flucytosine and the azoles.

Reservoir

Candida spp. are normal flora of the gastrointestinal and urogenital tracts and skin of mammals and birds. However, *C. albicans* can invade any organ of the body and cause disease.

Transmission

Most *Candida* diseases arise from an endogenous source; that is, they are caused by a commensal strain that invades the host after disruption of the normal barrier. The bovine udder becomes infected via the teat canal by way of administered medication, during milking, by cow-to-cow spread, or from the environment. Use of concurrent broad-spectrum antibiotics encourages proliferation of *Candida*, increasing the risk of infection. Moreover, as neutrophils are key in destruction of blastospores, any condition that causes impaired neutrophil concentration of function results in a predisposition to the development of candidiasis. Hematogenous spread has also been reported as leading to systemic infections with this yeast.

Pathogenesis

Mechanisms

Chitin, mannoprotein, and lipids are possible adhesins in human candidiasis; several extracellular matrix proteins have been shown to be the receptor. Germ tube formation is correlated with experimental pathogenicity, but the role of mycelium formation in virulence is under dispute. Proteases and phospholipases are believed to be virulence factors. Cell wall glycoproteins have endotoxin-like activity.

Pathology

Candidiasis most frequently affects the mucous surfaces on which the agent is normally found, possibly the anterior digestive tract from the mouth to the stomach; it typically remains confined to areas of squamous epithelium. The urogenital tract, skin, and claws can be involved as well. Occasional peritonitis, respiratory, intestinal, and septicemic infections occur.

On epithelial surfaces, candidiasis forms whitish to yellow or gray plaques, marking areas of ulceration with varying degree of inflammation. Diphtheritic membranes may form in the gut or respiratory tract, and abscesses may form in the viscera. Granulomatous lesions are rare. Inflammatory responses are predominantly neutrophilic.

Disease Patterns

Birds

Avian candidiasis affects chickens, turkeys, pigeons, and other birds. Crop mycosis (thrush) in avian species is caused by *C. albicans*. The existing digestive tract of birds can be affected; and mortality is very high with this disease condition.

Swine

In the alimentary tract of pigs, candidiasis is seen as ulcerative lesions that may rupture.

Equine

In the alimentary tract of foals, candidiasis is seen as ulcerative lesions that may rupture. Equine genital infections cause infertility, metritis, and abortions.

Cattle

Pneumonic, enteric, and generalized candidiasis affect calves on intensive antibiotic regimens. Candida mastitis in dairy cows is typically mild and self-limiting, ending in spontaneous recovery within about a week. Bovine abortions have been reported.

Dogs and Cats

Localized infections in dogs and cats are characterized by non-healing, ulcerative lesions of the oral, upper respiratory, gastrointestinal, and/or genitourinary mucosae. Rarely, dogs and cats may develop disseminated disease; clinical signs on presentation will typically reflect involvement of a specific organ system.

Other Animals

Lower primates and marine mammals may acquire mucocutaneous candidiasis.

Epidemiology

The common agents of candidiasis are commensal with most warm-blooded species. Disease is linked to immune and hormonal inadequacies, reduced colonization resistance (a measure of the "health" of the normal flora), or intensive exposure of weakened hosts or vulnerable tissues. These conditions account for susceptibility of infants, diabetics,

subjects on antibiotic and steroid regimes, patients with indwelling catheters, and mammary glands of lactating cows.

Immunologic Aspects

Immunoincompetent individuals are preferred targets for infection.

Neutrophils (primarily) and activated macrophages form the chief defense against candidiasis. The role played by opsonins (antibody; complement) is to facilitate phagocytosis. Macrophages are activated by gamma interferon secreted by TH1 cells stimulated by interleukin 12 from macrophages actively engaged in phagocytosis.

There is no vaccine for candidiasis.

Laboratory Diagnosis

In exudate, *Candida* appears as yeast cells (blastoconidia) or (pseudo)hyphae. All forms are demonstrable in unstained wet mounts, or in fixed smears stained with Gram's stain, Romanowsky-type stains (Wright's, Giemsa), or fungal stains – for example, PAS and GMS.

C. albicans grows well on blood or Sabouraud agar, with or without inhibitors. Other *Candida* spp. may be inhibited by cycloheximide.

Incubation at 37 °C for ≥2 hours of a lightly inoculated tube of serum will produce germ tubes if the isolate is *C. albicans*, which also produced chlamydospores on cornmeal-tween 80 agar (see Figure 43.6). Yeast identification kits are commercially available.

Molecular and serological methods for diagnosis of candidiasis are not routinely done in diagnostic laboratories. However, DNA-based tests are available in the reference laboratories.

Treatment, Control, and Prevention

Correcting the conditions underlying clinical candidiasis may in itself lead to recovery. Corneal and cutaneous infections are usually treated with a combination of systemic and topical antifungal medications.

In poultry, copper sulfate in drinking water is a traditional treatment. Nystatin can be given in feed or water. It is also used topically in mucosal and cutaneous forms of candidiasis of mammals, as are amphotericin B, itraconazole, and miconazole. Fluconazole (preferred) or flucytosine is useful for treating dogs or cats with lower urinary tract candidiasis.

In disseminated forms, oral fluconazole or flucytosine are drugs of choice. Susceptibility testing is advisable. Combined flucytosine-amphotericin B is sometimes used in humans and occasionally in animals.

Other Yeasts

Geotrichum candidum

This yeast-like fungus occurs widely in nature and causes the rare disease in animals called geotrichosis. The diagnostic relevance of this fungus in clinical samples is always questionable. However, it is known to cause infections of the mucous membranes of the alimentary tract, respiratory tract, and mammary glands of humans and animals including cattle, pigs, horses, dogs, and avian species. The organism grows rapidly on fungal growth medium and is identified based on colony characteristics and microscopic examinations of culture. It is susceptible to amphotericin B and flucytosine.

Trichosporon beigelii

It is a soilborne, yeast-like, imperfect fungus that causes trichosporonosis. It is an opportunist fungus that causes deep-seated and superficial infections in immunocompromised animals and humans. Colonies are initially creamy and smooth on Sabouraud agar and may take five to seven days of incubation at 37 °C. Definitive identification is based on assimilation tests, growth characteristics, and microscopic appearance. Treatment is similar to that for other yeast infections.

Acknowledgments

Dwight C. Hirsh and Ernst L. Biberstein contributed the material for this chapter in the 1st and 2nd editions of this textbook, and much of that information has been retained here.

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44

Dermatophytes

M.M. Chengappa and Lisa M. Pohlman

Dermatophytes are molds capable of parasitizing only keratinized epidermal structures: superficial skin, hair, feathers, horn, hooves, claws, and nails. Those that have a sexual reproductive phase belong are ascomycetes. Dermatophyte infections are called ringworm or dermatophytoses. Occasionally, yeast and saprophytic fungi cause cutaneous infections mimicking infections caused by dermatophytes; hence, the generic term *dermatomycoses* is used to represent all fungal infections of the skin.

Descriptive Features

Morphology

In their nonparasitic state, including culture, dermatophytes produce septate, branching hyphae collectively called mycelium. The asexual reproductive units (conidia) are found in the aerial mycelium. These units may be either macroconidia: pluricellular, podlike structures up to 100 µm long; or microconidia: unicellular spheres or rods less than 10 µm in any dimension. The shape, size, structure, arrangement, and abundance of conidia are diagnostic criteria. Hyphal peculiarities – spirals, nodules, rackets, chandeliers, and chlamydoconidia (chlamydospores) – are more common in some species than others, but they are rarely diagnostic. Colony characteristics and pigmentation are useful in dermatophyte differentiation.

In the parasitic state, only hyphae and arthroconidia (arthrospores), another asexual reproductive unit, are seen. Except in size ranges, which overlap among dermatophyte species, arthroconidia are indistinguishable from species to species.

Sexual spores (ascospores) are absent in the parasitic phase.

The distinguishing features of the three genera of dermatophytes – *Microsporum*, *Trichophyton*, and *Epidermophyton* – are shown in Table 44.1. Only *Microsporum* and *Trichophyton* affect animals consistently. *Epidermophyton* is seen primarily in humans.

Growth Characteristics

The traditional medium for propagating dermatophytes (and other pathogenic fungi) is Sabouraud dextrose agar containing 2% agar, 1% peptone, and 4% dextrose. Its acidity (pH 5.6) renders it mildly bacteriostatic and selective. The selectivity is enhanced by the addition of cycloheximide (500 µg/ml), which inhibits saprophytic fungi, and gentamicin and tetracycline (100 µg/ml of each), or chloramphenicol (50 µg/ml), which inhibit bacteria. Dermatophytes are aerobes and nonfermenters. Some attack proteins and deaminate amino acids. They grow optimally at 25–30 °C and require several days to weeks of incubation.

Some dermatophytes in skin and hair (but not in culture) produce a green fluorescence due to a tryptophan metabolite that is visible under ultraviolet light (366 nm), frequently referred to as a Wood's light or Wood's lamp. Of animal dermatophytes, only *Microsporum canis* produces this green fluorescence.

Resistance

Dermatophytes are susceptible to common disinfectants, particularly those containing cresol, iodine, or chlorine. They survive for years in inanimate objects or environment.

Variability

There are a number of strains of each species of *Microsporum* and *Trichophyton*, making construction of effective immunizing products difficult. However, there

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Table 44.1 Features of dermatophyte genera.

	Microsporum	Trichophyton	Epidermophyton
Macroconidia	Usually present	Often absent	Present
Walls	Thick	Thin	Thick
Surface	Rough	Smooth	Smooth
Shape	Spindle, cigar	Club (slender)	Club (broad)
Microconidia	Often absent	Usually present	Absent
Sexual form	*Nannizzia*	*Arthroderma*	None known

is a live attenuated vaccine for ringworm in cattle caused by *Trichophyton verrucosum* that is available in Europe.

Ecology

Reservoir

One speaks of geophilic, zoophilic, and anthropophilic dermatophytes when discussing dermatophytes having a soil, animal, or human reservoir, respectively. Table 44.2 shows the important dermatophytes of animals. Animal dermatophytes can infect humans through direct contact, whereas human dermatophytes rarely infect animals.

Transmission

Dermatophytes are disseminated by direct and, owing to their persistence on fomites and premises, indirect contact.

Pathogenesis

Mechanisms

Proteolytic enzymes (elastase, collagenase, keratinase) may determine virulence, particularly in severe inflammatory disease. Localization in the keratinized epidermis has been attributed to the lack of sufficient available iron elsewhere. This may account for the frequent arrest of dermatophytoses by inflammatory responses (through the influx of iron-binding proteins) and by enzyme inhibitors.

The infectious unit, a conidium, enters via a defect in the stratum corneum. Germination is triggered by unknown cues. The germ tube develops into hyphae branching among cornified epithelium. Portions of hyphae differentiate into arthroconidia. This growth pattern in the hairless skin predominates with some dermatophytes (*Microsporum nanum* and *Trichophyton rubrum*). Hair invasion, which is prominent in most animal ringworm, begins with germination of a spore near a follicular orifice. Hyphal strands grow into hair follicles along outer root sheaths and invade growing hairs near the living root cells. Hyphae grow within the hair cortex, in the outer parts of which arthroconidia form and accumulate on the surface of the hair. The pattern of accumulation of arthroconidia outside the hair shaft is called ectothrix, whereas the accumulation of arthroconidia inside the hair shaft is called endothrix formation.

Pathology

The pathogenic process begins with colonization, during which the events just described occur but evoke little host response. There may be hypertrophy of the stratum corneum with accelerated keratinization and exfoliation, producing a scurfy appearance and hair loss. In dogs with *M. canis* infections, this is often the main effect. In adult cats there may be no signs. Adult cats are known to carry *M. canis* spores on their skin without exhibiting any clinical signs or lesions of ringworm.

The second phase begins at about the second week with inflammation at the margin of the parasitized area. Manifestations range from erythema to vesiculopustular reactions and suppuration. Mild forms are seen in *T. verrucosum* infection of calves. Severe reactions are typical in *Trichophyton mentagrophytes* infection of dogs and *Microsporum gypseum* infection of horses. Local plaques ("kerion") may resemble certain skin tumors, especially in dogs. The inflammatory reaction may arrest the mycotic infection but become the primary problem through secondary suppurative bacterial infection.

The circular pattern of the lesions and their inflamed margins suggested the terms ringworm and tinea (Latin for worm).

Disease Patterns

The patterns of dermatophytosis in domestic animals are summarized in Table 44.3.

Ringworm generally regresses spontaneously within a few weeks or months, unless complicated by secondary bacterial infections or constitutional factors. The agents may persist after clinical cure.

Table 44.2 Important dermatophytes of animals.

Species	Principal hosts	Fluores-cence	Arthro-spores	Cultural features	Macroconidia	Microconidia
Microsporum canis	Dog and cat	+	Ectothrix	White to buff; reverse: yellow to orange	Spindle-shaped	Few, sessile
Microsporum gypseum	Dog, horse, cat	–	Ectothrix	Buff; reverse: orange brown to yellow	Ellipsoidal, septa 2–6	Sessile or on short sterigmata; clavate
Microsporum nanum	Swine	–	Ectothrix	White to buff; reverse: red	Obovoid to ellipsoidal ovate	Clavate
Trichophyton mentagrophytes	Many domestic and wild animal species	–	Ectothrix	Granular, light buff to tan; reverse: variable red, yellow	Occurrence variable; spindle or clavate; 5–6 septa	Abundant, pyriform, or clavate, sessile
Trichophyton verrucosum	Cattle, sheep	–	Ectothrix	Deeply folded, white to brilliant yellow	Requires thiamine; long and thin-walled; rare	Abundant with thiamine; singly ovoid; pyriform or clavate
Trichophyton gallinae	Fowl	–	Ectothrix	Radial folds, white to pale rose; reverse red	Infrequent; club-shaped and clavate	Singly on hyphae, pyriform to clavate
Trichophyton equinum	Horse	–	Ectothrix	White, cottony, yellow edge; old colony velvety to cream-tan; reverse: yellow to red-brown	Rare; clavate Requires nicotinic acid	Many, spherical to pyriform

Table 44.3 Important dermatophyte infections in domestic animals.

Host	Agent	Nature of lesions
Horse	*Trichophyton equinum*	Dry, scaly usually noninflammatory (unless secondarily infected)
	Microsporum gypseum	Often suppurative under alopecic thickened areas
	Microsporum equinum	Resembling *T. equinum* lesions
Cattle	*Trichophyton verrucosum*	Painless, thick, white plaques, local alopecia
Swine	*Microsporum nanum*	Tannish, crusty, spreading centrifugally on trunk; painless, margins slightly inflamed. No hair loss.
Dog	*Microsporum canis*	Typically noninflammatory, scaly, alopecic patches, occasional kerion
	Trichophyton mentagrophytes	Often spreading, extensively scaling to inflammatory lesions, secondary suppuration
	Microsporum gypseum	As *T. mentagrophytes*
Cat	*Microsporum canis*	Often subclinical in adults. Generally noninflammatory, except in young kittens, may become generalized in debilitated kittens.
	Trichophyton mentagrophytes	As in dogs
Chicken	*Microsporum gallinae*	Generally affects unfeathered portions. Whitish chalky scaling on comb and wattles, noninflammatory.

Epidemiology

Dermatophytoses often affect the young. The extent and severity are influenced by environmental factors. Crowding of animals or assembling of large numbers is often associated with increased prevalence. Improvement in calves often follows their release from damp, dark, crowded winter quarters to the outdoors.

Infected individuals of the same species perpetuate the important dermatophytoses of animals. Sporadic occurrence of the soil-derived *M. gypseum* infections contrasts with the endemic to epidemic but bland infections of swine with geophilic *M. nanum*.

The major agents of animal ringworm are globally distributed.

Immunologic Aspects

Immune Mechanisms of Disease

The major antigens associated with dermatophyte infections are the keratinases (elicit cell-mediated responses) and glycoproteins (carbohydrate moieties stimulate antibodies; protein moieties stimulate cell-mediated responses).

Antibody-mediated and cell-mediated hypersensitivities occur in the course of dermatophytoses. Their onset generally coincides with that of the inflammatory phase of the infection and may contribute to its manifestations. Production of skin lesions is facilitated due to the release of interferon gamma in the host.

Sterile inflammatory skin lesions (phytids) occur in human ringworm infections. They are allergic reactions to circulating fungal antigens.

Recovery and Resistance

Antibodies play at best a limited role in resistance. Evidence favors cell-mediated mechanisms as decisive in protection and recovery.

Recovered individuals resist reinfection, although local reactions may be more acute and intense than on primary exposure. Acquired resistance varies in degree and duration with host, dermatophyte species, and possibly anatomical area.

Artificial Immunization

Mycelial *T. verrucosum* vaccines, inactivated, and live avirulent, are used in Europe in cattle. They are credited with reducing the number of infected herds and new infections. Mixtures of *Microsporum* and *Trichophyton* – either as a live, attenuated vaccine, or a killed product – have been disappointing in protecting cats from developing dermatophytosis, though they do appear to restrict spread in individual cats. Although live vaccines appear to elicit a better immune (protective) response, the presence of numerous strains of *Microsporum* and *Trichophyton* make manufacturing such products difficult.

Laboratory Diagnosis

Direct Examination

In 50–70% of cases, hairs and skin scales infected with *M. canis* or *Microsporum audouinii* (in humans) may emit a bright greenish-yellow fluorescence under ultraviolet light, for example, a Wood's lamp ($\lambda = 366$ nm).

Microscopic Examination

Skin scrapings and hair are examined microscopically for the presence of hyphae and/or arthroconidia. The scraping should include material from the margins of any lesion and the full thickness of the keratinized epidermis. The hair is plucked, so as to include the intrafollicular portion. The sample is placed on a slide, flooded with 10–20% potassium hydroxide, covered with a coverslip, and heated gently. The treatment clears the sample (makes it "transparent") while leaving fungal structures and enough of the hair and epidermis intact to reveal the agent in its relation to the parasitized structures.

Microscopic examination should begin under low power (100×) and subdued light. At higher magnification (400×) of such hairs, individual, spherical arthroconidia or arthrospores are recognizable. These arthrospores are mostly ectothrix in nature in dermatophytes of veterinary significance (see Table 44.2).

Stains and penetrating and wetting agents (permanent ink, lactophenol cotton blue, dimethyl sulfoxide) improve visualization. Calcofluor white reagent imparts fluorescence to fungal structures and facilitates diagnosis where a fluorescent microscope is available.

Culture

Scrapings are planted onto and into the surface of selective media (Sabouraud agar with chloramphenicol and cycloheximide, Dermatophyte Test Medium [DTM], Rapid Sporulation Medium [RSM]), which are incubated at 25 °C (room temperature) for up to four weeks. Samples suspected of containing *T. verrucosum* are incubated at 37 °C. On DTM and RSM, an alkaline reaction suggests the presence of a dermatophyte. (Dermatophytes, if given a choice of glucose and protein, will usually digest the protein first, leading to alkaline products; saprophytic fungi will most often utilize glucose, leading to acid by-products. Caution: After the preferred substrate is utilized, the microorganism will make use of the other, shifting the pH in the other direction.)

Suspicious growth is examined microscopically. The adhesive side of a clear cellophane tape strip is pressed gently on the suspect colony (taken from RSM, or Sabouraud agar with chloramphenicol and cycloheximide – dermatophytes do not sporulate well on DTM) and mounted in a drop of lactophenol cotton blue on a slide and examined microscopically (see Table 44.1 and Figures 44.1–44.3). Colony color (front and back), texture, and rate of growth are important for identification of dermatophytes. Also important are the size and shape of macro- and microconidia of dermatophytes. In the absence of diagnostic conidia, as in the case of *Trichophyton* spp., auxotrophic tests are used for speciation.

Knowledge of the source (host species and type of lesion) aids significantly in provisional identification of animal dermatophytes.

Molecular Techniques

A number of DNA-based techniques are available for direct examination of clinical samples, or for use in identification of an isolated dermatophyte. These techniques include determination of the sequence of DNA encoding specific products (e.g. chitin synthase 1 gene), determination of the sequence of the internal transcribed spacer region, determination of the sequence of the DNA encoding large ribosomal subunit (28S), and generation of unique DNA fragments following arbitrary or randomly primed polymerase chain reactions. These techniques are not routinely used in diagnostic laboratories.

More recently, a versatile mass spectrometry technique known as MALDI-TOF has begun to be used in diagnostic

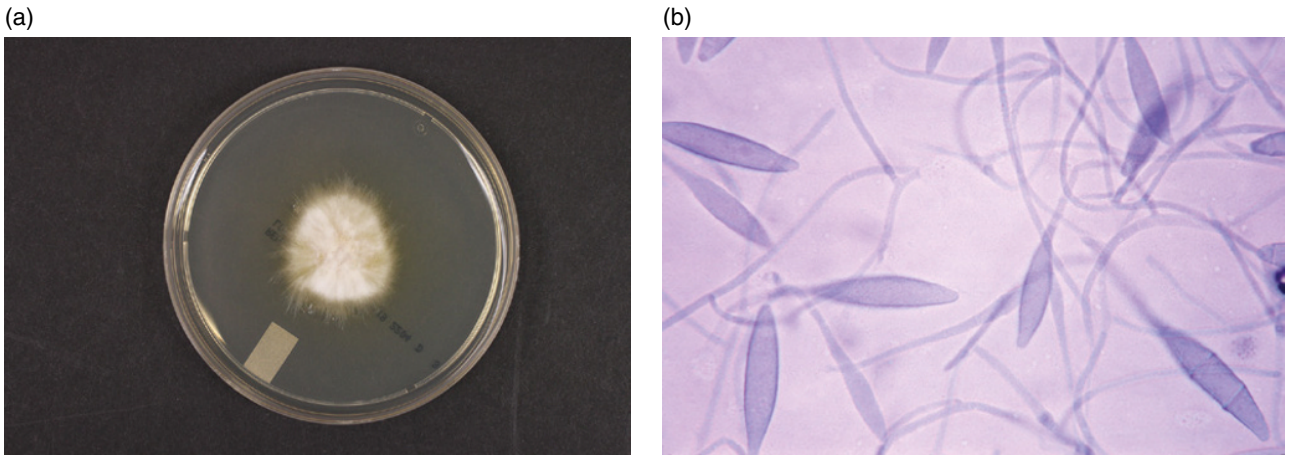


Figure 44.1 (a) Characteristic cottony white to buff color colony of *Microsporum canis* on Sabouraud dextrose agar. (b) Lactophenol cotton blue mount of *M. canis* from Sabouraud dextrose agar culture. Spindle-shaped macromedia, 400×.

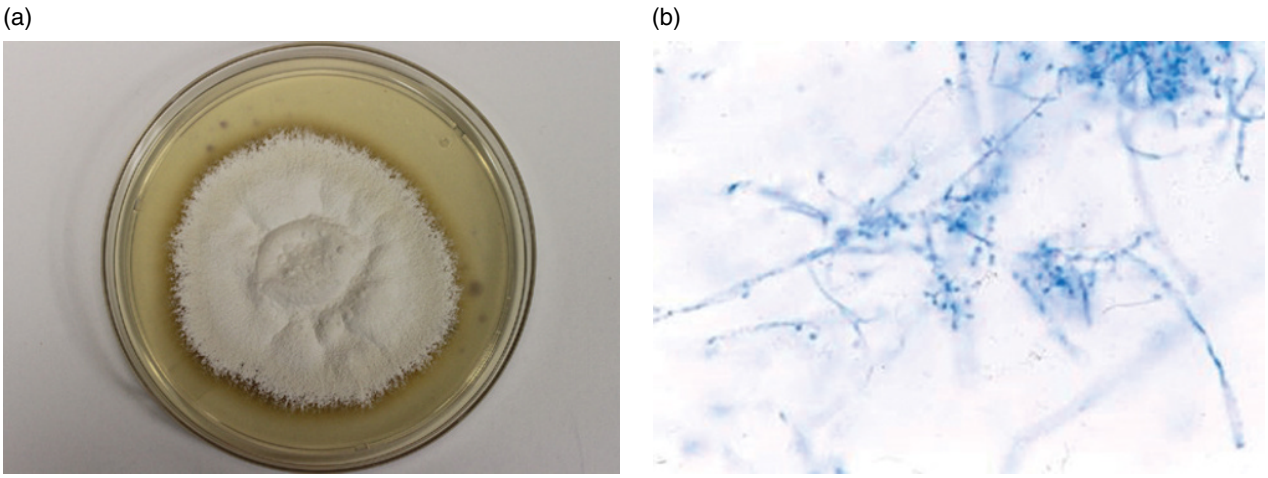


Figure 44.2 (a) Characteristic granular light buff to tan color colony of *Trichophyton mentagrophytes* on Sabouraud dextrose agar. (b) Lactophenol cotton blue mount of *T. mentagrophytes* with numerous oval to pyriform-shaped microconidia, 400×.

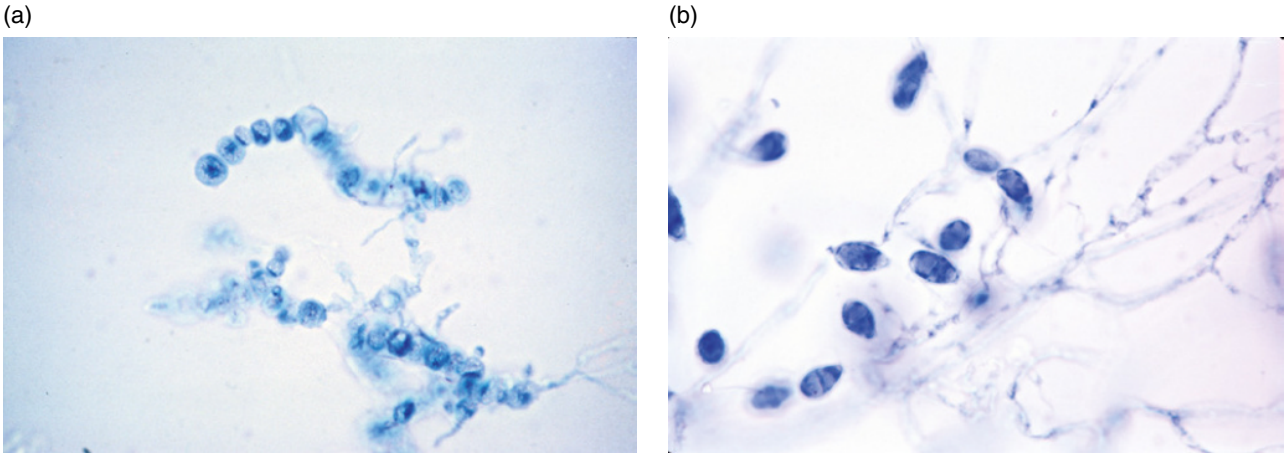


Figure 44.3 (a) *Trichophyton verrucosum*. Lactophenol cotton blue mount from Sabouraud dextrose agar culture grown at 37 °C. Macromedia, 400×. (b) *Microsporum nanum*. Lactophenol cotton blue mount from Sabouraud dextrose agar culture. Pear-shaped macromedia, 400×.

laboratories for the identification of important fungal agents that cause diseases in humans and animals. MALDI-TOF stands for Matrix-Assisted Laser Desorption Ionization – Time Of Flight. This is a highly sensitive method used by many laboratories for rapid identification of important bacteria and fungi of veterinary significance. The limitation of the technology is that identification of new bacteria or fungi is possible only if the database contains peptide map fingerprints of the type of strains of specific genera, species, or subspecies.

Treatment and Control

Combined topical and systemic treatment is often preferable. Topical antifungal agents include, but are not limited to, products containing lime sulfur, miconazole, enilconazole, ketoconazole, itraconazole, and thiobendazole. Terbinafine (an allylamine antifungal that inhibits ergosterol biosynthesis, and concentrates in skin, nails, and hair), and itraconazole (especially effective in cats) are often drugs of choice for systemic therapy in small animals. Fluconazole and ketoconazole are sometimes used in small animals, but may be less effective systemic therapeutic options for dermatophytosis. Ketoconazole use in cats is controversial as adverse side effects (gastrointestinal, hepatotoxic effects) are more common in cats.

Antifungal orchard spray is effective on ringworm of large animals (Captan 45% powder, 2 tablespoons/gal). Affected areas are first clipped. In large animals, two applications at biweekly intervals are recommended. Contact with human skin should be avoided. Thiabendazole is used on large animals. Lime sulfur dips (usually twice weekly) in combination with systemic antifungal medication is often effective in small animals.

Povidone-iodine (Betadine) and chlorhexidine (Nolvasan), available as lotions and ointments, are general antiseptics with antifungal action. Lime sulfur solution and miconazole/chlorhexidine shampoo are effective topical treatments for dogs and cats.

A thorough cleanup of premises with an iodine, chlorine, or phenol-containing disinfectant is essential to prevent fomite contamination. Sodium hypochlorite and enilconazole also have been shown to be effective antifungal disinfectants. Utensils and equipment are disinfected with Captan or Bordeaux orchard spray.

Identification of carriers in kennels and catteries can be attempted by culture of hair coat brushings. The Wood's lamp is useful in population screening of cat colonies where *M. canis* is the only concern. Infected individuals should be isolated and treated. Exposed animals are treated prophylactically.

Successful vaccination is widely practiced on European cattle. A live attenuated strain (*T. verrucosum*) appears to be most immunogenic. Neither live attenuated vaccines nor killed products have been effective in preventing dermatophytosis in cats.

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Original chapter written by Drs. Ernst L. Biberstein and Dwight C. Hirsh.

Further Reading

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45

Agents of Subcutaneous Mycoses

Lisa M. Pohlman and M.M. Chengappa

Whether a fungus is categorized as mold or yeast is based upon the microscopic appearance in tissue or on routine culture media (the asexual stage). If hyphal structures are observed, the fungus is termed a mold; if a single-celled budding structure is observed, the fungus is termed a yeast. On routine culture media, molds will have a "fuzzy" or wooly appearance, and yeast will be bacteria-like in its colonial morphology and consistency. Some pathogenic fungi will produce either hyphal structures or yeast-like structures, depending upon the conditions in which they are growing. Such fungi are called dimorphic fungi, which are discussed below and in Chapter 46.

This chapter deals with dimorphic fungi and fungus-like microorganisms affecting skin and subcutis. We will discuss *Sporothrix schenckii*, which is the cause of sporotrichosis in a variety of animal species, but more frequently in people, horses, dogs, and cats; *Histoplasma capsulatum* var. *farciminosum*, the cause of epizootic lymphangitis in equids (horses, donkeys, mules); the agents of oomycosis (Aphanomyces, Lagenidium, Pythium, and Saprolegnia), which cause a variety of diseases in fish and mammals; and miscellaneous conditions involving the skin and subcutis, including chromoblastomycosis, phaeohyphomycosis, and mycetoma. Systemic mycoses, of which skin lesions may be one manifestation, are described in this chapter.

Sporothrix schenckii

Sporotrichosis is a relatively rare disease caused by *Sporothrix* species (usually *S. schenckii*, although several other causative species have recently been reported) that are saprophytic, dimorphic fungi. In immunocompetent humans, this disease usually manifests as a chronic, ulcerative lymphangitis of skin and subcutis. Systemic (disseminated) disease occurs occasionally in immunocompromised human patients (e.g. alcoholics; individuals affected with the human immunodeficiency virus [HIV]). In immunocompetent horses and dogs, the disease is usually limited to the cutaneous or cutaneolymphatic form, and organisms are typically sparse or rare within the lesions. Disseminated disease is very rare in horses and dogs unless the patient is immunosuppressed. Conversely, most cats with sporotrichosis will develop cutaneolymphatic or disseminated disease regardless of their immune status at the time of infection. Further, in cats, organisms are typically abundant and found easily within the lesions and exudates.

Descriptive Features

Morphology and Staining

S. schenckii is a dimorphic fungus; that is, it exhibits a different morphology depending upon the conditions of growth. At room temperature (25 °C, on Sabouraud agar), *S. schenckii* grows as a mold. This so-called saprophytic phase consists of septate hyphae, with oval or tear-shaped conidia (2–3 μm by 3–6 μm) in clusters on conidiophores and along hyphae. At 35–37 °C (in tissue or on rich media, e.g. blood agar, incubated at that temperature), it exists as budding pleomorphic yeasts (characterized typically by unique "cigar bodies," but yeasts can also be round-shaped) measuring up to 10 μm in the longest dimension. The yeast phase stains with Gram's stain, and either phase accepts the Romanowsky-type stain (e.g. Wright's or Giemsa) (Figure 45.1) or fungal stains (periodic acid-Schiff, Grocott methenamine silver, Gridley).

Cellular Constituents and Products

S. schenckii possesses a typical fungal cell wall containing chitin and ergosterol. Various glycoconjugates found upon the wall have adhesive properties (see Section 45.2.3).

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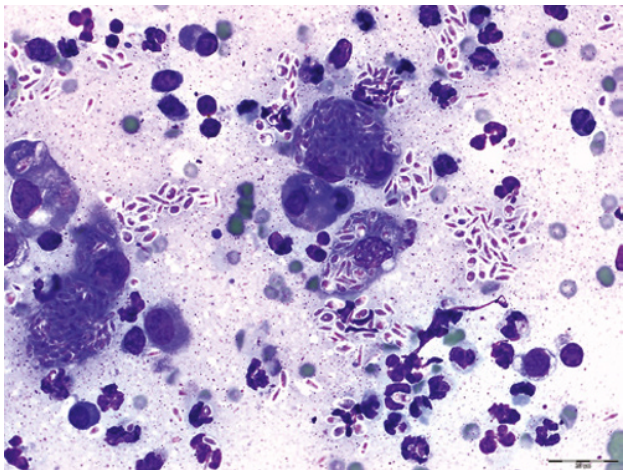


Figure 45.1 Exudate from a cutaneous sporotrichosis lesion in a cat. Note the pleomorphism of the yeast cells; shapes vary from round- to oval- to cigar-shaped (cigar bodies). Modified Wright's stain. 1000×.

Cellular Products of Medical Interest

Adhesins

The cell wall of *S. schenckii* contains glycoconjugates with affinity for extracellular matrix proteins (fibronectin, laminin, and Type II collagen). This interaction does not involve an "Arg-Gly-Asp" (RGD) sequence.

Cell Wall

The cell wall of *S. schenckii* contains several substances that may play a role in the virulence of this microorganism. These include lipid, melanin, peptide-rhamnomannan, and sialic acid:

1) **Lipid**. The lipid portion of the cell wall of *S. schenckii* inhibits phagocytosis by monocytes and macrophages.
2) **Melanin**. Melanin found in the cell wall protects *S. schenckii* from the effects of reactive oxygen intermediates within the phagolysosomes of phagocytic cells. Melanin is a free radical scavenger (it reduces the toxicity of the hydroxy radicals, superoxides, and singlet oxygen radicals found within the phagolysosome).
3) **Peptide-rhamnomannan**. The peptide-rhamnomannan fraction of the cell wall acts as an immunosuppressive substance by suppressing the liberation of pro-inflammatory cytokines by phagocytic cells.
4) **Sialic acids**. Sialic acids found in the cell wall inhibit uptake of *S. schenckii* by phagocytic cells. Sialic acid directs complement proteins toward the degradative pathway, rather than generating the effective opsonizing fragments and anaphylotoxins needed to generate an effective inflammatory response.

Proteinases

S. schenckii produces two proteinases, I and II. The significance of these enzymes in the pathogenesis of sporotrichosis is unclear. However, these enzymes are believed to hydrolyze human stratum corneum cells *in vitro*.

Growth Characteristics

After several days on Sabouraud agar at room temperature, initially moist, off-white to black colonies develop that become wrinkled and fuzzy. On blood agar at 35–37 °C, whitish, smooth yeast colonies appear within a few days. Colonies at room temperature on potato dextrose agar are black and wrinkled.

Variability

Examination of mitochondrial DNA (by restriction length polymorphism analysis) shows that there are at least 20 different types or strains of *S. schenckii* (1–20).

Ecology

Reservoir

The organism prefers soil rich in decaying and organic matter; it has also been isolated on many live plants throughout the world. Occasionally it is isolated from claws of clinically healthy cats, mucous membranes of healthy animals, and animal products. Disease has been reported in people, dogs, cats, horses, mules, donkeys, goats, cattle, rats, mice, hamsters, foxes, birds, camels, dolphins, armadillos, and chimpanzees. The disease is most common in cats, dogs, and horses, where it occurs in multiple forms. Human infections are often associated with rose gardening (and often called a "rose gardener's disease") because infection usually occurs via a traumatic puncture wound after handling plants and plant materials. More correctly, however, any thorny plant should be regarded as a possible source of infection. Infection via inhalation of spores has also been reported, but this is much less common.

Transmission

The mycelial form of the organism enters the tissue via traumatic inoculation and then converts to the yeast form, where local proliferation of the organism occurs to result in ulcerated, exudative, cutaneous nodules that involve the dermal and subcutaneous tissues. These discharging lesions may be contagious, especially in cats, where the

numbers of microorganisms in the exudates from this species are quite large. The organism has also been isolated from the nails of affected cats, and from clinically healthy cats in contact with infected individuals. Rare cases of internal infection are due to inhalation or ingestion.

Pathogenesis

Post inoculation, local proliferation of the organism and the associated inflammatory reaction results in draining wounds that first appear similar to cat-bite abscesses or cellulitis. These wounds, which are refractory to antibiotic therapy, progress to become ulcerated cutaneous nodules that involve the dermal and subcutaneous tissues. Subsequently the affected area becomes ulcerated (Figure 45.2) and forms large crusts.

The typical inflammatory reaction is pyogranulomatous, with a purulent center surrounded by epithelioid and multinucleated macrophages, and, peripherally, lymphocytes and plasma cells. However, upon removal of the crusts, there is often a purulent exudate. The infectious process follows subcutaneous lymph channels, producing suppurating ulcers at intervals. These heal slowly but often re-erupt. Lymphatics often become thickened. In immunocompetent patients (except cats), the disease is usually limited to the cutaneous or cutaneolymphatic form. Again, with the exception of cats, disseminated disease is very rare unless the patient is immunosuppressed. Conversely, most cats will develop cutaneolymphatic or disseminated disease regardless of their immune status at the time of infection and thus dissemination to viscera, joints, bones, and the central nervous system is common in cats.

Proteinases are possible virulence factors, and proteinase inhibitors have been shown to suppress nodule formation.



Figure 45.2 Ulcerated lesions from a cat with sporotrichosis.

Cell wall constituents and adhesins delay elimination of the microorganism.

Epidemiology

Sporotrichosis is acquired from the nonliving environment, but transmission from suppurating lesions is possible. The zoonotic potential of infection from cats to people is well established. The occurrence of infection transmission from dogs and horses to people is not as well documented. It is reported that the abundance of organisms in the lesions, exudates, feces, and under the nails of infected cats, and the relative lack of organisms in the lesions of dogs is the reason for this difference. However, transmission from cats to people has been reported even when the cat's lesions have had very few organisms, and transmission from dogs has not occurred when owners have been exposed to lesions with abundant organisms. Therefore, it is likely that there are other factors that affect zoonotic potential, and not simply the number of organisms in a lesion. Transmission from person to person does not occur.

Immunologic Aspects

Cell-mediated reactivity is significantly related to resistance. No artificial immunization procedures exist.

Laboratory Diagnosis

Direct examination of exudates is often unrewarding except with feline specimens or specimens from immunocompromised patients, which generally contain abundant yeast cells (round-shaped organisms and cigar bodies) (Figure 45.1). If organisms are present in the cytological preparation, identification of the organism is straightforward assuming the classic oval to cigar-shaped yeast forms (cigar bodies) are seen. However, in rare instances when only the round-shaped yeast forms are present, these organisms can be difficult to distinguish from *H. capsulatum*. In tissue sections, *S. schenckii* has also reportedly been misidentified as *Cryptococcus neoformans*. This error is likely due to an artifact of sample preparation in which there is contraction of the cytoplasm from the cell wall, resulting in the impression of a large clear capsule. With other hosts, fungal stains (see above) and immunofluorescence may help detect yeast cells.

The agent grows readily in culture. Definitive diagnosis requires demonstration of both phases. Serological tests (yeast cell and latex agglutination, agar gel diffusion) have

been of limited use in animals. Molecular techniques utilizing the polymerase chain reaction (PCR) with primers designed to amplify segments of the gene encoding the chitin synthase 1 have been used to demonstrate the fungus in clinical samples, as well to identify isolates (useful when an isolate resists the mold-yeast conversion).

More recently, a versatile mass spectrometry technique known as MALDI-TOF has begun to be used in diagnostic laboratories for the identification of *S. schenckii*. MALDI-TOF stands for Matrix-Assisted Laser Desorption Ionization – Time Of Flight.

Treatment and Control

The cutaneous form responds generally to the oral administration of sodium or potassium iodides. The azole drugs, especially itraconazole, are effective. Terbinafine (an allylamine antifungal agent that inhibits ergosterol biosynthesis) has shown some success in treating human patients with the cutaneous form of sporotrichosis. Amphotericin B and flucytosine are used on deep and disseminated forms.

Histoplasma capsulatum var. *farciminosum*

H. capsulatum is a dimorphic fungus existing as a mold at 25–30 °C (saprophytic phase) and as a yeast at 37 °C (parasitic phase). Classically this fungus has been divided into three varieties: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. capsulatum* var. *farciminosum*. Recently, via molecular techniques combined with the geospatial distribution of their sources, *Histoplasma* spp. have been grouped into eight clades (two North American clades, two South American clades, one Australian, one Indonesian, one African, and one Eurasian clade) and it is currently unclear whether the division into the three varieties is still valid. Because there does not seem to be a useful alternative until the status of this organism is resolved and because the classic division of varieties is still the most common, the classic terminology will be used here.

The variety *farciminosum* causes epizootic lymphangitis (pseudoglanders), a chronic pyogranulomatous disease typically involving the skin and lymphatics that mainly affects horses, donkeys, and mules but has also been reported in camels, cattle, cats, dogs, and at least one wild badger. In mice, guinea pigs, and rabbits, experimental infections have been established. Rarely, internal organs may be involved. Epizootic lymphangitis is discussed below.

The varieties *capsulatum* and *duboisii* cause histoplasmosis, a systemic fungal disease that will be discussed in Chapter 46.

Descriptive Features

Morphology and Staining

H. capsulatum var. *farciminosum*, a dimorphic fungus, produces budding yeasts (2–3 µm by 3–4 µm) in tissue, and usually sterile hyphae in its mycelial form when grown at 25 °C or at room temperature. Exceptionally, arthroconidia, chlamydoconidia, and spherical, thick-walled macroconidia are seen.

The yeast phase is best demonstrated with a Romanowsky-type stain (e.g. Wright's or Giemsa) or fungal stains (periodic acid-Schiff, Grocott methenamine silver, Gridley).

Growth Characteristics

H. capsulatum var. *farciminosum* grows on common laboratory media (Sabouraud glucose, infusion, and blood-supplemented media with or without cycloheximide and antibacterial agents) over a broad temperature range. The optimal temperature for mycelial growth is 25–30 °C, and it takes several weeks to form a cottony, white to brown colony. Pigmentation parallels the abundance of macroconidia. The yeast phase requires rich media (e.g. glucose cysteine blood agar; brain heart infusion blood agar) and temperatures of 34–37 °C, and it takes several days to form a cream to tan, yeast-like colony. Conversion of mold to yeast on blood-containing agar requires incubation at 37 °C under 15–20% CO_2. Several passages may be needed to convert the mold to the yeast phase.

Resistance

H. capsulatum var. *farciminosum* is quite resistant to physical and chemical agents. It survives in soil at ambient temperatures for months (several weeks in corrals and stables) and at refrigerator temperature or in a desiccated form for years.

Ecology

Reservoir

The primary reservoir for *Histoplasma* species is nitrogen-rich soil.

Transmission

Infection is most commonly through skin wounds, but *H. capsulatum* var. *farciminosum* can also invade mucous membranes such as nasal mucosa or conjunctiva, and sexual transmission was described from stallions to mares. Infective organisms may be in the yeast form and come from skin lesions, or nasal and/or ocular exudates of infected animals, or they may be in the mycelial from the environment (e.g. soil). Fomites such as grooming or harness equipment are also a source of infection. Organisms spread most readily where large numbers of equids are assembled in high concentrations in tropical and subtropical climates as warm, moist conditions allow the organism to survive in the soil for months. Arthropods may play a part. Respiratory and localized gastrointestinal infections, which are rare, likely develop subsequent to inhalation or ingestion of the organism, respectively. However, experimental studies have not reliably been able to produce gastrointestinal disease with oral administration of organisms.

Pathogenesis

The first symptom is a painless, freely moveable skin nodule that over time enlarges, becomes abscessed, and eventually bursts, resulting in an ulcerated lesion. Although some lesions seem to heal spontaneously, more commonly they continue to ulcerate and grow, with periods of granulation, and partial healing, followed by the development of new lesions. Typically, adjacent lymphatics develop nodules along their course, showing the same alternating activity. Regional lymph nodes develop abscesses that drain by sinus tracts to the outside, but fever is uncommon. Hematogenous spread and visceral involvement that can ultimately result in death is possible, especially in animals that are in poor condition and have limited access to veterinary care. Histologically, the process evolves from suppurative to granulomatous (pyogranulomatous), marked by lymphocytes, macrophages, and giant cells, to eventual fibrosis. Yeast cells occur extracellularly and intracellularly, especially in macrophages.

Cutaneous disease is the most commonly reported syndrome, and these cases may present with or without respiratory and/or ocular disease. In mild cases, the skin lesions remain localized to one area of the body with lesions most commonly seen on the head, neck, and limbs. In extreme cases, lesions may spread to affect most of the body. Cycles of exacerbation and partial healing may gradually resolve in two to three months in some animals. Other cases may become chronic. In these cases, over time, animals with non-resolving, severe, skin lesions lose condition, and eventually become anorectic and debilitated.

Epidemiology

Endemic areas include parts of Africa, much of Asia including India, Japan, and Pakistan, and the Mediterranean littoral. The epidemiology of *H. capsulatum* var. *farciminosum* infection is unclear. Manifestations vary with a geographic area. Seasonal peaks suggest arthropod transmission.

The disease mainly affects equids but cattle, camelids, cat, dogs, and other animals may also be affected. Young horses (less than six years of age) are most susceptible.

Immunologic Factors

The role of immune mechanisms in pathogenesis or resistance is not known, but cell-mediated immunity is probably the key host defense. Skin sensitivity develops following exposure, even in the absence of disease. Circulating antibodies, demonstrable by indirect fluorescent antibody, agar gel diffusion, an enzyme linked immunosorbent assay (ELISA), or by the serum agglutination tests are indicative of infection.

Laboratory Diagnosis

Differential diagnosis includes sporotrichosis (see above, *S. schenckii*), ulcerative lymphangitis produced by *Corynebacterium pseudotuberculosis*, *Candida* (Chapter 43), and *Cryptococcus* (Chapter 43). Direct examination of stained cytology preparations (Wright's or Giemsa stained) or biopsy material (hematoxylin–eosin, periodic acid-Schiff, Grocott methenamine silver) may reveal intracellular (within macrophages) or extracellular characteristic yeasts. It is important to note that the yeast form of *H. capsulatum var. farciminosum* is indistinguishable from *H. capsulatum var. capsulatum*.

H. capsulatum var. *farciminosum* grows on Sabouraud glucose agar with inhibitors (cycloheximide and chloramphenicol). Mycelial extracts contain genus-specific antigens demonstrable by agar gel diffusion or by the serum agglutination test. Growth patterns and microscopic morphology differentiate *H. capsulatum* var. *farciminosum* from *H. capsulatum* var. *capsulatum* (Chapter 46). Samples from skin lesions should be collected by aspiration of unruptured nodules to avoid contamination by environmental fungi, whenever possible.

More recently, a rapid nested PCR method targeting the internal transcribed spacer (ITS) region of the rRNA operon

was validated for application to equine clinical samples and could detect *H. capsulatum var. farciminosum* directly in clinical samples from horses, including exudates from skin lesions and nasal discharges. This assay outperformed diagnosis based on microscopic observation of organisms.

Treatment and Control

In developed countries, histoplasmosis is typically treated with azoles (itraconazole, fluconazole, voriconazole) or amphotericin B.

In impoverished countries, where the disease is most common, less expensive iodides (with or without surgical excision and/or griseofulvin) have been relatively successful. Amphotericin B has been used with some success.

When epizootic lymphangitis is diagnosed or suspected, veterinarians should follow their national and/or local guidelines for disease reporting. At the time of writing this chapter, in May 2021, Epizootic lymphangitis was not on the reportable disease list in the United States.

Oomycosis

Oomycosis is caused by a member of a group of eukaryotic microorganisms belonging to the Kingdom Stramenopiles (which also contains the diatoms and brown algae). They are saprophytic microbes that usually live in water and moist soil. Though they are morphologically similar to fungi (they produce hyphae in tissue and grow as mold-like colonies on media *in vitro*), they are not members of the kingdom Fungi. Members of this group are the agents of several plant and animal diseases. Historically, the most notorious is Phytophthora, the cause of the Irish potato famine, which is responsible for killing oak trees along the Pacific coast of North America. The following genera are associated with disease in animals: *Aphanomyces* (ulcerative disease of fish and crustaceans), *Lagenidium* (pyogranulomatous disease of dogs and cats identical clinically to pythiosis), *Pythium* (pyogranulomatous disease, called pythiosis, of a variety of animal species), and *Saprolegnia* (systemic disease of cultured salmonids). The discussion that follows is focused on *Pythium insidiosum*, the cause of pyogranulomatous conditions (pythiosis) of dogs ("swamp cancer"), horses ("Florida horse leeches"), cattle, cats, and humans.

Cutaneous Pythiosis (Swamp Cancer – Florida Horse Leeches)

The genus *Pythium* contains over 85 species, and only *P. insidiosum* is associated with disease in animals. *P. insidiosum* causes ulcerative fibrogranulomatous or pyogranulomatous and eosinophilic cutaneous and subcutaneous lesions in horses, cattle, dogs, and cats, and gastrointestinal disease in dogs. It has also been reported as a cause of arteritis, keratitis, or periorbital cellulitis in people. It is mainly seen in tropical or subtropical areas – in North America, this disease is seen most frequently diagnosed along the Gulf Coast. Young, large breed dogs; cats less than one year of age; and horses are the species most commonly affected. The agent is an aquatic oomycete with wide (4 μm), sparsely septate hyphae. Lesions in horses are typically large, exudative swellings, usually on extremities, ventral trunk, or head. The nasal mucosa may be involved. Hyphae are demonstrable within granulomatous coagula (termed kunkers or "leeches" in horses) consisting of necrotic macrophages (including epithelioid and multinucleated macrophages) and eosinophils.

Diagnostic methods include cytology (in which broad, poorly septate, and branching hyphal elements are seen among the pyogranulomatous and eosinophilic inflammation) (Figure 45.3), serology (ELISA), molecular techniques (utilizing primers designed to amplify *P. insidiosum*–specific DNA by means of PCR), immunohistochemical examination of exudates, and culture.

Cultural techniques are tedious and time consuming, and involve the growth of a mold-like microorganism on Sabouraud dextrose agar or brain heart infusion agar after 24–48 hours at 30 °C. Identification requires demonstration of motile zoospores and/or reaction of extracts of the isolate with reference antisera. The PCR-based assay mentioned is used to establish the presence of *P. insidiosum* in

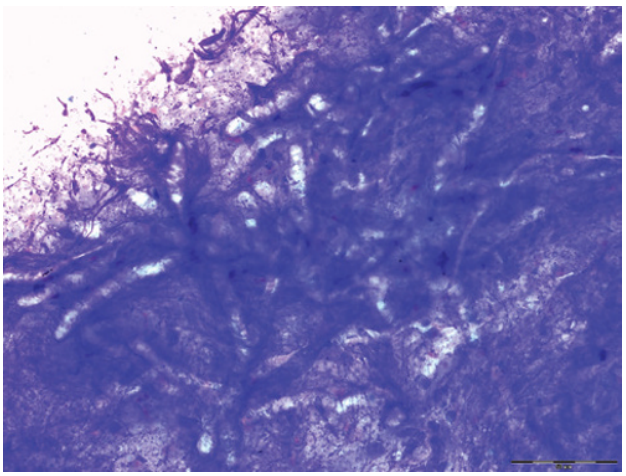


Figure 45.3 Image of a fine-needle aspirate cytology preparation, from a cutaneous, ventral abdominal mass from a dog with pythiosis. Note the unstained hyphal structures highlighted by the deeply stained necrotic and cellular debris. Modified Wright's stain. 1000×.

clinical samples as well as for identification of isolates. The PCR assay also successfully identifies members of the genus *Lagenidium* (another oomycete), which produces a similar clinical picture in dogs and cats.

Early diagnosis is the key to successful treatment. Treatments include aggressive surgical excision with wide margins with or without antifungal therapy (a combination of itraconazole and terbinafine is the recommended choice of antifungal therapy and has been shown to be more effective than amphotericin B or itraconazole therapy alone). Immunotherapy utilizing killed whole organisms or extracts has shown promise in horses and humans, but efficacy has been poor in dogs.

Chromoblastomycosis and Phaeohyphomycosis

Chromoblastomycosis and phaeohyphomycosis are caused by dark-pigmented (dematiaceous) fungi. Most of the roughly 70 species implicated belong to the genera *Alternaria, Bipolaris, Cladosporium, Cladophialophora, Curvularia, Exophiala, Fonsecaea, Phaeoacremonium*, and *Phialophora*. In chromoblastomycosis, the fungal elements in tissue are large (<12 μm), pigmented "sclerotic bodies." Infections in which hyphae are present are called phaeohyphomycosis.

Chromoblastomycosis is rare in nonhuman mammals, but occurs in frogs and toads. Phaeohyphomycosis is seen sporadically in cats, dogs, horses, cattle, and goats and may be systemic. *Cladophialophora bantiana* is the fungus most commonly seen in dogs and cats, with central nervous system localization frequently observed.

The agents, soil- and plant-associated saprophytes, enter through the skin and multiply subcutaneously, causing pyogranulomatous reactions. No tissue colonies or granules are seen. Nodular or larger swellings develop that may ulcerate and discharge pus.

Diagnosis is by biopsy and culture. Sclerotic bodies (chromoblastomycosis) and hyphae (phaeohyphomycosis) are seen in stained (hematoxylin–eosin, periodic acid-Schiff, Grocott methenamine silver) biopsy sections. Culture, on Sabouraud agar without inhibitors, often requires lengthy incubation. The resulting colonies range from olive to brown to black, depending on the color of the species of the fungus involved.

Lesions are excised, but may recur. Medical treatment (flucytosine, itraconazole, amphotericin B, ketoconazole) has given mixed results.

Eumycotic Mycetoma

Swelling, granule formation, and discharging sinus tracts are characteristics of mycetoma. They may be associated with bacteria, most notably an actinomycete such as members of the genus *Nocardia* or *Actinomyces* (actinomycotic mycetoma) or a fungus (eumycotic mycetoma). Mycetomas are occasionally reported in cattle, horses, dogs, and cats. The cutaneous form is often nodular and is associated with similar nasal lesions.

Fungi associated with eumycotic mycetoma fungi include *Pseudallescheria boydii*, *Cochliobolus spiciferus* (the sexual forms of *Scedosporium apiospermum* and *Bipolaris spicifera*, respectively), and *Curvularia geniculata*. All are saprophytes that presumably enter via a wound. It is not clear what triggers this course of pathogenesis, because the same agents can cause other pathological patterns.

Fungal colonies are surrounded by suppuration bordered by granulomatous reactions. Sinus tracts carry pus and granules, consisting of microorganisms and inflammatory components, to the surface. The color and texture of granules vary depending on the species of fungus involved in the mycetoma. The processes are slowly progressive, involving adjacent tissues.

Treatment is excision if possible. Antifungal agents (azoles, amphotericin B) have been disappointing.

Rhinosporidiosis

The cause of this disease is *Rhinosporidium seebri*. This fungus occurs in nature and has not been cultivated on fungal media, but has been propagated in cell culture. It causes a slow-growing, chronic, granulomatous type of infection of the nasal cavity in horses, cattle mules, dogs, cats, goats, and wild waterfowls. Infection, though rare, occurs in humans. Formation of cauliflower-like growths, also known as polyps, is characteristic of infection caused by this fungus. The disease occurs mostly in tropical and subtropical countries; however, sporadic cases have been reported in the United States. Diagnosis is based on gross lesion and microscopic examination of tissue sections or discharge from polyps. On tissue section, the presence of large sporangia (200–300 μm) filled with endospores is sufficient to make a definitive diagnosis of this disease. On cytological preparations, however, sporangia are rarely seen, and the numerous endospores are the typical finding (Figure 45.4). Surgical excision, usually involving rhinotomy, is the treatment of choice, but many recur. Topical povidone-iodine treatment post surgery has been reported to minimize the chance of recurrence.

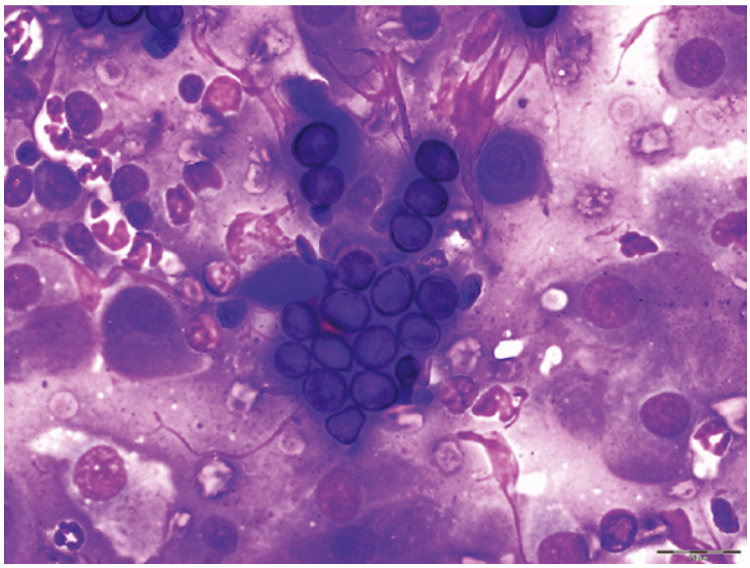


Figure 45.4 Impression smear of cauliflower-like growth in the nasal cavity of a dog. Note the central clump of endospores among the pyogranulomatous inflammation. Modified Wright's stain. 1000×.

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Agents of Systemic Mycoses

Lisa M. Pohlman and M.M. Chengappa

Whether a fungus is categorized as a mold or a yeast is based upon the microscopic appearance in tissue or on routine culture media (the asexual stage). Microscopically, if hyphal structures are observed, the fungus is termed a mold; if single-celled budding structures are observed, the fungus is termed a yeast. On routine culture media, molds will have a "fuzzy" or wooly appearance, and yeast will be bacteria-like in its colonial morphology and consistency. Some pathogenic fungi will produce either hyphal structures or yeast-like structures, depending upon the conditions in which they are growing. Such fungi are called dimorphic fungi. These fungi are discussed below and in Chapter 45.

The agents of most systemic (or "deep") mycoses are saprophytic fungi. Morphologically and ecologically diverse, they share disease-related features:

1) Many are dimorphic; that is, their saprophytic and parasitic phases differ morphologically. *Coccidioides, Histoplasma, Blastomyces*, and *Paracoccidioides* grow as molds in their inanimate habitat. In tissue, *Coccidioides* produces sporangia, whereas the others grow as budding yeasts.
2) Infection is usually by inhalation.
3) Host factors are often the decisive disease determinants. Some mycoses (aspergillosis, mucormycosis) are seen primarily in immunocompromised animals.
4) Lesions tend to be granulomatous to pyogranulomatous. After primary pulmonary infection, the course of disease is determined by the effectiveness of cell-mediated immune responses. If these are inadequate, dissemination may occur to bone, skin, gastrointestinal tract, central nervous system, abdominal viscera, etc.
5) Systemic mycoses are noncontagious. Although the agent is often demonstrably shed, it fails to infect individuals on contact.
6) Because the inflammatory lesions are granulomatous to pyogranulomatous, all have the potential to cause hypercalcemia in the patient. This hypercalcemia occurs as a result of the response to granulomatous inflammation in which activated macrophages are stimulated to convert vitamin D precursors to the active form of vitamin D (i.e. calcitriol) in an unregulated manner. This chapter discusses the dimorphic fungi *Coccidioides immitis, Histoplasma capsulatum*, and *Blastomyces dermatitidis*, together with the mold *Aspergillus*. The fungus *Pneumocystis* and the alga *Prototheca* are briefly mentioned.

Coccidioides

Members of the genus *Coccidioides* are dimorphic fungi. The genus *Coccidioides* contains two species, *immitis* and *posadasii*. The disease coccidioidomycosis is caused by both. *Coccidioides immitis* and *Coccidioides posadasii* differ in their preferred geographic habitats. Both species occur only in the western hemisphere in the Lower Sonoran Life Zone, apparently as a result of that area's peculiar soil properties and temperature and rainfall patterns. *C. immitis* is found in the Central Valley of California in the United States (mainly the San Joaquin Valley), whereas *C. posadasii* is found in non-California locations (Texas, New Mexico, Arizona in the United States, and in South America). Of domestic animals, dogs are most frequently affected, and horses are occasionally affected. Less commonly infections are also reported in cats, swine, sheep, cattle, human, and nonhuman primates, and some 30 species of nondomestic mammals.

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Descriptive Features

Morphology, Structure, and Composition

In the soil, *Coccidioides* is a mold made up of slender septate hyphae that give rise, on thicker secondary branches, to chains of infectious arthroconidia (arthrospores, arthroaleuriospores, arthroaleurioconidia). These are bulging, barrel-shaped, thick-walled cells, 2.0–4.0 µm by 3.0–6.0 µm, separated by empty cells (disjunctors), through which breaks occur when arthroconidia are dispersed.

In tissue, arthroconidia grow into spherical sporangia with birefringent walls, "spherules" (10–100 µm diameter), which by internal cleavage produce several hundred "endospores" (2–5 µm diameter) (Figure 46.1). The walls disintegrate, allowing dissemination of the endospores, each of which may repeat the cycle or, on a nonliving substrate, give rise to mycelial (a collection of hyphae) growth. Though only arthroconidia are naturally infectious, endospores can experimentally initiate disease. Sexual spores are not known.

"Coccidioidin" in supernatants of mycelial *Coccidioides* broth cultures is largely polysaccharide, but contains some amino acid nitrogen. It is used in cutaneous hypersensitivity and serologic tests. "Spherulin," a lysate of cultured spherules, is also used in skin tests.

Cellular Products of Medical Interest

Adhesins

SOWgp (for spherule outer wall glycoprotein) is a proline-rich glycoprotein adhesin on the surface of spherules. SOWgp has an affinity for extracellular matrix proteins (laminin, fibronectin, and collagen). SOWgp stimulates a strong TH2 lymphocyte response, resulting in elevated levels of antibody and suppression of the protective cell-mediated immune response. Mutants unable to produce SOWgp have greatly reduced virulence.

Miscellaneous products are as follows:

1) **Beta-glucosidase 2**. Bgl2 (for beta-glucosidase 2) is an enzyme secreted by the endospores of *Coccidioides* that most likely plays a role in endospore morphology. Antibodies (IgM isotype) to Bgl2 are made early in the infectious process, and are useful diagnostically (detected by the precipitin test) to signal that a recent infection has occurred.
2) **Chitinase 1**. Cts1 (for chitinase 1) is one of several chitinases that are involved in the formation of, and release of, endospores from the spherule. An important target for the Cts enzymes is the "segregation apparatus" (a weblike mesh of chitin-containing structures within the spherule). Antibodies (IgG isotype) to Cts1 are made late in the infectious process (in disseminated disease), and are useful diagnostically (detected by the complement fixation [CF] test).
3) **Beta-1, 3 glucanosyltransferase**. Gel (for glucan-elongating glucanosyltransferase) is located on the surface of endospores. Gel elicits a strong TH1 lymphocyte response, resulting in elevated levels of gamma interferon followed by protection against disseminated disease (activates macrophages).
4) **Serine proteases**. These enzymes play a role in the stimulation of the inflammatory response elicited by *Coccidioides*. These enzymes digest elastin, collagen, and immunoglobulins.
5) **Urease**. Though its role in virulence is unknown, the enzyme urease (Ure) elicits a strong protective immune response following stimulation of TH1 lymphocytes (activates macrophages).

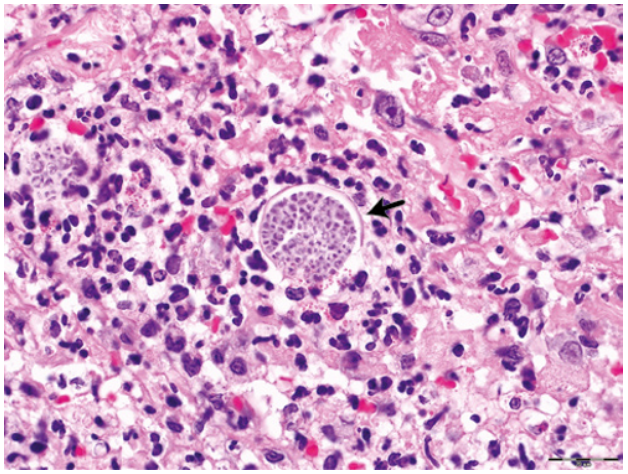


Figure 46.1 Tissue section from a skin lesion on a dog. A large *C. immitis* spherule containing endospores (arrow) surrounded by pyogranulomatous inflammation. Hematoxylin–eosin stain. 1000×.

Growth Characteristics

Coccidioides grows on simple media over a broad temperature range. On Sabouraud or blood agar, growth is mycelial. Over several days, initially dull gray colonies develop with sparse aerial mycelium, which gradually becomes more abundant. Arthroconidia are produced in five to seven days.

Spherules (sporangia) can be demonstrated *in vitro* by growth at 40 °C in media containing casein hydrolysate, glucose, biotin, glutathione, and a salt mixture (spherule medium).

Ecology

Reservoir

Coccidioides inhabits the soil in the Lower Sonoran Life Zone, including parts of California in North America (*C. immitis*) and the southwestern portion of the United States, Mexico, Central America, the northern and western rim states of South America, Argentina, and Paraguay (*C. posadasii*). High prevalence is associated with an annual rainfall of 5–20 in. and mean summer and winter temperatures of over 80 and 45 °F, respectively.

Arthroconidia resist drying and tolerate heat and salinity better than do competing soil organisms. In summer heat, *Coccidioides* survives in soil layers nearer the surface than do its competitors. When conditions favor growth again after rains, *Coccidioides* repopulates the superficial soil layers first, ensuring its widespread dispersal. Arthroconidia are dispersed by wind. Rodent burrows are heavily concentrated with arthroconidia in endemic areas. Dogs are frequently exposed to the fungus when they sniff the burrows.

Transmission

Infection is mainly by inhalation of arthroconidia. The organism is highly infectious, and very few arthroconidia need be inhaled to produce disease. Primary cutaneous infections are rare.

Pathogenesis

Mechanisms and Pathology

Following inhalation, the barrel-shaped arthroconidia round into spherical-shaped endospores, which, following enlargement and internal cleavage, differentiate into multinucleate spherules (10–100 μm) containing hundreds of endospores (2–5 μm), a process that takes several days. Spherules rupture, releasing endospores, and the cycle is repeated. Arthroconidia, endospores, and spherules trigger an inflammatory response in the lung (serine proteases are partly responsible). Arthroconidia and endospores are engulfed but not killed by phagocytes, and are conveyed to the hilar lymph node, where another inflammatory focus develops (as well as more spherules). Inflammation is stimulated in part by serine proteases, which are liberated during the growth of the fungus. Normally, cell-mediated immune responses arrest the process at this stage following stimulation of TH1 lymphocytes that activate macrophages, permitting destruction of the endospores. Antibodies to Bgl2 are made during this phase of the infectious process. With inadequate cell-mediated immunity (caused by SOWgp-directed immunomodulation, genetic makeup, infectious dose), dissemination can occur to bones, skin, abdominal viscera, the heart, genital tract, and eye (and rarely in animals to the brain and meninges). Gross lesions are white granulomas varying from miliary nodules to irregular masses. Peritoneal, pleural, and pericardial effusions occur. Antibodies to Cts1 are made during this phase of the infectious process. Microscopically, the predominant response is pyogranulomatous: to arthroconidia and endospores, the response is suppurative; and to spherules, the response is granulomatous, with epithelioid macrophages the chief component, admixed with multinucleated giant cells, lymphocytes, and neutrophils (Figure 46.1).

Disease Patterns

Dogs

Disseminated disease of dogs is the pattern seen most commonly by veterinarians. The complaints are lassitude, anorexia, and loss of condition. There may be respiratory signs (including cough), fever, and lameness due to bone involvement or arthritis, or discharging sinuses from deep lesions.

Cats

Disseminated disease in cats typically presents with less osseous and more visceral involvement. Skin lesions are the most frequent reason for presentation to the veterinarian.

Horses

As with cats, there is usually less osseous and more visceral involvement.

Cattle, Sheep, and Swine

In these species, the disease is usually asymptomatic, limited to lungs and regional lymph nodes, and undiagnosed until slaughter.

Humans

Naturally occurring asymptomatic coccidioidomycosis occurs frequently in humans. Clinical symptoms range from a flu-like illness to severe pneumonia and death. Excavator operators, agricultural and construction workers, and archeologists are at high risk for the disease when working in endemic areas given their exposure to disturbed soil. Those who are immunocompromised (e.g. acquired immunodeficiency syndrome [AIDS] patients), Filipino and African American patients, as well as women in the third trimester of pregnancy are reported to be at highest risk for disseminated disease.

Epidemiology

In all species, overt disease is the exception. The highest prevalence of canine systemic coccidioidomycosis is observed in male dogs, four to seven years of age, with the highest number of illnesses seen from January to March and May to July. These peaks may represent seasonal stress and increased exposure, respectively. Geographic and climatic factors have been mentioned. Young Boxer dogs and Doberman pinschers appear particularly susceptible.

Immunologic Aspects

Cell-mediated hypersensitivity, as determined by skin tests, develops within one or more weeks of exposure and can persist indefinitely. Its presence is an indicator of resistance to progressive disease. Its absence is the rule in disseminated infections, and its return is a favorable sign.

IgM antibody (specific for Bgl2), demonstrable by latex particle precipitation, appears temporarily after infection and usually disappears. IgG antibody titers (specific for Cts1), which are detectable by CF and immunodiffusion (ID) tests, rise in disseminated disease and remain high (1:16) until the process is brought under control.

No vaccine is presently available.

Laboratory Diagnosis

Direct Examination of Specimens

Animal fluids and tissues are examined for spherules by wet mount in saline containing 10% KOH. Spherules are 10–100 µm in diameter, have a thick wall (<2 µm), and contain endospores when mature (see Figure 46.1). Free endospores look indistinct in wet mounts but are recognizable in fixed smears stained by a fungal stain (e.g. periodic acid-Schiff [PAS], Gridley, or Gomori methenamine silver stains).

Cytological preparations, such as aspirates or imprints of lesions, stained with Romanowsky-type stains, typically reveal the pyogranulomatous inflammation and may have the characteristic large (diameter of 10–100 µm), double-contoured, clear to blue spheres that have variably visible round endospores inside. It is the presence of endospores and the extreme variation in size of the spheres that differentiates this organism from *B. dermatitidis*. Stained tissue sections (hematoxylin and eosin [H&E] (Figure 46.1), Gomori methenamine silver, Gridley) show both the agent and the characteristic lesion.

Culture

Blood agar and Sabouraud agar with or without antibiotics are inoculated, tape-sealed, and incubated at 37 and 25 °C, respectively. All processing of cultures is done under a microbiological safety hood. Mycelial growth should be evident within a week and is examined for the presence of barrel-shaped arthroconidia in a lactophenol cotton blue wet mount. Colonies are white and cottony with tan, brown, pink, or yellow pigmentation. The isolate can be reconverted to the sporangial phase by animal inoculation or cultivation in a spherule medium (see Section 46.2.3). Culturing and handling of culture should be avoided if a proper biocontainment facility is not available. Contamination of the laboratory with arthrospore could easily occur if the culture is not handled appropriately.

A commercially available "exoantigen" test kit furnishes prepared antisera to *Coccidioides*, *H. capsulatum*, and *B. dermatitidis* to be tested against extracts of suspect cultures in an ID agar plate, where precipitation lines develop between extracts and their homologous antisera.

Serology

Detection of antibodies to *Coccidioides* spp. is of value because tests for organism detection have low diagnostic sensitivity. Quantitative gel ID assays for IgG or IgM are typically used. Depending on the antigen preparation technique used for the assay, tube precipitation detection of IgM, or CF of IgG antibodies is reported.

In dogs, false negatives from CF are rare but false positives are common, and there is no clear correlation between the magnitude of the IgG titer and the severity of the illness. Therefore results must be interpreted in light of other clinical findings.

In cats, the CF test is positive in most cats that have the disease.

Molecular Methods

Most molecular methods used to identify or detect *Coccidioides* make use of primers specific for sequences contained within the DNA encoding ribosomal RNA. These sequences are amplified using the polymerase chain reaction (PCR).

Treatment and Control

The high cost of therapy as well as the stage of disease at the time of presentation are limiting factors in the successful treatment of coccidiomycosis. Most dogs and cats present with advanced disease.

For dogs and cats, supportive therapy and antifungal treatment (fluconazole or itraconazole) are required. Given orally over a period of months (usually a minimum of six months), they have effected permanent cures. Toxic effects are relatively minor except during pregnancy, when fetal deaths may occur. Surgical management, such as amputation or enucleation, may be required to control disease in some cases. Historically, ketoconazole has been used successfully but it is no longer the drug of choice due to the higher likelihood of toxicities and its lower activity. In addition to monitoring liver enzymes during treatment, the CF titer is used to monitor the effect of treatment.

For refractory cases, treatment with deoxycholate or amphotericin B, either alone or in combination with an azole, is recommended.

The prognosis depends on the severity of disease at presentation, the extent of dissemination, and owner compliance with long-term treatment. Patients with localized disease have the best prognosis.

Vaccines are not available.

Histoplasma capsulatum

H. capsulatum is a dimorphic fungus existing as a mold at 25–30 °C (saprophytic phase) and as a yeast at 37 °C (parasitic phase). This fungus has three varieties: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. capsulatum* var. *farciminosum*. The variety *farciminosum* causes epizootic lymphangitis (pseudoglanders), a chronic pyogranulomatous disease of equine skin and is discussed in Chapter 46. Varieties *capsulatum* and *duboisii* cause histoplasmosis, a systemic fungal disease of mammals. The variety *duboisii* is found only in Africa, whereas the variety *capsulatum* is found worldwide, and is the most common cause of histoplasmosis. In this chapter, histoplasmosis will be discussed without regard to this varietal distinction; that is, *H. capsulatum* var. *capsulatum*, and *H. capsulatum* var. *duboisii* will be regarded as *H. capsulatum*.

Descriptive Features

Morphology, Structure, and Composition

The free-living form of *H. capsulatum* consists of septate hyphae bearing spherical to pyriform microconidia 2–4 µm in diameter, and "tuberculate" macroconidia, thick-walled spheroidal cells 8–14 µm in diameter and studded with finger-like projections (Figure 46.2). In animal hosts or appropriate culture, the mold becomes a yeast consisting of round or oval, singly budding cells that measure 2–4 µm in diameter. A sexual, ascomycetous state, *Ajellomyces capsulatus*, has been described.

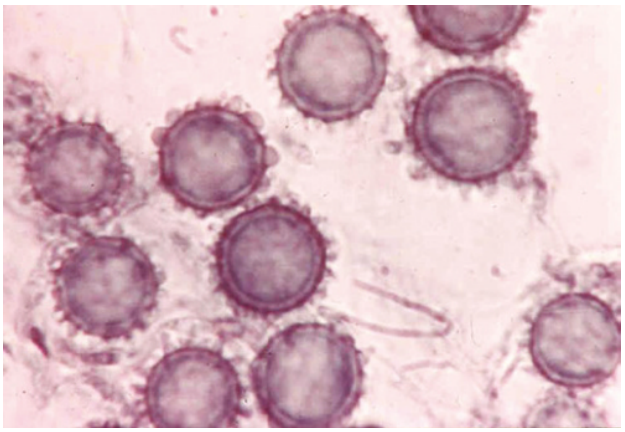


Figure 46.2 *Histoplasma capsulatum*, mycelial phase. Lactophenol cotton blue mount of a culture grown at 25 °C. "Tuberculate" macromedia studded with finger-like projections, 400×.

Histoplasmins, which are used in immunodiagnosis, are obtained from mycelial culture filtrates. They contain polysaccharides, with variable admixtures of glycoproteins and cellular breakdown products. Mycelial and yeast phases differ in cellular constituents, some of which (e.g. cell wall glucan) have been related to virulence.

Cellular Products of Medical Interest

Adhesins

Inhaled microconidia and yeast-phase parasitic forms are recognized by, and bind to, beta-2 integrins on the surface of neutrophils and macrophages. The fungal structure involved is unknown. Likewise, an unknown adhesin is responsible for the adherence of *H. capsulatum* to fibronectin receptors on dendritic cells. Adherence in this fashion to neutrophils, macrophages, or dendritic cells allows the fungus to enter the cell without triggering an effective oxidative burst and the generation of reactive oxygen and nitrogen intermediates.

Miscellaneous Products

H. capsulatum produces a variety of products that may play a role in histoplasmosis:

1) **Calcium-binding protein (**Cbp**)**. The yeast (parasitic) phase of *H. capsulatum* produces a Cbp that permits the yeast phase to grow in low-calcium environments (the phagolysosome) by efficiently chelating calcium and delivering it to the fungus. In addition, because Cbp

chelates available calcium within the phagolysosome, this impedes the effectiveness of several calcium-requiring lysosomal enzymes. Normal acidification of the phagolysosome is also a calcium-dependent event. *H. capsulatum* mutants unable to produce Cbp are avirulent.

2) **H antigen**. Host immune responses to H antigen were originally used as a diagnostic tool in the diagnosis of histoplasmosis. Subsequently, H antigen was found to be a beta-glucosidase that elicits a cell-mediated (protective) immune response to the yeast (parasitic) phase of *H. capsulatum*.
3) **Iron acquisition**. Iron is an absolute growth requirement for *H. capsulatum* (as it is for all life-forms). *H. capsulatum* acquires iron in several ways: production of hydroxamate siderophores capable of removing iron from host iron-binding proteins (transferrin, lactoferrin); expression of a hemin-binding receptor on the surface of yeast (parasitic) forms; glutathione-dependent ferric reductase that reduces Fe(III) to Fe(II), thereby releasing it from host iron-binding proteins; and several poorly defined iron reductases on the surface of the yeast phase.
4) **M antigen**. Host immune response to M antigen was originally used as a diagnostic tool in the diagnosis of histoplasmosis. Subsequently, M antigen was found to be a catalase enzyme, which plays a role in the survival of the yeast phase within the phagolysosome.
5) **Melanin**. Melanin is produced by *H. capsulatum*. Melanin is a free radical scavenger (reducing the toxicity of hydroxy radicals, superoxides, and singlet oxygen radicals found within the phagolysosome).
6) **Phagolysosome acidification**. Normal phagolysosomes have a pH <5, a pH that optimizes the activity of many of the digestive enzymes found in this environment. *H. capsulatum* raises the pH of the phagolysosome to 6–6.5, thereby reducing the activity of these lysosomal enzymes. How the fungus does this is unknown.

Growth Characteristics

H. capsulatum grows on common laboratory media over a broad temperature range. The optimum range for mycelial growth is 25–30 °C. The cottony aerial mycelium is white, brown, or intermediate. Pigmentation parallels abundance of macroconidia. The yeast phase requires richer media (e.g. glucose cysteine blood agar) and temperatures of 34–37 °C. Growth may take a week or more before characteristic colonies are seen.

H. capsulatum survives at ambient temperatures for months and at refrigerator temperature for years. It withstands freezing and thawing, and tolerates heating for more than an hour at 45 °C.

Variability

Classically this fungus has been divided into three varieties: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. capsulatum* var. *farciminosum*. Recently, via molecular techniques combined with the geospatial distribution of their sources, *Histoplasma* spp. have been grouped into eight clades (two North American clades, two South American clades, one Australian, one Indonesian, one African, and one Eurasian clade) and it is currently unclear whether the division into the three varieties is still valid. Because there does not seem to be a useful alternative until the status of this organism is resolved and because the classic division of varieties is still most common, the classic terminology will be used here.

The varieties *capsulatum* (worldwide) and *duboisii* (Africa) produce histoplasmosis, and the variety *farciminosum* causes epizootic lymphangitis (pseudoglanders) of equids (see Chapter 45). Although *H. capsulatum* var. *capsulatum* is found worldwide, its central focus is in the Americas. Genetically, the variety capsulatum is divided into six classes: Classes 1 and 2 are found in North America; Class 3 is found in Central and South America; Class 4 is found in Florida (North America); and Classes 5 and 6 are found in human patients with AIDS from New York (North America) and Panama (Central America), respectively.

Ecology

Reservoir

Although most concentrated in the Mississippi and Ohio River watersheds of North America, *H. capsulatum* occurs sporadically worldwide. It is found in the topsoil layers, especially in the presence of bird (mainly starlings in North America and chickens in South America) and bat guano, which provide both enrichment (nitrogen) and inoculum. Birds are mainly passive carriers, whereas bats undergo intestinal infectious processes. *H. capsulatum* is favored by neutral to alkaline soil environments with an annual rainfall between 35 and 50 in. and mean temperatures between 68 and 90 °F.

Transmission

Transmission is mostly by inhalation of microconidia or hyphal fragments, possibly by ingestion, and, rarely, by wound infection.

Pathogenesis

Mechanisms and pathology. Microconidia, hyphal fragments (from the environment), or yeast cells (from an

intraphagocytic cell environment) attach to macrophages in the lung by way of a beta-2 integrin. It is immediately phagocytosed, a minimal respiratory burst occurs, and a phagolysosome results following fusion with lysosomes. Microconidia and hyphal elements differentiate into yeast. Survival within the phagolysosome is related to modulation of the phagolysosome pH (around 6–6.5), secretion of M antigen (a catalase), secretion of Cbp, melanin, and successful acquisition of iron from intracellular iron stores. The yeast multiplies within the phagolysosome, ultimately killing the cell, which releases the yeast cells (which continue the cycle). Intracellular multiplication continues until an effective cell-mediated immune response occurs, resulting in activated macrophages, which effectively control the multiplication of the yeast.

Early events and lesions resemble those of tuberculosis. Thoracic lymph nodes become enlarged, and lungs may contain grayish white nodules. The histological response varies from suppurative to granulomatous inflammation. Caseation necrosis and calcification are common.

In disseminated disease, lymph nodes and parenchymatous organs are enlarged and may contain gross nodular lesions. There may be ulcerations of skin and mucous membranes, abdominal and pleural effusions, and involvement of the central nervous system (including eyes), skin, and bone marrow. The inflammatory exudate consists of macrophage elements colonized by yeast cells (Figure 46.3).

Disease Patterns

Histoplasmosis can occur in almost any animal species, but dogs and cats are the most commonly affected. Most affected dogs and cats have disseminated disease by the time they present to the veterinarian. A wide range of nonspecific clinical signs are seen in these patients including depression, fever, lethargy, anorexia, weight loss, diarrhea, dehydration, and anemia. Hepatomegaly, splenomegaly, mesenteric lymphadenitis, and ascites may cause abdominal distention. Coughing is relatively common in dogs but not in cats; however, both species often exhibit dyspnea and abnormal lung sounds. Clinical signs resulting from gastrointestinal involvement are very common in affected dogs, but rare in affected cats.

Disease patterns in human patients parallel those described for cats and dogs.

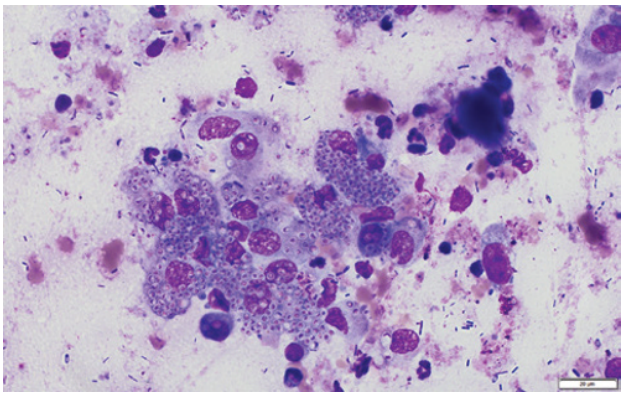


Figure 46.3 Rectal scraping from a dog with systemic histoplasmosis. Note the numerous intraphagocytic yeast cells that are round to oval and ~2 µm in diameter. Modified Wright's stain. 1000×.

Epidemiology

Subclinical infections with histoplasmosis are common in dogs, cats, and humans in endemic areas. Clinical disease is most prevalent in dogs aged two to seven years, in early autumn (September–November) and later winter to early spring (February–April). No sex predilection is reported, but the pointing breeds, Weimaraners, and Brittany spaniels have the greatest risk. Disseminated histoplasmosis in humans, dogs, and cats is found in association with immunosuppression.

Immunologic Aspects

Recovery and resistance are governed by cell-mediated immune responses, while circulating antibodies have no apparent protective function. Recovery from histoplasmosis appears to confer immunity.

No vaccines are available. However, certain antigens of *H. capsulatum* appear to protect mice against experimental challenge.

Laboratory Diagnosis

Direct Examination

Blood films, buffy coat smears, and cytological preparations such as aspirates and tissue impressions are stained with a Romanowsky-type stain (e.g. Giemsa, or Wright's) and examined for intraphagocytic yeast cells that are round to oval (not cigar-shaped) and approximately 2–4 µm in diameter (approximately ¼ to ½ the size of a RBC) (Figure 46.3). The yeasts stain light blue and have a pink- to purple-staining, eccentrically placed nucleus. Organisms are observed primarily within macrophages, but may also be seen within neutrophils, as well as in monocytes in peripheral blood. Fungal stains (PAS, Gridley, or Gomori methenamine silver) may be used to aid visualization, but are not routinely used on cytological preparations.

In sections stained with hematoxylin–eosin, *H. capsulatum* appears as tiny dots surrounded by halos. A duplicate fungal stain (e.g. PAS, Gridley, or Gomori methenamine silver) can be helpful.

Immunofluorescence has been used to identify the yeast in tissue and exudates.

Culture

Specimens are inoculated onto blood agar and Sabouraud agar, with and without inhibitors, and incubated in jars or plastic bags at room temperature for up to two months.

Colonial growth may look reddish-wrinkled before the appearance of cottony brownish to white mycelium.

Microconidia and macroconidia are demonstrated in lactophenol cotton blue wet mounts. Dimorphism must be proved by conversion to the yeast phase by incubating plates at 37 °C or by IV injection of mice. Mice will die within a few weeks. Their macrophages will contain the yeast forms.

A commercially available "exoantigen" test kit furnishes prepared antisera to *Coccidioides*, *H. capsulatum*, and *B. dermatitidis* to be tested against extracts of suspect cultures in an ID agar plate, where precipitation lines develop between extracts and their homologous antisera.

Serology

Antibody assays (gel ID or CF) and antigen assays (ELIZA) are available for diagnosis of histoplasmosis. Antibody assays are reported to have poor diagnostic sensitivity, with "false" positives occurring in recovered animals. Antigen assays are considerably more reliable but should be used in conjunction with other clinical findings. One study reported that none of the 26 cats without histoplasmosis had positive urine antigen tests and 17 of the 18 cats with known disease has a positive urine antigen test.

Molecular Techniques

Molecular methods used to identify or detect *H. capsulatum* make use of primers for specific sequences contained within the DNA, for example, genes encoding ribosomal RNA, or those encoding the M protein. These sequences are amplified using PCR. These tests are not used routinely in the veterinary diagnostic laboratory.

Treatment and Control

Azoles (itraconazole, fluconazole, and ketoconazole) and amphotericin B have been used successfully in the treatment of some cases of canine and feline histoplasmosis. Itraconazole and fluconazole are preferred to ketoconazole as they as less likely to cause toxicity and are more effective.

The duration of treatment is variable depending on the severity of the infection and the patient's response. Response to therapy is monitored by frequent evaluation for resolution of clinical signs and radiographic lesions and abnormalities in hematologic and biochemical profiles. When treatment is not continued for the appropriate length of time (at least four to six months), relapses are common. The prognosis for disseminated cases is guarded to grave.

Avoidance of bat caves and areas inhabited by avian species is recommended in endemic areas. However, animals housed exclusively indoors have been diagnosed with the disease in endemic areas.

Blastomyces dermatitidis

B. dermatitidis is a dimorphic fungus existing as a mold in the soil (saprophytic stage), and as a yeast in tissue (parasitic stage). It causes the systemic fungal disease called blastomycosis, which occurs most commonly in the eastern third of North America. Cases of the disease arise sporadically in Africa, Asia, and Europe. People and dogs are the species most frequently affected by blastomycosis. However, the disease has been reported in many animals including horses, cats, ferrets, and numerous wildlife species.

Descriptive Features

Morphology, Structure, and Composition

In the saprophytic phase (in soil or on artificial media at 25–30 °C), the hyphae of *B. dermatitidis* produce conidiophores with spherical or oval smooth-walled conidia 2–10 µm in diameter. In tissue or on blood agar at 37 °C, the agent is a thick-walled yeast 5–20 µm in diameter that reproduces by single buds attached by a broad base. The fungus has a sexual form, *Ajellomyces dermatitidis*.

Cell wall extracts contain 3–10 parts of polysaccharide to 1 part of protein. Protein is highest in the mycelial phase, while chitin is highest in the yeast phase. The lipid content of *B. dermatitidis* is higher than in other fungi. Lipid, protein, and chitin levels vary directly with virulence.

Cellular Products of Medical Interest

Adhesin

The yeast phase of *B. dermatitidis* produces a protein adhesin termed Bad1 (for Blastomyces adhesin 1), formally called

WI-1. Bad1 has two functions related to virulence of the fungus: (i) Bad1 adheres to beta-2 integrins on the surface of phagocytic cells, triggering uptake of the yeast, invoking a minimal respiratory burst, and resulting in little generation of reactive oxygen and nitrogen intermediates; (ii) Bad1 downregulates the production of pro-inflammatory cytokines (particularly tumor necrosis factor [TNF]) by the affected macrophages. The amino acid sequence of Bad1 is homologous to the invasion protein produced by Yersinia. *B. dermatitidis* mutants that do not produce Bad1 are avirulent.

Miscellaneous Products

B. dermatitidis produces a variety of extracellular enzymes and products that may be involved with disease production. These include proteases, phosphatases, esterases, and glycosidases. Culture filtrates are leukotactic for human neutrophils. The role played by these products in the production of disease is unknown.

Growth Characteristics

B. dermatitidis grows on most media at room temperature and 37 °C. Colonies develop from within two to over seven days. Mold colonies, formed at ambient temperatures (25–30 °C), are cottony white to tan depending on the abundance of conidia. Yeast colonies develop on blood agar at 37 °C. They are opaque off-white to tan, with a rough surface and a pasty consistency.

Variability

The two recognized serotypes are related to the geographic origin of the strains. Similar geographic relationships are seen when PCR-based fingerprinting systems are used.

Ecology

Reservoir

Soil is believed to be the reservoir for *Blastomyces*. Moisture, low pH, animal waste, and decaying vegetation appear to favor colonization. Humidity promotes release of conidia.

Transmission

Blastomycosis most commonly begins by inhalation of microconidia. Percutaneous infection probably occurs in dogs with organisms entering tissue via traumatic inoculation, but generally, solitary lesions should be considered part of a systemic disease.

Pathogenesis

Microconidia hyphal fragments are inhaled and convert to the yeast form within the alveolar spaces of the respiratory tract. The yeast expresses Bad1, which is followed by uptake (with minimal respiratory burst) by phagocytic cells. Downregulation of TNF production by affected macrophages delays stimulation of cell-mediated immunity (which is necessary to kill the yeast). In dogs, an inflammatory response involving macrophages and neutrophils and resulting in pyogranulomatous lesions occurs in the terminal bronchioles, followed by similar reactions in satellite lymph nodes. Blastomycosis is more often progressive than histoplasmosis and coccidioidomycosis. Dissemination is believed to occur via vascular and lymphatic routes. Preferred sites in the dog are the lymph nodes, skin, bones, bone marrow, eyes, subcutaneous tissue, brain, testes, and external nares. Less common sites of dissemination are the mammary glands, prostate, liver, heart, mouth, vulva, and urogenital tract. Although organisms usually enter the body via the lungs, occasionally lung lesions have resolved by the time the animal is presented to the veterinarian. The nodular lesions can be tubercle-like, with mononuclear cell types predominating, or there may be numerous neutrophils admixed. Liquefaction and caseation occur, but calcification and encapsulation are exceptional.

Signs include skin lesions and respiratory distress with fever, depression, anorexia, and weight loss. Locomotor disturbances result from bone or joint infection or – rarely – central nervous system involvement. Ocular disease is common in disseminated blastomycosis. Most dogs with multiple organ system involvement die within months. Evidence for a benign form is uncertain. Although much less common, the disease in cats resembles that in dogs.

Epidemiology

The highest prevalence in dogs is from spring to fall. Male dogs less than four years of age are most often affected. In general large-breed dogs are more often affected than small-breed dogs. No sex, age, or breed predisposition has been identified in cats. Though generally noncontagious, one human infection resulted from a dog bite.

Immunologic Aspects

Impaired cell-mediated responsiveness may explain canine disseminated blastomycosis.

Humoral and cell-mediated responses occur as a result of infection. Cell-mediated immunity is decisive in determining resistance. Artificial immunization is not available.

Laboratory Diagnosis

Direct Examination

B. dermatitidis can be demonstrated in wet mounts of exudates and tissue smears as thick-walled yeast cells with single buds attached by a broad base. In section, intracellular yeasts may be found. Cytological preparations such as aspirates and tissue impressions are stained with a Romanowsky-type stain (e.g. Giemsa, or Wright's) and examined for yeast cells that are approximately 5–20 µm in diameter, have a thick, retractile, double-contoured cell wall (Figures 46.4 and 46.5). Broad-based budding of organisms may be seen in some preparations; however, most organisms are singular. Blastomyces organisms are differentiated from *Cryptococcus* species by their lack of a clear capsule, their deeply basophilic cell wall, and by the presence of broad-based budding. Fungal stain (PAS, Gridley, or Gomori methenamine silver) can also be applied to aid visualization of the organisms but is not routinely done on cytology.

In sections stained with hematoxylin–eosin, *B. dermatitidis* appears as thick-walled yeast cells with single buds attached by a broad base. A duplicate fungal stain (e.g. PAS, Gridley, or Gomori methenamine silver) can be helpful.

Culture

Cultures on Sabouraud agar (with or without inhibitors) are incubated at the ambient temperature (25–30 °C) for up to three weeks. It is difficult to obtain the yeast form on primary isolation, but it is relatively easy to convert the mold to the yeast phase by increasing the temperature to 37 °C.

A commercially available "exoantigen" test kit furnishes prepared antisera to *Coccidioides*, *H. capsulatum*, and *B. dermatitidis* to be tested against extracts of suspect cultures in an ID agar plate, where precipitation lines develop between extracts and their homologous antisera.

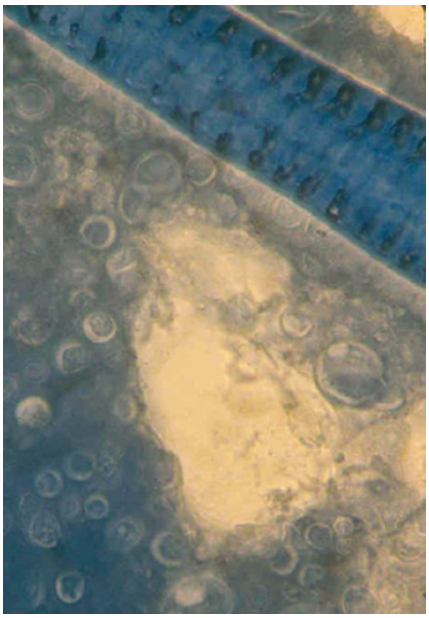


Figure 46.4 *Blastomyces dermatitidis*. Yeast phase, skin scraping of dog. Hematoxylin–eosin stain, budding yeast cell, 1000×.

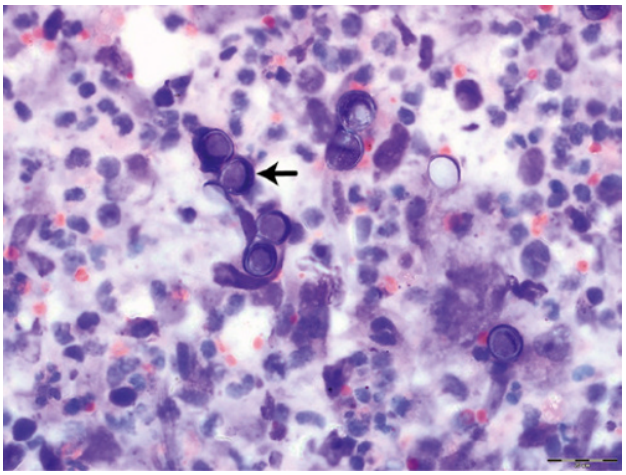


Figure 46.5 Exudate from the center of a necrotic lymph node from a dog with blastomycosis. Note the dark-staining *Blastomyces dermatitidis* yeasts characterized by the thick, double-contoured cell wall (arrow). Modified Wright's 1000×.

Serology

The antigen assay that detects the *Blastomyces* cell wall galactomannan had 93.5% sensitivity from urine samples, and 87% specificity from serum, in dogs with confirmed blastomycosis. Of 43 control dogs tested that did not have the disease, only one false positive result was obtained.

Although gel ID antibody assays are available, this assay has an unacceptable sensitivity for the diagnosis of blastomycosis in dogs and cats.

Molecular Methods

Molecular methods used to identify or detect *B. dermatitidis* make use of PCR primers for specific sequences contained within DNA encoding ribosomal RNA (e.g. 28S RNA; the internally transcribed spacer region). These sequences are amplified using PCR. PCR is not widely available for the detection of blastomycosis in dogs and cats.

Treatment and Control

For treatment of blastomycosis, itraconazole has largely replaced ketoconazole because it is effective in many cases and has fewer side effects. The duration of treatment is based on clinical assessment but is typically at least three months, though often six months and may take more than a year, Fluconazole is less effective and so is not generally recommend unless itraconazole is not tolerated or is unavailable. Amphotericin B is also effective and may be considered for use in dogs with severe disease that are not responding to azole therapy. Precautions must be taken to avoid exposure when handling animals or tissues suspected to be infected with blastomycosis, even though transmission from animals to humans is extremely rare.

Aspergillus spp.

Members of the genus *Aspergillus* are ubiquitous saprophytic molds with opportunistic pathogenic patterns that depend on impaired, overwhelmed, or bypassed host defenses. Of some 900 species, *Aspergillus fumigatus* is most frequent in animal and human infections.

Descriptive Features

Morphology and Composition

Aspergillus spp. are molds consisting of septate hyphae and characteristic asexual fruiting structures that are borne on conidiophores. Conidiophores are hyphal branches originating in a foot cell in the vegetative mycelium and ending in an expanded vesicle. The vesicle is covered by a layer or layers of flask-shaped phialides, from which chains of pigmented conidia (the asexual reproductive units) arise (Figure 46.6). They give the fungal colony its color.

In tissue, only hyphae are seen. In aerated cavities (e.g. nasal passages, air sacs, cavitary lesions), fruiting structures may be found.

Fruiting bodies are important diagnostic features of *Aspergillus* spp. by which species are identified.

Cellular Products of Medical Interest

Adhesins

Members of the genus *Aspergillus* produce a number of surface proteins (conidial and hyphal) that bind to extracellular matrix proteins (collagen, fibronectin, fibrinogen, and laminin).

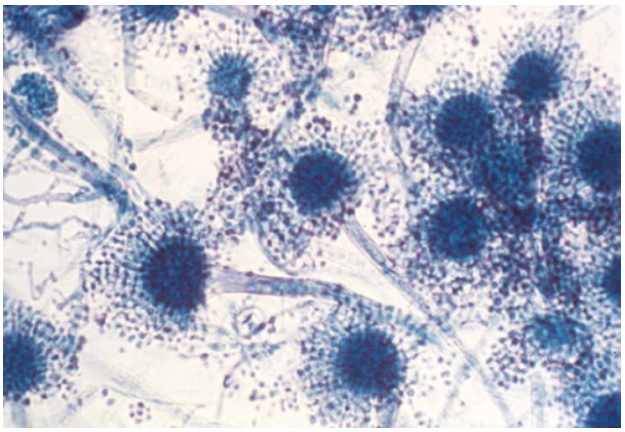


Figure 46.6 *Aspergillus fumigatus*. Lactophenol cotton blue mount from Sabouraud dextrose agar culture, 400×.

Cell Wall

The cell wall of *Aspergillus* displays a "pathogen-associated molecular pattern" that is recognized by Toll-like receptors on the surface of host macrophages. Binding to these receptors leads to the secretion of pro-inflammatory cytokines.

Extracellular Enzymes

Aspergillus produces a number of enzymes that have the potential to function *in vivo* to break down host tissue. These include elastase, proteases, and phospholipases. In addition, members of this genus produce catalases that reduce the effectiveness of peroxides generated by phagocytic cells.

Iron Acquisition

Aspergillus produces several hydroxamate siderophores (a ferrichrome and a fusarinine) needed to acquire iron from host iron-binding proteins (transferrin, lactoferrin).

Pigment (Melanin)

Conidia of *Aspergillus* are pigmented. The pigment, melanin, is a free radical scavenger (reducing the toxicity of hydroxy radicals, superoxides, and singlet oxygen radicals found within the phagolysosome).

Growth Characteristics

Aspergilli grow on all common laboratory media over a wide range of temperatures (up to 50 °C). Their biochemical activities have not been clearly related to virulence or utilized diagnostically.

Aspergilli thrive in the environment. Some are highly resistant to heat and drying. Most do not grow in cycloheximide-containing fungal media.

Ecology

Reservoir

Aspergilli are present in soil, vegetation, and feed, and secondarily in air and water and objects exposed to them. *A. fumigatus* becomes predominant over competing microbiota in fermented plant material (e.g. hay, silage, compost). Animal disease outbreaks are often traced to such sources.

Transmission

Aspergillosis is acquired from environmental sources, generally by inhalation or ingestion. Most *Aspergillus* mastitis follows intramammary inoculation. Intrauterine infections in cattle result from dissemination of subclinical lung or intestinal infections.

Pathogenesis

Mechanism

Following their deposition in tissue or on a surface (adhesins hinder removal), recognition (by way of "pathogen-associated molecular pattern") by phagocytic cells triggers an inflammatory response. Inflammation, along with the release of fungal elastase, proteases, and phospholipases, results in tissue damage. Pigment and catalase delay destruction by phagocytic cells.

Allergenic factors, which are recognized in human aspergilloses, are insufficiently documented in animal disease.

Pathology

In pulmonary infection, suppurative exudate accumulates in bronchioles and adjacent parenchyma. It surrounds colonies of mycelial growth, which may extend into blood vessels and produce infected thrombi and vasculitis, leading to dissemination. Infection may also spread directly into adjacent air spaces. Granulomas develop; they are grossly visible as grayish white nodules and consist of mononuclear cells and fibroblasts. In older lesions, colonies are fringed by acidophilic clubs (asteroid bodies), which resemble actinomycosis.

Lesions in avian lungs are caseous nodules. On serous membranes, caseous foci are covered by macroscopic mold colonies, accompanied by thickening of the membranes (e.g. air sacs). The cellular response is acute suppurative to chronic granulomatous.

Bovine abortion results from hematogenous seeding of placentomes, which is possibly a response to a growth factor in placental tissue. There is hyphal invasion of blood vessels producing vasculitis and a necrotizing, hemorrhagic placentitis. The fetus undergoes disseminated infection with signs of emaciation and dehydration. Lymph nodes, viscera, and brain may be involved. Ringworm-like plaques on the fetal skin are often seen.

On mucosal surfaces (e.g. nasal passage, trachea), mold colonies form on top of necrotic tissue, which is surrounded by a hemorrhagic zone.

Disease Patterns

Pulmonary and disseminated infections, frequently involving kidneys and the central nervous system, occur in most species.

Avian

Avian aspergillosis, which affects many species of birds, sometimes in epidemics, reflects heavy exposure or severe stress on domestic flocks or pet bird operations, or the effects of oil spills on marine birds. The disease is usually a respiratory tract infection, sometimes with hematogenous dissemination. The disease is also called "brooder pneumonia" in chicks and poults. The young bird acquires the disease by inhaling a massive dose of spores in the brooder. Signs are inappetence, listlessness, weight loss, dyspnea, sometimes diarrhea, and abnormal behavior and posture. The eyes are often affected. Mortality may approach 50%, especially in young birds. In mild cases, only gasping and hyperpnea may be seen. The course varies from a day to several weeks.

Ruminant

Bovine abortions usually occur late in pregnancy and resemble abortions due to other causes. Fetal skin plaques occur also in other mycotic abortions.

Mastitis due to *A. fumigatus* is reported at an increased rate, especially from Europe. It is usually chronic progressive, producing abscesses in the udder, and occasionally causes diarrhea in calves.

Dogs and Cats

Aspergillosis of mucous membranes (sinonasal aspergillosis) is predominantly caused by *A. fumigatus* and occurs commonly in dogs (any breed can be affected but large non-brachycephalic breeds are most commonly affected), rarely in cats (though brachycephalic breeds, especially Persians and Himalayans, are predisposed). It is a noninvasive, acute to chronic disease of the nasal passages and paranasal sinuses. It is manifested by sneezing and unilateral or bilateral persistent nasal discharge that is unresponsive to medical treatment. Purulent nasal discharge may be seen due to secondary bacterial infection.

Aspergillus felis causes sino-orbital aspergillosis (SOA) in cats though brachycephalic breeds, especially Persians and

Himalayans, are predisposed. In SOA, the fungus extends through the orbital bone from the submucosal tissue of the nasal cavity and sinuses to invade the retrobulbar space.

Aspergillus terreus and *Aspergillus deflectus* are the most common causes of disseminated aspergillosis in dogs, particularly German shepherd dogs. Rhodesian ridgebacks are also predisposed. Osteomyelitis is a common feature. Intestinal aspergillosis presenting as diarrhea has been reported in cats.

Horses

Aspergillus infection of the cornea is the leading cause of keratomycosis.

Vague upper respiratory signs may point to an aspergillosis of the equine guttural pouch. Intestinal aspergillosis may cause diarrhea in foals.

Humans

Aspergillus spp. could be a primary or secondary pathogen in humans, depending on the immune status of the individual. Tissues involved include the lungs, bones, sinuses, ear skin, and meninges.

Miscellaneous Species

Infrequent infections involving the lungs occur in other animals as well.

Epidemiology

The intensity of exposure is a significant feature in animal aspergillosis. Bovine abortion outbreaks are often related to moldy fodder. Aspergillosis in chicken flocks commonly coincides with the use of heavily contaminated litter.

Stress aspects are usually recognizable in outbreaks. Avian aspergillosis is seen under conditions of poor husbandry. In oiled seabirds, there is severely impaired thermal regulation. In pregnant cattle, advanced gestation combined with low-quality feed and poor weather and housing add up to a severe challenge.

Canine nasal aspergillosis occurs, especially in young dogs of dolichocephalic breeds.

A T lymphocyte deficiency may exist.

In keratomycosis of horses, the frequent history of topical antibacterial, and steroid treatment suggests immunosuppression and impaired colonization resistance.

Immunologic Aspects

Circulating antibody with no demonstrably protective role may be present in dogs with nasal aspergillosis (see under Section 46.24). Cell-mediated immunity is believed to be the major factor in limiting the dissemination of infection.

Immunization procedures are not available.

Laboratory Diagnosis

Direct Examination

Cytological preparations using Romanowsky-type stains (e.g. Wright's or Giemsa) may provide strong evidence of aspergillosis based on the presence of characteristic septate fungal hyphae that have parallel sides and are dichotomously branching at a 45° angle. Other fungi (*Penicillium*, *Pseudallescheria*, *Paecilomyces*) also present a similar picture but are rare.

Hyphae, fruiting heads, and conidia can often be demonstrated in samples either in wet mounts in 10% KOH or with calcofluor white.

For histopathology, fungal stains (PAS, Gridley, or Gomori methenamine silver) are best; Gram is of limited use and is not recommended.

Note that conidia may occur in air passages or other exposed sites in the absence of infection.

Isolation and Identification

Aspergillus is readily cultured. Because it is a ubiquitous contaminant, interpretation of positive cultures is often problematic. The presence of the agent must always be correlated with pathological and clinical findings. Identification of agents rests upon the morphological features and growth characteristics of isolates.

Serology

Serological tests may be useful adjuncts to the diagnosis of aspergillosis, but sensitivity and specificity are variable between tests and are often low. Because the tests that are available are species specific, it is necessary to know which *Aspergillus* to expect. For example, *A. fumigatus* is most commonly seen in nasal aspergillosis of dogs, whereas disseminated aspergillosis in that species is either *A. deflectus* or *A. terreus*. An ID kit for antibody detection is commercially available for detection of antibodies to *A. fumigatus*. An enzyme-linked immunosorbent assay and a counter immunoelectrophoresis test are also available for the serodiagnosis of aspergillosis in humans.

Molecular Methods

Primers designed to amplify DNA encoding ribosomal RNA (including the "internal transcribed spacer"

region) are available for demonstration or identification of members of the genus. Amplification of DNA is by PCR. Unfortunately, the diagnostic value of these assays is currently limited due to the unacceptable rates of false positive or low sensitivity.

Treatment, Control, and Prevention

The sino-nasal form in dogs requires thorough debridement until no visible fungus is evident and subsequent thorough lavage (if the cribriform plate is intact) followed by topical instillation of clotrimazole or enilconazole into the nasal passages and sinuses. Itraconazole (given orally) has been successfully used to treat nasal aspergillosis when topical treatment was not possible.

Azole therapy (most commonly itraconazole, but also voriconazole and posaconazole) with or without concurrent amphotericin B has been beneficial in treating sino-orbital and disseminated aspergillosis. Fluconazole is not recommended due to *Aspergillus* species resistance.

In horses, itraconazole as well as oral potassium and topical natamycin have been used effectively in cases of *Aspergillus* infection. In addition, to treat gutturomycosis, surgical exposure and curettage are recommended.

For bovine mastitis, combined intra-arterial and intramammary injection with miconazole has been effective.

For intestinal infections in pigs, foals, and calves, oral nystatin is recommended. Keratomycosis is treated topically with antimycotic ointments and solutions.

Avoidance of massive exposure requires elimination of cattle feed, particularly hay and silage that has undergone noticeable deterioration. *A. fumigatus* reaches high concentrations only under conditions of "biologic heat" generation, after other microbiota are eliminated. With poultry litter, proper storage and frequent changes of litter can prevent such buildup.

Other Saprophytic/Opportunistic Fungal Pathogens

Rhizopus spp., *Rhizomucor*, *Absidia* spp., *Mucor* spp., and *Mortierella* spp. cause mycotic bovine abortion and gastrointestinal infections marked by ulcerative lesions and mesenteric lymphadenitis as well as respiratory and hematogenous infections affecting various viscera and the central nervous system in ruminants, swine, and dogs. They are secondary to stress, such as dietary changes and inadequacies, antibiotic suppression of the gastrointestinal flora, concurrent infections, recent parturition, or trauma. Several cases of infections caused by these agents have been reported in humans and nonhuman primates. These agents grow rapidly on Sabouraud agar at room temperature. Amphotericin B is the preferred drug for treatment in both animals and humans.

Paecilomyces spp. are associated with disseminated canine and feline paecilomycosis, in which bone involvement and a respiratory epidemic in captive turtles have been reported. *Conidiobolus* spp. and *Basidiobolus* spp. cause nasal granulomas and subcutaneous infections in horses, mules, dogs, sheep, nonhuman primates, and humans. Agents are identified by direct examination of clinical samples and by cultural techniques. Considerable expertise is needed for species identification in the laboratory. Itraconazole, fluconazole, 5-fluorocytosine, and amphotericin B have provided effective therapy. *Diplorhinotricum gallopavum* causes dactylariosis in turkeys and chickens. It is a central nervous system disease with high morbidity and approximately 20% mortality. Treatment is not generally viewed as practical, but good sanitation of the poultry house and the hatchery is essential for control of this disease.

Pneumocystis

Members of the genus *Pneumocystis* are saprophytes of low virulence, capable of causing pneumonia in immunocompromised individuals. They have only been isolated from affected hosts (e.g. humans, dogs, cats, horses, pigs, goats, ferrets, mice, rats, chimpanzees, and monkeys). Though controversial, at present there seem to be six, likely very host-specific, species*: jiroveci* (affecting human patients), *carinii* and wakefieldiae (both affecting rats) murina (mice), oryctolagi (rabbits), and canis (affecting dogs); the latter requires further genetic study. The disease seems to be transmitted from infected to susceptible host, primarily through airborne droplets. Thus, *Pneumocystis* is a host-specific fungus that is contagious between members of the same species but is not zoonotic.

Spread is through aerosols. Affected hosts are usually immunocompromised, though there are reports of healthy animals (with no evidence of immunosuppression or underlying condition) developing *Pneumocystis* pneumonia when housed with known infected animals. Cavalier King Charles Spaniels and miniature dachshunds are overrepresented.

The fungus has not been grown in a cell-free culture system. Diagnosis is made by examination of material from the alveolar spaces, which is usually concentrated by cytocentrifugation and stained with Romanowsky-type stains (e.g. Giemsa, Wright's). On these cytological preparations, intracellular (within alveolar macrophages) and extracellular round-shaped cysts (approximately 4–7 μm in diameter) that contain 4–8 basophilic bodies arranged

in a circle are observed. Numerous ovoid- to crescent-shaped trophozoites (approximately 1–2 μm) are also typically seen. Silver stain (e.g. Gomori methenamine silver) can also be used to aid visualization of the organism. DNA primers have also been designed to amplify specific segments of the fungal genome, so that detection and identification are possible using PCR.

PCR assays are available to detect *Pneumocystis* from bronchoalveolar lavage fluid from humans and dogs. The sensitivity of PCR is reported to be greater than that of direct examination of cytological preparation.

Treatment includes one or more of the following: trimethoprim-sulfonamides (treatment of choice), dapsone, atovaquone, pentamidine, clindamycin, or trimetrexate/leucovorin.

Protothecosis

Prototheca is a unicellular alga lacking chlorophyll. It multiplies by endosporulation, producing roughly spherical cells that are 8–25 μm in diameter. Six species are currently recognized: *Prototheca zopfii, Prototheca wickerhamii, Prototheca blaschkeae, Prototheca stagnorum, Prototheca ulmea,* and *Prototheca cutis. P. zopfii* and *P. wickerhamii* cause the vast majority of disease worldwide. They grow on fungal media (without cycloheximide) at 25 and 37 °C, respectively, into white to tannish dull colonies in less than a week and are differentiated serologically and by carbohydrate assimilation tests.

Prototheca spp. are widespread in nature. Exposure is by ingestion, percutaneously, or, in dairy cows, by intramammary injection.

Disease occurs in dogs, cats, cattle, deer, bats, snakes, fish, and humans. In dogs, it is usually disseminated and accompanied by hemorrhagic diarrhea. Central nervous system involvement and eye lesions are frequent. Immunodeficiency is suspected. In cats and humans, cases to date have been cutaneous. Cats have single or multiple skin nodules but are otherwise in good health. In cattle, chronic progressive mastitis develops. Tissue reactions are pyogranulomatous.

The agent is easily cultured and can be demonstrated in unstained wet mounts from specimens or in cytological preparations stained with a Romanowsky-type stain (Wright's or Giemsa) or fungal (PAS, Gridley, or Gomori methenamine silver) stain.

For localized cutaneous lesions, surgical excision is curative. Treatment is challenging, and the prognosis is poor for patients with disseminated disease. Amphotericin B and ketoconazole are used in people. Amphotericin B has been used in dogs, but relapse is common with removal of the drug or there is complete failure of response. Outcomes using azoles is consistently poor.

Acknowledgments

Dwight C. Hirsh and Ernst L. Biberstein contributed the material for this chapter in the first and second editions of this textbook, and much of that information has been retained here.

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Part IV

Viruses

47

Parvoviridae

Rebecca P. Wilkes

The family Parvoviridae is a large and diverse family of viruses and currently comprises three subfamilies: Parvovirinae, Hamaparvovirinae, and Densovirinae. The subfamily Parvovirinae contains viruses of veterinary importance and is the focus of this chapter. This subfamily has 10 genera: *Protoparvovirus, Erythroparvovirus*, *Artiparvovirus*, *Loriparvovirus*, *Dependoparvovirus*, *Amdoparvovirus*, *Aveparvovirus*, *Copiparvovirus*, *Tetraparvovirus*, and *Bocaparvovirus*, and at present contains 75 species. Parvoviruses have a broad range of impacts on their hosts, differing from severe clinical disease to subclinical or nonpathogenic infection. For example, parvoviruses detected in rodents, pinnipeds, and primates most commonly cause subclinical infections, whereas parvovirus infections in dogs and cats may be fatal. In general, clinical disease caused by parvoviruses is more severe in fetuses, neonates, and young animals.

The parvoviruses are mainly species specific and named according to their host/reservoir species; however, some parvovirus species have altered their host range to create pandemic viruses, such as occurred with canine parvovirus (CPV). With the advent of next-generation sequencing, "new" parvoviruses are regularly discovered. Despite epidemiological studies, any possible association with the clinical signs of most of these newly described viruses remains unclear. Considering that many parvoviruses are clinically silent, the significance of newly identified parvoviruses must be determined.

The name *parvovirus* is derived from the Latin term *parvus*, which means "small." Parvoviruses are some of the smallest viruses known (18–26 nm). These viruses are nonenveloped, have icosahedral symmetry, and contain a linear single-stranded DNA genome of approximately 4–6 kb. In both terminal sequences of the virus, a complex palindromic hairpin structure is located and required for DNA replication. The polarity of the genome strand is either negative sense only or a proportion of individually encapsidated negative and positive strands. The genomes of Parvovirinae viruses contain two major expression cassettes, with open reading frames (ORFs) on the left-hand side coding for non-structural (NS) proteins, whereas those from the right-hand side give rise to the structural proteins (VPs). The largest of the NS proteins, traditionally designated NS1, contains a highly conserved domain and is essential for virus replication. Based on its conservation, this gene sequence is used to define family taxonomy. The capsid is composed of VPs, which control binding to host cells and define the host range. Interestingly, parvoviruses have high levels of genetic diversity and evolve at rates approaching those observed for RNA viruses. However, just a small number of mutations in the capsid protein gene that alter residues on the surface of the capsid can allow infection of a new host, which results in a new virus. These genetic differences in the capsid gene of *Protoparvovirus* between strains also make sequencing of this gene useful for characterizing these parvovirus strains in animals that can be infected by multiple parvoviruses. The ability of some animal species to be infected by multiple parvoviruses also allows for recombination between parvovirus strains, which adds to increased genetic variation. Changes in parvoviruses over time (as described for CPV, feline parvovirus, and porcine parvovirus [PPV]) have led to variants and questions regarding the efficacy of current vaccines in protecting against these newer wild type strains.

Regarding immune response against parvoviruses, the capsid protein is a major target of the host antibody response. Neutralizing antibodies produced against the capsid protein are important for protection because they block attachment of the virus to host cells. A useful

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characteristic of these viruses is that they hemagglutinate (HA) red blood cells, and an antibody response following infection results in the generation of hemagglutination inhibition (HAI) antibodies, which correlate with viral neutralization. HA and HAI are useful assays for the study of pathogenesis, protection, and prevalence.

The icosahedral virion and lack of an envelope confers great stability to the virus particles, and parvoviruses are among the most stable viruses known. They are resistant to inactivation by pH, organic solvents, extreme temperatures, desiccation, and many commercial disinfectants. This makes contaminated fomites a significant source of parvovirus infection. A 5% solution of household bleach (sodium hypochlorite) is an effective and practical virucidal disinfectant for these viruses. However, bleach and products in the bleach family are inactivated by organic material (requiring use of a disinfectant to clean prior to use of bleach), and these products do not work well on porous surfaces. Accelerated hydrogen peroxide (Accel/Rescue) and potassium peroxymonosulfate (Virkon®/Trifectant®) are also effective against parvoviruses, even in the presence of organic material. Unlike bleach, these products can be used effectively on unsealed surfaces, carpet, and furniture. Despite label claims of efficacy, independent studies have repeatedly shown that quaternary ammonium compounds do not reliably inactivate parvoviruses. In addition, it is important to note that alcohol-based hand sanitizers are ineffective against parvoviruses. Handwashing using soap and water is necessary for physical removal of the virus. For outside areas, dilution of virus by soaking with water and maximum exposure to direct sunlight (UV inactivates parvoviruses) is important. Accelerated hydrogen peroxide can also be used to spray contaminated areas. Ideally, it is best to wait 6–12 months before exposing an unvaccinated animal to these areas if possible.

Because of the simplicity of these viruses (including lack of viral DNA polymerase), actively dividing cells are necessary for productive viral replication for the majority of parvoviruses, and replication occurs in the nucleus of the host cells, resulting in large intranuclear inclusion bodies in infected cells. The need for dividing cells results in a strict cellular tropism that includes fetal tissues, intestinal crypt cells, and cells in the bone marrow and lymphoid tissues, with subsequent clinical signs such as fetal death, hemorrhagic diarrhea, and leukopenia, respectively.

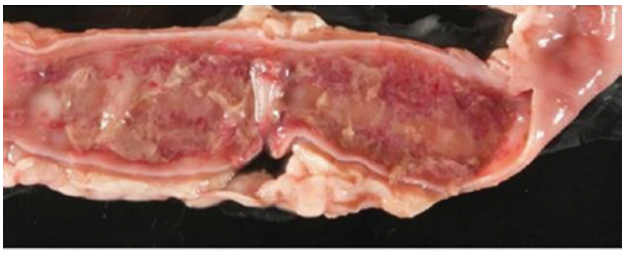
Although the list of parvoviruses continues to expand, the importance of most of these newly discovered viruses is unknown. However, there are several well-known parvovirus pathogens of veterinary importance that will be discussed, as well as some more recent additions worth noting.

Feline Panleukopenia

Disease

Feline panleukopenia, also known as feline distemper and feline infectious enteritis, is a highly contagious, acute viral disease of cats, caused mainly by feline parvovirus. In contrast to CPV enteritis of dogs, hemorrhagic diarrhea occurs in only 3–15% of cases of feline panleukopenia. However, as in dogs, diarrhea is caused by destruction of villus crypt cells and villus atrophy, with subsequent prevention of absorption of nutrients (Figure 47.1). Lethargy, anorexia, and fever are the most notable signs in some cats. As its name suggests, leukopenia is prominent and diagnosed in 65–75% of cases, and is characterized by neutropenia and lymphopenia. Thrombocytopenia, diagnosed in about 55% of cases, is due to megakaryocyte destruction or disseminated intravascular coagulation (DIC). Anemia is usually mild unless associated with severe intestinal blood loss and occurs in up to 50% of cases. Even with treatment, feline panleukopenia has

(a)



(b)

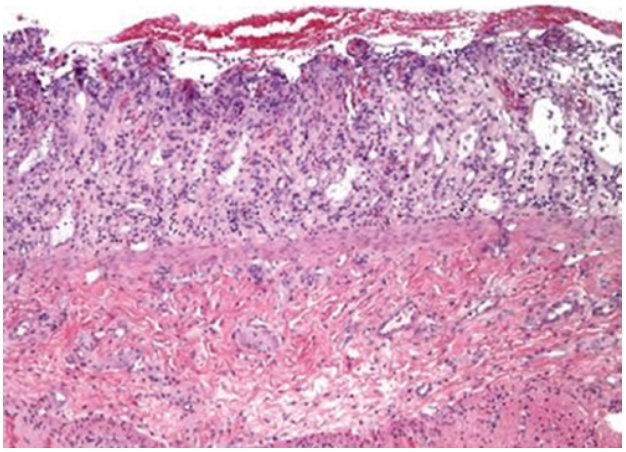


Figure 47.1 (a) Parvoviral necrotizing enteritis in the small intestine of a kitten. The mucosa is reddened and coated with fibrinous exudate, especially over atrophied submucosal Peyer's patches. (b) The primary lesion is destruction of crypt epithelium. The remaining crypts are dilated and lined either by attenuated or by hypertrophied and poorly differentiated enterocytes. *Source:* Photos courtesy of Dr. Margaret A. Miller, Purdue University.

a high mortality rate of 50–80%. Poor prognostic indicators include low leukocyte count (white blood cell count below 1000 cells per ml of blood is associated with a grave prognosis) or low platelet count. Hypoalbuminemia/hypoproteinemia due to a protein-losing enteropathy or hypokalemia due to diarrhea and/or metabolic alkalosis from loss of chlorine (hypochloremia with associated hyponatremia) through vomiting at presentation are also poor prognostic indicators. Secondary bacterial infections often occur as a result of a compromised immune system. The incubation period following infection is short (2–10 days). Pregnant queens may experience intrauterine infection with the feline panleukopenia virus, which leads to neonatal death or congenital abnormalities of the central nervous system manifested by cerebellar ataxia in kittens after birth. Cats of all ages are susceptible, but infection of healthy seronegative mature cats usually results in mild or no apparent disease. Subclinical infection of young adult cats may be common. Determinants of clinical disease include age, immune status, and coinfections with intestinal parasites, viruses, and bacteria.

Etiologic Agent

Feline parvovirus is a member of genus *Protoparvovirus*, species *Carnivore protoparvovirus 1*, which includes closely related parvoviruses CPV, mink enteritis virus (MEV), and raccoon parvovirus (RPV). Feline parvovirus virus (FPV) is a typical parvovirus with regard to virion structure, genomic organization, replication requirements, and physical/chemical properties, as previously described. FPV causes disease in cats but not dogs; however, CPV (particularly variants 2a, 2b, and 2c), which is ancestrally related to FPV (discussed in detail in Section 47.2), is capable of infecting felines. These CPV strains cause a disease indistinguishable from feline panleukopenia in cats, though this is uncommon, accounting for approximately 5% of cases.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

The host range of FPV includes domestic and wild felids (suborder Feliformia) and some wild canids (suborder Caniformia), such as foxes, but not domestic dogs. Infected cats serve as the principal reservoir, and the virus occurs worldwide. The major means of infection is fecal–oral because of shedding of large quantities of virus in the feces from infected cats, but the virus is shed in all excretions, including saliva, urine, and vomitus. Less commonly, the virus can be contracted through the respiratory tract through inhalation of aerosolized virus particles. Physical contact with FPV-contaminated utensils, cages, and bedding results in infection and subsequent transmission that rapidly spreads in contained housing situations. The virus is highly stable in the environment, making it more difficult to control.

Pathogenesis and Pathology

The virus replicates in the oropharyngeal lymphoid tissues and then travels via the bloodstream (producing a transient viremia) to infect additional cells in the body. Feline parvovirus requires actively dividing cells that are in the S phase of the cell cycle, particularly hematopoietic cells located within the bone marrow and lymphoid tissues (thymus, spleen, and lymph nodes), or cells of the intestinal crypts. Infection of hematopoietic and lymphoid cells leads to severe and protracted leukopenia, affecting all white blood cell types and leading to atrophy of lymphoid tissues, whereas infection of crypt cells leads to marked destruction of the intestinal epithelium with resultant malabsorption diarrhea. Severe infections often result in death of the animal due to secondary infections or severe dehydration. Histological lesions of acutely infected animals are characterized by necrosis of the epithelium of the intestinal crypts and marked destruction and depletion of lymphocytes in the lymph nodes, thymus, and spleen. Animals that survive a mild infection undergo regenerative lymphoid hyperplasia followed by recovery.

In utero infection of late-term fetuses results in infection of the brain and eye because of the high degree of proliferative activity in these organs, resulting in kittens born with central nervous system (CNS) or ocular deficits. Late-term fetus infection results most commonly in cerebellar hypoplasia. In addition, the Purkinje cells of the cerebellum continue to replicate in neonates, and FPV infection in neonates less than 10 days of age can also result in cerebellar hypoplasia. These kittens experience tremors of the head and have difficulty walking and maneuvering. Infection in early gestation can result in abortion, mummified fetuses, or stillborn kittens.

Host Responses to Infection

Following infection, hemagglutination-inhibiting and neutralizing antibodies appear in cats approximately one week after infection. High levels of antibodies are generally present after 10–12 days that can persist in cats for several years, but duration of immunity is considered lifelong following infection. Lactogenic immunity based on maternal antibodies protects kittens against viral infection; however, these antibodies also interfere with active immunization by modified live viral vaccines. Cellular immune responses are also generated during infection and likely are important in limiting virus replication during acute infection.

Laboratory Diagnosis

Kittens or young cats presenting with the clinical signs of high fever, anorexia, depression, presence of severe leukopenia or vomiting, diarrhea, and dehydration should be considered for a presumptive diagnosis of feline panleukopenia. Shedding of virus in feces may occur in the absence of clinical signs (subclinical infections), or before clinical signs of disease are detected. In experimental infections, virus shedding occurred in urine for as long as 21 days post infection and in feces for up to 6 weeks, although most cats stopped shedding virus in feces after 3 weeks. *Carnivore protoparvovirus 1* DNA persists for long periods in the tissues of animals that have recovered from infection, and the virus can produce latent infection of peripheral blood mononuclear cells, as demonstrated by successful culture of FPV and CPV from the peripheral blood mononuclear cells of healthy cats with high virus-neutralizing titers. The following methods can confirm the diagnosis:

1) Viral antigen can be detected in feces by enzyme-linked immunosorbent assay (ELISA), including commercially available rapid tests. These commercially available tests are labeled for detection of CPV and detect the three CPV variants (2a, 2b, and 2c) that may cause feline panleukopenia, but these tests can detect feline parvovirus as well. Although considered highly specific, these tests have reduced sensitivity compared to polymerase chain reaction (PCR) assays. False negative results may occur. In addition, vaccination can interfere with these antigen tests, but interference differs by test. False positive results may occur for at least 14 days after vaccination, depending on the test used. The results should always be interpreted in light of vaccination history and clinical signs of the animal.
2) Viral DNA can be detected in feces or tissues by PCR. Real-time PCR assays can provide semi-quantitative results (through use of cycle threshold values – Ct values). PCR reactions are highly sensitive and specific. Vaccine virus shedding will be detected by PCR, so vaccination history is important for diagnosis. Virus from infection may be detected in feces by PCR over six weeks later. Because PCR detects viral DNA and does not distinguish between active and inactive virus, care must be taken in interpreting the results from an infection control standpoint.
3) The virus may be detected in tissues (e.g. lymphoid, small intestine) from dead animals by antigen-capture ELISA, immunofluorescence staining, or immunohistochemistry with an FPV-specific antisera conjugate. In general, these methods are less sensitive than PCR. Infection of small intestine is segmental, so infected segments must be tested to detect the virus.
4) Parvoviruses can be detected in feces by electron microscopy (EM). EM is no longer widely available, but this method will detect all the different strains of parvoviruses that have been associated with feline panleukopenia. Parvoviruses have a characteristic size and shape that allows their detection by EM (Figure 47.2), but routine EM will not differentiate between different parvoviruses.
5) The virus may be isolated in cell culture from filtered feces or tissue samples, using cultured feline kidney cells. This is not routinely done for diagnostics because virus isolation takes several days (up to two weeks for a negative result). However, virus isolation is important for characterization of new parvovirus strains.
6) Serology of FPV, including HI, ELISA, or indirect immunofluorescence (IFA), is generally not recommended for diagnosis because a single titer cannot differentiate between maternally derived antibodies (MDA), vaccine associated titer, and infection. Paired sera and a fourfold rise in titer are required to confirm the diagnosis or seroconversion, if evaluating response to vaccination. Serology is routinely used to screen for vaccine titers. The magnitude of the titer that constitutes a "protective" titer can be test and lab specific, so the results must be interpreted carefully.

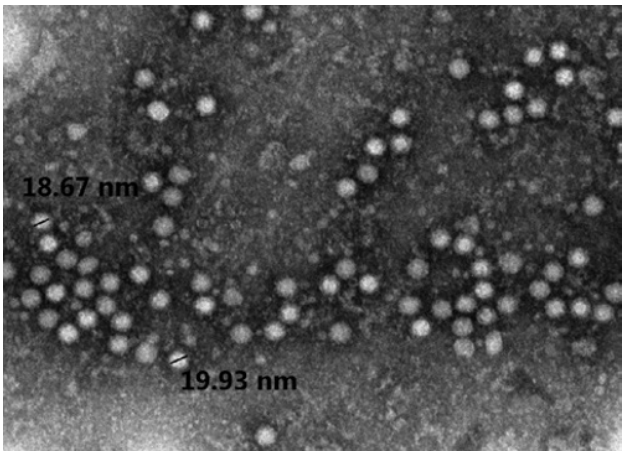


Figure 47.2 Electron micrograph of parvovirus. *Source:* Photo courtesy of Ms. Katrina Miller, Purdue University.

Treatment and Control

Supportive therapy directed to restoration of fluid and electrolyte balance will increase the survival rate of clinically affected animals; however, there is no specific treatment that eliminates FPV from the infected animal. Parenteral

glucose supplementation may be required in addition to intravenous crystalloid therapy. Hypoalbuminemic cats (albumin <20 g/l) require plasma, synthetic colloids (e.g. hetastarch), or typed whole-blood transfusion if there is concurrent anemia. Plasma and heparin therapy are appropriate for treatment of DIC. Antimicrobial therapy is essential, because overwhelming sepsis associated with translocation of gastrointestinal (GI) tract bacteria and profound immunosuppression are a leading cause of death. Antimicrobials with efficacy against gram-negative, gram-positive, and anaerobic bacteria should be chosen and given intravenously.

Isolation of infected animals and barrier precautions are necessary to prevent spread of this highly contagious virus. In addition to appropriate cleaning of contaminated environments and care to prevent fomite transmission, vaccination is paramount to prevention and is key to controlling this disease. Numerous efficacious inactivated and modified live FPV vaccines are commercially available. Vaccination of pregnant queens and kittens <4 weeks of age with a modified live virus vaccine should be avoided because of the theoretical concern of causing cerebellar hypoplasia. The FPV genome has remained relatively stable since its discovery as a virus in 1928. However, current wild type strains have been shown to have lower cross-reactivity with vaccine-induced neutralizing antibodies. It is unlikely that there will be complete lack of cross-protection provided by vaccination, but strain differences could potentially have some effect on the protection provided by maternal antibodies and the time at which kittens are susceptible to infection. Maternal antibodies can also interfere with the immunization of young kittens, and this needs to be considered as part of the vaccination regime. The passive antibody typically wanes around 4–12 weeks of age, and multiple vaccinations, beginning at 6 weeks of age and given 3–4 weeks apart, are required to try to prevent infection. An immunity gap, during which the maternal antibody level is too low to be protective but high enough that it inhibits sufficient response to a modified live vaccine, is when vaccinated kittens may become infected. Thus, vaccinations should continue until kittens reach at least 16–20 weeks of age because of this interference, followed by an additional booster at 1 year of age (or 6 months of age depending on at what age the last booster was administered in the kitten series). Then vaccinations should be given every three years thereafter, based on the current recommendations. FPV vaccines do provide cross-protection to CPV, although the neutralizing activity of the antibodies from these FPV vaccines is reduced against the CPV variants. The clinical significance of this is unknown.

Canine Parvovirus

Disease

Parvoviral disease in dogs is a relatively new disease of canines that evolved from FPV, or more likely, a closely related virus. It is somewhat similar to FPV disease in felines, but hemorrhagic diarrhea is a more prominent sign in dogs with CPV. CPV is not always fatal, but when it does kill, death is usually the result of dehydration and shock, along with the effects of septic toxins produced by the intestinal bacteria and/or the intestinal bacteria that have translocated into circulation. Young dogs between six weeks and six months old, unvaccinated, or incompletely vaccinated dogs are most at risk for infection. German Shepherds, Rottweilers, Doberman Pinschers, English Springer Spaniels, and American Staffordshire Terriers also have an increased risk of contracting parvovirus, although it is not clear why these dog breeds are at a higher risk than others. Neonates lacking maternal antibody protection sometimes develop myocarditis without clinical signs of enteritis; cardiac susceptibility to CPV is likely related to an initial high frequency of cardiomyocyte DNA synthesis in newborn animals. Cases of myocarditis, which had been declining as a result widespread use of highly effective vaccines, are now on the rise, potentially due in part to an anti-vaccination movement.

Etiologic Agent

CPV is a member of the genus *Protoparvovirus*, species *Carnivore protoparvovirus 1*. It was originally designated CPV2 to differentiate it from the genetically unrelated virus CPV1, which is the minute virus of canines that has been associated with neonatal mortality and is now designated *Carnivore bocaparvovirus 1* in the genus *Bocaparvovirus*. CPV disease was first described in North America in 1978, but retrospective serological studies indicate that the virus was rapidly spreading around the world in the 1970s and it caused a global pandemic. The original virus strain (CPV2) differs by only 6 amino acid residues in the VP2 gene region compared to FPV. This provided the original virus the ability to bind to canine cells but not to feline cells. The source of CPV has been suggested to be an FPV-like virus. Frequent cross-transmission of FPV-like and CPV-like viruses between wild carnivore species suggests that FPV and CPV may have evolved independently from an ancestral sylvatic parvovirus. To date, the exact evolution and origin of CPV2 remain elusive. The original CPV2 strain infects dogs of all breeds and other members of the family *Canidae*, such as wolves, foxes, and coyotes. CPV2 was replaced in 1979–1980 by CPV2a, which has different antigenicity and receptor

binding than its parent. CPV2a has an extended host range and is capable of infecting a number of carnivores including felines. Recent cross-species transmission data suggest that RPVs and subsequent canine–raccoon passages may have served as intermediates to the evolution of the CPV2a lineage and their extended host range. Two additional antigenic variants (CPV2b and CPV2c) have subsequently evolved, and these three variants (2a, 2b, and 2c) co-circulate in varying proportions in different geographic regions around the world. All three antigenic variants have retained the feline host range. To date, whether there is a greater infection or virulence by one variant over the others is not well established, neither in experimental systems nor by field observations. It is likely that all three variants have similar pathogenic potential, which can increase depending on particular animal conditions (age, immunologic status, stressing factors, etc.).

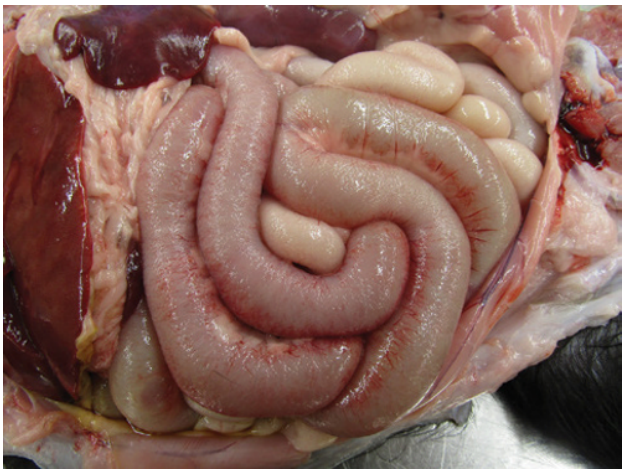


Figure 47.3 Opened abdominal cavity of an 8-week-old Labrador Retriever puppy with CPV. The small intestine is turgid, and the serosa has a "ground glass" appearance with scattered petechiae. *Source:* Photo courtesy of Dr. Grant N. Burcham, Purdue University.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

CPV infection of dogs, other members of the family *Canidae*, as well as most carnivores, is prevalent throughout the world. Parvovirus-infected dogs excrete infectious virus in their feces, and the virus is readily transmitted between dogs by the fecal–oral route. Fomite transmission is a significant source of infection.

Pathogenesis and Pathology

The pathogenesis of CPV infection of dogs is similar to that of panleukopenia virus infection of cats, although cerebellar hypoplasia and atrophy are not recognized as a consequence of *in utero* infection of dogs. Myocarditis is a potential consequence of parvovirus infection of young pups, whereas it is not described in panleukopenia virus-infected kittens.

Ingestion or inhalation of secretions from animals infected with the virus is the most common means of transmission. The virus replicates in pharyngeal lymphoid tissues and then travels via the bloodstream to other areas of the body. Virus replication requires infection of rapidly dividing cells in the intestinal epithelium and lymphoid tissues, including thymus, tonsils, retropharyngeal, and mesenteric lymph nodes, and spleen. Fecal excretion of virus begins as early as the third day post exposure and peaks soon thereafter. Most dogs stop excreting the virus by the twelfth day post infection.

Hemorrhage is the most striking lesion of parvovirus enteritis in dogs, occurring in the lumen of the small bowel, with accompanying enlargement and edema of the mesenteric lymph nodes. The enlarged mesenteric lymph nodes can be felt as an abdominal mass on palpation, along with thickened, painful intestinal loops due to edema (Figure 47.3). Gross myocarditis lesions that appear as mottled white streaks within the myocardium are indicative of cardiac involvement in young puppies.

Histological lesions resulting from CPV infection are confined to organs with large populations of rapidly proliferating cells, such as the small intestine, lymph nodes, and bone marrow. Necrosis of crypt epithelium and atrophy and fusion of intestinal villi are the most frequent findings in the small intestine, with lesions most severe in the ileum and jejunum. Due to damage of the intestinal epithelium, and the likelihood of neutropenia, there is a high risk of bacterial translocation, secondary bacterial infections, and possible septicemia. Regeneration of the intestinal epithelium occurs in dogs surviving the acute phase of enteric infection. CPV infects lymphocytes in the thymic cortex and germinal centers of lymph nodes, which results in lymphocytolysis and cellular depletion in lymphoid tissue. The myocardial form of CPV infection of the ventricular myocardium results in myofiber degeneration and necrosis that is accompanied by infiltration of mononuclear cells.

Host Response to Infection

The immune response of canines following CPV infection is similar to that of felines. HAI and neutralizing antibodies appear in dogs approximately one week after infection. High levels of antibodies are generally present after 10–12 days, and these antibodies can persist for the life of the animal. Lactogenic immunity based on maternal antibodies protects a puppy against viral infection; however, these antibodies

also interfere with active immunization by modified live or inactivated CPV viral vaccines. Cellular immune responses are also generated during infection, and are likely important in limiting virus replication during acute infection.

Laboratory Diagnosis

Laboratory diagnosis of CPV disease is very similar to feline parvovirus disease. Puppies or young dogs presenting with the clinical signs of high fever, anorexia, depression, vomiting, diarrhea, and dehydration, or the presence of severe leukopenia should be considered for a presumptive diagnosis of CPV disease. The diagnosis can be confirmed by one of the following laboratory methods:

1) The viral antigen may be detected in feces from live animals by antigen-capture ELISA. Commercially available immunochromatographic tests demonstrate very high specificity (up to 100%). These tests are not as sensitive as molecular methods, due to the low amounts of virus shed in the feces during the very early or late stages of infection and/or the early presence of high CPV antibody titers in the gut lumen that may sequestrate most viral particles. False negatives do occur. False positives are also possible with some of these tests, due to detection of vaccine virus shedding in the feces for approximately two weeks after vaccination with a modified live virus vaccine.
2) PCR amplification of viral nucleic acid from feces is a sensitive and specific method for detection. It is important to note that vaccine virus shedding will be detected by PCR. Real-time PCR may be useful for distinguishing vaccine shedding from infection, based on Ct value (vaccine virus shedding is at a lower amount and for a shorter time period with respect to field strains). Virus has been detected in feces of infected dogs by PCR for months post infection, but it is important to note this is detection of virus DNA by PCR and not necessarily detection of infectious virus particles. Despite this shortcoming of PCR, it has been used to provide a general idea of environmental contamination, but the results must be interpreted carefully. EM, though not widely available, can also be used detect the virus in feces.
3) The virus may be detected in tissues (e.g. small intestine, lymphoid tissues, myocardium) from dead animals by antigen-capture ELISA, immunofluorescence staining or immunohistochemistry with a CPV-specific antisera conjugate, or PCR.
4) The virus may be isolated in cell culture from filtered feces or tissue samples using primary cell cultures of canine or feline fetal lung and kidney, as well as continuous cell lines such as canine cell line A72 and feline cell line CRFK.
5) Serodiagnosis following disease or vaccine antibody level monitoring may be accomplished by HAI or SN (less common) testing. Other antibody tests that are useful to monitor exposure include ELISA or indirect immunofluorescence. Paired sera and a fourfold rise in titer are required to confirm the diagnosis or seroconversion following vaccination.

Treatment and Control

Severe cases of canine parvoviral disease are characterized by marked dehydration and metabolic acidosis. Supportive therapy directed to restoration of fluid and electrolyte balance and to the prevention of secondary bacterial infections will increase the survival rate of clinically affected animals; however, there is no specific treatment that eliminates CPV from the infected animal. While oseltamivir, a neuraminidase (NA) inhibitor, has been used for the treatment of CPV, the drug does not directly inhibit the virus because the virus lacks an NA. Supportive therapy includes the use of intravenous crystalloid therapy (and colloids if hypoalbuminemic: protein-losing enteropathy), broad-spectrum antibiotics, injectable anthelminthics (intestinal parasitism is a common comorbidity) and antiemetics, and gastroprotectants in vomiting animals. Implementing early nutrition may decrease the length of hospitalization. Early nutrition, via a nasogastric or esophagostomy tube, is correlated with earlier clinical improvement and significant weight gain over delaying offering food until vomiting has ceased for 12 hours. Early enteral nutrition may also improve gut barrier function, limiting the translocation of bacteria or endotoxins.

Prevention of infection is the key to controlling this disease. Vaccination, isolation of dogs that survive infection, and meticulous decontamination of premises that have housed infected animals are critical for containment of this highly transmissible virus. Household bleach at a 1:20 dilution or commercial products labeled for use against parvovirus provide effective virucidal activity against CPV and should be used extensively.

Numerous efficacious inactivated and modified live CPV vaccines are commercially available. Puppies should receive a dose of CPV vaccine beginning at six weeks (no younger than four weeks of age) and given every two to four weeks. The last puppy vaccination should be given between 14 and 16 weeks of age or older (20 weeks in situations with high incidence), regardless of how many doses were received earlier, to develop adequate protection. Dogs should receive a booster within 1 year of the primary vaccination course given as a pup or at any time point between 26 and 52 weeks of age, with subsequent revaccinations given at 3-year intervals.

There has been significant debate regarding the effectiveness of current vaccines, which contain either the original CPV2 strain or CPV2b, in protecting against the newer CPV variants (particularly CPV2c). Nevertheless, even considering all case reports of vaccination failures to date, there is no definitive evidence for the complete absence of cross-protection from clinical disease between old and variant CPVs. Thus, the primary cause of widespread virus circulation despite extensive vaccination is still represented by interference of MDA in vaccinated pups. Interfering levels of MDA are transmitted by bitches to their offspring through colostrum and, to a lesser extent, milk. The passive antibody typically wanes around 4–12 weeks of age, and multiple vaccinations are required in order to immunize puppies immediately prior to the "susceptibility window" and ensure uniform protection. In addition, in spite of proper vaccination, a small percentage of dogs do not develop protective immunity and remain susceptible to infection. Other causes of vaccine-related failures include vaccine storage or administration errors or other less likely explanations (Figure 47.4).

Until a puppy has received its complete series of vaccinations, pet owners should use caution when bringing their pet to places where young puppies congregate, such as doggy daycare, parks, pet stores, and puppy classes. Even if no direct dog-to-dog contact occurs, fomite transmission is possible.

Porcine Parvovirus

Disease

PPV is ubiquitous among swine throughout the world and is enzootic in most herds in the United States. The virus is considered to be one of the major causes of reproductive failure in swine worldwide. Infection of pregnant dams with PPV causes reproductive failure or stillbirth, mummification, embryonic death, and infertility (SMEDI) syndrome. Disease occurs most commonly in gilts that are not properly vaccinated prior to integration into the herd for service. Clinically this is seen as large numbers of mummified fetuses, an increase in the number of returns to estrus, small litters, failures to farrow, decreased farrowing rate, and rarely abortion. Infection of nonpregnant swine or male swine with PPV may result in a subclinical moderate and transient lymphopenia, independent of sex and age, between 5 and 10 days after the initial infection. Regardless of the presence or absence of a lymphopenia, the infection results in development of a rapid and lifelong immune response to the virus with no apparent clinical disease in these animals. Reproductive losses are typically low in vaccinated herds, but PPV can cause devastating abortion storms in unvaccinated herds, in situations in which the vaccine was administered incorrectly, or with the emergence of new antigenic types not well controlled with current vaccines. PPV does not cause diarrhea in swine.

Etiologic Agent

The virus is a member of the Parvovirinae subfamily and *Ungulate protoparvovirus 1* species. PPV is a typical protoparvovirus and has a similar appearance and genome organization to FPV and CPV. Though several genotypes have been described, only one serotype has been identified, and all isolates of PPV that have been compared have been found to be antigenically similar to each other. A field strain described in Germany (PPV-27a) and similarly related strains are the most prevalent wild type strains in several countries. These 27a-like isolates are genetically divergent from vaccine strains and considered to be more virulent than older strains. It has also been shown that vaccination with current vaccines does not prevent infection with these variants but does protect from clinical disease.

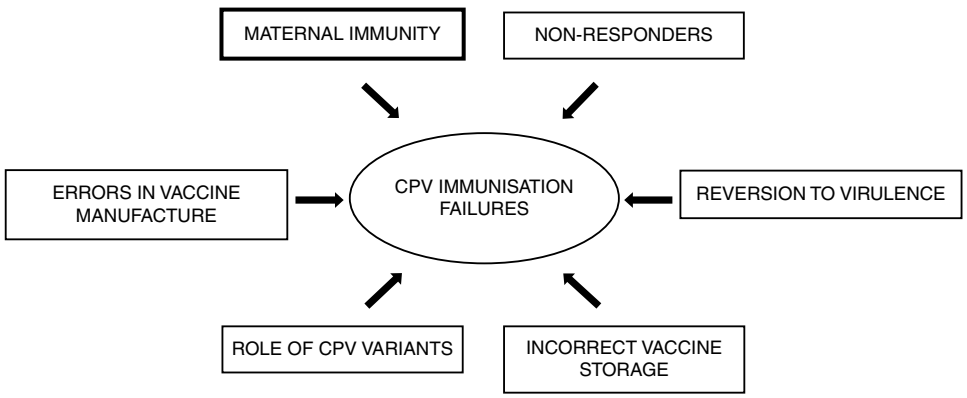


Figure 47.4 Schematic representation of causes of immunization failures after canine parvovirus vaccination. The thickness of the border of each box is proportional to the impact of the relative factor. *Source:* Reproduced from Decaro and Buonavoglia (2020).

Host–Virus Relationship

Distribution, Reservoir, and Transmission

PPV appears to be limited to swine, is ubiquitous in swine herds throughout the world, and serosurveillance data indicate that infection is very common. PPV is maintained in the environment due to shedding of the virus in oral secretions and feces following infection. The virus can also be spread through placental fluids along with infected fetuses. Exposing pigs to other pigs or boots, clothing, or equipment that have been around other pigs greatly increases the risk of disease transmission. There is evidence that *in utero* infection and immunotolerance may occur and persistently infected animals can shed the virus intermittently or continuously for prolonged periods of time, but the significance of this phenomenon is unknown. Venereal transmission through infected semen is also a mechanism of PPV transmission. Boars may also mechanically transmit the virus.

Transplacental infection of embryos and fetuses is the result of dams failing to develop active immunity prior to pregnancy. Surprisingly, surveys show that many herds with endemic infection contain some animals that are susceptible. Passive immunity persists in some gilts beyond the time they are old enough to be bred (gilts with high passive antibody titers are at risk). This interferes with their development of an active immune response to natural or vaccine exposure (active immunity usually develops following exposure of gilts to parvovirus in the environment as passive immunity wanes). Gilts with little or no immunity can be infected during their first pregnancy. If infected during pregnancy, the virus may cross the placenta and infect some or all of their embryos or fetuses. However, if this happens, once a sow has a "parvo litter," she would then have what is considered to be lifelong immunity, and the problem is unlikely to recur.

Pathogenesis and Pathology

PPV infection of nonpregnant, seronegative swine results in no apparent clinical disease and is accompanied by a vigorous antibody response. Even experimental PPV inoculation in boars, gilts, and sows does not produce macroscopic lesions. Gross lesions of PPV-infected pregnant dams have not been reported.

The primary replication of PPV occurs in lymphoid tissues, and the virus is distributed systemically via a viremia. It is not well understood how the PPV crosses the placenta and reaches the fetus, because six tissue layers completely separate the sow and the fetal blood circulation. As the virus is able to remain infectious after phagocytosis by macrophages, it likely can cross the porcine epitheliochorial placenta using macrophages to infect the fetus. The high mitotic rate present in the cells of the tissues of the fetus provides the perfect environment for virus replication. Pathological sequela caused by PPV are related mainly to the gestational period in which infection occurs. PPV infection occurring early in gestation (<30 days) is not always apparent as it often results in embryonic death and fetal resorption, which may be misinterpreted as generalized infertility and return to service. However, if the fetus becomes infected between approximately 30 and 70 days of gestation, death and mummification (resulting from dehydration of tissues) generally occur. If the fetus becomes infected after the 70th day of gestation, an immune response is generated and piglets are born with anti-PPV antibodies. These late-gestation fetal infections may be subclinical or may result in offspring that fail to thrive (Figure 47.5).

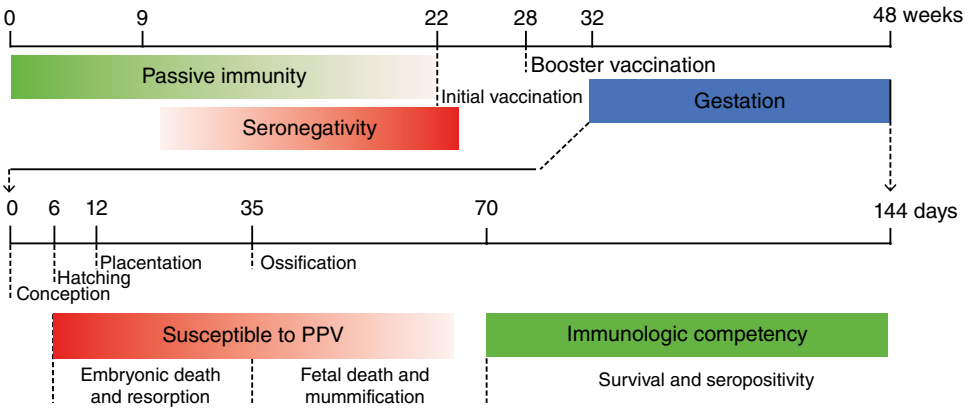


Figure 47.5 Major time points of porcine parvovirus (PPV) infection and immune response. The consequences of the intrauterine infection depend on the time of the infection. The embryos are susceptible to the infection (red line) until the development of immune competence around day 70 day of gestation (green line). Maternally derived antibodies protect the piglets passively (green line) until 9–22 weeks of age. The animals should be inoculated first after the depletion of maternal antibodies, and long-term immunity is maintained by booster vaccination. *Source*: Reproduced from Mészáros et al. (2017).

Microscopic lesions following experimental infection of sows include extensive cuffing of myometrial and endometrial vessels with mononuclear cells and focal accumulation of lymphocytes in the uterus. Microscopic changes in the fetus are often nonspecific, but can include foci of necrosis and mononuclear cell infiltration in organs such as the liver, heart, kidney, and cerebrum. In the fetus, histopathological changes tend to be widespread in the tissues, and the major lesions represent necrosis of cells in developing organ systems. Hemorrhages are present in subcutaneous tissues and muscle masses. Necrosis and mineralization are common in the lungs, kidneys, liver, skeletal muscle, and the heart.

Host Responses to Infection

No clinical disease is observed when PPV infection of seronegative, nonpregnant swine occurs. Following infection, the animals become viremic and mount a vigorous antibody response that provides lifelong immunity in most cases. Immune dams provide high levels of lactogenic immunity to their offspring through colostrum. Young pigs remain resistant to infection with PPV until the maternal antibody wanes, usually around three to six months of age. Reproductive disease may occur if seronegative gilts become infected while pregnant.

Laboratory Diagnosis

PPV should be considered in the differential diagnosis of reproductive failure when there is evidence of embryonic or fetal deaths. A hallmark of PPV is litters with dead fetuses of varying sizes, including mummified fetuses, along with stillborn and healthy pigs born to first-parity gilts. Abortions, unless the sow aborts a whole litter of mummified fetuses, is not likely with PPV infection. If PPV is suspected, the following assays can be used to confirm the diagnosis:

1) ELISA is the most frequently applied test to detect PPV-specific antibodies and is generally used for diagnosis if fetal tissues are unavailable. A recombinant NS1 protein-based DIVA test (Differentiating Infected from Vaccinated Animals) was developed to distinguish vaccinated (inactivated vaccine) from infected pigs. The HAI or SN assays can also be used to quantify antibodies. A positive titer only means previous exposure. Acute and convalescent samples from the dam, with a fourfold increase in titer, is needed to confirm acute infection. Otherwise, comparing titers from unmated gilts to those exhibiting signs of infertility may also be useful for diagnosis. Detection of precolostral antibodies in stillborn pigs is also diagnostic. However, serology is more commonly used to track vaccine antibody levels in adult swine.
2) Detection of virus DNA by PCR in fetal tissues confirms the diagnosis. PCR can also be used to detect PPV in semen.
3) Detection of viral antigen in fetal tissues by immunofluorescence or immunohistochemistry are also options but tend to be less sensitive and specific than PCR.
4) Virus isolation is possible but not always successful, depending on the condition of the fetal tissue when obtained. The virus can be propagated in a variety of cell lines such as ESK (embryonic swine kidney), PK-15 (pig kidney), SK6 (swine kidney), STE (swine testicular epithelioid) and SPEV (swine embryo kidney).

Treatment and Control

There is no treatment for reproductive failure caused by PPV infection. Losses from PPV now are well controlled, largely by vaccination, and the clinical effects of PPV are now less prevalent. Vaccination is the preferred method of controlling PPV infection in the breeding herd. The currently available vaccines against PPV are based on inactivated whole-virus preparations of PPV NADL-2 (cluster A) and similar strains, and were isolated approximately 40 years ago. These vaccines are effective against homologous infections, but do not prevent infection and virus shedding after challenge with the antigenically heterologous 27a (cluster D) strain. However, they can protect the fetus against disease. Likely the best way to prevent PPV infection is to vaccinate all susceptible breeding stock twice, two weeks apart, several weeks before breeding using killed vaccines. The first vaccine is generally given to gilts at the age of 170–180 days (between weeks 22 and 28) or 30 days before insemination, with the second dose administered 15 days later (Figure 47.4). This should stimulate an active immunity in the dams that will protect the developing fetuses. Vaccination must be carefully timed to ensure that animals are immunized after passive antibodies wane but before breeding occurs. Revaccination of sows 10–15 days after each farrowing is also recommended. Vaccination of boars annually or every six months to prevent infection of sows has been suggested, but this is off-label use of the vaccines (not labeled for use in boars).

Additional Porcine Parvoviruses

PPV are spread throughout multiple genera associated with the subfamily Parvovirinae (Table 47.1), with many of these recently identified, such as the bocaparvoviruses.

Table 47.1 Parvoviruses identified from pigs.

Virus	ICTV current classification
Porcine parvovirus 1	*Ungulate protoparvovirus 1*
Porcine parvovirus 2	*Ungulate tetraparvovirus 3*
Porcine parvovirus 3 (porcine hokovirus)	*Ungulate tetraparvovirus 2*
Porcine parvovirus 4	*Ungulate copiparvovirus 2*
Porcine parvovirus 5	*Not classified*
Porcine parvovirus 6	*Ungulate copiparvovirus 4*
Porcine bocavirus 1	*Ungulate bocaparvovirus 2*
Porcine bocavirus SX	*Ungulate bocaparvovirus 3*
Porcine bocavirus H18	*Ungulate bocaparvovirus 4*
Porcine bocavirus 3	*Ungulate bocaparvovirus 5*
Porcine bufavirus	*Ungulate protoparvovirus 2*

Formerly used nomenclature and the current classification based on the International Committee on Taxonomy of Viruses (ICTV).

Most of the bocaparvoviruses were identified in fecal and/or respiratory samples by virus discovery approaches, although some, less commonly, have also been detected in serum and tissues. Epidemiological studies indicate that many bocaparvoviruses are widespread in their respective host populations, but are rarely pathogenic. As with the bocaparvoviruses in pigs, the significance of these other parvoviruses in pigs is unknown.

Aleutian Disease in Mink

Disease

Unlike MEV, which causes severe enteritis in mink and is a member of *Carnivore protoparvovirus 1* with CPV and FPV, Aleutian disease virus (ADV) is an unrelated parvovirus that causes immune complex disease, mink plasmacytosis. Aleutian disease is a protracted disease characterized by gradual weight loss, poor reproduction, oral and gastrointestinal bleeding, renal failure and uremia, and high mortality. It is of economic importance in mink farming, though it infects wild populations as well. Costs to farmers and the industry include the loss of breeding animals and kits, losses due to infertility, reduced litter sizes and abortions, low-quality fur, restrictions on international trade, and costs related to control measures. ADV virulence differs markedly between virus strains and with the genetics of the host animal, particularly affecting inbred mink with the desirable homozygous recessive Aleutian (gray) coat color.

Etiologic Agent

ADV is the sole member of species *Carnivore amdoparvovirus 1*, which is one of five currently recognized species in the *Amdoparvovirus* genus. Additional viruses in this genus infect small carnivores including foxes, skunk, raccoon dogs, and red pandas. ADV strains can be categorized roughly into four classes based on their pathogenicity to mink: non, low, moderate, and high. No official strain-differentiation criterion currently exists for ADV, and all isolates are antigenically similar. All strains infect both Aleutian and non-Aleutian mink; some strains cause disease in only Aleutian mink, whereas others induce severe symptoms and lesions in mink of all genotypes. ADV can infect other mustelids such as weasels, ferrets, badgers, or otters, and highly pathogenic ADV strains are sometimes pathogenic in these species (particularly ferrets and striped skunk) but are more often asymptomatic and non-persistent. Skunk amdoparvovirus (*Carnivore amdoparvovirus 4*) also sometimes infects mink.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Because of animal trading within the fur industry, the virus, which originated in North America, is currently distributed worldwide. The infection is widespread in the wild as well as in farmed animals. Transmission occurs *in utero* and by direct or indirect contact with infected mink. Infected animals shed the virus in saliva, feces, and urine, and the virus is transmitted by fecal–oral or possibly respiratory routes. Indirect contact with fomites including contaminated feed, water, air, pens, equipment, and vehicles is an important source of transmission. Infection may also be transmitted by the iatrogenic route (e.g. toenail clippers), biting, and possibly insects. Intensive mink farming practices facilitate the occurrence of coinfections with different parvovirus strains (farms and even individual mink may be infected with multiple strains), favoring viral recombination, which produces variants that can spread through the surrounding wild populations. ADV in pet ferrets is receiving attention because asymptomatic animals are carriers of the virus and are capable of transmitting it at ferret shows and other gathering places.

Pathogenesis and Pathology

In newborn seronegative mink kits, ADV causes acute interstitial pneumonia, killing type II alveolar cells, which leads to a fatal respiratory distress syndrome. Kits that survive the infection develop classical Aleutian disease. Kits

with antibodies received from a seropositive dam do not develop pneumonia; instead, ADV infection in these kits results in a prolonged and non-progressive Aleutian disease with mild lesions because the antibodies, though unable to neutralize ADV, restrict viral replication in the lungs.

The clinical signs and lesions of Aleutian disease are actually a result of the host immune response to the virus, rather than being caused by virus itself. ADV replicates rapidly in experimentally infected mink, and virus titers are observed 10 days post inoculation. However, the disease process is slowly progressive and can take up to a year to manifest and produce noticeable symptoms. Most animals become persistently infected and harbor the virus in the viscera, blood, and urine for the remainder of their lives. In adult mink, the primary sites for replication are B-lymphocytes, macrophages, and dendritic cells in lymphoid organs. The entry of ADV into target cells is likely to occur via antibody-dependent enhancement (ADE), where virus-antibody complexes bind to cellular Fc-receptors that facilitate viral entry into the cell and subsequent delivery to tissues. Infected mink do not exhibit symptoms until several weeks or months after they are infected, at which time loss of appetite, decreased activity, weight loss, tarry diarrhea, and a rough coat are observed. In all cases, the animals are immunocompromised and subject to secondary infections. Once the symptoms become obvious, death of the mink is certain.

Gross lesions include splenomegaly, glomerulonephritis, and enlarged mesenteric lymph nodes. Histological lesions are characterized by plasma cell infiltration in the kidneys, liver, spleen, lymph nodes, and bone marrow. Arteritis and glomerulonephritis that are classic signs of the disease are caused by deposition of immune complexes and the subsequent inflammatory response (type III hypersensitivity reaction).

Infection in pet ferrets occasionally results in clinical presentation very similar to adult mink infection, with a variety of nonspecific clinical signs such as progressive weight loss, weakness, and ataxia. Ferrets may show nonapparent clinical signs until shortly before death. A severe hypergammaglobulinemia is the most consistent finding on blood work. A presumptive diagnosis is based on clinical signs and hyperglobulinemia. Ferrets may have no lesions upon necropsy, or infrequently they may have hepatomegaly, splenomegaly, and lymphadenopathy. Membranous glomerulonephritis has also been described. The most consistent histological finding is periportal lymphocytic infiltrates. Bile duct hyperplasia and periportal fibrosis have also been reported. Most ferrets that become infected remain asymptomatic and clear the virus over time.

Though not generally considered zoonotic, there has been a recent report of possible human disease associated with ADV. In two separate incidents, mink ranchers with vascular disease and microangiopathy were found to have ADV antibodies and viral nucleic acid.

Host Responses to Infection

Viremia develops five days to four weeks post infection and appears to persist in Aleutian mink infected with highly pathogenic strains, whereas mink (especially non-Aleutian) infected with low-pathogenicity strains may develop only transient or intermittent viremia. However, the onset of infection and viremia, and the level of virus replication vary greatly among individuals. Antibody response following infection reaches very high levels, and although abundant, these antibodies are unable to neutralize the virus. The antibodies bind with circulating virus to form infectious immune complexes. Following an antibody response, the viremia seems to decrease. Aleutian mink tend to have higher gamma globulin and IgG levels, and the antibody response in this genotype invariably leads to fatal disease, unlike in non-Aleutian mink. However, in non-Aleutian mink (and in other susceptible species), especially when infected with strains of low pathogenicity, the hypergammaglobulinemia may be transient and the level of ADV-specific antibodies is lower and decreasing. Regardless, antibodies persist for several years and possibly for life.

Laboratory Diagnosis

The antibody response following infection can be detected by many serological tests, including IFA and counterimmunoelectrophoresis (CIE). Both assays are useful for identifying infected carrier animals that may be shedding virus and need to be culled or quarantined from naïve mink. Immunofluorescence, immunohistochemistry, and PCR assays may be used to detect the virus in body secretions and tissues (e.g. spleen). Only a few strains have been adapted to replicate in cell culture, specifically feline renal epithelial cells (CRFK). As the cultivation of field strains in cell culture is often unsuccessful, virus isolation is not typically attempted.

Treatment and Control

There is no effective treatment. Immunization of mink carrying the Aleutian gene with inactivated virus vaccine increases the severity of the disease (ADE). Thus, there is no available vaccine, and affected farms have to implement laborious eradication programs to establish an ADV-free status. Because of the environmental stability of the virus,

eradication is difficult. Pens and equipment should be steam cleaned and cleaned with a 5% bleach solution or other appropriate disinfectant that is parvocidal. Maintaining a disease-free status can be challenging because reintroduction of the virus from the outside, via commercial routes or from the wild, where the virus cannot be eradicated, can quickly reestablish epidemics. For both mink and ferrets, biosecurity is the only effective control mechanism for maintaining seronegative animals. Secure physical separation between domestic and wild mink populations should be in place to minimize transmission. Aleutian disease is controlled through a test and slaughter program. Mink to be kept for breeding stock should be tested in late fall (before selection of breeding stock and pelting) and in January or February (before breeding). All animals entering an established seronegative mink ranch need to be screened for antibody prior to entry. If seropositive animals are found on a mink ranch, they should physically separated and maintained apart from seronegative animals to prevent horizontal transmission and then culled at harvest. Although immunosuppressive therapy with prednisone or cyclophosphamide can be used to treat Aleutian disease, these drugs have not been used in mink farming due to their high cost. Thus, medical treatment and supportive care is usually applied only to important breeding animals and pets such as ferrets. Despite treatment, the prognosis of Aleutian disease is poor.

Rodent Parvoviruses

Disease

Rodent parvoviruses are common in both laboratory and wild rodents, and most commonly cause subclinical infection, but *in utero* infections of rats, mice, and hamsters at critical stages of gestation with the appropriate strain of parvovirus can result in cerebellar hypoplasia, as seen with feline panleukopenia. Neonatal infection with some of the minute virus of mice (MVM) strains can induce runting, sudden or premature death, and disruption of hematopoiesis. Of the rat parvoviruses, the main virus reported to cause clinical disease is Kilham's rat virus (RV), which causes cerebellar injury, hemorrhagic encephalopathy, and hepatitis in young rats and outbreaks of peri-testicular and intra-abdominal hemorrhage in older rats. To date, none of the mouse parvovirus (MPV) infections cause clinical signs, even in immunodeficient mice, but can cause persistent lymphocytotrophic infection and immune dysfunction. The main importance of these viruses from a veterinary medicine standpoint is their confounding effect on research. Research associated with immunology, transplantation, hematopoiesis, and oncology can be disrupted through the virus's effects on the host immune response, including aberrant T-cell proliferation responses and function and acceleration of T-cell-mediated rejection of tumors, skin allografts, and syngenetic skin grafts.

Etiologic Agent

Parvoviruses of veterinary importance in rodents are classified in the genus *Protoparvovirus*, species *Rodent protoparvovirus 1*, which includes viruses of both mice and rats such as MVM, mouse parvoviruses 1–3 (MPV), Kilham's RV, and H-1 virus in rats. Two additional rodent parvoviruses in this genus include *Rodent protoparvovirus 2* (rat parvovirus 1) and *Rodent protoparvovirus 3* (rat bufavirus SY-2015).

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Rodents shed virus in urine, feces, and oronasal secretions, with transmission through urine or feces being the most common mode of transmission. Animals born from infected dams are protected by MDA for the first few weeks of life but are then infected horizontally. The ability of the parvovirus to persist in the environment means that exposure to fomites such as contaminated feed, bedding, and materials are an important means of transmission. Exposure to wild rodents or contaminated laboratory rodents can be the source of an outbreak. Contaminated murine biological products may also cause parvoviral infections.

Pathogenesis and Pathology

Rodent parvoviruses destroy diving cells, but with a more limited spectrum compared to other parvoviruses. These viruses have a primary tropism for hematopoietic and lymphoid tissues, and infections are usually subclinical. The host response varies with age, genetic background, and immune status, particularly for the MVM virus strains. Clinical disease from RV occurs when the virus is introduced to a naïve population. In newly infected breeding colonies, RV may cause decreased fertility, fetal resorption, small litters, and runting of pups. RV infection in juvenile and young adult male rats may result in lethal disease from hemorrhage and necrosis of brain and gonads. These lesions are a result of viral destruction of vascular endothelial cells and megakaryocytes. Lesions associated with RV infection include cerebellar hypoplasia, hemorrhagic infarction, thrombosis of multiple organ systems, focal necrosis, hypertrophy, and vacuolar degeneration of hepatocytes. No pathology has been associated with other rodent parvovirus infections.

Host Responses to Infection

Rodent parvoviruses, particularly MPV and MVM, can cause persistent infection, even in the presence of high titers of neutralizing antibodies. Therefore, some experimental manipulations, such as those that cause immunosuppression, can cause virus reactivation and shedding, which can lead to additional virus-caused immunosuppression that will affect experimental results due to modification of the biological response. Notably, humoral immunity to MVM does not protect against MPV infection, and vice versa. Recent studies indicate that several factors, including age, viral dose, coinfection with other pathogens, mouse strain, and for some strains even gender, influence the rate of seroconversion to parvoviruses as well as the duration of viral shedding.

Laboratory Diagnosis

Serology (HI, IFA, ELISA, and multiplexed fluorometric immunoassay [MFIA]) remains the most effective method of screening populations for viral infections. However, owing to the lack of seroconversion in some mouse strains as well as the possibility of persistent infections, more emphasis has been put on PCR as a diagnostic tool. Although PCR is sensitive and specific, it requires access to tissues usually obtained during necropsy. The preferred tissue for testing is mesenteric lymph nodes, but PCR may be performed on the spleen, tissue culture cells, or transplantable tumor cells. MVM and MPV can be detected by PCR in fecal samples, but the mesenteric lymph nodes yield a higher percentage of positive samples and the duration of virus shedding is variable, so use of feces for testing is limited to general use in routine testing. Parvoviruses often contaminate animal biological products, and regular testing of tumors, cell lines, and infectious disease stocks is necessary. Cell lines, transplantable tumors, and other biological products should be tested with PCR before being inoculated into animals. Sentinel mice exposed to soiled bedding from cages can be used for detection of the virus, but the threshold level of virus required to induce infection is influenced by the age of the mouse, its genotype (some strains of mice are more susceptible to infection), and even the type of caging. Thus, selection of the appropriate mouse strain is important.

Treatment and Control

Quarantine and barrier control must be rigorous to prevent introduction. Similar to other parvoviruses, these are very stable in the environment, and horizontal transmission by fomites (contaminated feed, bedding, materials) is an important means of transmission. Quarantine or sanitation of incoming animals, testing of biological materials, and sterilization of food by autoclaving or irradiation (e.g. 50 kGy) should be applied. If parvovirus is detected in a colony, virus elimination is done by depopulation, meticulous disinfection, and introduction of a new founding stock that is free of virus and/or antibodies. Colonies that have had parvovirus infections cannot always be repopulated by cesarean section and the use of foster mothers. Hysterectomy rederivation and embryo transfer have proved successful in parvovirus eradication, and should be pursued whenever possible.

Avian Parvoviruses

Disease

Avian parvoviruses of most concern are those that infect waterfowl and include the goose parvovirus (GPV)-related group and the Muscovy duck parvovirus (DPV)-related group that replicate in rapidly diving cells in the growing tissues of goslings and ducklings. Both of these viruses cause a fatal disease known as Derzsy's disease. GPV causes this disease in geese and Muscovy ducks, but Muscovy DPV infects only Muscovy ducks and hybrid duck breeds, but not geese. Derzsy's disease is characterized by watery diarrhea, lethargy, anorexia, and prostration. Infections in older birds are generally asymptomatic, but may be associated with ascites or loss of feathers.

Etiologic Agent

GPV and DPV are autonomous parvoviruses within the *Anseriform dependoparvovirus 1* species in *Dependoparvovirus,* along with adeno-associated viruses of mammals that are reliant on the presence of a helper virus for their efficient replication.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Apart from geese and Muscovy ducks (and some hybrid duck breeds), Derzsy's disease has not been reported in other avian species. The virus is found in all the major goose and Muscovy duck farming countries of Europe and the Far East (China, Taiwan, Vietnam, and Japan). Serological evidence suggests that GPV is present in several species of wild geese in Europe. Muscovy DPV has been isolated from an outbreak among Muscovy ducks in California. GPV/DPV is excreted in large amounts in

the feces of infected birds, resulting in rapid spread by direct and indirect means. Outbreaks of GPV are often initiated in susceptible goslings after vertical transmission of the virus via eggs laid by infected breeder geese, and subclinically infected geese may act as carriers. Infected eggs are often the source of the virus when outbreaks occur in countries or regions formerly free of the disease.

Pathogenesis and Pathology

After primary infection, the virus replicates in the intestinal wall and, after a short viremic phase, reaches the heart, liver, and other organs. In acute cases, lesions are commonly found in the heart, which has a pale myocardium and is characteristically rounded at its apex. The liver, spleen, and pancreas may be swollen and congested. In more prolonged cases, a sero-fibrinous perihepatitis, and pericarditis, is present with large amounts of fluid in the abdominal cavity. Pulmonary edema, necrotizing hepatitis, and catarrhal enteritis may also be present. In older birds, the disease follows a more protracted course, characterized by ocular and nasal discharge, a profuse white diarrhea, and weakness. The eyelids and uropygial glands are red and swollen. Birds that survive the acute stage show profound growth retardation, with loss of feathers and reddening of the skin, particularly on the back. Pathological lesions include ascites, hydropericardium, hepatitis, and myocarditis. Necrotic enteritis with ulceration of the mouth and pharynx are less common, depending on the presence of secondary infection. DPV signs resemble those seen with GPV but with the addition of sciatic neuritis and polioencephalomyelitis.

The main histological lesions are pronounced degenerative changes in myocardial cells with associated loss of striation, fatty infiltration, and the presence of scattered Cowdrey type A intranuclear inclusions. Similar histological changes are also found in intestinal and smooth muscle cells. In the liver, the predominant lesions are degeneration of hepatocytes with vacuolation and fatty infiltration. Small, eosinophilic inclusion-like bodies are sometimes seen in the cytoplasm of the vacuolated hepatocytes. Changes in the pancreas consist of shrunken, necrotic acinar cells with fatty infiltration. Some lymphoblastic processes are occasionally observed in the spleen, bursa of Fabricius, and thymus.

Host Responses to Infection

Depending on the age of the affected birds and the pathogenicity of the virus strain, the disease may present as acute, sub-acute, or chronic infection. The acute form can result in 100% mortality in birds less than one week old and 10–60% mortality in birds one to three weeks of age. Older geese and Muscovy ducks show no clinical signs but respond antigenically. There is limited antibody-driven cross-protection between GPV and DPV.

Laboratory Diagnosis

The virus neutralization test in embryonated goose or Muscovy duck eggs or primary cell cultures can be used to detect the presence of GPV neutralizing antibodies. There is also an agar gel diffusion test and an ELISA to test for the presence of antibodies. PCR can be used to detect the viral DNA, and Sanger sequencing can be used to differentiate the strains. Immunofluorescence is available to detect the presence of antigen in embryos, and virus isolation can be performed in 10–15-day-old embryonated goose or Muscovy duck eggs.

Treatment and Control

There is no specific treatment; antibiotic therapy is used to prevent secondary bacterial or fungal infections. Because many outbreaks of GPV are directly attributed to transmission of the disease by congenitally infected goslings during hatching, the practice of incubating and hatching eggs that have originated from different breeding flocks should be discouraged. Geese that have survived an outbreak should not be used for breeding purposes. Only eggs from known parvovirus-free flocks should be incubated together, and good hatchery hygiene should be maintained. Vaccines are available and are widely used in countries where the disease is endemic. The application of vaccines can effectively prevent the prevalence of Derzsy's disease in geese and Muscovy ducks. Vaccines are used to develop adequate immunity during the first four to five weeks of life by immunization of breeder birds. Protection is transferred to the progeny via the egg yolk. Eggs should be imported only from countries that can guarantee the absence of GPV/DPV parvovirus.

Additional Avian Parvoviruses

Recently a variant GPV was identified as the cause of the emerging short beak and dwarfism syndrome (SBDS) in ducklings, characterized by growth retardation with beak atrophy and protruding tongue (Figure 47.6), infecting Cherry Valley ducks (Pekin ducks) and mule ducks in mainland China.

Ducks with SBDS show strong growth retardation with beak atrophy, shortening of leg bones, enteritis, and

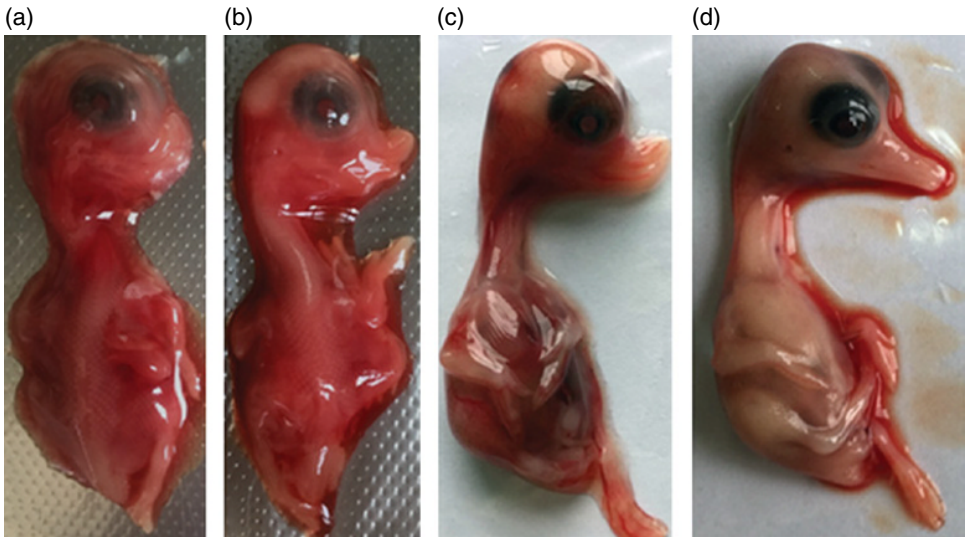


Figure 47.6 The pathogenicity of variant goose parvovirus to duck embryos. (a) and (b) Duck embryos died between 24 and 48 hours after inoculation with the virus. (c) Duck embryos died between 48 and 72 hours after inoculation with the virus. (d) Control group inoculated with saline. In contrast to the control group, duck embryos inoculated with the GPV variant showed beak abnormalities. *Source*: Reproduced from Li et al. (2018).

paralysis. Although the mortality rate has been 2–6% in affected flocks, the morbidity rate was 10–30% and even 50% in some regions, which caused great economic losses to the waterfowl industry in China, and outbreaks continue to occur. No pathological changes were found in the internal organs of affected ducks, though viral antigens could be found in most organ tissues such as the heart, liver, spleen, kidney, pancreas, and intestine. Experimentally infected birds exhibited remarkable growth retardation, anorexia, and diarrhea similar to naturally infected birds. All major duck species have been shown to be susceptible to the disease.

The parvoviruses infecting chickens and turkeys are currently classified into the genus *Aveparvovirus*, species *Galliform aveparvovirus 1*. These two viruses are genetically closely related, suggesting the possibility of interspecies transmission. However, the chicken parvovirus and turkey parvovirus lack the ability to cross-infect heterologous hosts (highly adapted to their respective host species). Susceptibility of birds to poultry parvoviruses is age related. Birds excrete virus in feces as early as four days of age, and the virus persists on contaminated surfaces and in litter. These viruses have been associated with enteric disease syndromes in turkeys and broiler chickens. The cause of these syndromes is complex and polymicrobial, including viruses, bacteria, and protozoa. The role of parvoviruses in enteric disease syndromes is not completely understood, and the virus can be detected in the healthy avian gut. Other viruses associated include rotavirus, reovirus, astrovirus, coronavirus, and adenovirus. Based on studies, it appears that the small intestine and local inflammatory cells are the primary targets of the aveparvoviruses, but the viruses can be found in several other tissues, potentially due to generalized infection. Detection is mainly by molecular methods PCR. Presently there is no commercial vaccine.

Equine Parvovirus

A newly discovered parvovirus in horses is the equine hepatitis parvovirus, which has been assigned to the species *Ungulate copiparvovirus 6* in the genus *Copiparvovirus*. This virus is now generally thought to be the cause of Theiler's disease and subclinical hepatitis in horses. Theiler's disease (also known as equine acute hepatitis necrosis, serum sickness, and post-vaccinal hepatitis), is commonly associated with the administration of equine biological products (which have now been shown to be contaminated with this virus), but can also occur in horses that have not received such treatments. Identification of this "new" virus in a case of equine serum hepatitis has been followed up with experimental infection. In a study including 10 horses, 8 of 10 experimentally infected horses developed hepatitis. One horse showed clinical signs of liver failure. The onset of hepatitis was temporally associated with seroconversion and a decline in viremia. Virus shedding occurred in oral and nasal secretions, as well as in feces. Transmission could be demonstrated with oral inoculation but not by nasal, vertical, or mechanical

transmission (horse fly bites). Liver histology and in situ hybridization showed lymphocytic infiltrates and necrotic equine parvovirus-infected hepatocytes, which is an unusual target for a parvovirus that requires dividing cells. High seroprevalence in horses without liver disease suggests the virus may be widespread in the equine population. Additional work is needed to further evaluate the pathogenicity of this virus.

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48

Circoviridae

Pablo Piñeyro and Sheela Ramamoorthy

Family Circoviridae

Circoviruses are extremely small DNA viruses with non-enveloped icosahedral capsids. They are characterized by the presence of covalently closed, circular, ambisense, single-stranded DNA genomes that encode oppositely oriented open reading frames (ORFs) and replicate by the rolling circle replication mechanism (Figure 48.1). The circoviral genome encodes just two major viral proteins; the capsid and replicase proteins. Currently two genera, *Circovirus* and *Cyclovirus*, are assigned to the Circoviridae family by the International Committee on the Taxonomy of Viruses. The differentiation at the genus level is based on the location of the viral origin of replication (Ori). The Ori of cycloviruses are located on the capsid protein encoding strand, whereas the Ori of circoviruses are located on the replicase encoding strand. Circoviruses infect a large number of mammalian, bird, and fish species. With the advent of metagenomic sequencing technology, the number of species assigned to both the *Circovirus* and *Cyclovirus* genera has expanded considerably. Currently, there are 52 species assigned to *Cyclovirus* and 49 species assigned to *Circovirus*, respectively. Circoviruses are an exception to the general paradigm that RNA viruses have a higher rate of mutation than DNA viruses, as the rates of mutation are similar to those of RNA viruses. In addition, recombination between strains is also common, substantiating the vast genetic diversity of the Circoviridae family. Differentiation at the species level is based on the species infected or the species in which the virus was first detected. Circoviruses of veterinary importance include beak and feather disease virus (BFDV) and porcine circovirus (PCV) types 2, 3, and potentially the newly discovered PCV4.

Currently, information on cycloviruses is limited to genome sequence records as obtained by metagenomic sequencing in arthropods, mammals, and birds. Cycloviruses have not been cultured in the laboratory thus far, and their biology is yet to be explored. It is not also clear if the species in which they were identified are also the definitive hosts.

Anelloviruses are also single-stranded, small DNA viruses, which have a ubiquitous distribution. They were formerly members of the Circoviridae family, but were recently assigned to a separate family based on differences in genome arrangements. The Anelloviridae family consists of two genera of veterinary interest: gyroviruses (chicken anemia virus) and torque teno viruses.

Physical and Biological Properties of Circoviruses

Most of the scientific information regarding circoviruses is derived from the experimental study of porcine circoviruses. The virions are extremely small, range between 17 and 32 nm in size, and have typical icosahedral symmetry. As circoviruses are non-enveloped, disinfectants aimed at dissolving lipids such as those utilizing the bases of alcohol, chlorhexidine, iodine, and phenol are ineffective against circoviruses. Virus inactivation requires alkaline disinfectants (sodium hydroxide), oxidizing agents (sodium hypochlorite), or quaternary ammonium compounds. Circoviruses are stable at a pH of 3.0 and at temperatures of 56 °C for 1 hour, or 70 °C for up to 15 minutes. Therefore, individual capsomeres can assemble into virus-like particles under appropriate conditions *in vitro*. Irreversible denaturation of the viral protein structures requires exposure to high temperatures for long periods.

Attachment and entry into permissive cells, which can support viral replication, is an essential first step for productive viral infection and is facilitated by the capsid protein. Following entry, viruses are transported to the nucleus, where viral replication occurs. The viral genome interacts with host cell DNA polymerases to replicate itself

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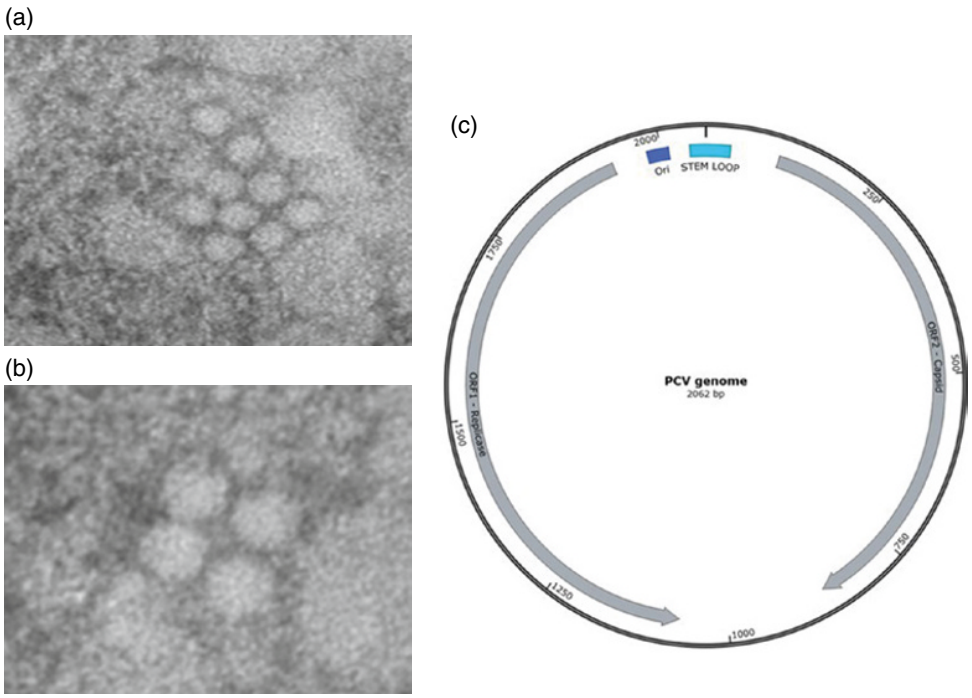


Figure 48.1 Structure of circoviruses. (a) and (b) Electron micrographs of negative-stained, cell-culture-derived Porcine circovirus 2, containing clusters of icosahedral virions of about 20 nm diameter. (c) Diagrammatic representation of the circoviral genome, ranging from 1700 to 2000 bp in size, depicting the two major ambisense ORFs shown as solid arrows running in opposite directions. The intergenic region containing the origin of replication and the stem loop structure located at the 5′ end of the ORFs are in blue; they are necessary for replication. *Source:* North Dakota State University, Department of Microbiological Sciences.

by the rolling circle mechanism, while viral mRNA transcripts are produced by transcription. Translocation of the transcripts to ribosomes and viral protein production occurs in the cytoplasm. Viral proteins traffic back to the nucleus to associate with the nascent viral DNA, leading to the final assembly of infective viral particles. As circoviruses do not encode their own polymerases, they depend on the host cellular machinery for replication. They multiply to high titers in cells in the S phase undergoing active replication, *in vivo* and *in vitro*. However, they are noncytolytic in cultured cells in that they do not produce visible cytopathic effects.

Avian Circoviruses

Although BFDV is a significant pathogen of Old and New World parrots, non-passerine birds, including ducks, geese, swans, gulls, pheasants, and pigeons, and a few passerine birds, including canaries and finches, can also be infected with circovirus species. Pigeon, goose, and canary circoviruses have the same genomic characteristics as other circovirus and are classified as members of the genus *Circovirus*. Pigeon circovirus and goose circovirus have a worldwide distribution. Their infection is associated with lethargy, anorexia, running, and poor racing performance. Canary circovirus produces abdominal distention and failure to thrive. Doves and Mulard ducks experience feather loss, dystrophy, growth retardation, and high mortality. Young birds are commonly affected. Typically, infection results in immunosuppression and atrophy of the cloacal bursa and to a lesser extent of the spleen. Adult and young finches have nasal discharge, dyspnea, anorexia, depression, and high mortality. Histological lesions include bursal lymphoid depletion, sinusitis/rhinitis, tracheitis, bronchopneumonia, myocarditis, nephritis, and splenitis. Detection of the virus can be done by observing the presence of the virus in characteristic intracytoplasmic inclusion bodies in the mononuclear cells of the bursa of Fabricius by transmission electron microscopy, detection of viral DNA in tissues by *in situ* hybridization (ISH), or polymerase chain reaction (PCR). Circoviruses are associated with but not confirmed as causative agents of fading chick syndrome in ostriches, which is clinically characterized by anorexia and diarrhea.

Beak and Feather Disease

Clinical Signs

BFDV affects Old and New World parrots. The first identification of BFDV occurred in 1984 when a large number of icosahedral viral particles were detected by electron

microscopy in skin sections of captive Australian parrots with severe alopecia and claw and beak deformities. Infection with BFDV can remain undetected or present mild clinical signs. However, when infection is chronic it is ultimately fatal, and is characterized by a long-term incubation with progressive feather dystrophy. The disease most commonly affects immature and fledgling birds less than five years old, although older birds can also be affected. Classical lesions appear after the first molt. The lesions are characterized by symmetrical and generalized loss of feathers, including contour, tail, and down feathers. Alopecic areas result from follicular necrosis and replacement by dystrophic feathers that are segmentally retained in the feather sheath. Beak deformities due to fractures and palatine necrosis followed by abnormal elongation or overgrowth are common features of the disease. Systemic clinical signs including lethargy, depression, diarrhea, and immunosuppression leading to death can also be observed. Occasional leukopenia and non-regenerative anemia have been reported in parrots. However, the severity of the clinical signs can vary with age, species, the virulence of the viral strain, and the route of viral exposure.

Etiologic Agent

Like other members of the family Circoviridae, BFDV possesses a small, circular, ambisense single-stranded DNA (ssDNA) genome of approximately 2 kb length. The genome encodes two major proteins: a replication initiation protein (1) and a capsid protein (cap). The cap is genetically diverse and is highly prone to mutation, although antigenically there is no evidence for different serotypes.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Beak and feather disease (BFD) is the most common viral disease of wild Psittaciformes (cockatoos, lorikeets, and parrots). It is recognized as a threat to endangered psittacine birds in Australia, New Zealand, and South Africa. In Africa, BFD has been identified in captive *Agapornis* sp. and wild cape parrots, *Poicephalus robustus*. The disease is also widespread in captive collections of psittacine species in Europe and the United States. The virus is normally shed in feces and feather dander with a high risk of nasal exposure or oral ingestion of virus particles. In addition, chicks can be infected with regurgitated crop contents. Vertical transmission of the disease is suspected to also play a role in the spread of infection. In some species, including lovebirds and orange-bellied parrots, the disease can be subclinical. These species are considered responsible for shedding and maintaining the virus in the environment and infecting susceptible bird species.

Host Response to Infection

Pathogenesis and Pathology

A characteristic feature of the BFD virus is its affinity for rapidly dividing cells, including those of feather follicles, beak, claws, lymphoid tissues, and the intestinal epithelium. Microscopic examination is consistent with necrosis and inflammation in dystrophic feathers. Lymphoid depletion is also noted, possibly as an indirect effect of the infection. The presence of characteristic intra intracytoplasmic ("botryoid") inclusions is commonly seen in follicular epithelium (Figure 48.2). Due to immunosuppression, secondary bacterial and fungal infection is common. Therefore, systemic signs including leukocytosis are common, although they might not directly result from the viral infection.

Laboratory Diagnosis

The diagnosis of psittacine beak and feather disease (PBFD) should be based on clinical signs and the presence of gross and histological lesions. Chronic alopecia associated with follicular necrosis and the presence of botryoid inclusion bodies on follicle biopsies is confirmatory. *In situ* detection of the virus can be confirmed by

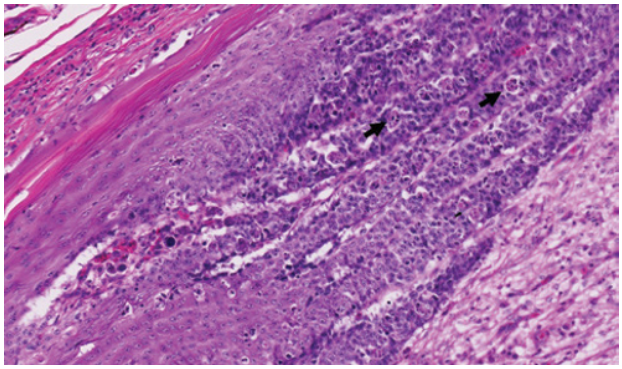


Figure 48.2 PBFD-associated necrotizing and degenerative folliculitis. Severe epithelial degeneration and necrosis of all epithelial strata of the feather follicle. Epithelial cells present pyknosis and karyorrhexis in addition to fewer apoptotic nuclei. Intracytoplasmic botryoid inclusion bodies (arrow) are consistent with the presence of BFDV. *Source:* Picture courtesy of Dr. Oscar Fletcher. Department of Population Health & Pathobiology, College of Veterinary Medicine, NC State University.

immunohistochemistry (IHC) and ISH, electron microscopy of the affected follicle, and detection of the viral nucleic acid by PCR. Hemagglutination (HA) and HA inhibition assays can also be used for viral and antibody detection, respectively.

Treatment and Control

Currently, there are no specific treatment or prevention methods. Commercial vaccines are not available. Only a few promising experimental vaccines based on virus-like particles have been evaluated. Serological studies suggest that the prevalence of the disease in wild birds is high. The chronic nature of the disease, in addition to high numbers of subclinically infected birds, may perpetuate the virus in the environment. The virus can be transmitted both vertically and horizontally. Therefore, quarantine procedures are required after introducing cockatoos in breeding colonies. Eradication from commercial flocks after new introductions is difficult, resulting in euthanasia and depopulation of the flock.

Porcine Circoviruses

Disease

PCV types 2, 3, and 4 are associated with a wide range of clinical signs in pigs that include wasting, lymphadenopathy, respiratory, reproductive and gastrointestinal signs, and reproductive failure. They are collectively known as porcine circovirus associated disease (PCVAD).

Etiologic Agent

Porcine circovirus type 1 (PCV1) was the first discovered PCV. It was found to contaminate the porcine kidney cell line, PK-15, and was widely prevalent but not associated with clinical disease. In the mid-1990s, widespread outbreaks of a wasting disease in weanling piglets (PMWS) were determined to be caused by a pathogenic PCV variant, which was designated as porcine circovirus type 2 (PCV2). Subsequently, PCV2 was isolated in cell culture. Experimental infection of pigs with PCV2 resulted in development of PCV2-associated lesions including lymphadenomegaly and lymphoid depletion, with the detection of PCV2 antigen in major organs and lymphoid tissue. These findings demonstrated Koch's postulates for PCV2 and established its role as a primary pathogen of pigs. The newly identified PCV2 is approximately 60% genetically similar to PCV1. The viral replicase protein encoded by ORF1 is conserved between PCV1 and PCV2, whereas the capsid protein is not. Therefore, PCV1 and PCV2 are serologically distinct. Although PCV2 was designated as the etiologic agent of PMWS, it is noteworthy that PCV2 and all other subsequently identified PCV types are detected in both healthy and sick animals. Currently identified PCV2 subtypes include PCV2a–f, with PCV2a, b, and d being associated with clinical disease. Vaccines against PCV2 are mostly based on the PCV2a subtype. They were introduced in 2006 in the United States and were very effective in preventing clinical disease. However, PCV2a was quickly replaced by PCV2b as the most predominant subtype, and since 2014, PCV2d has now become the most widely prevalent subtype globally.

In 2015, a new PCV type, designated PCV3, was discovered by metagenomic sequencing of samples collected from pigs showing signs of porcine dermatitis and nephropathy syndrome (PDNS), and from cases of reproductive failure myocarditis, respiratory disease, and multisystemic inflammation. Similar to PCV1 and PCV2, PCV3 is widely prevalent in global swine herds, both in healthy and morbid animals. A 4th PCV type, designated PCV4, was discovered in 2020 in cases of respiratory and enteric diseases and PDNS. PCV3 and 4 are genetically and serologically distinct from PCV1 and PCV2. PCV1 and PCV2 are easily cultured in porcine kidney cells, whereas *in vitro* culture of PCV3 and PCV4 remains challenging. The demonstration of Koch's postulates has been achieved for PCV3, but the detailed biology and pathogenesis of PCV3 and PCV4 are yet to be explored. Coinfection of pigs with more than one PCV type or subtype, or with other infectious agents such as swine influenza A viruses (SIAVs) or the porcine reproductive and respiratory disease syndrome virus (PRRSV), which have similar clinical manifestations, is very common. Therefore, accurate diagnosis requires testing for multiple pathogens.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Porcine circoviruses are widely prevalent in production swine herds and in feral swine populations, and hence are considered endemic in swine-producing countries worldwide. Both PCV2 and PCV3 are detected in several nonporcine species such as bovines, canines, deer, rodents, and humans. However, it is not clear if these species are actually the definitive hosts of PCV2 or PCV3. Rodent models used for experimental PCV2 infection indicate that PCV2 can infect and replicate in rodents but does not cause PCV2-associated lesions in them. Mink circovirus, which is closely related to PCV2, has been detected in cases of diarrhea and reproductive failure of mink. It is considered the

primary etiologic agent of mink refractory diarrhea syndrome. PCV2 infections are long term and chronic in nature. Infected pigs shed virus in oronasal fluid, urine, blood, semen, and feces. Given the highly stable nature of PCV2, it can remain in contaminated environments for extended periods and promote horizontal transmission via contaminated feed, water, and fomites. Horizontal transmission is considered to be the primary mode of PCV transmission, whereas vertical transmission and transmission via contaminated semen have also been experimentally demonstrated for PCV2.

Host Response to Infection

Antibody responses to PCV2 can be detected within 7–10 days post infection and increase in magnitude over 4–6 weeks post infection. Viremia increases in the first three to four weeks of infection and then declines in recovering animals as neutralizing antibody levels increase between two and five weeks post infection. Hence, virus-neutralizing antibody responses are important for protection against PCVAD. Maternal antibodies can be detected in the serum of suckling piglets until about three to five weeks after birth. Cell-mediated immunity also plays an important role in clearance of the virus, as protection against infection has been reported in the absence of strong antibody responses, and piglets with maternal antibodies are not always protected. However, in natural infections, the host immune response is unable to completely clear viral infection, leading to the establishment of a chronic state of infection. The depletion of B and T lymphocytes compromises the ability of the infected host to mount an adequate immune response to PCV2 and other coinfecting agents, and therefore is an important mechanism of immunopathology in pigs with clinical PCVAD. In addition, hypersensitivity reactions expressed as PDNS are the result of the formation and accumulation of immune complexes due to dysregulation of the immune system in clinically infected pigs. The exact trigger for the development of clinical disease is at yet unidentified but is suspected to be caused by stress and other coinfections.

Pathogenesis and Pathology

Since the first description of PCV2 as the causative agent of post-weaning multisystemic wasting (PMWS), it has been associated with numerous disease syndromes. International consensus is still needed regarding a nomenclature that can integrate all these clinical manifestations associated with PCV2. Collectively, in Europe, the term *porcine circovirus diseases (*PCVD*s)* is used, whereas the term *porcine circovirus associated diseases (PCVADs)* is used in the United States. Although PCV2 produces systemic disease (PCV2-SD), also known as PMWS, it can also produce subclinical infections (PCV2-SI). It has also been implicated as a potential causative agent of PDNS, as the primary cause of interstitial pneumonia or lung disease (PCV-LD), as part of the porcine respiratory disease complex (PRDC), as the cause of diarrhea in grower-finisher pigs – known as PCV2 enteric disease (PCV2-E) – and as the cause of reproductive failure in sows (PCV2 reproductive disease [PCV2-RD]). These disease manifestations and their diagnoses are now described.

PCV2 Systemic Disease (PCV2-SD)

PCV2-SD or post-weaning multisystemic wasting disease syndrome (PMWS) was the first described and recognized manifestation of PCV2 infection. Grower-finisher pigs from 5 to 16 weeks of age are mainly affected. Morbidity varies from 4% to 60%, and mortality can range from 4% to 20% depending on farm type, husbandry, and coinfections. Characteristic clinical changes include growth retardation, weight loss, skin pallor or jaundice, dyspnea, and diarrhea. Lymphadenitis and lymphadenomegaly are the hallmarks of pathological changes for PCV2-SD. However, non-collapsed lungs, gastric ulcers, colonic edema, and white spotted kidneys are also commonly observed. Histologically, lymphoid depletion, granulomatous inflammation, and the presence of basophilic intracytoplasmic botryoid inclusion bodies are often seen in most of the lymphoid tissues, including Peyer's patches, the spleen, thymus, and tonsil. Lymphoid depletion caused by PCV2 is linked to lymphopenia, specifically depletion of peripheral CD8+ and CD4+CD8+ double-positive T lymphocytes and B lymphocytes. Other histological changes include interstitial nephritis, lymphoplasmacytic periportal hepatitis, and interstitial pneumonia. Differences in severity of clinical signs or lesions are not associated with the different PCV2 subtypes that have evolved over time.

PDNS

This clinical presentation of PCV2 affects grower and finisher pigs but can also affect adult animals. The incidence of PDNS in production swine is about 1–2%, but the mortality rate is higher and can reach 100% in pigs older than three months and 40–50% in growers. Clinical signs are nonspecific; anorexia, weight loss, depression, and normal temperature to mild pyrexia. The onset of the clinical signs is characterized by the presence of well-demarcated to coalescing red-to-purple macules and papules located on the hind limbs and perineal region that progress to the ventral abdomen. The kidneys are enlarged and have cortical petechia. Other gross abnormalities include subcutaneous edema and enlarged and hemorrhagic lymph nodes. Histological lesions are characterized by dermal and epidermal necrosis

associated with vascular necrosis, dermal thrombosis, lymphoplasmacytic perivasculitis, necrotizing glomerulitis, and interstitial nephritis with occasional intratubular proteinaceous casts. Although PCV2 seems to be necessary, it alone is not sufficient to induce PDNS, as there are no differences in the PCV2 viral load in pigs with or without PDNS, or with mild lesions of PCV2-SD. Infection with PRRSV and torque teno virus (TTV) without PCV2 is also reported to induce lesions resembling PDNS. The newly recognized virus, PCV3, has also been strongly associated with PDNS. Although the pathogenesis of PDNS is not clearly understood, kidneys in PDNS-affected animals have increased IgG1 + IgG2 and IgM levels, complement factors C1q and C3, and increased numbers of CD8+ lymphocytes. The detection of immunoglobulin and complement factors in the affected vessels and glomeruli suggests that a typical type III hypersensitivity reaction is central to the pathogenesis of PDNS.

PCV-2 Reproductive Disease (PCV2-RD)

Clinical signs of PCV2-RD include abortions associated with increased rates of mummified, macerated, stillborn, and weak-born piglets. Coinfection with other pathogens might exacerbate PCV2-RD. A number of pathogens such as porcine parvovirus (PPV), PRRSV, and PCV3 have also been reported in association with PCV2-RD. All gestational periods are affected by PCV2-RD. Viral antigen can be detected in early gestation associated with embryonic death and irregular returns to estrus. Mid-gestation PCV2-RD is associated with mummified fetuses and abortions. Late pregnancy PCV2-RD is associated with mummies, stillborn, weak-born piglets, and delayed farrowing. Histological lesions are characterized by myocardial lymphocytic infiltration and occasional fibrosis. PCV2 antigen can be detected in the myocardium and other tissues, including the liver, kidney, and lung. No specific differences in the manifestation of reproductive failure are attributed to the different PCV2 subtypes. Experimental infection of fetuses by intrauterine inoculation or artificial insemination with PCV2 contaminated semen obtained from clinical cases of PCV2-RD, PCV2-SD, and PDNS, did not result in differences in virus replication or tissue tropism in the infected fetuses. Experimental fetal infection also did not reproduce the clinical outcome present at the source of collection. Infected boars can shed PCV2 in semen. Semen is an essential source of viral dissemination.

PCV2 Respiratory Disease (PCV2-LD) and PRDC

PCV2 plays a vital role as a primary pulmonary pathogen causing interstitial pneumonia. It can also predispose to secondary pulmonary infections and contribute significantly to PRDC, which is multifactorial and affects pigs from 8 to 22 weeks of age. Several agents including PCV2, SIAV, PRRSV, pseudorabies virus (PRV), porcine respiratory coronavirus (PRCV), *Mycoplasma hyopneumoniae, Pasteurella multocida, Streptococcus suis*, and *Actinobacillus pleuropneumoniae* contribute to PRDC. Of these agents, PCV2, PRRSV, and *M. hyopneumoniae* are the most important coinfecting agents in the field. Regardless of the PCV2 subtype, the presence of PRRSV enhances PCV2-associated lesions, increasing viral load in serum and the amount of antigen in tissue. PCV2 lesions and viremia are also enhanced by *M. hyopneumoniae* coinfection. Clinical signs are dyspnea, cough, lethargy, anorexia, and fever. Common gross lesions are interstitial pneumonia with interlobular septal edema. Microscopically, PCV2 can be detected in interstitial macrophages and bronchiolar lamina propria by IHC and ISH. Pulmonary changes can be observed in both PCV2-LD and PCV2-SD. Differentiation is based on histological findings, as pathognomonic lymphoid lesions are more often observed in PCV2-SD but not in PCV2-LD.

PCV2 Subclinical Infection (PCV2-SI)

Subclinical infection is perhaps the most common clinical presentation in post-weaning pigs. The high rate of PCV2-SI prevalence correlates directly with the high rates of detection of PCV2 DNA and antibodies in animals with no clinical signs, and the detection of PCV2 in retrospective studies long before the first description of PMWS. Immunologic mechanisms that trigger either PCV2-SD or PCV-SI manifestations in infected pigs are unknown. Perhaps the only observable clinical manifestation associated with PCV2-SI is growth retardation. Subclinically infected pigs are generally seropositive, with a low viral load in the range of $<10^5$–10^6 PCV-2 genome copies/ml of serum. The economic losses due to PCV2-SI at the farm level are actually higher than the economic impacts due to overt PCV2-SD, mainly due to the decrease in daily weight gain and reduced vaccine efficacy. It is suggested that the efficacy of PCV2 vaccines is impaired in subclinically infected pigs; however, vaccination programs can improve growth performance in farms with PCV2-SI.

PCV2-Enteric Disease (PCV2-ED)

Diarrhea is often observed in infected pigs 16 to 22 weeks of age. Clinical signs are nonspecific and cannot be differentiated from other enteric diseases affecting growers and finisher pigs. Diarrhea is yellow and can progress to dark-red, with growth retardation and wasting. Morbidity varies from 10% to 20%, and mortality is 50% to 60% in PCV2-ED infections. The diagnosis of PCV2-ED is appropriate for clinical diarrhea, with hallmark microscopic lesions in Peyer's patches but not in other lymphoid tissues, and demonstration of PCV2 DNA or antigen in lesions. Gross lesions include enlarged mesenteric lymph nodes and

increased thickness of the intestinal mucosa. Histological lesions of PCV2-ED include granulomatous inflammation of Peyer's patches characterized by lymphoid depletion and replacement of the follicle architecture by macrophages, histiocytes, and occasional multinucleated giant cells with rare grape-like intracytoplasmic inclusion bodies. PCV2 antigen or DNA can be detected in histiocytes in Peyer's patches, submucosa, and crypt epithelium by IHC or ISH, respectively.

PCV3-Associated Disease

Since the initial reports regarding the detection and characterization of the newly emerged PCV3, it has been detected globally and retrospectively in cases from pigs of all ages with a range of clinical presentations. Similar to PCV2, PCV3 is associated with both subclinical infections and a range of overt clinical signs. The main PCV3 clinical presentations are linked to reproductive failure and PDNS-like lesions. Sows display PDNS-like skin lesions, increased mortality, and reproductive problems, including increased abortions and percentage of mummies, and decreased conception rates. Dermal histological changes resemble those observed in PDNS associated with PCV2 infection. Dermatitis and epidermitis associated with lymphoplasmacytic perivasculitis and proliferative glomerulonephritis, cortical tubule attenuation, and interstitial fibrosis are the hallmarks of PCV3 infections. In addition, bronchointerstitial pneumonia and granulomatous lymphadenitis with positive immunofluorescence in macrophages are observed in affected sows. Multisystemic inflammation, including lymphoplasmacytic myocarditis, interstitial nephritis, and lymphoplasmacytic periarteritis in the intestinal serosa affects animals between 3 and 10 weeks of age. In addition, viral replication occurs in the myocardium, smooth muscle arteries of the heart and kidney, and trophoblasts of the placenta. Histological lesions observed in experimental studies are less severe than in field cases, suggesting that PCV3 infection alone may not be sufficient to cause clinical disease. It may act as a cofactor that enables other pathogens to exacerbate disease severity. Numerous retrospective studies have demonstrated that subclinical PCV3 infection occurs in animals of all ages, with the absence of evident clinical signs, but detectable prolonged viremia, viral replication in tissues, and multisystemic inflammation. Coinfections of PCV3 with multiple swine pathogens are relatively common, but those with PCV2, PRRSV, or PPV are most commonly reported. The epidemiology and transmission of PCV3 are poorly understood. Experimental and field studies have demonstrated that PCV3 can be shed in oral fluids, nasal secretions, feces, colostrum, and semen. Viremia can be detected for more than seven weeks post infection, suggesting that virus-neutralizing antibody responses are inadequate. PCV3 has been detected in numerous domesticated and wild animal species, including cattle, dogs, mice, wild boar, chamois, roe deer, ticks, and mosquitoes. Although it is not clear if these species are the definitive hosts of PCV3, the findings suggest the potential for multiple reservoirs and cross-species transmission.

The detection of a fourth PCV type, PCV4, by metagenomic sequencing in pigs with PDNS and respiratory and enteric signs led to the discovery and classification of PCV4 as a new PCV type. However, Koch's postulates have not yet been demonstrated for PCV4, and detailed information on the disease manifestations of PCV4 is not yet available.

Laboratory Diagnosis

The diagnosis of PCV2 infections is rather complex due to the large number of clinical presentations. In addition, the virus is highly prevalent and ubiquitous in most swine-producing countries. Therefore, both the clinical context and laboratory diagnosis are equally important for evaluation. Currently, numerous laboratory techniques are available for PCV2 detection. Direct detection of viral DNA is common and involves methods such as conventional PCR assays, restriction fragment length polymorphism (RFLP), quantitative PCR (qPCR), and ISH. Cyber green and TaqMan qPCR are most commonly used for viral quantification in tissues and fluids. In addition to molecular techniques for viral detection, viral sequencing is commonly used for molecular epidemiology studies. Traditional Sanger sequencing methods are being increasingly replaced by next-generation sequencing (NGS) techniques as they do not require prior sequence information. ISH allows for a direct observation of PCV2 nucleic acid in lesions. Different hybridization approaches and protocols are currently available, but most of them target the PCV2 ORF2. Probes that are specific to different subtypes can help to differentiate between them. Detection of viral antigen in tissues is accomplished by IHC with monoclonal antibodies generated against the PCV2 ORF2. However, IHC techniques do not allow subtype differentiation.

Serological diagnosis is based on antibody detection with enzyme-linked immunosorbent assays (ELISA). The specific reactivity of serum antibodies with the PCV2 capsid protein is measured using several assay formats such as indirect or blocking ELISAs, and microbeads-based assays, which can detect antibodies to several viruses in one composite assay. All the serological techniques used can detect PCV2 antibodies generated during natural infection but do not allow the differentiation of antibodies induced by infection versus vaccination.

The field diagnosis of PCVAD should consider the main clinical presentation on the farm and the results of laboratory tests equally. The diagnosis of PCV2 subclinical infection (PCV2-SI) is challenging and controversial. By definition, a farm that presents evidence for virus circulation in pigs by PCR and evidence of infection by the presence of PCV2 antibodies in the absence of overt clinical signs should be considered subclinically infected. Lymphoid lesions are minimal or absent in PCV2-SI, with low to no *in situ* detection of virus by IHC/ISH. There is not a real consensus regarding the viral load necessary to define PCV2-SI. However, a range of less than 10^5 to 10^6 genomic copy numbers/ml of serum without clinical signs is consistent with PCV2-SI.

The diagnosis of PCV2-SD (PMWS) is based on three basic criteria. Clinically, the presence of animals that have growth retardation; that are associated with respiratory distress, enlarged lymph nodes and occasionally jaundice; and that do not respond to antibiotic treatment is indicative of PCV2-SD. Typical histological lesions include lymphoid depletion, lymphohistiocytic granulomatous lymphadenitis with occasional multinucleated giant cells, and characteristic botryoid inclusion bodies. Finally, *in situ* detection of PCV2 by IHC/ISH is necessary. Significant increase in post-weaning mortality associated with the presence of clinical signs characteristic of PCV2-SD has been proposed as a requisite criterion for a positive PCV2-SD herd definition.

A diagnosis of PDNS does not necessarily include etiologic criteria. Rather, it is based on the presence of characteristic morphological changes and clinical signs. The presence of macula that progress to necrotizing and hemorrhagic dermatitis and enlarged, swollen kidneys and lymph nodes with multiple cortical petechia are diagnostic features. Histological lesions are characterized by severe necrotizing vasculitis and fibrinous glomerulonephritis. Due to the chronic nature of the PDNS lesions, the detection of PCV2 antigen or nucleic acid *in situ* is rare.

The diagnosis if PCV2-RD should be based on the clinical presentation and detection of PCV2 in fetal tissues. Increased rates of abortions, stillbirth, and mummification above the historical values in the farm indicate PCV2-RD. Confirmation of nucleic acids in fetal tissues as well as histological lesions including lymphoplasmacytic and necrotizing myocarditis coupled with *in situ* detection of PCV2 by IHC/ISH provides a definitive diagnosis. The diagnosis of PCV2 lung disease (PCV2-LD) needs to be included within the typical PRDC diagnostic workup request. The presence of histiocytic interstitial pneumonia in association with the detection of PCV2 by PCR or ISH/IHC can be suggestive of either PCV2-LD or PCV2-SD. Enteric disease due to PCV2 (PCV2-ED) needs to be differentiated from other enteric diseases affecting grower and finisher pigs. Enteric signs in the presence of granulomatous enteritis, lymphocytic depletion in Payer's patches, and detection of PCV2 by IHC/ISH in the absence of other diagnostic features of PCV2-SD should provide a confirmatory diagnosis of PCV2-ED.

Treatment and Control

Commercial vaccines against PCV2 are highly effective in preventing clinical signs of PCVAD and reducing production losses. Hence, vaccination against PCV2 is widely deployed in production swine herds. The available vaccines contain either the inactivated whole virus, inactivated chimeric PCV1-2a/b viruses, or recombinant PCV2 capsid antigen. The first dose is administered at three weeks of age, followed by a booster at six weeks of age for the two-dose regimens, and a single dose is administered at three weeks for the one-dose regimen. Sow vaccinations are administered at five and two weeks pre-partum. A combined piglet and sow vaccination regimen has the best outcome for preventing infection but can be limited by the cost of vaccination. Due to the multifactorial nature of the PCVAD, the presence of the clinical signs can be reduced with good husbandry practices such as optimal stock density, temperature, and humidity of the grower-finisher barns. As PCV2 is hardy and can contaminate the environment for extended periods of time, instituting strict measures for sanitation with disinfectants which are effective against PCV2, preventing age mixing, following an all-in-all-out policy, and early segregation of clinically affected animals can prevent transmission on farm premises and reduce clinical disease. Strict vaccination programs for coinfecting agents including PRRS, *M. hyopneumoniae*, PPV, and SIAV have also been established to reduce PCV2-associated clinical manifestations.

Canine Circovirus

Canine circovirus (CaCV) was first discovered in 2012. Subsequent retrospective studies showed that this virus has circulated in dogs since 2009. The circular CaCV genome is 2063 nt in size. Its organization is similar to other members of Circoviridae, including an ambisense organization with two major ORFs encoding a replication-associated and capsid proteins. The CaCV replicase proteins are 42–54% similar to the Rep proteins of porcine and avian circoviruses, and most closely related to PCV1. The CaCV capsid is <30% similar to all other known circovirus capsids. Thus, the CaCV strains cluster with PCV1 and PCV2 phylogenetically. Together they form a distinctive clade of mammalian circoviruses distinct from bird circoviruses.

CaCV infections in dogs are reported in several countries, including the United States, Italy, Germany, Turkey, China, Taiwan, Colombia, Brazil, and Argentina. Detection of CaCV in wild animals including wolves, foxes, and badgers has also been reported. The virus was first reported in dogs with no clinical history. Later CaCV was associated with canine hemorrhagic diarrhea, vasculitis, granulomatous lymphadenitis, and acute gastroenteritis. Although CaCV is epidemiologically associated with canine diarrhea, its pathogenicity remains controversial as other enteric or respiratory pathogens including canine parvovirus and canine distemper virus are often co-detected in clinical cases. Serological studies with CaCV capsid protein specific ELISAs reveal high levels of seroprevalence in dogs over three years of age. Antibodies are developed between 9 and 12 days post infection in experimental infections. However, it is not clear if antibodies are protective against disease. Clinical diagnosis is based on CaCV detection and the presence of characteristic lesions. Viral detection in clinical specimens is achieved by conventional PCR, electron microscopy, quantitative real-time PCR (qPCR), IHC, and ISH.

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Asfarviridae and Iridoviridae

Melissa Kennedy, Gustavo Delhon, D. Scott McVey, Hiep Vu, and Manuel Borca

Asfarviridae

The family *Asfarviridae* includes just one genus, *Asfivirus*, of which African swine fever virus (ASFV) is the type and, currently, sole species.

ASFV

African swine fever (ASF) is a highly contagious hemorrhagic disease of domestic and some species of wild swine caused by ASFV. Disease in ASFV-infected pigs ranges from peracute to chronic and inapparent. In sub-Saharan Africa, the primary natural reservoirs of the virus are warthogs and arthropod vectors. Incursions of the virus into populations of domestic and wild swine typically result in extensive outbreaks of acute ASF, and the subacute and chronic forms appear after the virus becomes established in the pig population. Whether endemic or newly introduced, ASF has a profound impact on economics and food security, but it is not a zoonotic disease. Given that several species of wild and domestic pigs as well as tick vectors are susceptible, it continues to be a global threat (Figure 49.1).

Disease

Acute, severe ASF has a high mortality, often approaching 100%, and death may occur prior to the development of clinical signs. The disease is characterized by high fever and leukopenia, often followed by the appearance of erythema (red areas on the skin), and cyanosis, weakness, accelerated respiration, and pulse, vomiting, bloody diarrhea, and nasal and conjunctival discharges. Subacute ASF is characterized by less dramatic clinical signs and death or recovery in three to four weeks. Affected pigs typically experience a high fever; abortion is common and may be the only sign of illness. Pigs with chronic ASF fail to thrive (stunting and emaciation) and exhibit swollen joints and lameness, skin ulcerations, and pneumonia.

Etiologic Agent

Virion Structure and Antigenic Properties

The ASF virus is a large enveloped DNA virus (Figure 49.2). The viral particle is 175–215 nm in diameter, spherical to pleomorphic and consists of a nucleoid containing the viral genome successively surrounded (from the center to the outside) by a core shell, inner capsid or matrix shell, inner membrane, outer capsid, and the outer envelope (Figure 49.3). The outer capsid is round and exhibits icosahedral symmetry (T = 189–217). The genome is a single large (170–190 kb) molecule of double-stranded DNA that includes approximately 150–170 open reading frames and sequence-complementary ends. Virions contain more than 60 different proteins, including a large number of enzymes and proteins required for replication. The viral genome also encodes proteins that appear to modulate the protective host antiviral response.

To date, greater than 24 ASFV genotypes have been identified based on the sequences of the p72 capsid protein gene and central variable region tandem repeats. The greatest genetic variation occurs in regions where the sylvatic cycle is present. Eight distinct antigenic types of ASFV have been identified based on hemagglutination inhibition

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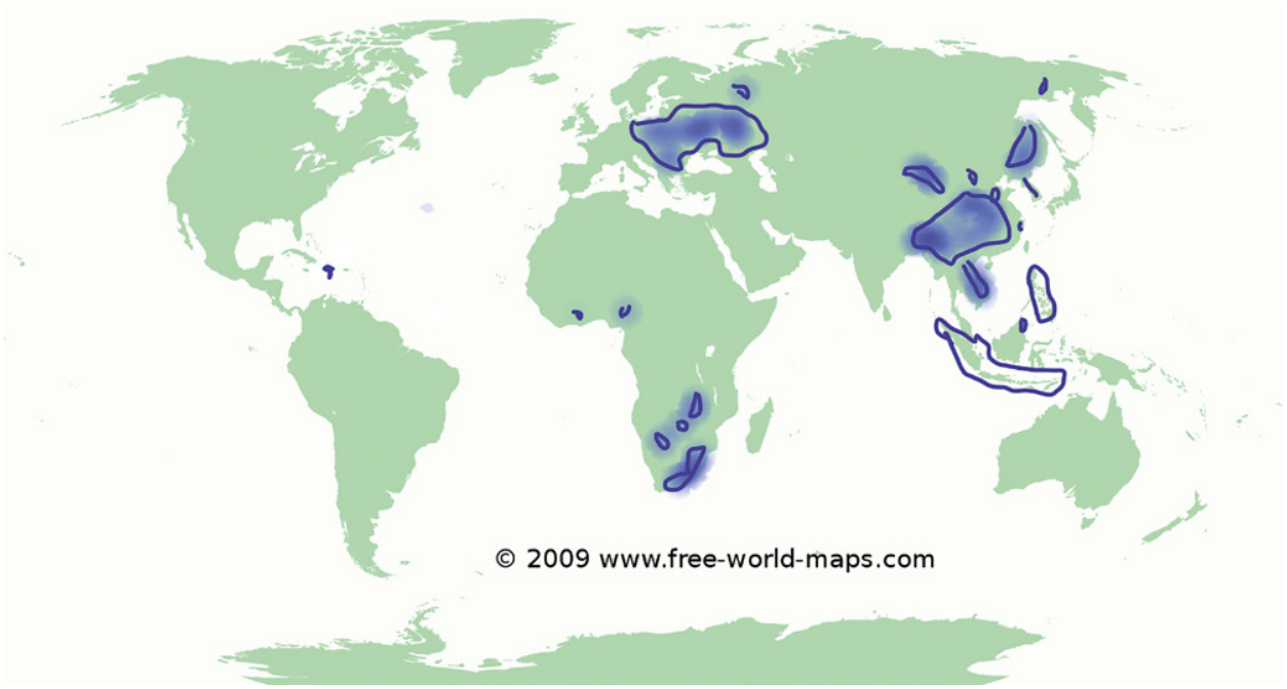


Figure 49.1 Global distribution of ASF infection in wild and domestic swine, 2007 – 2022. Blue-shaded areas are regions of known and suspected ASFV infection.

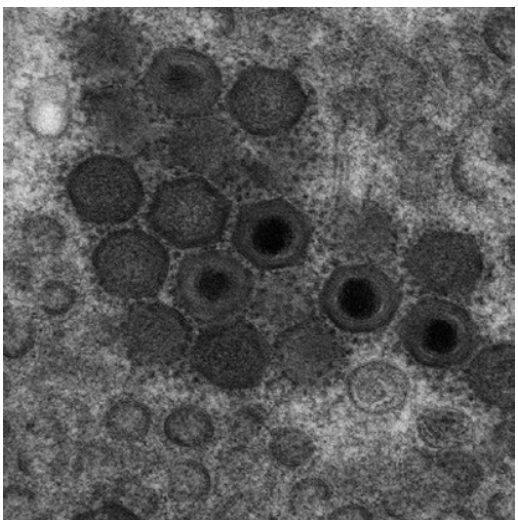


Figure 49.2 Transmission electron microscopy of a section of tissue culture cells infected with African swine fever virus (ASFV) (58 000×). *Source:* Courtesy of I.C. Pang.

assay (HAI) serological typing. Viruses within a serogroup appear to cross-protect against one another. ASFV strains can vary markedly in their virulence in swine.

Resistance to Physical and Chemical Agents

ASFV is stable in tissues and excretions. The movement of infected animals or animal products, contaminated transport vehicles, and animal feed made from food waste are the most common means of maintaining viral circulation once the virus is introduced. ASFV can withstand pH over a considerable range (pH 4–13). The virus is inactivated by heating to 60 °C for 20 minutes and by lipid solvents, and some disinfectants (paraphenylphenolic disinfectants are very effective against the virus).

Infectivity for Other Species and Culture Systems

ASFV infects domestic and wild pigs (including European wild boars), warthogs, giant forest hogs, and bush pigs. Soft ticks of the genus *Ornithodoros* transmit the virus as biological vectors. Only domestic pigs, feral pigs, and European wild boars show clinical disease, whereas African wild pigs do not.

In vivo, ASFV replicates in pig macrophages and specific lineages of reticular cells, and can be propagated *in vitro* on cultures of swine bone marrow cells, monocytes, and alveolar macrophages. The virus can also be adapted to various established cell lines (porcine kidney, VERO, and baby hamster kidney) although the process of adaptation is usually accompanied by significant genomic changes.

Distribution, Reservoir, and Transmission

ASF was first described in European domestic pigs in Kenya in the early 1900s, and subsequently throughout

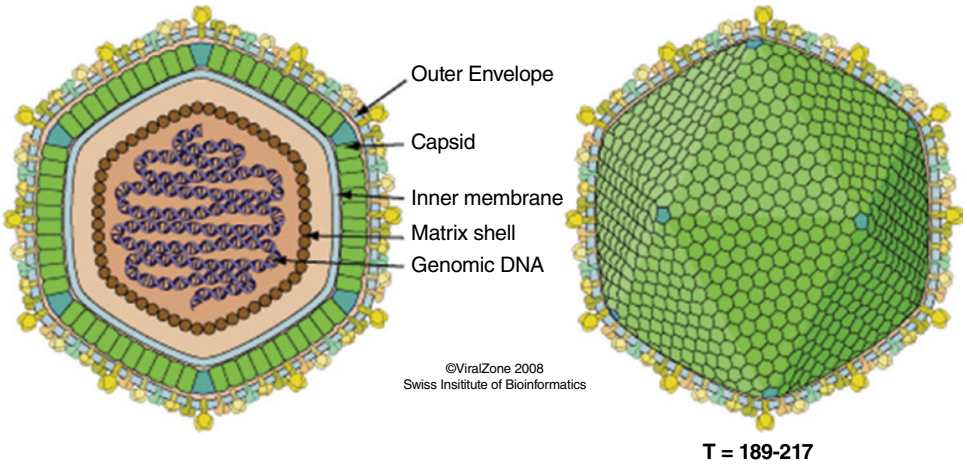


Figure 49.3 Asfivirus structure. *Source:* Courtesy of ViralZone.

much of Africa. Currently ASF is endemic in more than 20 countries of sub-Saharan Africa and on the Italian island of Sardinia. The disease spread outside of Africa for the first time in 1957 when it appeared on the Iberian Peninsula, and subsequently has occurred in Mediterranean Europe (Spain, France, Italy, Malta, and Sardinia), northern Europe (Belgium and the Netherlands), the Caribbean (Cuba, the Dominican Republic, and Haiti), and South America (Brazil). In 2007, ASF outbreaks and continuing epidemics began in the Caucasus region and now occur in the Russian Federation, Europe, China, Vietnam, and the Dominican Republic, representing a serious threat to the swine industry worldwide. After almost 40 years since the last report in the Americas, ASF was diagnosed in the Dominican Republic in July 2021.

The ASF virus exists in two distinct cycles of infection: first, a sylvatic cycle in ticks and wild pigs in Africa; and, second, epidemic, and endemic cycles in domestic swine and wild boars. The reservoirs of the sylvatic cycle of ASFV infection in Africa are persistent or inapparent infections in African wild pigs (warthogs in particular) and the soft tick vector. Vertical transmission of ASFV in the tick vectors makes them an especially efficient reservoir of the virus. The virus spreads into domestic swine through the bites of infected ticks, or by ingestion of tissues from carrier swine. ASFV is then readily transmitted to susceptible pigs by direct contact, including aerosols and fomites. The virus is easily transmitted over long distances because of its stability in infected tissues, including uncooked and some cured pork products. Throughout the history of ASF, epidemiological evidence has shown that the vast majority of outbreaks that have occurred in ASF-free zones were mainly the result of feeding food waste products from infected pigs to susceptible pigs. In addition, soft ticks become infected with the virus after they feed on viremic swine, and thus they can become reservoirs of the virus after it incurs into previously noninfected regions. Pigs that survive infection with the ASFV become virus carriers.

Host–Virus Relationship

Pathogenesis and Pathology

Following oral or nasal exposure of domestic pigs to the ASF virus, virus replication initially occurs in the upper respiratory tract with subsequent dissemination to adjacent lymph nodes and then systemic spread via leukocytes, erythrocytes, or both in the lymph and blood; this occurs within two to seven days post infection and corresponds closely with the onset of pyrexia. The virus replicates in macrophages; thus, the highest titers of the virus occur in those tissues in which macrophages are most abundant, such as lymphoid tissues.

Acute severe ASF is characterized by edema and hemorrhage within internal organs, particularly the lymph nodes and spleen, which can be very large and intensely hemorrhagic. Pulmonary edema and intestinal congestion and hemorrhage are also common. The lesions of subacute ASF are similar but less pronounced, whereas animals with chronic ASF may show fibrinous pericarditis and pleuritis, lobular consolidation of the lungs, swollen joints, and patchy necrosis of the skin (Figure 49.4). Lesions in aborted piglets are relatively nonspecific but may include disseminated petechial hemorrhages.

Microscopic lesions are most pronounced in the lymphoid tissues and include extensive necrosis, and apoptosis of both lymphocytes and mononuclear phagocytic

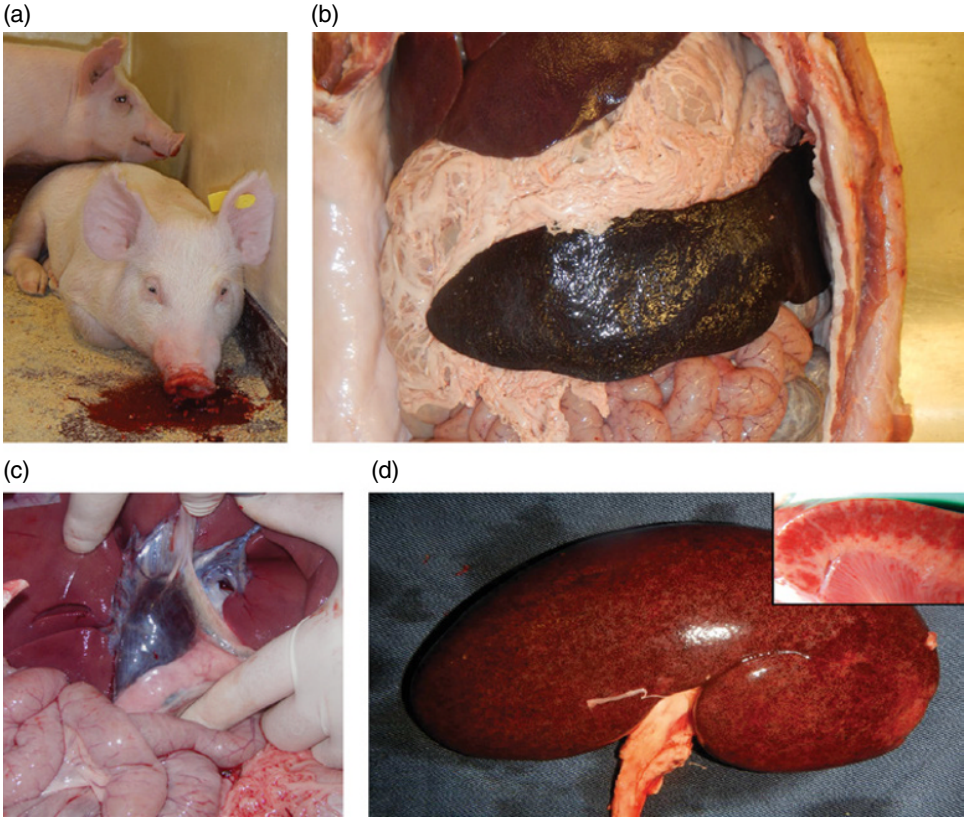


Figure 49.4 Clinical signs and gross lesions of fulminant (late stage) African swine fever (ASF) in domestic pigs. (a) Epistaxis, hematemesis, and other forms of hemorrhage are common in the late stages of ASF. (b) Splenomegaly. (c) Gastrohepatic lymph node hemorrhage and congestion. (d) Renal cortical hemorrhage apparent on the intact and cut (inset) cortical surfaces. *Source:* Images courtesy of Jonathan Arzt, Foreign Animal Disease Research Unit, ARS/USDA.

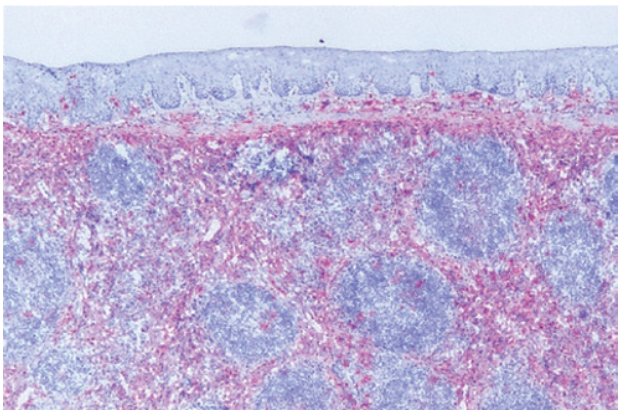


Figure 49.5 Soft palate tonsil of a domestic pig with fulminant African swine fever (ASF). African swine fever virus (ASFV) is identified with red staining in this immunohistochemical stain. ASFV localizes predominantly to inter-follicular tonsillar regions, with less virus in follicles and epithelium, consistent with viral tropism for histiocytic cells. *Source:* Image courtesy of Jonathan Arzt, Foreign Animal Disease Research Unit, ARS/USDA.

cells, which is responsible for the marked lymphopenia in infected pigs (Figure 49.5). Endothelial cell necrosis and thrombosis of the pulmonary vasculature is common in fulminant cases of acute ASF. During the clinical phase of infection, virus and viral DNA can be detected in all fluids, including secretions and excretions, with the highest titers in the blood, where virus is associated with red blood cells.

Host Responses to Infection

Pigs that survive infection with ASF virus develop immunity that protects them against a lethal challenge with strains of the same serogroup but not against strains of different serogroups. The correlates of immune protection remain uncertain. Both cell-mediated and humoral immune responses are important for protective immunity. Depletion of CD8+ T cells from pigs that were immunized with an avirulent ASFV strain abrogated the protective immunity that otherwise would fully protect pigs from a lethal ASFV challenge, clearly demonstrating the importance of T cells in ASFV immune protection. Passive transfer of ASFV hyperimmune sera to naïve pigs results in a solid protection against a lethal ASFV infection, indicating that antibody alone can protect pigs from ASFV. However, the effectivity of sera collected from ASFV-infected pigs in inhibiting virus infectivity in an

in vitro remains a highly controversial topic. The exact mechanisms of antibody-mediated protection remain elusive. Likewise, the viral proteins that elicit protective immune responses remain unknown. This knowledge gap has complicated the development of an effective ASF vaccine.

Diagnosis and Control

Laboratory Diagnosis

Laboratory tests are required to distinguish between classical swine fever (CSF)/hog cholera (also currently a foreign animal disease in the United States) and ASF because the two diseases cause very similar signs and lesions in susceptible pigs, including fever, high mortality, and hemorrhages within internal organs. Salmonellosis and erysipelas are endemic diseases that can also resemble ASF. Tissues submitted should include spleen, liver, lymph nodes, and EDTA blood. Virus isolation can be used to identify ASFV, and then confirmed with hemadsorption. Immunofluorescent or immunohistochemical staining of sections of tissue from affected pigs using ASFV-specific antisera provides a rapid method of diagnosis (Figure 49.5). Techniques based on polymerase chain reaction (PCR) can also be used to rapidly identify the presence of ASF virus genomic material. All ASFV testing is controlled by the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) in the United States, and the National Animal Health Laboratory Network (NAHLN) labs in collaboration with the Foreign Animal Disease Diagnostic Laboratory (FADDL). A positive diagnosis is World Organisation for Animal Health (OIE) reportable. There is ongoing active surveillance and passive surveillance.

Treatment and Control

There is currently no effective vaccine or treatment for ASF. Vaccine development has been hindered by limited knowledge of aspects of ASF immunity, the extent of virus strain variation, and the viral proteins responsible for protective immunity. Eradication of the disease is accomplished by slaughter and disposal of all exposed pigs after the virus occurs in new areas. These drastic measures, however, may not prevent the virus from spreading to the local populations of soft ticks and wild pigs. Eradication procedures involve not only slaughtering all pigs on the premises but also treating the premises with insecticides and disinfectants containing O-phenylphenol with surfactants and must remain free of livestock for at least a month. Prior to restocking, susceptible sentinel animals should be placed on the premises to confirm eradication of the virus.

In endemic areas, contact between domestic and wild swine must be avoided. Early detection is critical for control of outbreaks in domestic swine. Localized disease eradication with the establishment of ASF-free zones is beneficial and can enhance support of larger-scale eradication programs.

Iridoviridae

The family *Iridoviridae* includes two subfamilies, Alphairidovirinae, which encompass viruses that mainly infect cold-blooded vertebrates, and Betairidovirinae, with viruses that mainly infect invertebrates. Alphairidoviruses are grouped into three genera: *Lymphocystivirus* and *Megalocityvirus,* which cause important diseases of fish, and *Ranavirus*, which comprises significant pathogens of reptiles and amphibians as well as fish. Betairidoviruses are grouped into four genera – *Chloriridovirus*, *Decapodiridovirus*, and *Iridovirus* – with viruses infecting insects and crustaceans.

Iridoviruses are enveloped viruses that have icosahedral symmetry with a virion diameter of 120–200 nm but occasionally up to 350 nm. The genome is a single molecule of double-stranded DNA between 100 and 300 kb pairs. Iridoviruses, like asfarviruses, are structurally complex with large numbers of virus-specific proteins (at least 36) encoded by the genome. A single protein constitutes the majority of the outer capsid. The double-stranded DNA genome is circular and terminally redundant, with many of the internal cytosine residues being highly methylated in most viruses. Viral replication occurs in the nucleus and cytoplasm of infected cells. These viruses are resistant to drying, and can persist in water for months. A definitive diagnosis is best made by viral isolation and/or characterization of the genome by molecular biology techniques.

The lymphocystis virus infects a wide variety of fish and causes unsightly wart-like cutaneous lesions. These lesions consist of benign proliferations of hypertrophied, virus-infected cells on the skin, peritoneum, and mesentery. The lesions typically resolve with minimal mortality. The virus spreads by direct contact through abrasions, especially when fish are crowded; thus, disease caused by the lymphocystis virus is especially important in fish raised in aquaria and in some commercial aquaculture operations. Two strains of lymphocystis disease virus (LCDV) have been described, with LCDV-1 being associated with flounder and LCDV-2 being associated with dabs.



Figure 49.6 Cop's gray tree frog (*Hyla chrysoscelis*) larvae experimentally exposed to ranavirus. The body and legs are swollen and there is hemorrhage in the legs, consistent with ranaviral disease.

In contrast, viruses in the *Ranavirus* genus have been associated with high mortality in both farmed and free-ranging fish. These pathogenic iridoviruses causing fatal systemic disease are closely related to frog virus 3, the latter serving as the prototype ranavirus. Members of this genus have been reported to cause epizootic hematopoietic necrosis and systemic hemorrhagic disease in fish (Figure 49.6). Ranaviruses of amphibians are virulent for larval anurans, though susceptibility varies among different species. Recent widespread die-offs in amphibian populations may be the result of a combination of host immunity, natural, and synthetic stressors, and the emergence of novel strains. Megalocytiviruses affect a wide variety of tropical marine and freshwater fish, leading to systemic infection with significant mortality. The commercial trade and movement of fish, amphibians, and reptiles may contribute to the emergence of new iridoviruses.

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Papillomaviridae and Polyomaviridae

Mohamed A. Abouelkhair and Melissa Kennedy

Papillomaviridae

The papillomaviruses are widespread among mammals, having been identified in cattle, sheep, goats, deer, elk, horses, rabbits, dogs, monkeys, pigs, opossums, mice, elephants, and several species of birds. The Papillomaviridae are currently divided into two subfamilies: *Firstpapillomavirinae* with 52 genera and *Secondpapillomavirinae* with one genus. Members of the Papillomaviridae primarily infect mucosal and keratinized epithelia, and despite co-evolutionary history with their hosts, some papillomaviruses are pathogens of their natural host species. Virus-induced papillomas (warts) are benign, hyperplastic epithelial proliferations of the skin or mucous membranes that may undergo malignant transformation in certain circumstances. Some papillomaviruses also cause proliferations of mesenchymal tissues in the skin, with or without associated epithelial proliferations. Those proliferations with exclusively epithelial proliferation are papillomas, whereas those with proliferation of both mesenchymal (fibrous tissue) and epithelial tissue are termed fibropapillomas. Papillomaviruses are highly species specific, and thus are generally contagious only to the animal species in which they naturally occur. Virus-induced cutaneous papillomas (warts) are common in horses and cattle and infrequent in dogs, sheep, and goats. Oral papillomas are most common in young dogs and rabbits. In domestic livestock, genital papillomas are relatively common. In some cases, papillomaviruses may play a role in neoplastic development, as occurs in humans.

Papillomaviruses have naked icosahedral capsids approximately 60 nm in diameter (Figure 50.1). The viral genome is a single circular molecule of double-stranded DNA that encodes between 8 and 10 proteins; 2 are structural (L1 and L2), and the remainder are nonstructural proteins (designated E1–E8) that are essential for virus replication. Replication of papillomaviruses is dependent on and intimately linked to the growth and differentiation of cells in stratified squamous epithelium.

Bovine Papillomaviruses

Bovine papilloma virus (BPV) types are distinguished on the basis of their antigenic and nucleotide sequence homologies. They can be further distinguished on the basis of the nature of the lesions that they cause in cattle. This has led to the establishment of five genera of bovine papillomaviruses: *Xipapillomavirus*, which is epitheliotropic; *Deltapapillomavirus*, which causes fibropapillomas; *Epsilonpapillomavirus*, which contains properties of both xi- and deltapapillomaviruses; *Dyokappapapillomavirus*; and *Dyoxipapillomavirus*. However, most bovine papillomaviruses are referred to by type number, of which there are at least 18. Cutaneous fibropapillomas (warts) caused by papillomaviruses Types 1, 2, and 5 occur commonly in calves less than two years old. They appear most frequently on the head, especially in the skin around the eyes. They may also appear on the sides of the neck and less commonly on other parts of the body. They begin as small, nodular growths that then grow rapidly into dry, horny, whitish, cauliflower-like masses that eventually regress spontaneously. The histological appearance is a variable mixture of proliferating dermal fibrous tissue and overlying epithelium. Infectious papillomas (without a fibrous tissue component) that occur on the skin and teats of dairy cattle are also associated with infection by bovine papillomaviruses, as are some epithelial proliferations (polyps) that occur in the bladder and gastrointestinal tract, particularly those that affect the esophagus, forestomachs, and intestines.

Fibropapilloma is a papillomavirus-induced tumor that occurs on the penis of young bulls and the vagina and

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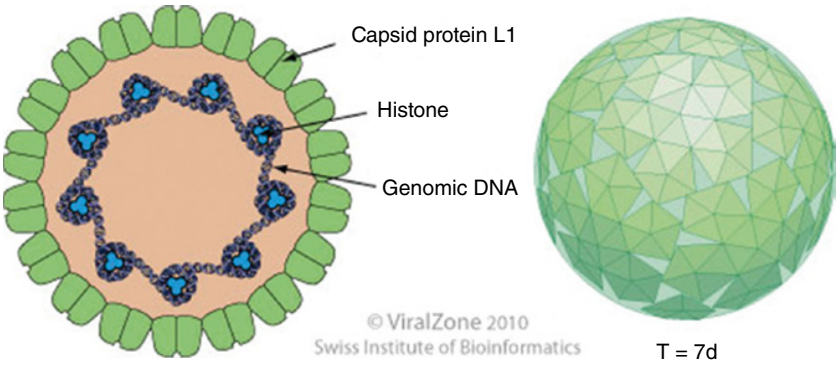


Figure 50.1 Schematic diagram representing the 72 capsomers in a T = 7 arrangement of a Papillomaviridae capsid. *Source:* From ViralZone, (n.d.) https://viralzone.expasy.org.

vulva of young heifers. These are fleshy, raised multinodular proliferations that consist of abundant fibrous tissue covered by epithelium of variable thickness.

Host immune responses eventually control papillomavirus infections because most warts persist for variable periods and usually spontaneously regress. The host is then immune to reinfection with the same virus, but not to other types of bovine papillomavirus. Occasionally, they may evolve into cancers of epithelial or mesenchymal origin. In cattle grazing on bracken ferns, BPV-2 and 4 have been associated with urinary bladder and upper gastrointestinal tumors, respectively. Conversely, DNA of BPV have been identified in normal bovine skin using polymerase chain reaction (PCR), indicating it can persist in a latent state causing no clinical signs. Treatment of bovine papillomatosis with finely ground wart tissue suspended in a 0.4% formalin solution has been used for many years to combat outbreaks of the disease, but it is difficult to evaluate the efficacy of this procedure as the disease is self-limiting and its duration varies between individual animals. A significant proportion of vaccinated animals apparently fail to reject their warts after vaccination with autologous tumor preparations. Cattle vaccinated with the L2 structural protein of bovine papillomavirus Type 4 did not develop alimentary papillomas when challenged with that virus type.

Equine Papillomavirus and Equine Sarcoids

Skin warts of horses are not as common as those affecting cattle. They develop most often on the nose and around the lips of young horses, appearing as small, elevated, papillary (horny) masses. Aural plaques are raised pale nodules on the inner aspects of ear. The plaques are neither pruritic nor painful, but they do not spontaneously regress, unlike cutaneous papilloma. The causative virus is spread by direct contact of infectious material through wounds and cutaneous abrasions. The virus can be experimentally transmitted to horses by intradermal inoculation of a suspension of wart tissue, but not to other animal species. Equine papillomas are usually self-limiting and disappear spontaneously in nine months, although they can progress to squamous cell carcinoma in rare instances. Natural infection provides solid immunity.

Sarcoids are common skin tumors of horses, mules, and donkeys that grossly and histologically resemble fibropapillomas of cattle. They are believed to be the most common equine skin tumor, affecting about 2% of the worldwide population. Interestingly, the genome of bovine *Deltapapillomavirus 1* and *2* is present within some of these tumors, whereas that of equine papillomavirus is not. Sarcoids have been reproduced by direct inoculation of bovine papillomavirus into susceptible horses. Flies may act as vectors as they move between wound sites on different horses. Alternatively, BPV infection may be transmitted via stable management practices, such as the sharing of contaminated tack, or it may be passed into existing wounds from contaminated pasture. The tumors range from being largely epithelial in nature to intensely fibroblastic, and the histological diagnosis is dependent on demonstrating both epithelial and mesenchymal components to the tumor. Sarcoids are frequently multiple in affected horses, and they commonly are ulcerated. A definitive diagnosis can be made by biopsy; however, acquiring the sample carries the risk of triggering an uncontrollable expansion of the lesion. Sarcoids were traditionally left without treatment, but as they almost invariably become larger and more difficult to treat, early intervention is strongly recommended to avoid long-term sequelae. Treatment may involve topical or intra-lesional chemotherapy or immune modulators, surgical procedures, or radiotherapy. Appropriate treatment selection is largely dictated by the location and type of the lesion. Recurrence after surgical removal is common due to incomplete

removal, but metastasis has not been described; thus, they are not malignant tumors despite their locally aggressive behavior. Recurrent sarcoids become increasingly difficult to manage and convey a far worse prognosis than those appropriately treated in the first place.

Canine Oral Papillomavirus

Sixteen papillomaviruses from three different genera have been identified in domestic dogs, with Canis familiaris papillomavirus (CPV) Types 1 and 6 belonging to the *Lambdapapillomavirus* genus, CPV Types 2, 7, and 13 to the *Taupapillomavirus* genus, and the remainder to the *Chipapillomavirus* genus. CPV-1 (formerly canine oral papillomavirus) and, possibly, CPV-13 induces warts in the mouths of dogs (Figures 50.2 and 50.3). The warts generally develop on the lips and spread to the buccal mucosa, tongue, palate, and pharynx. The warts are usually benign and disappear spontaneously within eight weeks. Occasionally, the papillomas may interfere with eating, or may compromise breathing necessitating excision. Dogs recovered from the infection develop immunity to reinfection. The infection is highly contagious. Concentrated populations, diverse animal admissions, direct animal contact, and fomites, among other factors, can contribute to the creation of an ideal environment for canine papillomavirus transmission. Dogs that are immunosuppressed have been reported to be more susceptible to development of papillomatosis.

Warts have been experimentally transmitted by rubbing pieces of wart tissue on scarified mucous membranes of susceptible dogs. Under such conditions, the incubation period was from four to six weeks. Infectious venereal papillomas (warts) and inverted abdomen papillomas (warts) (Figure 50.4) have also been described in dogs.



Figure 50.2 Oral papilloma of a dog. *Source:* From Lange and Favrot (2011). With permission of Elsevier.

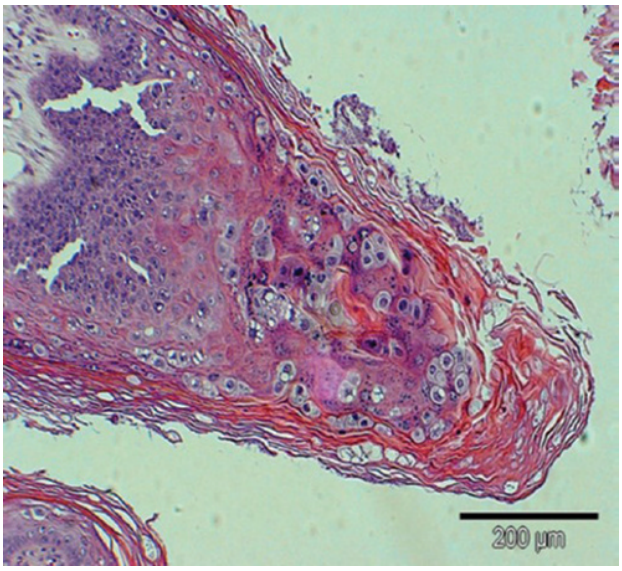


Figure 50.3 Histopathology of an oral papilloma of a dog. *Source:* From Lange and Favrot (2011). With permission of Elsevier.



Figure 50.4 Inverted papilloma on the abdomen of a dog. *Source:* From Lange and Favrot (2011). With permission of Elsevier.

Azithromycin, a macrolide antibiotic, has been shown to be effective for treatment of human papillomatosis. Recent studies indicate that it may also be effective in treating canine papillomatosis. Although the mechanism of this activity is unknown, it may aid resolution in chronic cases.

Feline Papillomavirus

Four *Felis catus* papillomaviruses (FcaPVs) have been completely sequenced, one as a *Lambdapapillomavirus* (FcaPV-1),

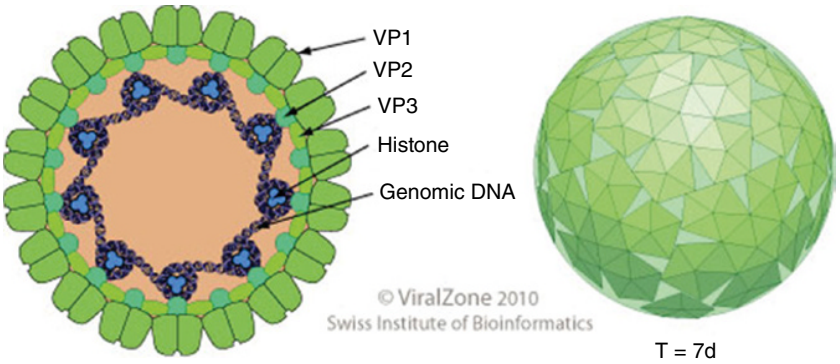


Figure 50.5 Schematic diagram representing a T = 7 arrangement of a Polyomaviridae capsid. *Source:* From ViralZone, (n.d.) https://viralzone.expasy.org.

one as a *Dyothetapapillomavirus* (FcaPV-2), and two belonging to *Taupapillomavirus* (FcaPV-3 and 4). Recently, FcaPV-2 has been associated with both cutaneous viral plaques and Bowenoid in situ carcinomas (BISCs). These skin lesions are often of little clinical significance, but may occasionally progress to squamous cell carcinomas (SCCs).

Polyomaviridae

Polyomaviridae are currently divided into six genera: *Alphapolyomavirus*, *Betapolyomavirus*, *Deltapolyomavirus*, *Epsilonpolyomavirus*, *Gammapolyomavirus*, and *Zetapolyomavirus*. Polyomaviruses have not been associated with diseases of domestic animals, with the notable exception of an avian polyomavirus that causes an acute generalized infection in fledgling budgerigars. Polyomaviruses are nonenveloped and 40–45 nm in diameter, with an icosahedral capsid (Figure 50.5) and a genome of a single molecule of circular double-stranded DNA. Although polyomavirus replication is similar to that of papillomaviruses, coding region transcription occurs from both DNA strands in the case of polyomaviruses (Figure 50.6) and from one strand in the case of

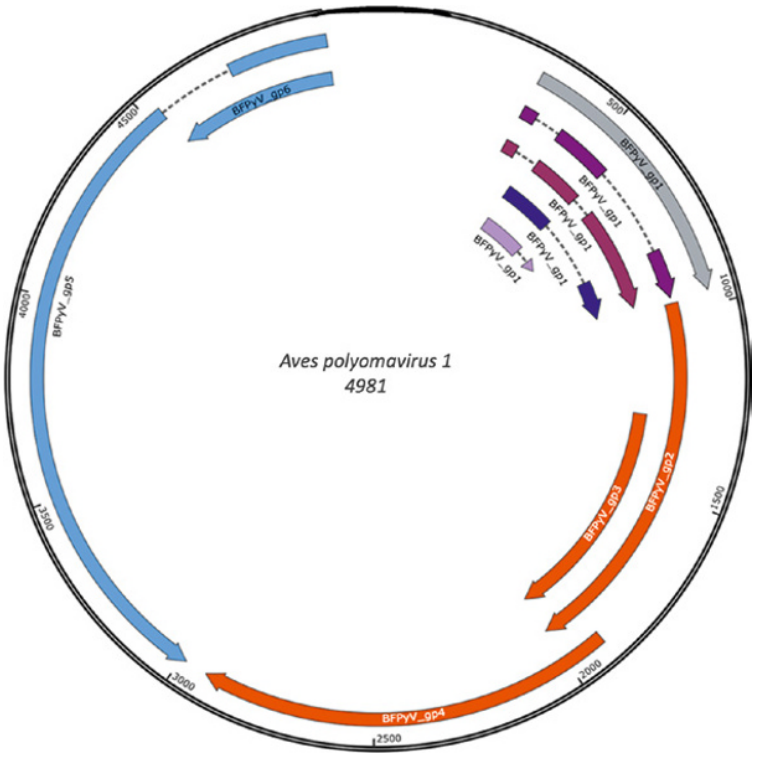


Figure 50.6 Genomic structure of *Aves polyomavirus 1* virus.

papillomaviruses. The genome encodes at least three structural and five nonstructural proteins.

Aves polyomavirus 1 (genus *Gammapolyomavirus*) is the agent of budgerigar fledgling disease, a disease characterized by abdominal distention, feather abnormalities, and acute death. Avian polyomaviruses have a wider host range than mammalian polyomaviruses, which have definite, if not absolute, host-species specificity. *Aves polyomavirus 1* has been found in species other than psittacines, such as finches and crows. The virus is transmitted horizontally and vertically. It targets endothelial cells and macrophages but may be found in any tissue. Nestling and juvenile birds are the most susceptible, and asymptomatic adults are the major source. Diagnosis is through clinical signs and PCR detection of the virus. There is no treatment.

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Adenoviridae

Yunjeong Kim and Kyeong-Ok Chang

Adenoviruses are large, non-enveloped viruses with a double-stranded 26–45 kb DNA genome. The virus genome and proteins are surrounded by icosahedral capsids that are composed of 252 capsomers. Fiber, a component of capsid protein, protrudes from the capsid, and initiates viral attachment to target cells (Figure 51.1). Adenoviruses replicate in the nucleus of infected cells and produce at least 50 different proteins, some of which modulate the host cell environment to enhance virus replication. Adenoviruses comprise a large family of viruses, with new adenoviruses continuously being discovered. Adenoviruses have been isolated from various vertebrate hosts, including mammals, birds, reptiles, and fish, but the host range of adenovirus types are usually limited to a single or closely related species. Although adenovirus infections are often asymptomatic or subclinical, some adenoviruses are pathogenic.

The family Adenoviridae consists of five genera, *Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus,* and *Siadenovirus*. The members of each genus share a common group antigen. Adenoviruses in the genus *Atadenovirus* infect mammals, birds, and reptiles. Members of *Aviadenovirus* infect birds, and those of *Mastadenovirus* infect various mammals. *Ichtadenovirus* is a relatively new genus with only a single member, white sturgeon adenovirus. Members of *Siadenovirus* infect birds and reptiles. Table 51.1 lists the diseases of domestic animals caused by adenoviruses.

Physical and Chemical Properties of Adenoviruses

Adenoviruses are resistant to many common disinfectants such as lipid solvents (ether and chloroform) and alcohols. They are relatively stable in soiled material and tolerate a wide pH range (3–9). Viral infectivity is lost after heating at 50–60 °C for 10 minutes. Steam cleaning and iodine, phenol, sodium hydroxide-based disinfectants, quaternary ammonium, and sodium hypochlorite (household bleach) can inactivate the virus.

Treatment

Therapy is supportive and symptomatic as there is no specific treatment for adenovirus infections.

Infectious Canine Hepatitis

Disease

Infectious canine hepatitis (ICH) is a disease of dogs and wild canids. Widespread use of vaccines has substantially decreased the incidence of ICH, but it is occasionally observed in unvaccinated dogs or due to inadequate vaccination. Infections are generally asymptomatic. However, dogs of all ages may be affected, and ICH is more commonly seen in young dogs. The clinical course may vary from peracute cases resulting in death within one to two days of illness to chronic cases with hepatic failure lasting for months. Acute cases of about two weeks of duration are most common and have a mortality rate of 10–30%. Dogs with acute disease may exhibit fever, tonsillitis, inappetence, lethargy, vomiting, diarrhea, cough, widespread hemorrhage on the mucosal membrane, and hematuria due to vascular injury. Rarely, neurological signs such as seizure and ataxia may be observed. Severe ICH is most likely to occur in pups that are not immune to the virus. Corneal edema (blue eye), usually transient, may occur in some dogs recovering from ICH. Chronic hepatitis was observed in some experimentally infected dogs that

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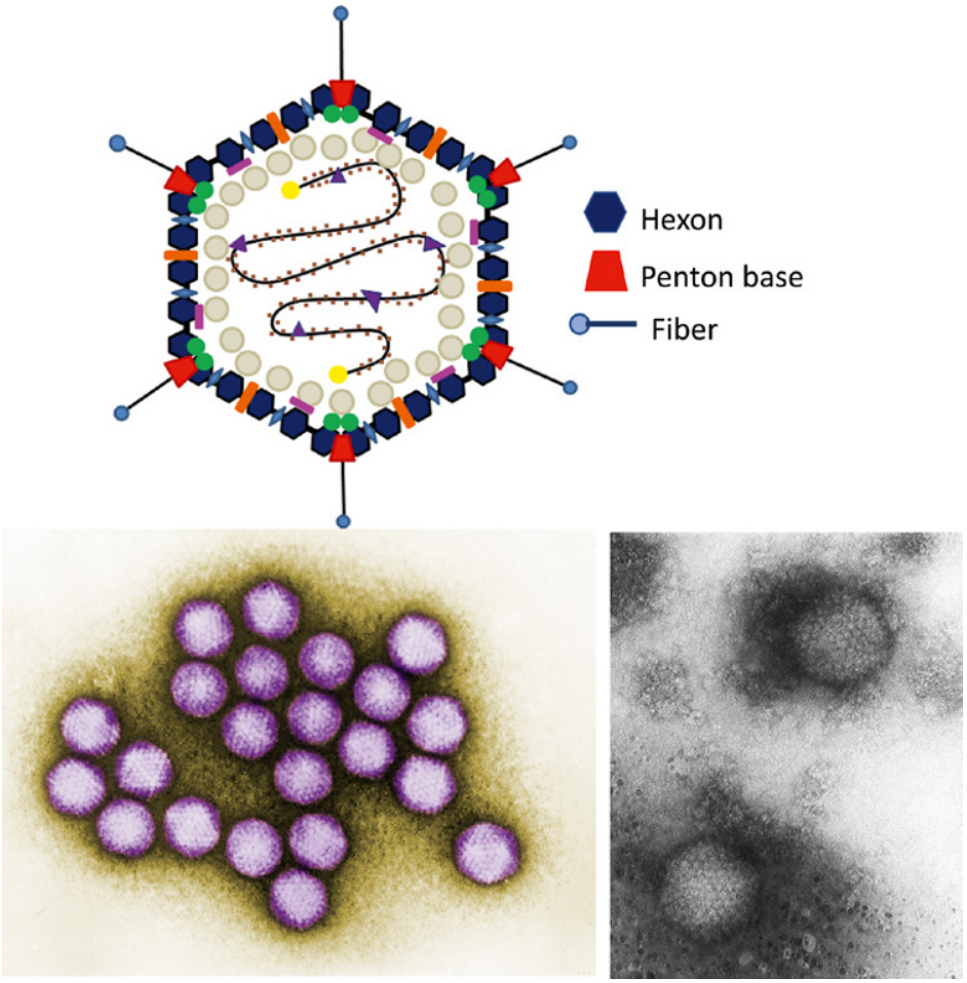


Figure 51.1 Schematic diagram and transmission electron micrograph images of adenoviruses. Top: Adenovirus particle consists of icosahedral capsids surrounding a linear dsDNA and core proteins. The capsids comprise the hexon, penton base, and fiber. This work is licensed under a Creative Commons Attribution 3.0. *Source:* It is attributed to Mary Miu, Yee Wage, and Chor Wing Sing. (2010) Anti-viral drugs for human adenoviruses. *Pharmaceuticals* 3(10):3343–3354. Bottom left: Transmission electron micrograph image of adenovirus virions is reproduced from CDC/Dr. G. William Gary, Jr. 1981. Viruses are digitally colored to show ultrastructural details. Retrieved from Public Health Image Library (PHIL). Bottom right: Transmission electron micrograph image of adenovirus virions. *Source:* Reproduced from CDC/Dr. John Hierholzer. 1974. Retrieved from Public Health Image Library (PHIL).

recovered, but there is little evidence of canine adenovirus being implicated in spontaneous chronic hepatitis in dogs.

Etiologic Agent

ICH is caused by Canine mastadenovirus A, previously canine adenovirus 1 (CAdV-1). CAdV-1 infection was first identified as a cause of epizootic fox encephalitis in foxes in 1925. Infected foxes may develop hepatitis, vasculitis, and encephalitis, and they may serve as potential wildlife reservoir with viruses circulating among foxes. Other wild canids including wolves, foxes, and coyotes are susceptible, and infection of bears, skunks, and otters has been reported. Antibodies to CAdV-1 have been detected in free-ranging terrestrial carnivores and marine mammals. CAdV-1 replicates well in canine kidney cells. Although some strains of CAdV-1 and Canine adenovirus type 2 (CAdV-2) are oncogenic in inoculated newborn hamsters, these viruses have not been associated with neoplastic disease in dogs.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

ICH has a global distribution, but clinical disease has become increasingly rare with proper vaccinations. The virus is transmitted by direct contact with urine (major mode), and less frequently, feces and saliva. Dogs shed viruses in urine for several months after infection.

Pathogenesis and Pathology

Following oropharynx infection, the virus initially replicates in the tonsils and spreads to regional lymph nodes and then

Table 51.1 Diseases of domestic animals caused by adenoviruses.

Virus	Type of disease
Atadenovirus	
Bovine atadenovirus D and E (include bovine types 4–6 and 8)	Conjunctivitis, pneumonia, diarrhea, polyarthritis
Duck atadenovirus A (Duck adenovirus 1)	Egg drop syndrome ’76
Psittacine atadenovirus A	Hepatitis, conjunctivitis, pneumonia, enteritis
Ovine atadenovirus D	Respiratory and enteric
Deer atadenovirus A (Odocoileus adenovirus-1)	Systemic vasculitis with hemorrhage and pulmonary edema
Mastadenovirus	
Bovine mastadenovirus A, B, C (include types 1, 3, 9, and 10)	Conjunctivitis, pneumonia, diarrhea, polyarthritis
Canine mastadenovirus A (Canine adenovirus type 1)	Hemorrhagic and hepatic (infectious canine hepatitis)
Canine adenovirus type 2	Respiratory
Equine mastadenovirus A and B (types 1 and 2)	Respiratory
Ovine mastadenovirus A, B, and C	Respiratory and/or enteric
Porcine mastadenovirus A, B, and C	Diarrhea or meningoencephalitis, or both
Siadenovirus	
Turkey siadenovirus A (turkey adenovirus 3)	Hemorrhagic enteritis of turkeys, marble skin disease in pheasants
Psittacine siadenovirus D and E	Hepatitis, conjunctivitis, pneumonia, enteritis
Aviadenovirus	
Turkey aviadenovirus B (Turkey adenovirus 1)	Respiratory
Duck aviadenovirus B (Duck adenoviruses 2 and 3)	Hemorrhagic liver and kidney
Psittacine aviadenovirus B and C	Hepatitis, conjunctivitis, pneumonia, enteritis
Fowl aviadenoviruses A–E	Hepatitis-hydropericardium syndrome, inclusion body hepatitis, adenoviral gizzard erosion

to the systemic circulation. Viremia results in rapid dissemination of the virus to all body tissues and secretions, including saliva, urine, and feces. The virus has a particular tropism for hepatocytes and endothelial cells, which produces the characteristic signs of the disease. Virus-induced injury to endothelial cells leads to disseminated intravascular coagulation and a generalized bleeding tendency. Bleeding is also a result of impaired synthesis of clotting factors in the liver. Bleeding in the oral cavity and ecchymotic hemorrhage in serosal surfaces and lymph nodes are observed. Infected dogs show marked leukopenia, protein urea, and increased levels of liver enzymes. Gross pathological findings include enlarged and congested liver, blood-tinged abdominal fluid, edematous and congested lymph nodes, and edematous gallbladder. Hemorrhages may be present on serosal surfaces. Characteristic intranuclear inclusion bodies (due to virus replication in the nucleus of infected cells) may be present in hepatocytes, vascular endothelium, and macrophages (Figure 51.2). Deposition of immune complexes may occur in some dogs recovering

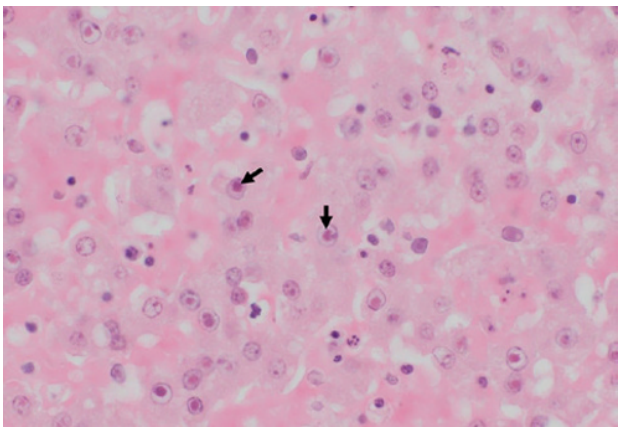


Figure 51.2 Histopathology of liver from a dog infected with canine adenovirus (H&A stain, ×400). Intranuclear adenoviral inclusion bodies (arrows) are present in infected hepatocytes. *Source:* Courtesy of Dr. Bradley Njaa, Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University.

from ICH, which manifests as corneal edema, anterior uveitis, or immune complex glomerulonephritis.

Host Response to Infections

Neonatal pubs or young pubs of one to four weeks of age that lack maternally derived immunity may develop fatal disease. Newborn pubs may collapse after a short illness of one to three days, and litter mortality may reach 100%. Clinical signs are vague and include anorexia, abdominal pain, inactivity, soft feces, incoordination, and coma. Infection of older pubs and adult dogs is generally asymptomatic. Recovery from ICH, regardless of the severity of illness, results in long-lasting immunity that likely is lifelong.

Diagnosis

ICH is suspected in young, unvaccinated dogs with fever, gastrointestinal, and hepatic disease, or corneal edema. Antemortem laboratory diagnosis include detection of virus or virus genome (using PCR) from affected tissue or body secretions. Histological examination of the liver shows hepatic necrosis with intranuclear inclusion bodies. Immunohistochemistry for adenovirus antigen can be performed on the liver. Serological testing to detect antibodies to adenovirus include ELISA, indirect immunofluorescence assay, virus neutralization, or hemagglutination inhibition. A fourfold rise in titer between acute and convalescent serum samples needs to be demonstrated for diagnosis by serology.

Treatment and Control

Therapy is limited to supportive and symptomatic treatment. Control is achieved by vaccination and strict sanitation of affected premises with quarantine of exposed dogs. Live attenuated CAdV-2 vaccines induce heterologous protection against CAdV-1. Maternal antibody is an important component of immunity that protects neonates from ICH. Vaccinations are administered every 2–4 weeks, beginning at 6 weeks of age, until pups are at least 16 weeks old to ensure that maternal antibodies do not interfere with active immunization of pups.

Canine Adenovirus Type 2 Infection

CAdV-2, a member of the genus *Mastadenovirus*, is one of the causative agents of infectious tracheobronchitis (kennel cough) in dogs. CAdV-2 infection is usually mild and restricted to the upper respiratory tract, including nasal mucosa, pharynx, trachea, and bronchi, but interstitial pneumonia may occur in some cases. Secondary bacterial infection or coinfection with other viral pathogen, such as distemper virus, may complicate the disease. CAdV-2 has also been isolated from the gastrointestinal tract, and CAdV-2-associated enteritis has been reported in dogs. Unlike CAdV-1, CAdV-2 does not produce generalized disease or renal and ocular lesions and is not excreted in urine. CAdV-2 vaccines do not produce post-vaccination ocular lesions, and CAdV-2 is antigenically indistinguishable from CAdV-1. Therefore, CAdV-2 vaccines are used to provide cross-protection against ICH. Various laboratory diagnostic methods include virus isolation, hemagglutination, virus neutralization, and PCR detection of virus nucleic acids.

Bovine Adenovirus Infection

There are currently 10 recognized serotypes of bovine adenoviruses, which belong to two genera, *Atadenovirus* and *Mastadenovirus*. Serological surveys indicate that bovine adenovirus infection is common in cattle worldwide. Bovine adenovirus has been isolated from calves with pneumonia but is also frequently isolated from apparently normal cattle. The virus infection is usually clinically inapparent or causes mild respiratory or enteric disease in immunocompetent animals. However, various factors such as virus serotypes, host immunity, stress, and concurrent infection likely influence clinical disease. In immunosuppressed animals, severe pneumonia may occur. Some serotypes have been implicated in enteric disease and death in young cattle. Immunity after natural infection is long-lasting. Diagnostic methods include detecting rising antibody titers, viral isolation, or detection of virus nucleic acid by PCR. Bovine adenoviruses can be isolated from rectal, nasal, or conjunctival swabs or infected tissues. Most types do not produce characteristic cytopathogenic effects until after several blind passages. Histopathology of infected tissues may show the characteristic intranuclear inclusion bodies. No licensed vaccines are available.

Equine Adenovirus Infection

There are two serotypes, Equine adenovirus (EAdV) 1 and 2, which are also called Equine mastadenovirus A and B, respectively. EAdV-1 infection occurs worldwide and is usually associated with upper respiratory tract disease and conjunctivitis. EAdV-2 has been isolated from foals with diarrhea and appears to be associated with gastrointestinal infections. Arabian foals with severe combined immunodeficiency (SCID) are highly susceptible to infection with EAdV-1. Foals with SCID infected with adenovirus develop progressive, fatal pneumonia, and generalized adenovirus infections. Laboratory diagnostic methods include PCR-based equine acute respiratory panel tests for bacterial and viral pathogens, which includes EAdV. Virus isolation may be attempted from infected tissues or nasal and ocular

swab samples in cell culture of equine origin. The virus can be identified by electron microscopy or by immunofluorescence or immunohistochemical staining of infected tissues. Serological assays to detect antibodies include viral neutralization and the hemagglutination-inhibition test. There is no commercially available vaccine.

Porcine Adenovirus Infection

Porcine adenoviruses are members of the genus of *Mastadenovirus*, and five serotypes are currently recognized. Serological survey indicates that porcine adenoviruses are widespread among pig populations, and the virus is commonly isolated from the gastrointestinal tract of normal swine. Virus infection is usually subclinical, but can be associated with enteritis, pneumonia, and conjunctivitis.

Avian Adenovirus Infection

Avian adenoviruses are a diverse group of viruses in *Aviadenovirus, Atadenovirus*, and *Siadenovirus* genera. Avian adenoviruses are commonly found in domestic and wild birds without apparent diseases. However, some adenoviruses cause specific diseases in different bird species, which include egg drop syndrome '76, hemorrhagic enteritis (HE) of turkeys, marble spleen disease (MSD), avian adenovirus splenomegaly (AAS), hepatitis-hydropericardium syndrome (HHS) (HHS), inclusion body hepatitis (IBH), and adenoviral gizzard erosion (AGE).

Egg Drop Syndrome '76

Disease

Egg drop syndrome causes significant economic losses from a transient decline in normal egg production in laying hens. It is characterized by the production of thin-shelled or shell-less eggs or eggs with other abnormalities. Layers show no apparent signs of illness.

Etiologic Agent

The causative agent is Duck atadenovirus A (previously Duck adenovirus 1) in the genus *Atadenovirus*. Only one serotype is known.

Infectivity for Other Species and Culture Systems

Reduced egg production associated with duck adenovirus infection has been reported in Japanese quails and turkeys. Natural and experimental infections of ducklings and goslings with respiratory disease have been reported, but it appears rare. Antibodies to duck adenovirus are commonly found in wild waterfowl and other aquatic birds. However, they are generally asymptomatic, or little is known about their clinical significance. Duck adenoviruses grow well in embryonated duck and goose eggs and in some cell cultures originating from ducks and chicks.

Distribution, Reservoir, and Transmission

Natural hosts appear to be waterfowl, including wild and domestic ducks and geese, which can be the source of virus transmission to chickens by direct contact or contact with water contaminated with droppings. In chickens, both vertical and horizontal transmission can occur by infected eggs or infected, apparently healthy birds. In horizontal infection, birds get infected mainly by the fecal–oral route, but fomite transmission by contaminated equipment or egg trays also occurs often.

Pathogenesis and Pathology

Chicks vertically infected through the egg may excrete viruses, but more often they remain latent until they reach sexual maturity, at which time viruses are shed in the eggs and droppings. After layers are infected by other infected layers or through contact with infected ducks or contaminated water, viruses replicate in lymphoid tissue and subsequently in the pouch shell gland, which coincides with the production of abnormal eggs (8–20 days after infection). Some may have transient diarrhea. Profuse virus replication occurs in the pouch shell gland, but not in the intestinal tract, and viruses in the droppings originate from secretions from the oviduct.

Host Response to Infections

In naïve layer flocks, the commercially usable egg production may drop by 10–50% for several weeks before returning to normal. In flocks with some existing antibody, the effect on egg production is usually small, and a series of small-scale disease episodes may be noticed.

Laboratory Diagnosis

Virus isolation using embryonated duck or goose eggs or in susceptible cell culture, PCR detection of virus nucleic acids, antigen-capture ELISA, or immunofluorescence can be done using the reproductive tissue (oviduct, uterus, or pouch shell gland) of the affected layers. In goslings and ducklings with respiratory infection, respiratory tract tissues are mainly used for detection of virus. In unvaccinated flocks, serological tests such as the hemagglutination-inhibition test using fowl red blood cells, ELISA, and serum neutralization are used. The double immunodiffusion test used to be the common assay. Emphasis should be placed on selecting recently affected birds to ensure accurate diagnosis. Young chickens infected through the egg or before sexual maturity may not develop antibodies until they mature.

Treatment and Control

There is no specific treatment. Implementation of biosecurity measures to reduce exposure to waterfowl and fomites can reduce the risk of virus transmission. Inactivated vaccines can prevent disease and decrease virus shedding. These vaccines are given prior to laying, usually between 14 and 20 weeks of age. Sentinel chickens can be used to monitor virus circulation in vaccinated flocks.

Turkey Siadenovirus A Infections in Poultry

Turkey siadenovirus A, previously Turkey adenovirus 3, is the causative agent of several disease syndromes in different bird species, which include HE of turkeys, MSD of pheasants, and AAS. Viruses responsible for these diseases are serologically indistinguishable. However, it appears that virus strains of varying virulence exist as the severity of clinical manifestation differs within the same bird species. The severity of clinical disease increases with the presence of secondary infection, which may follow immunosuppression caused by the initial virus infection. Splenomegaly is commonly observed.

Hemorrhagic Enteritis

HE is a disease of growing turkeys, typically affecting turkeys 6–12 weeks of age. Turkey poults of <3–4 weeks of age are generally resistant to clinical disease until maternal antibody levels wane. Infected birds show no apparent signs of illness or may manifest clinical signs such as depression and bloody droppings from gastrointestinal hemorrhage. The mortality during the acute phase varies among flocks, with an average of 10–15%. Surviving birds can be infected with a secondary bacterial infection due to transient immunosuppression, resulting in a second peak in mortality. The lesions include intestinal congestion and hemorrhages and enlarged spleen with lymphoid necrosis, reticuloendothelial cell hyperplasia, and typical basophilic intranuclear inclusion bodies. The fecal-oral/cloacal route is the main route of virus transmission, and viruses are readily transmitted by persons and equipment contaminated with virus-containing droppings. There is no evidence of vertical transmission.

Marble Spleen Disease

MSD is an acute respiratory disease of captive-reared pheasants aged three to eight months. Clinical signs include dyspnea, asphyxiation, and sudden death due to pulmonary congestion and edema. The mortality rate ranges from 2% to 15%. Secondary bacterial infections may occur due to immunosuppression. The histological features of splenomegaly are similar to those seen in birds with HE.

Avian Adenovirus Splenomegaly

AAS is a disease of broiler chickens 20–45 weeks of age. The affected birds develop lung hemorrhage and edema, and splenomegaly, with pathology similar to HE.

Laboratory Diagnosis

PCR detection of viral nucleic acid is commonly done. Isolation of virus is time-consuming due to limited options for propagating viruses. Serological tests such as agar gel immunodiffusion or ELISA are performed for confirmation.

Treatment and Control

Live vaccines for HE and MSD are used in drinking water at four to five weeks of age when maternal antibody levels wane. Secondary bacterial infections may be treated with antibiotics. Control is achieved by vaccination and strict sanitation, all-in, all-out management, and implementation of good biosecurity.

Fowl Aviadenovirus Infections in Poultry

Several serotypes and species of fowl aviadenoviruses are causative agents of important disease complexes in poultry. The diseases include hepatitis-hydropericardium syndrome, IBH, and AGE in chickens. Adenoviruses, particularly fowl aviadenoviruses, are also reported to cause quail bronchitis with a high mortality, especially in young quails <3 weeks of age.

Hepatitis-Hydropericardium Syndrome

HHS is predominantly caused by serotype 4 (species C) and usually affects three- to seven-week-old broiler chickens. Affected birds show nonspecific clinical signs, and often have a sudden spiking increase in mortality ranging from 2% to 40%. Discolored, enlarged liver with multiple necrotic or hemorrhagic foci and basophilic intranuclear inclusion bodies are seen. Accumulation of straw-colored fluid in the pericardial sac is a characteristic gross finding. Live and inactivated vaccines are used for boiler chickens.

Inclusion Body Hepatitis

IBH is caused by serotypes 2 and 11 (species D) and 7, 8, and 8b (species E), and mainly observed in three- to seven-week-old broiler chickens. Affected flocks have nonspecific clinical signs with a sudden increase in mortality of 1–10%. The main lesions include swollen, mottled liver with necrotic foci. Live and inactivated vaccines are used for boiler chickens.

Adenoviral Gizzard Erosion

AGE is an emerging disease in boiler chickens and caused by serotype 1 (species A). Affected chickens may not have

apparent clinical signs, but several natural outbreaks in broilers of 9–36 days of age with uneven growth, depression, and dull feathers have been reported in several countries. Gross lesions include multiple, focal gizzard erosions, and necrotizing ventriculitis, and basophilic intranuclear inclusion bodies in glandular epithelial cells of the gizzard are seen microscopically.

Distribution, Reservoir, and Transmission

Horizontal transmission mainly occurs by the fecal-oral route. Vertical transmission has been reported with certain serotypes associated with HHS and IBH.

Laboratory Diagnosis

A tentative diagnosis is made based on gross lesions and the mortality pattern, which is confirmed by PCR or histopathology by detecting typical microscopic lesions and basophilic intranuclear inclusion bodies.

Adenovirus Infection in Psittacine

Adenovirus infections are usually subclinical. However, several adenoviruses are associated with diseases in psittacine, and fowl aviadenoviruses have been mainly detected from diseased birds. Clinical signs include depression, anorexia, diarrhea, and cloacal hemorrhage. Some affected birds may be found dead. Gross lesions affecting the liver, spleen, intestines, and lungs are seen.

Ovine Adenovirus Infection

Adenoviruses are often isolated in clinically normal sheep, and high seroprevalence suggests that adenovirus infections in sheep are common. Seven serotypes have been identified, and clinical presentations are typically inapparent or mild. However, some adenoviruses in the genera *Atadenovirus* and *Mastadenovirus* have been associated with respiratory disease and enteritis, and lambs and kids are most often affected. Experimental infection of adenoviruses in lambs produced more severe pneumonia following coinfection with a bacterial pathogen than a single infection of adenovirus, which suggests that adenovirus may have a role in disease pathogenesis.

Adenovirus Hemorrhagic Disease of Deer

Deer atadenovirus A (previously Odocoileus adenovirus 1, OdAdV-1) causes adenovirus hemorrhagic disease of deer. Transmission is by direct contact between deer or contact with bodily fluid. Airborne transmission may also occur. Susceptible animals include mule deer, white- and black-tailed deer, Rocky Mountain elk, and moose. Fawns and juveniles are more susceptible to disease, and mortality can be high. Adenovirus-associated hemorrhagic disease was first described in outbreaks with a high mortality among black-tailed mule deer in California in 1993–1994, and subsequently identified in multiple states as sporadic cases or outbreaks. Virus infection can manifest as an acute systemic form or as a chronic localized form. In the acute form, localized or systemic vasculitis leads to pulmonary edema and hemorrhagic enteropathy with clinical signs such as dyspnea, foaming, or drooling from the mouth, diarrhea (can be bloody), and seizures. The chronic localized form manifests as oral ulcer/abscess, weight loss, and death. Diagnosis is based on the characteristic gross and histological lesions, immunohistochemistry, or PCR. It is difficult to isolate the virus in culture.

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Herpesviridae

Rebecca P. Wilkes and Jobin Kattoor

Herpesviruses have been found in virtually every species that have been investigated and cause significant disease in every domestic animal species, except sheep. Within this group of viruses, there is wide variation in biological properties including pathogenicity and oncogenic potential. Herpesviruses are morphologically similar, with a double-stranded DNA core and an icosahedral capsid consisting of 162 capsomeres, surrounded by a granular zone composed of globular proteins (tegument) and encompassed by a lipid envelope (Figure 52.1). The genome of herpesviruses is large, 125–290 kilobases (kb), and encodes many different proteins; functions of the proteins encoded by the viral genome include virus replication, virus structural proteins that are needed for cell attachment and cell-to-cell spread, and a variety of proteins that regulate cell growth and modulate the host's antiviral response.

Viral replication and encapsidation occur in the nucleus, and the envelope is obtained by budding through the inner layer of the nuclear envelope. Intranuclear inclusion bodies are a characteristic feature of infection (Figure 52.2). Infection with herpesviruses results in lifelong latent infection with subsequent recrudescence and intermittent or continuous shedding of virus.

Herpesviruses do not survive well outside of the host. Transmission usually requires close contact, but respiratory droplet spread is important in facilities with confined populations, such as cattle feedlots and animal shelters. The virus may survive longer in cool, moist environments, but latently infected animals serve as the reservoir for transmission. Even though it is rare, the virus is documented to transmit transplacentally to young ones from an infected dam.

Herpesviruses have been assigned to the order *Herpesvirales*, which contains three distinct families: *Herpesviridae* includes herpesviruses of birds, mammals, and reptiles; *Alloherpesviridae* includes the herpesviruses of fishes and frogs; and *Malacoherpesviridae*, which contains viruses isolated from oysters (Ostreid herpesvirus 1) and abalones (Haliotid herpesvirus 1). *Herpesviridae* consists of three major subfamilies, *Alpha-*, *Beta-*, and *Gammaherpesvirinae,* which were initially distinguished by host range, duration of replication, reproductive cycle, cytopathology, and latent infection characteristics. The *Alphaherpesvirinae* are generally highly cytopathic in cell culture, have a relatively short replication cycle (24 hours), and frequently cause latent viral infections in sensory ganglia. Most of these viruses are restricted in their host range, indicating that they evolved with their hosts over long periods of time. *Betaherpesvirinae* have a variable host range and a long replication cycle; infected cells often become enlarged (cytomegaly), hence their designation as cytomegaloviruses. Latency can be established in numerous tissues, including secretory glands, and lymphoreticular tissues. *Gammaherpesvirinae*, with some exceptions, tend to be tropic for B or T lymphocytes (lymphotropic), replicate in lymphoblastoid cells, and may cause lytic infections in certain types of epithelial and fibroblastic cells. Infection is frequently arrested at a prelytic stage with persistent and minimum expression of viral genome in the cell. Latency is established frequently in lymphoid tissue. The host range is narrow, with experimental hosts usually limited to the order of the natural host.

Equid Herpesviruses

All the nine equine herpesviruses (EHVs) isolated to date are alpha- or gammaherpesviruses (Table 52.1). EHVs 1–5 produce disease in horses, whereas EHVs 6–8 produce disease in donkeys and wild equids. EHV9 produces little or no illness in equid species, and zebra are thought to be the natural host and reservoir. The virus has not been found naturally in domestic horses. EHV9 has been shown to produce neurotropic pathogenicity in non-equids, including Thomson's gazelles, a reticulated giraffe, and a polar bear. Some additional gammaherpesviruses have been

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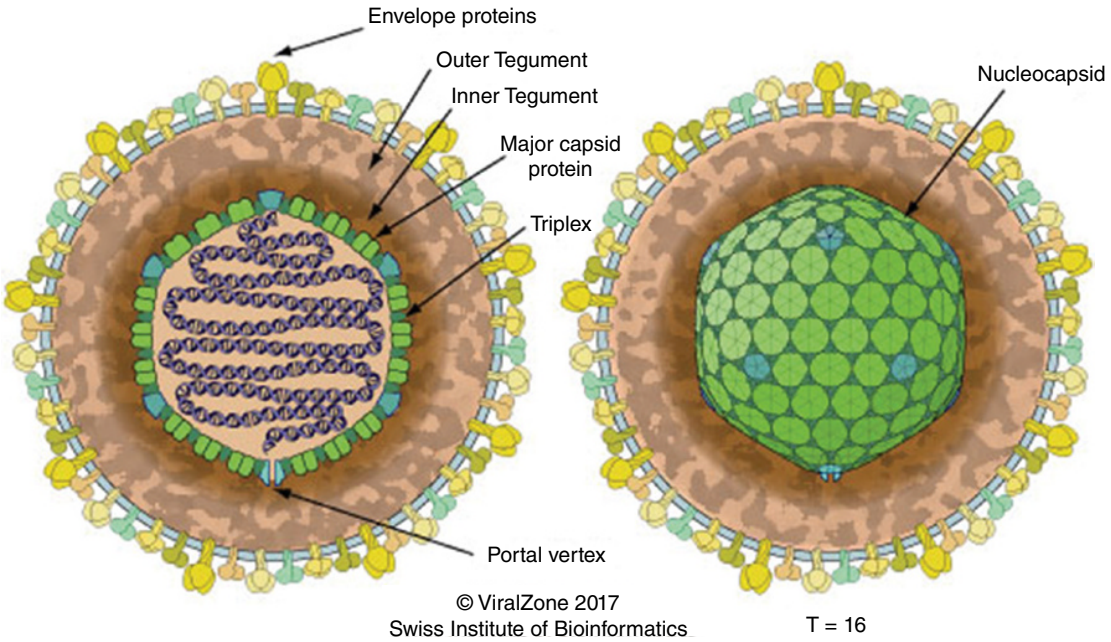


Figure 52.1 Herpesvirus structure: enveloped, spherical to pleomorphic, 150-200 nm in diameter, T=16 icosahedral symmetry. Capsid consists of 162 capsomers and is surrounded by an amorphous tegument. Glycoproteins complexes are embed in the lipid envelope. *Source:* Image and caption reproduced from ViralZone.

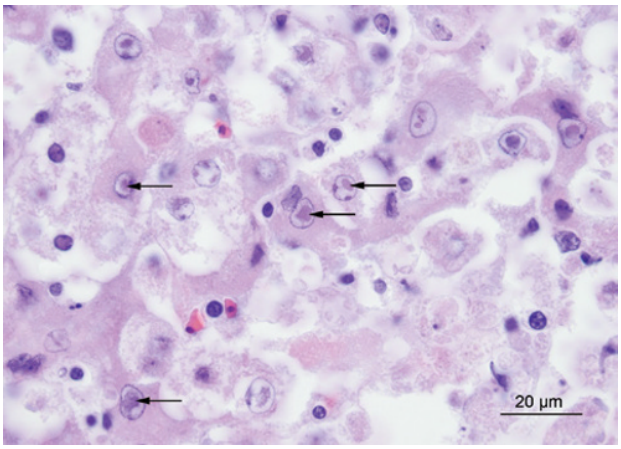


Figure 52.2 Photomicrograph of the liver (100×) stained with hematoxylin and eosin. Several nuclei with peripheralized chromatin and herpesviral eosinophilic intranuclear inclusion bodies are indicated by arrows. *Source:* Photo courtesy of Dr. Robert Donnell, The University of Tennessee.

isolated from donkeys with neurological disease or severe interstitial pneumonia, but these viruses have not been well characterized.

EHV1

Disease

EHV1 causes disease in horses, resulting in frequent outbreaks of respiratory disease, abortions, neonatal foal death, and myeloencephalopathy. Although abortion in mares may occur as early as four months of gestation, it most frequently occurs between the seventh and eleventh months of gestation and usually without any premonitory signs. Foals infected *in utero* may be born alive but are usually weak and die within two to three days. Naturally occurring respiratory disease due to EHV1 is characterized by fever, anorexia, nasal discharge of varying severity, and ocular discharge. Recrudescence with respiratory disease is common in young performance horses (under two years of age) during periods of stress (e.g. during periods when horses are comingled) that were infected within EHV-1 the first weeks or months of life. This can result in interruptions in athletic training programs. In addition, EHV1 infection can lead to chorioretinopathy, causing permanent lesions, which can be focal, multifocal, or rarely, diffuse, affecting the whole eye, in a substantial proportion of infected horses. Clinically, only diffuse lesions have a significant impact and cause loss of vision.

Equine herpesvirus myeloencephalopathy (EHM) with variable neurological signs ranging from ataxia, urinary incontinence and limb paresis to paralysis and death has been associated with certain strains of EHV1. EHM is a relatively uncommon manifestation of EHV1 infection because transfer of the virus to the central nervous system (CNS) endothelium from the bloodstream during viremia is not a common occurrence (typically 10% of infected horses develop neurological signs during EHM outbreaks). Recently, these outbreaks have been reported with increased frequency, and devastating losses have occurred. EHM results in extensive movement restrictions, disrupting breeding or training schedules and causing management difficulties at training centers,

Table 52.1 Equid herpesviruses.

Species	Genus	Common name
Equid alphaherpesvirus 1	*Varicellovirus*	Equine abortion virus
Equid gammaherpesvirus 2	*Percavirus*	NA[a]
Equid alphaherpesvirus 3	*Varicellovirus*	Equine coital exanthema (ECE)
Equid alphaherpesvirus 4	*Varicellovirus*	Equine rhinopneumonitis virus
Equid alphaherpesvirus 5	*Percavirus*	NA
Equid alphaherpesvirus 6	Unassigned	Asinine herpesvirus 1
Equid gammaherpesvirus 7	Unassigned	Asinine herpesvirus 2
Equid alphaherpesvirus 8	*Varicellovirus*	Asinine herpesvirus 3
Equid alphaherpesvirus 9	*Varicellovirus*	NA

[a] Not applicable.

racetracks, and horse events. Neuropathogenic strains in which the there is a change in an amino acid from asparagine to aspartic acid (N752D) in the viral DNA polymerase (ORF 30) have been identified. However, strains lacking this mutation are also associated with EHM.

Etiologic Agent

Physical, Chemical, and Antigen Properties

The equid herpesviruses have typical morphology and cannot be distinguished from each other based on their morphology. They are, however, antigenically distinct and can be distinguished by serological assays.

Resistance to Physical and Chemical Agents

The equid herpesviruses are inactivated by ether, acid (pH 3), drying, exposure to heat of 56 °C for 30 minutes, and detergents and commonly used disinfectants.

Infectivity for Other Species and Culture Systems

EHV1 usually infects only horses; however, ocular disease due to EHV1 characterized by vitritis, retinitis, and optic neuritis leading to blindness has been described in New World camelids (alpacas, llamas). EHV1 also produces encephalitis and acute death in these species. Abortions due to EHV1 have been described in cattle and zebras.

EHV1 can be propagated in equine fetal kidney, rabbit kidney, and L (mouse fibroblast) cells, resulting in the formation of cytopathic effect (CPE) and intranuclear inclusion bodies.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

EHV1 is prevalent in horses worldwide. Current estimates of the prevalence of latent EHV1 infection suggest a rate of 60%, though this number is likely higher because of the limitations of tests in detecting latent infections. For practical purposes, clinicians should presume that the majority of horses are latently infected with EHV1. The source of the majority of infections is likely from reactivation of virus and spread by direct contact with respiratory secretions from these latently infected horses. Transmission may occur by contact with virus-laden aborted fetuses or placentas, and it is possible that dogs, foxes, and carrion birds may carry infection with fragments of aborted fetuses from one farm to another. Therefore, aborted material should be removed as soon as possible and can be submitted to the lab for diagnostic purposes. Aerosol transmission through inhalation of respiratory droplets is considered less important than direct contact or spread through secretions on fomites.

Pathogenesis and Pathology

Primary EHV1 replication occurs in the upper respiratory tract epithelial cells, followed by replication in mononuclear cells and dendritic cells. The virus from the initial replication sites migrates to the local lymph nodes and beyond, resulting in leukocyte-associated viremia. Studies reveal that the herpesvirus uses the "Trojan horse" mechanism in leukocytes to disseminate in the blood circulation even though enough virus-neutralizing (VN) antibodies are present in the infected animal. This is achieved by the late expression of antigens (gC and gD) recognized by neutralizing antibodies in the infected leukocytes. This leukocyte-associated viremia in an acute infection has been shown to be a prerequisite for abortion and EHM by initiating replication of EHV1 in the endothelial cell lining of blood vessels in the pregnant uterus or CNS (Figure 52.3). A widespread infection of endometrial blood vessels results in severe vasculitis and multifocal thrombosis, which could result in an abortion of a virus-negative fetus. Less extensive uterine vascular pathology may allow focal transfer of virus across the utero-placental barrier and abortion of a virus-infected fetus. A near-term transplacental EHV1 infection could result in the birth of

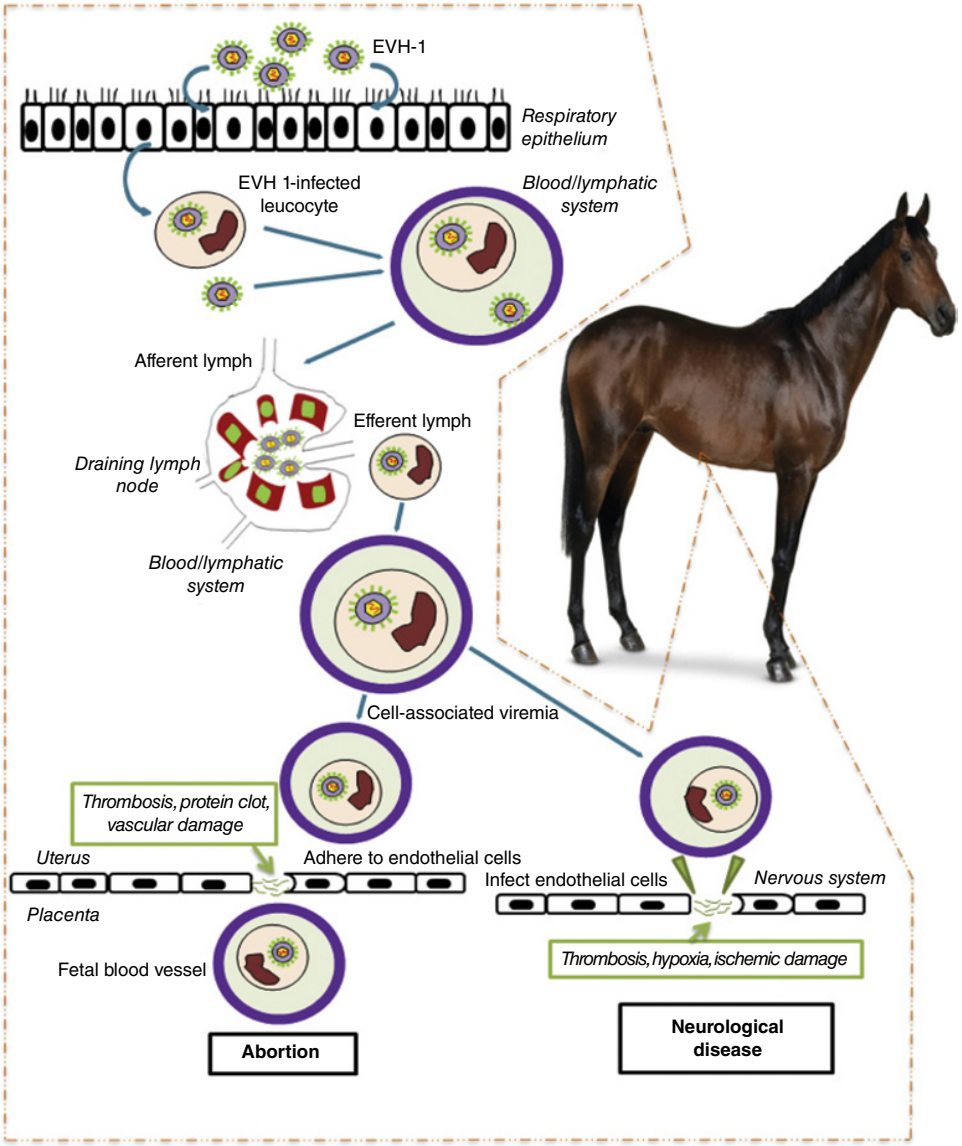


Figure 52.3 Schematic representation of EHV1 pathogenesis. *Source:* Reproduced from Khusro et al. (2020). With permission of Elsevier.

live infected foals, which usually die a few days afterward – a condition known as neonatal foal disease. These differences in abortigenic potential may relate to differences in the level of viremia or endothelial cell infection induced by different virus strains.

Macroscopically, the most prominent lesions of the virus-infected fetus are jaundice, lung and mucous membrane petechiation, subcutaneous and pleural edema, straw-colored fluid in abdominal and pleural cavities, splenic enlargement with prominent lymphoid follicles, and hepatomegaly with focal necrosis. The severity of the lesions may vary, and some aborted fetuses may fail to show any apparent gross lesions. Histologically, there is bronchiolitis, pneumonitis, severe necrosis of the splenic white pulp, and focal hepatic necrosis, all accompanied by intranuclear inclusion bodies. The early fetus (<3 months) shows little or no response to the viral infection. However, the fetus in its last four months of gestation shows a marked ability to react in a specific manner to the presence of the virus. Lesions in the fetus less than seven months of age differ from those in older fetuses, suggesting that the lesions represent a fetal response to the virus.

The neurological signs of EHM do not result from direct damage to neurons or glial cells by viral infection but instead result from diffuse, multifocal myeloencephalopathy secondary to vasculitis, hemorrhage, thrombosis, and ischemic neuronal injury due to viral replication in the endothelial cells lining the arterioles of the CNS. The lesions are focal, and neurological signs are associated with the sites and extent of CNS infection. The spinal cord gray

and white matter are most commonly affected. Clinical signs initially reflect lower motor neuron defects, including an initial dysuria, constipation, perineal analgesia, protrusion of the penis, locomotor dysfunction varying from mild ataxia to severe paralysis, and possible tetraplegia. When the cortex or brainstem is involved, encephalitic signs, including convulsions, may occur.

Host Responses to Infection

Virus persists in circulating leukocytes for as long as nine days following infection. Abortion in mares may occur as long as 90–120 days following infection. Both complement-fixing (CF) and VN antibodies appear in the sera of infected horses. In general, CF antibodies are demonstrable for six months after infection, with VN antibodies persisting longer. IgG antibodies against the viral envelope neutralize virus, whereas those against the nucleocapsid do not. Results from an experimental challenge study failed to reveal any significant relationship between pre-exposure VN antibody titer and the magnitude of post-infection EHV1 viremia. The intracellular location of EHV1 during most of its infection cycle within horses may limit the effectiveness of VN antibody in controlling cell-associated EHV1 viremia. Experimentally, pregnant mares with antibody to EHV1 may abort when challenged with EHV1.

Also, the concentration of antibodies prior to virus inoculation does not correlate with protection against challenge with neuropathogenic EHV1. The factors that determine development of EHM are not well understood but have been shown to be age (older horses, >5 years old, are more at risk), breed (ponies and smaller breeds are less commonly represented), and sex (females are more commonly affected) related. It was proposed that development of the neurological disease was based on the magnitude of cell-associated viremia, suggesting that a higher magnitude and duration of viremia would result in EHM. Neuropathogenic strains of EHV1 are thought to replicate more efficiently than non-neuropathogenic strains, and the quantitative load of circulating virus that follows infection by EHV-1 was defined as a major risk factor for post-exposure development of EHV-1 CNS disease. However, evidence to support this is contradictory, with some studies demonstrating similar levels of viremia in horses with EHM and control horses. Mechanisms associated with the development of EHM likely extend beyond high levels of viremia. A more recent study suggests that utilization of different receptors and the efficiency of attachment or entry of infected leukocytes into endothelial cells in response to host factors may play a role in the development of EHM. Although the specific immune mechanisms required for control of EHV1 neurological disease are largely unknown, data suggest that presence of a high frequency of pre-exposure EHV1-specific memory cytotoxic T lymphocytes is significantly correlated with protective immunity against development of EHM. A previous study also demonstrated an association between a high frequency of memory cytotoxic T lymphocytes and resistance to abortion in ponies exposed to EHV1. The relationship between immunity and EHM risk has not been defined and requires further study.

Laboratory Diagnosis

Virus culture and isolation from the nasal and nasopharyngeal swab and or buffy coat are considered the gold-standard test for EHV-1. In cases of abortion, diagnosis is based on characteristic lesions with intranuclear inclusions present in the fetal liver, spleen, lung, and thymus. The same tissues provide a good source of virus, which can be demonstrated by immunofluorescence or immunohistochemical staining of tissue sections. Histopathological examination of the brain and spinal cord is essential in confirming EHV1 infection in a horse with suspected EHM. Vasculitis and thrombosis of small blood vessels in the spinal cord or brain are consistent changes, and virus detection in the CNS is achieved using immunohistochemistry, in situ hybridization, or polymerase chain reaction (PCR). Virus isolation can be used to confirm the diagnosis in abortion cases, but isolation from neural tissues in EHM cases is difficult. Definitive antemortem identification is difficult in EHM due to the lack of consistent results, but an increase of protein concentration in cerebrospinal fluid and xanthochromia along with the demonstration of EHV-1 in blood or nasal discharge can be considered as a definitive diagnosis.

PCR has become the diagnostic test of choice due to its high sensitivity and specificity, and PCR detection of EHV1 is routinely performed on secretions from nasal or nasopharyngeal swabs or from uncoagulated blood samples. Detection of virus in the blood suggests active infection because latent virus should not be present in the blood. Real-time PCR and use of Ct values are helpful for evaluating the level of respiratory shedding of virus by testing a nasal swab. The lower the Ct value, the more the amount of virus being shed.

A variable region in the EHV1 DNA polymerase gene (ORF 30) involved in initial viral replication within cells has been linked to EHM. PCR assays based on ORF 30 have been developed and used to differentiate between EHV1 isolates from neurologically and non-neurologically affected horses. These tests are based on a single nucleotide polymorphism that changes the asparagine (N) amino acid residue to an aspartic acid residue (D) at position 752 in ORF30. D752 viruses are more commonly isolated from horses that suffered from neurological disease than are

N752. Targeted mutation of the D752 to the N752 genotype in a neurovirulent isolate resulted in attenuation of virulence, specifically reduced levels of viremia, reduced capacity to cause neurological disease, and reduced severity of other clinical signs. However, the genotyping of field isolates needs to be interpreted carefully, because between approximately 14% and 24% of EHV1 isolates from horses with EHM do not have this neuropathogenic marker. Of note, the N752 variants are also pathogenic and have been responsible for approximately 95–98% of abortion outbreaks in the United States, United Kingdom, and other countries; are the most common variants found in the field; and have been associated with EHM outbreaks. In addition, horses can be coinfected with both variants. Any horse with suspected EHM should be handled with rigorous disease control measures, regardless of the D/N752 genotype. However, strain characterization may be helpful to determine the possibility of increased risk for a neurological disease outbreak, given that the odds of neurological disease when infected with the neurotropic EHV1 genotype versus the non-neurotropic genotype are 162 times greater. Serology that demonstrates a fourfold or greater increase in serum antibody titer, using serum neutralization tests, on acute and convalescent samples collected 7–21 days apart provides presumptive evidence of infection. The mares' sera may, but does not invariably, demonstrate a rise in titer. Also, many horses with EHM do not exhibit a fourfold rise in serum neutralization titer, because titers rise rapidly and may have peaked by the time neurological signs appear. Although diagnosis using samples from an individual horse may be unrewarding or impossible, obtaining samples from in-contact horses (affected and unaffected) as well may work to show seroconversion to prove EHV1 was the disease-inciting agent. Importantly, many serology-based tests cannot distinguish between antibodies against EHV1 and EHV4. The presence of antibodies does not indicate protective immunity in horses, so "vaccine titers" are not useful.

Treatment and Control

Both modified-live and inactivated vaccines are commercially available and widely used; however, the vaccines do not prevent infection, the development of viremia, or the establishment of latency. Regular vaccination of mares has been shown to reduce the risk of EHV1-induced abortion, but no vaccine currently offers protection against the development of EHM, and it has been observed in horses regularly vaccinated against EHV1 at three to five monthly intervals with inactivated or modified-live vaccines. Horse owners must develop an understanding of the concept of boosting herd immunity to help protect individual horses rather than having an (as yet unattainable) expectation that the attending veterinarian can reliably protect an individual horse from developing potentially fatal EHM by administering one of the currently available vaccines. Because EHV1 is endemic in many equine populations, most mature horses have developed some immunity through repeated natural infection; thus, most mature horses do not develop serious respiratory disease when they become reinfected but may be a source of infection for other susceptible horses. Frequent vaccination of non-pregnant mature horses is not generally indicated. Pregnant mares should be vaccinated during the fifth, seventh, and ninth months of gestation with an inactivated vaccine licensed for prevention of abortion. The vaccine can also be administered during the third month of gestation and at the time of breeding. Vaccination of mares with an inactivated vaccine four to six weeks prior to foaling is commonly done to enhance antibodies in colostrum. This may decrease the incidence of respiratory disease in foals but will not prevent infection. Foals should be administered a primary series of three doses of inactivated or modified-live virus vaccine beginning at four to six months of age, with a four to six week interval between the first and second doses. The third dose should be administered at 10–12 months of age. Immunity is short-lived, so revaccination of adults at six-month intervals is needed for at-risk horses.

Thus, disease control measures are important for preventing the spread of virus. Limiting traffic in and out of brood-mare barns and weanling fields, minimizing stress to pregnant mares, and separating pregnant mares from the remaining population have been suggested to help prevent abortion disease. There should be an isolation period of at least three weeks of all mares introduced to a stud farm or returning after leaving the premises.

It is known that the nasal secretions of horses with EHM contain large amounts of replicating virus and these secretions in particular contribute to the spread of disease to other susceptible individuals. As a consequence, horses suspected of having EHM must be removed from the stable environment as quickly as possible and placed in strict isolation. Both aerosol and fomite transmission are modes of transmission, so biosecurity measures are important to prevent further spread. Quarantine of premises is required for a period of 28 days after the occurrence of any new cases. This period can be reduced to 14 days by testing nasal swabs from all horses by real-time PCR for 2–4 consecutive days. In the face of an outbreak, vaccination can be used in horses at increased risk of exposure. Booster vaccination leads to a rapid anamnestic response and reduced virus shedding, but prevention of EHM should not be expected.

The treatment of EHM is challenging, and the outcome is directly related to the severity of the neurological deficits in the affected horse. The management of affected animals is directed toward supportive nursing and nutritional care,

frequent bladder and rectal evacuation, and in some cases, reducing CNS inflammation with corticosteroids. There is poor understanding regarding the efficacy of steroid use in treating EHM, and thus use is reserved for cases presenting in recumbency or with severe ataxia (guarded prognosis for survival). Antiviral drugs are also unproven in terms of treating EHM, though there has been increased use. Valacyclovir shows greater promise than acyclovir in terms of bioavailability in horses. Currently, the effects of timing of valacyclovir administration relative to the onset of EHV1 infection or EHM development on treatment outcome are unknown. Affected horses that remain standing have a good prognosis, and improvement is generally apparent within a few days, though some horses may be left with residual neurological deficits.

EHV2

Disease

EHV2 is prevalent in horses worldwide and has been isolated from both clinically healthy and ill horses. It has been isolated from cases of superficial and chronic pharyngeal lymphoid follicular hyperplasia, mild respiratory disease, and horses with gastroesophageal ulcers, foals with pneumonia, and foals with keratoconjunctivitis. It has also been associated with granulomatous dermatitis and abortion. Considerable skepticism has been expressed by some concerning its role in disease. The relationship of EHV2 to disease causality is uncertain because of the ubiquity of the virus in horse populations worldwide. The fact that gammaherpesviruses are optimally adapted to their natural hosts means that significant clinical expression of infection is rarely encountered.

Etiologic Agent

Physical, Chemical, and Antigen Properties

EHV2 is a typical gammaherpesvirus.

Resistance to Physical and Chemical Agents

The virus is labile in the environment, and infectivity is destroyed by heat, drying, detergents, and common disinfectants.

Culture Systems

Primary rabbit kidney and equine dermal cells as well as hamster embryos can be used for virus isolation.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

EHV2 has been isolated from horses worldwide. Ninety-seven percent of horses less than one year old have antibodies to EHV2, and in one study, the virus could be isolated from the leukocytes of 88.7% of normal horses. Similarly, virus was isolated from 68 of 69 foals sampled once between one and eight months of age. Both healthy and clinically ill horses act as a reservoir for the virus. In ponies experimentally inoculated with EHV2, the virus was recovered up to 118 days post inoculation. The virus is latent in B lymphocytes and is frequently isolated from nasopharyngeal swabs following infection or viral recrudescence. Foals generally shed higher loads of EHV2 in nasal secretions early in infection, and progressively less with age.

Pathogenesis and Pathology

Little is known about the pathogenesis of EHV2. It is suggested that infection is initiated in tonsils, with replication in other sites based on an observed viremia and its cell-associated nature. Young foals are probably infected by their dams and can be infected despite the presence of maternal antibodies. The virus can then be spread horizontally to in-contact foals. Sequence heterogeneity exists among strains, and horses may be infected with more than one strain at a time. Even though DNA viruses are more stable than their RNA counterparts in terms of mutations and genetic heterogeneity, EHV2 has been shown to have distinct genetic lineages inside a single host, a possible mechanism to evade neutralizing epitope-mediated immune responses elicited by the host. One study suggests the heterogeneity may be due to positive selection, producing variation in a neutralization domain; however, strain variation and viral load have not been correlated to any clinical manifestation of disease. A recent study suggests that disease produced by EHV2 may be immunologically mediated, similar to infectious mononucleosis caused by the related gammaherpesvirus, Epstein–Barr virus (human herpesvirus 4). Fever in individual foals was associated with an increase in EHV2-specific responses of PBMC, though the results were not statistically significant. Several aspects of the immune response of the foals and EHV2 infection status were unrelated to occurrence of clinical disease in the foals. Although EHV2 has been associated with various forms of clinical disease in young horses and in experimental animal models of immunosuppression, the role of this virus in causing fatigue, poor performance, or immunomodulation requires further study.

Laboratory Diagnosis

There are no specific diagnostic features associated with EHV2 infection. The virus can be detected from nasal and pharyngeal swabs and from blood buffy coats by PCR assay. EHV2 is most frequently isolated from leukocytes, lymphoid tissue, and occasionally from trigeminal ganglia.

Rabbit kidney cells (RK13) and equine dermal cells are used for viral isolation. EHV2 slowly induces CPE in culture (detectable within 12–21 days) and cannot be differentiated from other equid gammaherpesviruses such as EHV5 solely from its growth characteristics.

Treatment and Control

Control methods have not been established, and a vaccine is not available for EHV2.

EHV3

Disease

ECE, caused by EHV3 (Equid alphaherpesvirus 3, commonly known as ECE – equine coital exanthema virus), is an acute, sexually transmitted disease characterized by the formation of papules, vesicles, pustules, and ulcers on the penis and prepuce of stallions and on the external genitalia and perineal skin of mares (Figure 52.4). The lesions usually heal after approximately 14 days. Lesions have occurred, infrequently, around the lips, external nares, nasal mucosa, and conjunctiva. The virus does not cause systemic disease, nor does it cause infertility or abortion. But the stallions may show decreased libido, and mares will show painful and frequent micturition. Also, secondary bacterial infections with *Streptococcus zooepidemicus* can aggravate the disease condition. The virus has potential serious economic consequences as a result of temporary disruption of mating activities in affected animals (specifically the thoroughbred breed) and the risk of iatrogenic EHV3 dissemination and outbreaks of ECE in artificial insemination and embryo transfer facilities.



Figure 52.4 Lesions of equine coital exanthema on the vulva and perineal skin of a mare. *Source:* Reproduced from Barrandeguy and Thiry (2012). With permission of Elsevier.

Etiologic Agent

Physical, Chemical, and Antigen Properties

EHV3 is a typical alphaherpesvirus and is antigenically, genetically, and pathogenically distinct from the other equid herpesviruses.

Resistance to Physical and Chemical Agents

The virus is labile in the environment, and infectivity is destroyed by heat, drying, detergents, and common disinfectants.

Infection for Other Species and Culture Systems

The only known reservoir for the virus is the horse, and EHV3 only replicates in cell cultures of equine origin.

Distribution, Reservoir, and Transmission

EHV3 was first isolated in 1968 concurrently in North America and Australia and is endemic in most horse breeding populations worldwide. The usual mode of transmission is venereal, but EHV3 can be spread without coitus. EHV3 can be potentially transmitted to the ejaculate through penile contact with an artificial vagina or sleeve and, consequently, by fresh or frozen semen. The infection may also spread via fomites, or insects may act as mechanical carriers. The vulva and vagina need not be damaged for infection to occur. It is suggested that in some infected stallions and mares, EHV3 becomes latent in the nonbreeding season and is reactivated during the breeding season. The anatomical site that harbors latent EHV3 is unknown, but it is hypothesized that the virus establishes a latent infection in sciatic and/or sacral ganglion cells.

Pathogenesis and Pathology

Viral replication is limited to the stratified epithelium of epidermal tissue present within the skin or at muco-cutaneous margins. The destruction of epithelium caused by the lytic virus infection elicits a vigorous, localized inflammatory response that gives rise to the formation of the characteristic cutaneous lesions of ECE. Microscopic examination of vulvar tissue demonstrates shallow erosions along with occasional typical intranuclear inclusions scattered in germinal epithelium or in nuclear remnants in necrotic areas.

Host Responses to Infection

Immunity to EHV3 has not been studied in detail; the horse responds to infection by EHV3 with the production of serum CF and VN) antibodies that reach maximal levels 14–21 days after infection.

Laboratory Diagnosis

ECE genital lesions in both mares and stallions are usually characteristic enough for a clinical diagnosis to be made

with reasonable certainty. A presumptive diagnosis can be confirmed in the laboratory by isolation of the virus. Detection of EHV3 DNA by PCR or demonstration of either seroconversion or a fourfold or greater rise in antibody titer in paired serum samples can also confirm diagnosis. Reactivation from latency can be associated with low or decreasing neutralizing antibody titers.

Treatment and Control

At the present time, ECE vaccines are not commercially available, and vaccination to prevent the disease has not been investigated. Treatment includes daily cleansing of the affected areas with antiseptics, using anti-inflammatory agents to reduce inflammation, and preventing secondary bacterial infections with antimicrobials. Recovery from ECE is complete in a matter of two to three weeks and occurs without permanent sequelae. Prevention of ECE requires examination of mares or the stallions before breeding or semen collection. Clinically affected animals should not be used until lesions have healed and the virus excretion has ceased. However, reactivation of the latent virus is not preventable and subclinical virus excretion does occur, making control of ECE outbreaks in breeding establishments difficult. Mechanical transmission of the virus can be prevented by early recognition of new clinical cases and strict adherence to hygiene procedures. Therapy with topical ganciclovir twice a day for 13 days showed reduced levels and duration of viral excretion and less severe lesions in experimentally infected mares. Further trials are needed to optimize the dose of the antiviral for a definitive formulation.

EHV4

Clinical manifestations of EHV4 are seen principally in foals and younger horses as protection from maternal antibody wanes. Though historically the clinical condition, equine rhinopneumonitis, was associated with EHV1, this terminology is now assigned to EHV4. Signs often include malaise and elevated temperature up to 105 °F, which may persist for two to five days; watery nasal discharge, which becomes mucopurulent in the later stages; congested conjunctiva; and, infrequently, enlarged submandibular nodes. Bacterial proliferation in nasal passages may be a contributory factor in the development of rhinopneumonitis. Clinically, respiratory disease caused by EHV1 and EHV4 are indistinguishable. Sporadic cases of equine abortion have also been associated with this virus. In contrast to EHV1, the pathogenesis of EHV4 has not been well studied. Leukocyte-associated viremia is not a consistent feature of EHV4 infection, restricting the virus to the respiratory tract. The agent appears to have worldwide distribution; however, it is infrequently isolated. EHV1 and 4 share many antigens, resulting in serological cross-reactivity between these viruses, and protection against EHV4 can be produced with EHV1 vaccines. There are also vaccines available that contain both EHV1 and 4. Both inactivated and modified-live vaccines are used, but results are not uniformly favorable. With a specific enzyme-linked immunosorbent assay (ELISA) now available, it is possible to distinguish EHV1 from EHV4 serologically. PCR assays can also distinguish between EHV1 and 4 infection.

EHV5

As a gammaherpesvirus, EHV5 is adapted to its natural host, so most infected horses are asymptomatic. It is assumed that foals become infected through the upper respiratory tract around the age of one to six months and the virus establishes latency in T and B lymphocytes. This virus is endemic in the horse population, is shed in nasal secretions, and can be isolated from peripheral blood mononuclear cells and lymphoid organs. EHV5 can also be cultured from samples of horses with clinical respiratory disease, and the virus has been associated with equine multinodular pulmonary fibrosis (EMPF), a novel pathological entity with characteristic gross and histopathological pulmonary lesions. Pulmonary interstitial fibrosis of alveolar parenchyma with intraluminal accumulation of neutrophils and macrophages within the alveoli and airways has been described. Eosinophilic intranuclear viral inclusion bodies are rarely seen in macrophages. The high correlation between the presence of EMPF and EHV5 DNA suggests that the virus is involved in the development of lung fibrosis. Experimental infection was shown to induce lung fibrosis in horses upon direct delivery of virulent EHV5 strains into the lungs. However, the choice of viral strain (genetic variability exists among strains), immunologic status of experimental animals, and inoculation route likely favored the outcome of disease. The exact pathogenic role this virus plays in EMPF is unknown; EHV5 may be an etiologic agent or cofactor in the development of EMPF. EHV5 can be isolated on RK13, equine fetal kidney, equine dermal, and Vero cells. Slow development of CPE is described over three to four passages with evidence of ballooning, resulting in syncytia formation on equine dermal cells. PCR assays have been developed to detect this virus.

Ruminant Herpesviruses

Bovine herpesviruses (BoHV), representing alpha- and gammaherpesviruses, are responsible for a wide range of conditions in ruminants, including neurological, genital, fetal, and

Table 52.2. Bovine herpesviruses.

Species	Genus	Common name
Bovine alphaherpesvirus 1	*Varicellovirus*	Infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV)
Bovine alphaherpesvirus 2	*Simplexvirus*	Bovine herpes mammillitis virus
Bovine gammaherpesvirus 4	*Rhadinovirus*	Not applicable
Bovine alphaherpesvirus 5	*Varicellovirus*	Bovine encephalitis virus
Bovine gammaherpesvirus 6	*Macavirus*	Bovine lymphotropic virus

respiratory diseases (Table 52.2). Alphaherpesviruses in the genus *Varicellovirus* have also been isolated from goats (Caprine alphaherpesvirus 1) and deer (Cervid alphaherpesvirus 1-3). Caprine alphaherpesvirus 1 produces disease in goats similar to bovine alphaherpesvirus 1 in cattle. Cervid alphaherpesvirus 2 has been associated with infectious keratoconjunctivitis in Eurasian tundra reindeer. Several gammaherpesviruses in the genus *Macavirus* are associated with malignant catarrhal fever in ruminants.

Bovine Herpesviruses

BoHV1 Disease

BoHV1 is a member of the Alphaherpesvirinae subfamily, and three well-defined subtypes, BoHV-1.1, BoHV-1.2a, and BoHV-1.2b, exist. BoHV1.1 mainly causes IBR and can also cause abortion; BoHV1.2a mainly causes IPV and can also cause abortion; and BoHV1.2b mainly causes IPV but does not cause abortion. Subtype 1 is the prevalent one in North America, South America, and Europe. BoHV1 infection in cattle may present as ocular, genital, and respiratory disease. The respiratory disease typically presents as rhinotracheitis (IBR), which may lead to severe and often fatal bronchopneumonia. Conjunctivitis is common. Occasionally, the cornea is involved, and a panophthalmitis may occur. BoHV1 can infect genitalia, resulting in balanoposthitis and vulvovaginitis (IPV). BoHV1 is considered the most common cause of viral abortion in North American cattle, accounting for 25–60% of the abortion storms. BoHV1 has been isolated from vesicular lesions on the udder and teats of a cow with mastitis. Previous reports of meningoencephalitis in young calves associated with BoHV1 are now being attributed to a different bovine herpesvirus, the BoHV5. Rarely can BoHV1 reach the brain of infected cattle, but a few cases of BoHV1-associated encephalitis have been reported in cattle.

Etiologic Agent

Infectivity for Other Species and Culture Systems

Although cattle appear to be the major species affected by BoHV1, the virus has been incriminated in swine vaginitis and balanitis and has been isolated from stillborn and newborn pigs. Approximately 11% of swine tested in parts of North America (Iowa and Texas) had antibodies to IBR. The virus has been isolated from red deer with ocular disease and could be reactivated in Malaysian buffalo by steroid administration. It does not appear that BoHV1 virus is a significant pathogen of goats. Only 3% of 1146 serum samples of captive ruminants in US zoos had antibodies to IBR virus.

BoHV1 virus can be grown in a wide variety of cells, including bovine, canine, feline, equine, ovine, rabbit, monkey, and human, where it produces a characteristic CPE.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

The disease occurs worldwide, but a few countries have successfully eradicated the infection by applying a strict culling policy. Cattle are considered the primary reservoir. Susceptible species can be defined as animals in which BoHV1 can establish a latent infection and include cattle, sheep, goats, and other species belonging to the subfamily Bovidae, such as wildebeest. The virus is transmitted by respiratory, genital, and conjunctival secretions of infected cattle for 10–17 days, with a peak at 4–6 days post infection. Direct contact (nose to nose) is considered to be the most important route of transmission within farms. BoHV1 may be transmitted in imported semen, either due to virus contamination of the semen or indirect transmission by personnel. Introduction of latently infected animals is the most important source of infection. Continuous introduction of susceptible animals to herds promotes ongoing virus circulation.

Pathogenesis and Pathology

IBR is commonly diagnosed in cattle in feedlots as a result of close contact between cattle and respiratory droplet transmission. The virus injures the respiratory mucosa, which becomes hyperemic, and lesions within the nasal cavity progress from areas of focal epithelial necrosis to large areas of ulceration, covered by a pseudomembrane composed of fibrin and cellular debris that results from an intense inflammatory response to the virus. The injury

predisposes the cattle to bacterial infection and subsequent bacterial bronchopneumonia (shipping fever complex). The virus can be recovered from nasal secretions for almost two weeks following infection. Although viremia is difficult to demonstrate, experimental infections have yielded virus from various organs, perhaps as a consequence of a leukocyte-associated viremia.

Conjunctivitis is common in BoHV1 infection. Typically, it presents with profuse lacrimation and occasionally extends into the cornea, resulting in a keratitis. In some cattle, a multifocal lymphoid hyperplasia may be seen in the palpebral conjunctiva.

Genital infections are most common in dairy cattle and most likely to transmit venereally, though mechanical transmission between bulls is possible in artificial insemination centers. Artificial insemination with infected semen may also produce genital infection. Lesions consisting of pustules and later fibronecrotic plaques are usually limited to the vulva and posterior vagina in the female. Similar lesions are seen on the prepuce of affected bulls. Lesions typically heal in 10–14 days, and many cases are subclinical. Respiratory disease has been produced with genital isolates, and genital lesions have been produced with respiratory isolates. Natural outbreaks of simultaneous respiratory and genital disease are rare. Abortion may be seen in pregnant cattle with IBR or occasionally following vaccination with a modified-live virus vaccine. The incubation period between infection of the dam and fetal death varies from 15 to 60 days. Because fetal death occurs several days before abortion, the fetus is often severely autolyzed. Fetal edema, especially of the fetal membranes, occurs along with extensive hemorrhagic edema in the perirenal tissue. Extensive hemorrhagic necrosis of the renal cortex is seen along with focal necrosis in the liver and usually in the lymph nodes. Some necrosis may be observed in placentomes, which are usually good sources of virus for isolation attempts.

Sites of latency are the trigeminal and sciatic ganglia following respiratory and genital disease, respectively. Recrudescent shedding of the virus has been observed naturally following stressful events (e.g. transport, inclement weather, parturition, poor husbandry/diet) and in response to corticosteroid administration.

Host Response to Infection

The waning of maternal immunity is associated with an increased risk of infection and seroconversion, so this results in a higher prevalence of antibodies against BoHV1 in adult cattle, in which the rate of seroconversion is lower due to herd immunity. The immune response to BoHV1 involves many factors in addition to the stimulation of neutralizing antibodies, most of which are directed toward surface glycoproteins. IgG and IgM antibodies appear seven days following exposure. BoHV1 infection impairs immunity, including loss of CD4+ T cells due to virus-mediated apoptosis, failure of CD 8+ T cell recognition of infected cells, and reduced interferon responses.

Laboratory Diagnosis

There are several different tests that can be used to detect the virus, including PCR, fluorescent antibody test, antigen ELISA, and virus isolation, and these are normally used only to confirm IBR infection in an animal with clinical signs. Tests for the virus are usually performed on swabs taken from the nose, eye (conjunctival), and oropharynx of an animal. Detection of BoHV1 in semen is possible by PCR-based techniques. Abortion may be difficult to diagnose as the fetus is often presented in an autolyzed condition, and detection will depend on the condition of the fetus. Immunohistochemical staining of aborted fetal tissues as well as PCR detection of viral DNA can be used for diagnosis. If the placenta is available and relatively fresh, virus isolation attempts can be made from the placentomes. Diagnosis based on serology may be difficult because animals often have high titers at the time of abortion, regardless of cause, making it difficult to demonstrate rising titers. Calves less than six months old may have maternal antibodies, resulting in positive test results.

Treatment and Control

Reduction of stress prior to and during transportation and after arrival on-farm can help minimize IBR disease. Introducing latently infected cattle is the highest risk activity for BoHV1 entry into a herd. Activities that allow direct/close contact between animals from different herds are also higher risk. Examples include mixing stock at housing, pasture, during transport, and at agricultural shows or marts, or sharing bulls between farms. Perimeter fencing should be intact to prevent mixing with animals from an outside farm. Indirect contact through contaminated fomites is also a potential source of infection (e.g. borrowing contaminated farm equipment from another farm). Movement of individuals with contaminated clothing or equipment between farms is also a possible source of the virus. A good biosecurity plan can prevent these introductions. Less likely sources of infection are embryo transfer and interspecies mixing, with other ruminant species serving as a potential source of infection.

Use of IBR vaccines (ideally in advance of movement or on arrival on farm) can help control the contribution of IBR to the respiratory disease complex. Both modified-live and inactivated vaccines are available for BoHV1 and are used extensively in areas where BoHV1 is

endemic. Breeding animals should be vaccinated prior to coitus (45 days prior to breeding is best) to prevent abortion. Modified-live vaccines should not be used in naïve pregnant dams due to the potential for abortion. Vaccination of caves (beef and dairy) is generally delayed until the animals are four to six months of age due to interference of maternally derived antibodies. Ideally, beef calves should be vaccinated 14–21 days prior to weaning for the most effective immune response, with a booster administered after weaning. If not administered before, vaccination should be administered at weaning. Vaccination should be performed after introduction to a feedlot. Neonatal calves (beef and dairy) can be vaccinated with an intranasal vaccine if the herd has a history of this disease in young calves. Bulls are generally vaccinated annually. Vaccination does not prevent infection or development of latency, but it does reduce the incidence and severity of disease. Vaccination also considerably reduces the amount of virus excreted during primary infection and following reactivation. Marker vaccines (particularly gE deletion mutants) are widely used in Europe. These vaccines are based on the absence of one or more glycoproteins in the vaccine that are normally present in the wild-type strains. After infection (but not after vaccination), an antibody response against the specific protein(s) can be detected using a protein-specific diagnostic test. This allow serological differentiation of naturally infected from vaccinated animals (DIVA). These vaccines are necessary for use in countries with BoHV1 control or eradication programs.

Eradication

Considering that infections with BoHV1 may be associated with changes in production parameters, ranging from fever, reduced growth rate and milk yield to an increased risk of abortion and death, many countries are seeking to eradicate this virus. In the European Union (EU), a number of countries or regions are considered free from BoHV1 following the implementation of EU-approved eradication programs. These countries include Austria, Germany, Denmark, Finland, Sweden, Jersey (United Kingdom) and Valle d'Aosta, and the Province of Bolzano (Italy). Switzerland and Norway are also considered free of infection. Other countries and regions within the EU are currently implementing eradication programs, including Belgium, Czech Republic, Luxembourg, and the province Friuli-Venezia Giulia in Italy. Based on regulations, certain guarantees are granted according to program status. These guarantees have an impact on the trade of live cattle from countries or regions not free from BoHV1, particularly when they do not have approved eradication programs. However, despite such measures to prevent reintroduction of virus, seropositive animals are still sporadically detected in regions of disease-free states, particularly in areas bordering endemically infected countries.

BoHV2 (Bovine Herpes Mammillitis Virus)

BoHV2 has been isolated from cattle with generalized skin disease (pseudolumpy skin disease, PLSD) and localized ulcerative mammillitis (bovine herpes mammillitis, BHM). PLSD is characterized by generalized superficial cutaneous nodules with central depressions over the entire body of affected animals. PLSD is clinically indistinguishable from lumpy skin disease (LSD), which is a disease of cattle caused by a poxvirus that is found in Africa, in several countries in the Middle East, Asia, and Eastern Europe. LSD is considered a foreign animal disease for the United States and is subjected to mandatory reporting in the United States and in European countries, so PLSD must be distinguished from LSD by identification of the causal virus. PSLD is widespread in Southern Africa, with sporadic reports from Australia, United Kingdom, United States, and Israel. Bovine herpes mammillitis is also a localized form of BoHV2 infection that causes erosive-ulcerative self-limiting lesions on the breast and nipples (Figure 52.5). It is primarily a disease of lactating dairy cows but can also affect cows about to calve and beef cattle. This disease has been described sporadically in the United States, Canada, Great Britain, Europe, Africa, and Australia. BoHV2 has also been associated with a more localized skin infection involving the dorsal portion of the head, periocular skin, and edges of the ears in dairy breed calves in the United States. The incubation period of BoHV2 is considered to be one to two weeks. The virus generally produces self-limiting disease, with spontaneous regression of lesions two to four weeks after onset, leaving behind areas of alopecia where crusts have fallen off the skin. Clinical disease regression is associated with seroconversion, but the virus does produce a latent infection. BoHV-2 DNA has been identified in trigeminal ganglia of cattle, but the site of latency has not been identified. BoHV2 will replicate in a wide range of cells, but bovine kidney cell culture is most widely used. Cattle appear to be primarily infected, but buffalo and giraffe are also affected by BHV-2. Mild experimental disease are produced in sheep, goats, and pigs.

Suggested modes of infection include transmission at milking, mechanically by biting flies, or activation of latent virus. Intact teat skin is refractory to virus penetration, indicating that some form of teat trauma precedes infection. Reactivation of latent virus is also a mode of transmission. The latent virus genome can be detected in the trigeminal ganglion of apparently healthy animals, and in the trigeminal ganglion, tonsils, and regional lymph nodes of experimentally infected animals. Intravenous exposure



Figure 52.5 Erosive and crusted lesions on the nipples (a) and (b) and extensive crusted areas on the abdomen (c) and (d) caused by Bovine herpesvirus 2. *Source:* Reproduced from Lanave et al. (2020). With permission of Springer Nature.

produces generalized skin lesions, which are characterized by a severe intercellular edema in the epidermis along with syncytia with intranuclear inclusions. An epidermal mononuclear cell and neutrophil infiltrate are present along with mononuclear and lymphocytic dermal perivascular infiltration.

The diagnosis of PLSD and mammillitis is based on clinical signs and detection of the organism by PCR or from direct electron microscopy of skin crusts. Seroconversion may be demonstrated with disease regression.

BoHV4

BoHV4 is distributed worldwide and is ubiquitous in cattle, causing mainly asymptomatic infection. Among ruminants, American buffalo, African buffalo, sheep, and goats support natural or experimental infection with BoHV4. BoHV4 has been isolated from cattle with a variety of inflammatory lesions, such as mammary pustular dermatitis, metritis, pneumonia, diarrhea, conjunctivitis, respiratory infections, interdigital dermatitis, and vaginitis. However, there is no proven causal role between the virus and these diseases, and the involvement of BoHV4 with these diseases is most likely in synergism with other pathogens. Recent reports show an increase of BoHV4 antibodies in abortion cases compared to asymptomatic cattle. The virus infects peripheral blood mononuclear cells, which allows it to be distributed throughout the body. In primary infection and during reactivation of latent virus, BoHV4 can be isolated from virtually all bovine tissues and organs. Although BoHV4's importance as a pathogen is unclear, the virus does have a strong association with post-partum metritis. Multiple genotypes have been isolated, and it is possible that genetic diversity of strains can be a factor for potential difference in the pathogenicity of strains and production of clinical disease. For control, post-parturient infected cows with metritis must be isolated because they can excrete high quantities of viruses in uterine exudates. The virus can be detected by PCR from exudates with uterine/vaginal swabs or from nasal swabs. BoHV4 is mainly transmitted by the respiratory route, so direct contacts between seropositive and seronegative animals must be prohibited to prevent spread. The virus can also be detected by isolation in bovine cells such as MDBKs, but it can be relatively difficult to isolate due to its slow replication cycle.

BoHV5

BoHV5 is an alphaherpesvirus responsible for nonsuppurative meningoencephalitis in young cattle and is closely antigenically and genetically related to BoHV1. Although a case of BoHV5-associated disease in Europe and some outbreaks in United States and Australia have been reported, the current geographical distribution of BoHV5 infection is mainly restricted to South America, especially Brazil and Argentina. However, as this virus is closely related to BoHV1 and currently available serological tests do not differentiate antibodies against each virus, the true prevalence of BoHV5 infection remains unknown. PCR-based methods can differentiate between the two viruses (samples used for detection of BoHV5 include nasal secretions, semen, or postmortem samples) and monoclonal antibodies can also be used for serological differentiation. Cattle are the natural hosts, and latently infected cattle are reservoirs of BoHV5. The trigeminal ganglion serves as the site for latency. Viral DNA and infectious virus have been detected in the semen of subclinically infected bulls. Based on experimental evidence, sheep and goats may be potential reservoirs of the virus. Natural

occurrence of BoHV5 antibodies has been reported in sheep. BoHV5 initially infects the epithelial cells at the portal of entry (either the nasal or vaginal mucosa). The initial replication of BoHV5 in the respiratory system leads to large amounts of virus shedding in the nasal secretions and constitutes a very efficient source of transmission during acute infection. After initial replication, the virus spread probably occurs by three routes: local dissemination, systemic spread by viremia, and neuronal spread. Systemic spread by viremia does not appear to be an important part of the pathogenesis of BoHV5, and BoHV5 infection induces either a subclinical infection or disease of moderate severity in adult cattle. However, it can induce lethal encephalitis in young animals (less than six months of age). Differences in virulence have been associated both with viral and host factors, especially the age and the immunologic status. Although BoHV5 and BoHV1 are related, they differ in their neuroinvasive and neurovirulence capacities. BoHV1 neuroinvasion usually does not go further than the first order neuron located in the trigeminal ganglion, where the latent infection is established, whereas BoHV5 is able to infect different regions of the brain. Neurological signs in infected calves include incoordination, circling, nystagmus, grinding of teeth, recumbence, and finally, death. The main macroscopic lesions reported following BoHV5 infection consist of softening of the parenchymal tissue, focal meningeal hemorrhages in the frontal and/or ventral areas, and hemorrhagic foci in the pons and left parietal lobe. Gross respiratory lesions, such as nasal congestion, petechial hemorrhages and congestion of the pharyngeal and laryngeal mucosa, as well as bronchopneumonia, have also been described. BoHV5 can be reactivated and re-excreted without clinical signs or cattle may develop clinical manifestations of encephalitis similar to those observed during acute infection. Specific BoHV5 vaccines remain at an experimental stage of development. Therefore, the use of BoHV1 vaccines is considered the best option to protect against BoHV5; however, several studies show that full protection against BoHV5 infection is difficult to induce with a BoHV1 vaccine, and it does not prevent BoHV5 latency.

AlHV1 and OvHV2

MCF, an often fatal infection of many species of *Bovidae* and *Cervidae*, is caused by five MCF-inducing viruses in subfamily Gammaherpesvirinae, genus *Macavirus*: Alcelaphine herpesviruses 1 and 2 (AlHV1, AlHV2), Caprine herpesviruses 2 (CpHV2), MCF virus (MCFV)–white-tailed deer (proposed CpHV3), and ibex-MCFV. *Giraffidae* and *Suidae* are also susceptible to infection. The disease has been documented in more than 30 ungulate species. Susceptibility varies by animal species. Disease in susceptible ruminants is polysystemic and is characterized by lymphoproliferation and inflammation oriented toward mucosal surfaces and blood vessels. AlHV1 is responsible for wildebeest-derived (or African) MCF, which occurs when cattle or other susceptible wild ruminants and wildebeest graze together. This form of the disease occurs in Africa and has occurred in zoos that house African ungulates. AlHV2 is the cause of MCF in hartebeest. The sheep-associated form of MCF has been described in cattle, bison, and deer outside of Africa and in zoos where animals have had contact with sheep. An etiologic agent for the sheep-associated form of MCF has not been isolated; however, MCF can be transmitted to cattle or bison by aerosolization of nasal secretions from sheep experiencing viral shedding of OvHV2, implicating OvHV2 as the cause of this form of MCF. The genome of OvHV2 has been completely sequenced, and it is genetically closely related to AlHV1. CpHV-2 is endemic in domestic and wild goats and has been documented to cause disease in several species, including white-tailed deer, sika deer, and pronghorn antelope. Alopecia, chronic weight loss, and dermatitis are the main clinical characteristics of MCF caused by CpHV2. The Ibex-MCF is responsible for several cases of MCF in bongo, an African forest antelope, and in anoa (midget buffalo). AlHV1 and OvHV2 are the most well studied of these viruses and are not recognized as significant causes of disease in their natural hosts, wildebeest and sheep, respectively.

AlHV1 MCF is associated with mixing wildebeest and cattle during periods when the wildebeest are calving. Newborn wildebeest calves shed virus in nasal and ocular secretions up to three months of age. Viral shedding also occurs in adult wildebeest given corticosteroid. There is no evidence for congenital infection of sheep with OvHV2; rather, lambs appear to become infected during the first year of life and typically do not shed virus until after five months of age. Although sheep-associated MCF in cattle has been associated with lambing, it appears that the newborn lamb does not play the same role as the newborn wildebeest. It also appears that all domestic US sheep carry OvHV2. Cattle are considered dead-end hosts for OvHV2 because the cow-to-cow transmission has not been demonstrated. OvHV2 represents a serious threat to American bison due to their high susceptibility to disease.

Both AlHV1 and OvHV2 produce similar clinical disease syndromes. However, lesions are variable, depending on the species affected, and the severity and length of clinical illness. Typical presentation is referred to as the head-and-eye form. Affected animals present with mucopurulent nasal and ocular discharge and bilateral corneal opacity resulting in photophobia and partial blindness, which is caused by panophthalmitis (Figure 52.6). Oral lesions may be present, consisting of multiple erosions preceded by



Figure 52.6 Cow with acute MCF showing lacrimation, salivation, mucopurulent nasal discharge, corneal edema, matting of facial hair, and epidermal necrosis of the muzzle. Nasal exudate becomes mucoid in character within a few days, with mucopurulent discharges from the nose, stertorous breathing, and often dyspnea. Muzzle epithelium is initially inflamed and later necrotic, resulting in encrustation, cracking, and often dislodging of affected epithelial patches to reveal the underlying inflamed subepithelium (arrowed). *Source:* Photo from CABI Invasive Species Compendium, with permission from Dr. Donal O'Toole, University of Wyoming.

diffuse hyperemia and profuse salivation. Erosions can occur anywhere along the alimentary tract, from the muzzle to the colon. Central nervous disturbances are frequent, and diarrhea is common. Death generally occurs within two to seven days after the onset of clinical signs.

Histological lesions are those of a lymphoproliferative disorder characterized by perivascular mononuclear infiltration, necrotizing vasculitis, and tissue lymphoid infiltration, resulting in enlarged, edematous, and potentially hemorrhagic lymph nodes. A recent report suggest that OvHV2-infected T cells, monocytes, and locally proliferating macrophages contribute to the vasculitis in MCF. The initial trigger resulting in leukocyte recruitment and activation is not yet known, but there is evidence that latently infected, activated endothelial cells play a role in this. Activated macrophages might then release the necessary pro-inflammatory mediators and, eventually, induce the characteristic vascular changes. History, clinical signs, and histopathology suggest the diagnosis of MCF. Confirmation of the diagnosis of AlHV-1 can be made by isolation of the virus from peripheral blood leukocytes in calf thyroid cells or by PCR detection of the viral DNA. OvHV2 has not been isolated in cell culture, but the viral DNA may be detected in the tissues of infected animals by PCR. PCRs specific for other MCF-associated viruses are also available. Currently, vaccines for AlHV1 and OvHV2 are in experimental stages. Control aims to prevent contact between susceptible hosts and viral carriers.

OvHV2-associated MCF has also been reported in swine. DNA was detected in the blood and semen of asymptomatic boars and from the brain of symptomatic sows and gilts with MCF that was probably transmitted by artificial insemination. Signs in symptomatic sows and gilts included depression, fever, anorexia, abortion, followed by neurological signs including ataxia, tremors, convulsions, and aggressive behavior, and death. Pigs that survived generally display a forelimb paralysis. Pigs are terminal hosts and are not believed to spread the virus.

CpHV1

CpHV1 is an alphaherpesvirus that is spread worldwide where goat husbandry is practiced. In the United States, neither the prevalence of seropositive goats nor the prevalence of disease associated with them is known. CpHV1 is genetically and antigenically related to BoHV1, and though the virus can infect cattle, it has not been associated with disease in cattle.

CpHV1 induces generalized, often lethal, infection in one- to two-week-old kids. Gross lesions are limited to the gastrointestinal tract with necrosis and ulceration in the rumen, cecum, and colon. Intranuclear inclusions are present in epithelial cells near the necrotic areas. In adult goats, the infection is mild or subclinical, with affected animals showing very slight signs of respiratory distress, vaginitis, balanoposthitis, or occasionally, abortion.

CpHV1 establishes latent infections, but, unlike other herpesviruses, its reactivation is extremely difficult to demonstrate under both natural and experimental conditions. In natural infection, CpHV1 reactivates during estrus, but only in animals with low neutralizing antibody titers. After a primary infection, neutralizing antibodies are detected in the serum 1–2 weeks after the infection; they reach a peak in the following third or fourth week and decrease slowly about 6–10 months later. If the animals are stressed at this stage, CpHV1 can reactivate and be shed, especially by the genital route. In response to the reactivation, humoral and cell-mediated immune responses are boosted. It should be noted that a residual sero-neutralizing activity persists even years after the primary CpHV1 infection.

The disease can be diagnosed by viral isolation from nasal secretions and fecal material. CpHV1 replicates in canine, rabbit, feline, equine, bovine, and lamb cells. PCR assays for DNA detection are also available. Serum neutralization is the gold standard for serological diagnosis, and an ELISA has also been developed.

No CpHV1 vaccine is presently available in the United States, and it is unlikely that such a vaccine will be

developed because of the small market size. Antibodies directed against BoHV1 will react with CpHV1; thus, it has been suggested that BoHV1 vaccines could be used in goats to protect against CpHV1 infection. However, a challenge study found that vaccinating goats with an attenuated BoHV1 vaccine induced only partial protection against CpHV1 infection. No studies have determined whether vaccination would protect against CpHV1 abortion.

SuHV1

Disease

Pseudorabies (also known as Aujeszky's disease virus), caused by Suid herpesvirus 1 (SuHV1), is a notifiable disease that causes substantial economic losses to the swine industry in countries where the virus is present in domestic pigs. The virus is most severe in younger animals. It commonly affects the nervous system, and the mortality rate varies from 5% to 100%. Infection of sows during mid- to late pregnancy can result in abortion, fetal death, mummification, or stillbirths. In adult pigs, severe nervous disorders are rare, and pseudorabies usually presents as a rather vague illness of transient pyrexia, dullness, inappetence, incoordination, and ataxia. Respiratory disease can also be seen in pigs of various ages but is most common in grower and finishing pigs. Inapparent or mild disease may be missed or misdiagnosed in older swine. Pseudorabies also occurs in several other species, including cattle, sheep, dogs, cats, and raccoons, in which the clinical signs are usually neurological and manifested by an intense pruritis.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

The pseudorabies virus (PRV) is an alphaherpesvirus that is designated SuHV1. Only one serotype has been identified; however, strain variability has been shown by restriction endonuclease digestion of viruses from different geographic areas. Attenuated strains have been demonstrated to have a deletion in their genome, suggesting that specific regions are associated with virulence.

Sensitivity to Physical and Chemical Agents

PRV is fairly sensitive to high temperatures and is stable in cell culture fluid between a pH of 6 and 8 at cooler temperatures. The virus has been observed to survive in nonchlorinated water for seven days and two days in an anaerobic lagoon. Chemicals that cleave chlorine appear to be the most effective disinfectants.

Infectivity for Other Species and Culture Systems

The disease occurs naturally in cattle, sheep, dogs, cats, and rats. In all but adult swine, the disease is almost always fatal; hence, other animals are essentially "dead-end" hosts. PRV is not readily transmitted to humans.

The virus replicates readily in cell cultures from many species and tissues, including cat, dog, cattle, badger, coyote, deer, buzzard, chicken, and goose.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Pseudorabies has been eradicated from domestic pigs in several parts of Europe, Canada, New Zealand, and the United States. However, it is recognized as one of the most important diseases of domestic pigs, particularly in regions with dense pig populations, including some remaining regions in Europe and Latin America, and Asia. The principal reservoir of PRV appears to be the pig, and transmission is frequently pig to pig. The virus is transmitted by ingestion and inhalation, and during coitus, the virus can be transmitted from boar to sow, or vice versa. The virus is not shed in urine or feces. Transmission can occur in a contaminated environment under crowded conditions.

Feral swine can transmit the virus to domestic swine, and endemic infections in wild swine populations in the United States and Europe represent a constant danger for reintroduction of PRV into free domestic pig herds and regions. The pig is the primary source of viral spread to other species. Cases in dogs have been linked to the consumption of feral swine tissues. Cats appear to be more sensitive, and infection in cats was observed in 51% of PRV-infected farms where cats were present.

Pathogenesis and Pathology

The virus replicates primarily in the upper respiratory epithelium, including the tonsillar tissue. The virus can be isolated from the brain 24 hours following infection, which suggests that the route of infection is via the axoplasm. Viremia is difficult to demonstrate; however, viral shedding may persist in nasal secretions for up to 14 days. Lower airway infection often results, and cardiac and splanchnic ganglia become involved.

The virus produces a nonsuppurative meningoencephalomyelitis with extensive damage to neurons, widespread perivascular cuffing, and gliosis. The brain stem is particularly affected, but lesions also occur throughout the cerebral cortex and cerebellum. There may be intranuclear inclusion bodies in all types of cells. In the respiratory form of the disease, necrotizing tracheitis and pneumonia occur, resulting in loss of epithelium in the airways and necrosis of alveolar cells.

Microscopic lesions in aborted fetuses include necrosis of many organs, but primarily liver, spleen, visceral lymph nodes, and adrenal glands. Intranuclear inclusion bodies are often present in degenerating hepatocytes, cells of the

adrenal cortex, and occasionally mononuclear phagocytic cells of the spleen and lymph nodes. Placental lesions are characterized by degeneration and necrosis of the trophoblasts and mesenchymal cells of the chorion.

Host Response to Infection

IgM antibodies are first detectable about the fifth day after infection, followed by measurable IgG antibodies about the seventh day, reaching maximum levels by the 12th to 14th day.

Laboratory Diagnosis

Because signs of the disease in swine vary widely with the age of the animal, the dose of virus received, the strain of virus, and the route of exposure, clinical diagnosis is often difficult.

In the laboratory, a definitive diagnosis of pseudorabies can be made by viral isolation or detection of viral DNA by PCR. Immunofluorescent staining of frozen tonsil or brain tissue can provide a rapid diagnosis. ELISA tests are used to differentiate antibody response to gene-deleted vaccines and field infection, and have been used in eradication programs. In an acute outbreak, serology may not be helpful, because of the time needed for antibodies to develop.

Prevention and Control

In PRV-free areas, biosecurity should be maintained to prevent contact between infected wild swine populations and domestic pigs. Yearly serological monitoring of domestic pigs is required for maintaining pseudorabies-free status. In areas with an endemic disease in domestic pigs, efforts should be taken to avoid the disease in a breeding herd. In infected herds, quarantine is the most urgent obligation, and it is recommended that the movement of swine be limited to slaughter only. Attenuated live vaccines are available and have been successful in reducing death losses in endemic areas. These vaccines do not prevent reinfection with virulent field virus or the shedding of virulent virus for variable periods. Latently infected and vaccinated animals may shed the virus for indeterminate periods while asymptomatic.

Inactivated vaccines are commercially available. Their principal use has been in susceptible sows in endemic areas to provide antibodies to the colostrum to protect newborn pigs during the first few weeks of life. In certified pseudorabies-free areas, vaccination is prohibited.

CaHV1

Disease

Canid herpesvirus 1 (CaHV1) causes a fatal systemic infection in newborn pups and relatively mild infections in older dogs. CaHV1 has been isolated from dogs with respiratory diseases, and, with other viruses and bacteria, may be involved in the "kennel cough" syndrome. CaHV1 may be associated with outbreaks of highly contagious ocular infection in susceptible adult dogs, in the absence of concurrent overt systemic disease. CaHV1 can induce genital lesions in male and female dogs. The affected animals appear healthy but often present with a history of infertility.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

CaHV1 is a typical alphaherpesvirus. There is no cross-neutralization between CaHV1 and the viruses of herpes simplex, PRV, or IBR. However, CaHV1 appears to be antigenically related to the herpes simplex virus and feline herpesvirus-1. Only one serotype of CaHV1 is recognized; however, restriction endonuclease analysis of CaHV1 isolates has detected genotypic differences between viruses. Currently, no correlation between different CaHV1 genomes and disease patterns has been demonstrated.

Infectivity for Other Species and Culture Systems

The host range for CaHV1 is restricted to domestic and wild canids. Limited growth occurs in human lung cells and calf, monkey, pig, rabbit, and hamster kidney cells.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

CaHV1 has a worldwide distribution. CaHV1 seroprevalence is high in many domestic canine populations, and primary infection is generally believed to occur at a young age in most dogs. The only known reservoir of CaHV1 in all geographic areas is the dog, with the possible exception of coyotes in the United States. Pups born to seronegative bitches are infected oronasally either from the bitch or from other infected dogs from viral shedding in venereal, ocular, or nasal secretions. It is documented that about 40–90% of the canine population is seropositive for CaHV1

Pathogenesis and Pathology

Clinical manifestations and disease severity during primary infection with many alphaherpesviruses, including CaHV1, are host age dependent. In pups less than four weeks of age, primary CaHV1 infection may result in severe generalized disease due to the hematogenous spread of the virus. This produces necrotizing vasculitis in many organs, and the disease is often fatal. Grossly, the kidneys may appear mottled, and there may be pulmonary congestion and edema, splenomegaly, lymphadenitis, and nonsuppurative meningoencephalitis. Widespread foci of necrosis and hemorrhages characterize the histological lesions in affected organs such as the kidney (Figure 52.7)

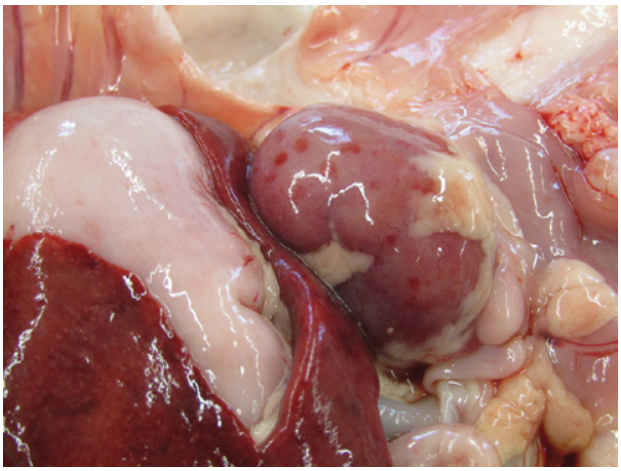


Figure 52.7 Renal hemorrhage in a female French Bulldog puppy with CaHV1. Kidneys contained multiple hemorrhagic foci 1–2 mm in size scattered throughout the cortices. *Source:* Photo courtesy of Dr. Grant N. Burcham, Purdue University.

liver, lung, and gastrointestinal tract. Intranuclear inclusions may be present in areas adjacent to necrotic lesions, specifically in the liver. Primary CaHV1 infection in older canids is frequently subclinical or results in a localized respiratory tract, genital mucosa, or ocular disease. Ocular lesions associated with primary CaHV1 infection in adult dogs include conjunctivitis and, less frequently, keratitis. Viremia is not typical of CaHV1 infection in immunocompetent adult dogs; however, a generalized disease resulting from viremia has been reported in an immunocompromised adult dog. Trigeminal ganglion, tonsils, liver, and parotid salivary glands are reported as the most common sites of viral latency.

Host Response to Infection

Neutralizing antibodies develop in dogs inoculated with CaHV1 and are detectable by day 7 post inoculation. Titers peak by day 21 and slowly decrease, persisting for approximately eight months post inoculation. Reactivation of CaHV1 in experimentally infected pups and dogs has been demonstrated with the administration of corticosteroids. Latency is established in neurons of the sensory ganglia. During viral reactivation, CaHV1 neutralization titers become detectable and elevate in as few as seven days, but rapidly decline in a matter of weeks following active infection resolution.

Laboratory Diagnosis

Clinical signs and histopathology can be useful in diagnosis. Definitive diagnosis is by viral isolation and more rapidly by immunofluorescent staining of affected tissues or by detection of genomic DNA by PCR.

Treatment and Control

Commercial CaHV1 vaccines are not available in the United States; a killed virus vaccine is available in Europe. Hyperimmune globulin may be useful but is difficult to obtain because the virus is poorly immunogenic. Removal or separation of infected animals should be considered. The optimal temperature for viral replication is approximately 33 °C. Due to the poor ability of puppies to control their body temperature until about four weeks of age, younger animals are susceptible to severe disease. Maintenance of normal body temperature in exposed puppies may have therapeutic value. Antiviral therapy has been evaluated for treatment of CaHV1-induced corneal ulcers. The efficacy of antiviral therapy is not established, but topical application of idoxuridine or trifluridine on corneal ulcers is reported to yield clinical resolution. Other treatment strategies using antiviral drugs such as cidofovir along with IL-2 inhibitors have been shown to produce early recovery from CaHV1-induced corneal ulcerations.

FeHV1

Disease

Felid herpesvirus 1 (FeHV1) is the cause of feline viral rhinotracheitis (FVR). Primary infection causes acute rhinitis and conjunctivitis (Figure 52.8), usually accompanied by fever, depression, and anorexia. Ulcerative keratitis is a frequent sequela to recurrent FeHV1 infection and should be considered the cause of corneal ulceration in cats unless proved otherwise. The virus has also been associated with ulcerative stomatitis, abortions, pneumonia, and facial dermatitis.



Figure 52.8 Kittens with conjunctivitis and rhinitis caused by FeHV1. *Source:* Photo courtesy of Dr. Rebecca E. Gompf, The University of Tennessee.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

FeHV1 is a typical alphaherpesvirus. Serological comparisons suggest that FeHV-1 from around the world belong to a single known serotype; however, differences in the clinical manifestations caused by various isolates have been observed. Viral DNA analysis suggests genetic differences between strains.

Sensitivity to Physical and Chemical Agents

The virus is inactivated by most commercially available detergents, disinfectants, and antiseptics. Virus in cell culture fluid loses 90% of its viability within six hours at 37 °C, six days at 25 °C, and one month at 4 °C. The virus is most stable at pH 6, and complete activity is lost in three hours at pH 3 and pH 9. FeHV can be recovered for up to 18 hours in a moist environment at 15 °C, but for less than 12 hours in a dry room.

Infectivity for Other Species and Culture Systems

Natural infections with FeHV1 have been observed only in the cat family. FeHV1 *in vitro* growth is limited to cells of feline origin. The virus propagates to high titers with demonstrated CPE in primary cell cultures of feline testicle, lung, and renal cells.

Host–Virus Relationships

Distribution, Reservoir, and Transmission

Serological studies show that FeHV1 is widespread in the feline population worldwide, with reported exposure rates of up to 97%. Cats serve as reservoirs. Healthy cats that are latently infected with FeHV1 may shed virus when stressed and following corticosteroid administration, and viral transcripts have been detected in trigeminal ganglia of latently infected cats. The major avenue for spread of FeHV1 is by direct cat-to-cat contact through infectious discharges, particularly respiratory secretions. Overcrowded conditions and close housing greatly increase the likelihood of viral transmission as FeHV1 is short-lived in the environment. Indirect or fomite transmission via a contaminated environment, personnel, or feeding and cleaning utensils appears important only in multi-cat situations such as catteries, shelters, and multi-cat households. Strict attention to hygiene should be sufficient to prevent this route of infection. Given that parturition and lactation have been shown to precipitate viral shedding, neonatal infection is highly probable, and the primary infection typically occurs in kittens. Transplacental infection has not been demonstrated in natural infections.

Pathogenesis and Pathology

FeHV1 has a tropism for the conjunctival, nasal, and pharyngeal epithelium. The pathogenesis of the infection differs with the route of inoculation. As FeHV1 infection is often manifested in the upper respiratory tract, experimental studies have been done by nasal and ocular routes. When introduced intranasally, the virus produces rapid, cytolytic infection of epithelial cells of the nasal passages. The virus generally persists in the upper respiratory tract for two weeks. Damage to the nasal turbinates in acute disease predisposes some cats to chronic rhinitis. In cases of chronic rhinosinusitis, the virus does not replicate, suggesting that the disease is perpetuated by immune-mediated mechanisms.

Although many cats develop conjunctivitis during the primary disease, very few develop corneal disease. Reactivation of FeHV1 latency is usually associated with conjunctivitis and may be accompanied by corneal epithelium invasion, which is associated with epithelial ulceration, initially of a pathognomonic dendritic form (Figure 52.9), but progressing rapidly to a larger irregular geographical form. Intranuclear inclusion bodies are numerous in the stratified squamous epithelium of the conjunctiva, with conjunctival and corneal lesions. Histologically, corneal ulcers reveal disorientation and degeneration of the epithelial cells, some of which contain nuclear inclusions. Many of these ulcers are slow to heal, leading to a more chronic evolution than seen in primary disease. Chronic keratitis is an immune-mediated condition that results from persistence of virus antigen in the corneal stroma and leads to corneal vascularization and infiltration of inflammatory cells. Formation of corneal sequestra can occur secondarily to any chronic ulcerative keratitis, including FeHV1 ulceration.

Host Response to Infection

In general, the immune response protects against disease, but not against infection, and mild clinical signs have been observed following reinfection only 150 days after primary infection. The primary immune response of cats to

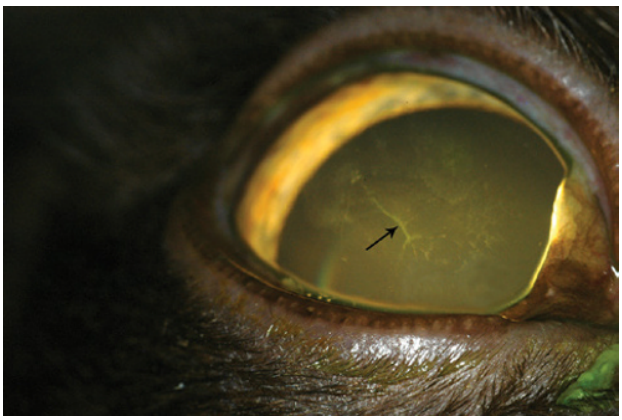


Figure 52.9 Dendritic corneal ulcer (arrow), pathognomonic for herpesviral infection. *Source:* Photo courtesy of Dr. Diane Hendrix, The University of Tennessee.

intranasal infection as measured by serum-neutralizing antibodies is not impressive. Antibodies usually persist for 1–3 months, although titers have been observed to fluctuate in a cat over a 12-month period. Correlation between the presence of antibodies and resistance to infection is not absolute. As with other alphaherpesviruses, cell-mediated immunity (CMI) plays a very important role in protection, because vaccinated cats without detectable antibody are not necessarily susceptible to disease. In contrast, seroconversion has been shown to correlate with protection against virulent FeHV1 challenge. In these cases, antibodies may serve as an indicator of cellular immune responses, because T lymphocytes are required for the maintenance of B lymphocyte function. Because FeHV1 is a pathogen of the respiratory tract, mucosal cellular and humoral responses are important. Although a correlation exists between FeHV1 antibodies and protection against clinical signs, there is no test available to predict protection in individual cats.

Laboratory Diagnosis

Immunofluorescent staining can demonstrate viral antigens in the tissues of infected cats. However, the test is not particularly sensitive, and the use of stains, such as fluorescein, may result in false positive results. PCR has become the mainstay for diagnosis of FeHV1 infections. Detection of virus may indicate either coincidental reactivation of latent infection, a consequential reactivation due to another disease process, or the cause of the disease. Feline herpesvirus 1 may also be isolated from tissue samples and from swabs of the ocular, nasal, or oropharyngeal mucosa. The virus can be cultured for 14–21 days after infection, but most consistently during the first week. Virus isolation is more difficult in chronic cases. VN antibodies can be detected in the serum of convalescent cats. Serology is complicated by vaccine virus, and positive titers are independent of clinical signs. Titers may be low in both acute and chronic disease. Serology, therefore, is only of limited value in the diagnosis of FeHV1 infection.

Treatment and Control

Both modified-live and inactivated parenteral vaccines are available. Subunit FeHV1 vaccines and modified-live intranasal vaccines are no longer available in Europe. Vaccination protects against disease, but not necessarily against infection. However, it can reduce virus excretion upon infection. Assessment of the duration of immunity is complicated: vaccination does not provide complete protection even shortly after vaccination and no protection against reactivation and shedding, and the degree of protection decreases with time. Even vaccinated cats remain at some risk because FeHV1 vaccines, both parenteral and intranasal, confer only partial immunity against clinical signs and no protection against reactivation/shedding. Maternally derived antibodies provide some degree of humoral protection in kittens up to the age of around eight weeks, and therefore, primary vaccination should be initiated around nine weeks of age, with a second vaccination two to four weeks later. Annual boosters are particularly important for cats in high-risk situations or environments. However, for cats in low-risk situations (e.g. indoor-only cats without contact with other cats), three-year intervals are recommended. Care should be taken to avoid introducing cats with developing, subclinical, or latent infections to a colony. The use of systemic antiviral drugs such as famciclovir, or topical treatment with cidofovir, or ganciclovir in combination with immune suppressive drugs such as ciclosporin, are promising among the treatments of FeHV1 infection.

McHV1

Macacine herpesvirus 1 (B-virus of macaques) causes a natural infection in *Macaca* – a genus of Old World monkeys (rhesus and cynomolgus species). The infection in monkeys is characterized by oral vesicles similar to the cold sores in humans caused by the herpes simplex virus. The disease, is common in macaques, is not fatal, and the virus may remain latent in the trigeminal and lumbosacral ganglia of infected animals. Direct contact is the most common means of viral spread in monkeys, because virus can be recovered from saliva and from the central nervous tissues of clinically asymptomatic, persistently infected animals.

The virus can cause fatal central nervous infection in humans and laboratory animals such as rabbits, and unweaned mice. Most human infections are caused by a bite from a monkey secreting infectious virus, although handling virus-infected primary monkey kidney cell cultures can also lead to infection. The incubation period in humans is 10–20 days. Usually, there is local inflammation at the bite site followed by vesicle formation and necrosis of the area. The virus reaches the CNS by peripheral nerves, and in the majority of cases, death occurs due to acute encephalitis or encephalomyelitis.

B-virus is morphologically similar to other alphaherpesviruses. It is readily inactivated by detergents. The virus can be cultivated on the chorioallantoic membrane of embryonated chick eggs, and in rabbit, monkey, and human cell cultures, producing intranuclear inclusion bodies and syncytia formation. Strong cross-reactivity exists between human herpes simplex viruses and B-virus.

B-virus infection can be diagnosed by isolating the virus from the central nervous tissues of fatal human cases. A PCR-based assay has been developed for viral identification. Diagnostic detection of herpes B specific

antibodies is difficult. An effective vaccine has not been developed. Rapid diagnosis and initiation of therapy, such as antiviral drugs like acyclovir, can benefit, and may prevent death or permanent disability in patients that survive. Caution and wearing of protective gear when handling monkeys remain the best ways to avoid infection.

Avian Herpesviruses

The avian herpesviruses in this section are all alphaherpesviruses and include infectious laryngotracheitis virus (ILTV), Marek's disease virus (MDV), and Pacheco's disease virus. ILTV and MDV primarily cause disease in chickens, whereas Pacheco's disease infects psittacine birds.

GaHV1

Disease

ILTV usually occurs as an acute disease in chickens and represents a serious problem in areas of intense poultry husbandry. The virus produces signs of respiratory distress and coughing that often produce a bloody discharge. Mild enzootic forms of ILTV infection may result in reduced egg production, conjunctivitis, and persistent nasal discharge with swollen nasal and infraorbital sinuses. The mild enzootic form is the most common form in modern poultry operations.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

ILTV is a typical alphaherpesvirus that is designated Gallid herpesvirus 1 (GaHV1), and is a member of the genus *Iltovirus*. There is one serotype of the virus, but genetic variation occurs among strains from different regions.

Sensitivity to Physical and Chemical Agents

ILTV is inactivated by 3% cresol, 1% sodium hydroxide, and 1% lye, 24 hours exposure to ether, and 10–15 minutes at 55 °C. The virus can be stored for lengthy periods by lyophilization and freezing.

Infectivity for Other Species and Culture Systems

ILTV is primarily a disease of chickens and is most common in birds 4–18 months of age. The disease has also been reported in pheasants and peafowl. Young turkeys have been infected experimentally, but natural infection rarely occurs in turkeys. Starlings, sparrows, crows, doves, ducks, pigeons, and guinea fowl have been found to be resistant to ILTV. There are no known wild bird reservoirs of this virus.

Chicken kidney monolayer cell cultures, embryonated chicken eggs, and chicken embryo (kidney, liver, and lung) cell cultures have been used to culture ILTV.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

ILTV has been identified in almost every country in the world; it occurs primarily in areas with high concentrations of chickens. ILTV continues to cause disease problems, especially in the Americas and Australia. Chickens are assumed to be the primary reservoir and mode of transmission, which occurs by direct contact through droplet infection of the ocular and respiratory secretions. Mechanical transmission can occur via contaminated equipment and litter. Egg transmission of ILTV has not been demonstrated. A carrier state can develop in birds with sublethal disease, and ILTV has been isolated from chickens two years after infection. Unvaccinated birds are susceptible to infection from vaccinated birds, and vaccinated birds may become carriers. Acutely infected birds represent a greater source of virus than clinically recovered carrier birds.

Pathogenesis and Pathology

ILTV in natural conditions enters through the upper respiratory tract and ocular tract. In the natural disease, the greatest concentration of ILTV is found in the trachea, and the virus replicates only in the nasal cavity, trachea, and lower respiratory tract. Latent virus has been demonstrated in the trigeminal ganglion. Viremia has not been reported.

Lethal infections of ILTV can occur due to asphyxiation, resulting from extensive diphtheritic membrane formation that plugs the tracheal bifurcation. Histopathology demonstrates fibrinous laryngotracheitis (Figure 52.10),

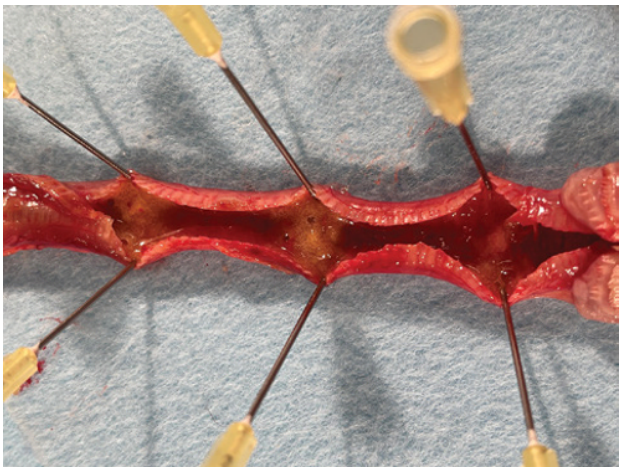


Figure 52.10 Tracheal lumen contains frank hemorrhage and aggregates of thick, yellow, mucoid debris consistent with infectious laryngotracheitis virus (ILTV). *Source:* Photo courtesy of Dr. Geoffrey Lossie, Purdue University.

with syncytia with large intranuclear inclusion bodies noted in the tracheal epithelium that are the basis for a strong presumptive diagnosis.

Host Response to Infection

The first signs of ILTV infection usually appear 6–12 days following natural exposure. Resistance to disease following the infection or vaccination usually persists for approximately one year. Infected birds develop precipitating and serum-neutralizing antibodies; however, the cell-mediated response appears to be important in resistance. Full immunity can be demonstrated in bursectomized chickens in the absence of a humoral response.

Laboratory Diagnosis

Virus can be isolated from tracheal and lung tissue in embryonated chicken eggs and cell culture, and ILTV DNA can be demonstrated by PCR. Immunofluorescent staining of trachea can demonstrate the presence of viral antigen for up to 14 days post infection. ELISA-based serology is widely used.

Prevention and Control

Use of live ILTV vaccine results in carriers, which can shed to nonvaccinated susceptible birds. Vaccine strains have been associated with outbreaks in some flocks following reversion of the strains to virulence. Because the virus can survive for 10 days at temperatures of 13–23 °C, the cleaning of infected premises is very important. Complete depopulation and disinfection of premises has been used to control the disease.

Use of attenuated vaccines is a common practice for breeding and egg production flocks, though vaccination does not protect against infection with virulent virus or development of latency. The development of genetically engineered vaccines holds promise for improved control strategies.

GaHV2

Disease

Marek's disease (MD), caused by Gallid herpesvirus 2 (Marek's disease virus [GaHV2]), is a lymphoproliferative disease, lymphoma being most common, of chickens that may involve numerous tissues. Most frequently peripheral nerves are affected. Prior to vaccine development, MD was responsible for heavy losses, and increased losses to MD in vaccinated flocks have suggested an evolution toward greater virulence.

Progressive paralysis of one or more extremities (Figure 52.11), incoordination, drooping wings, and lowered head position are the most common signs of MD. Mortality varies from 10% with mild MD to more than 50% in unvaccinated birds.

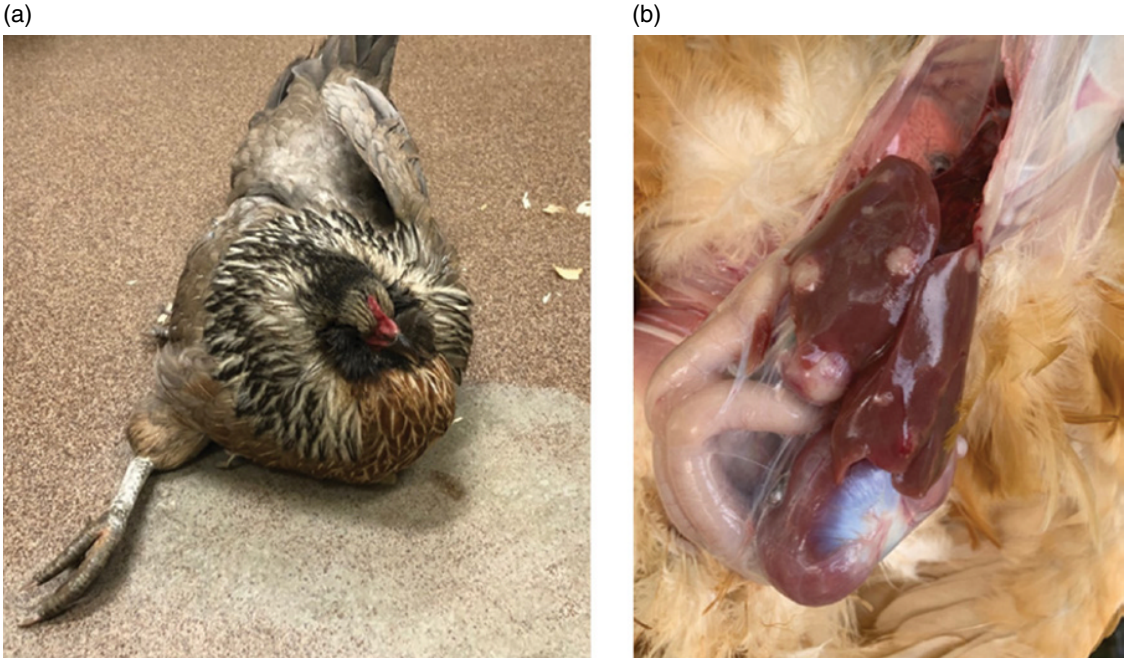


Figure 52.11 (a) Hen showing classic unilateral paresis/paralysis due to vagus nerve swelling/tumor infiltration due to Marek's disease. (b) Multifocal, discrete, nodular, tan firm swellings throughout the hepatic parenchyma due to lymphoma formation secondary to Marek's disease infection. *Source:* Photos courtesy of Dr. Geoffrey Lossie, Purdue University.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

GaHV2 is an alphaherpesvirus in the genus *Mardivirus*, and virulence varies greatly among strains.

Resistance to Physical and Chemical Agents

Cell-free virus is readily inactivated at temperatures greater than 37 °C and is only relatively stable at 25 °C (four days) and 4 °C (two weeks). MDV can be maintained for long periods at 27 °C. Virus is inactivated by pH 3 and pH 11. Infectivity of dried MDV-infected feathers is destroyed by chlorine, organic iodine, a quaternary ammonium compound, cresylic acid, synthetic phenol, and sodium hydroxide.

Infectivity for Other Species and Culture Systems

The chicken is the primary natural host for the MDV, and the disease is rare in other species except for quail. MD virus has not been shown to affect any non-avian animals. No etiologic link has been demonstrated between MDV and human cancer. The virus is most often cultivated on chicken or duck embryo fibroblast cells. Chicken kidney cells have also been used.

Host–Virus Relationships

Distribution, Reservoir, and Transmission

MD is a major disease of domestic chicken flocks worldwide. The virus tends to remain highly cell associated, but release of cell-free infectious virus is associated with productive infection of epithelial cells at the base of feather follicles, and birds are usually infected through inhalation of this cell-free virus in feather dander. The virus can persist in litter and poultry house dust. The virus is not transmitted *in ovo*.

Pathogenesis and Pathology

The incidence of MD is variable, depending on the strain of the virus and the age of the chicken. It usually occurs in chickens between 2 and 5 months old, and is commonly thought not to be seen in birds older than 22 weeks; however, disease has been observed in birds as young as 3–4 weeks and in 60-week-old laying hens. The virus primarily affects the nervous system, although visceral organs and other tissues may also be involved. Lesions involve peripheral nerves and spinal roots. The principal infected nerve trunk shows gross lesions consisting of a grayish-white swelling, which histologically is characterized by extensive lymphocytic infiltrations. Nerve enlargement is frequently unilateral. Edema may be present, and myelin degeneration of nerve sheaths may be apparent.

Ocular lymphomatosis is another possible outcome of MDV infection, with blindness resulting due to iris involvement. Histologically, a similar infiltration of lymphocytes is present, which can also occur in the optic nerve.

In the visceral form, lymphoid tumors of varying degrees of severity infiltrate the gonads, liver (Figure 52.11), lung, and skin. Affected chickens have enlarged visceral organs with white nodular or miliary foci. Occlusive atherosclerosis has been observed experimentally.

Host Response to Infection

The immune response to MDV is complex, in that both humoral and CMI develop in normal birds. Bursectomized birds survive experimental infection, suggesting that CMI is important. In chicks, passively acquired antibody is thought to limit the extent of infection rather than prevent it or clear the virus. Viral-specific antibodies appear within one to three weeks following infection, and neutralizing antibodies persist for the life of the bird. The genome of MD virus has incorporated *onc* genes, which result in transformation of infected T cells and production of T cell lymphomas following infiltration and proliferation of these transformed cells. Following infection, transient CMI suppression is common; it may persist in birds that develop neoplasms. Genetic resistance is correlated with birds that carry the B21 alloantigen of the B red blood cell group. The basis for this resistance has not been fully characterized.

Laboratory Diagnosis

On necropsy, gross lesions are common in peripheral nerves, root ganglia, and the spinal roots. Lymphomatous lesions are characteristically composed of small lymphocytes, lymphoblasts, and reticulum cells. Arterial lesions of atherosclerosis are often present. Confirmatory diagnosis is made by viral isolation or by antigen detection using fluorescent antibody or immunoperoxidase, or by detection of viral DNA by PCR. Antibodies can be detected by agar gel immunodiffusion, indirect immunofluorescence, and viral neutralization and ELISA assays.

Prevention and Control

Experimentally, MDV-free flocks can be maintained by strict isolation, constant surveillance, and frequent monitoring for virus and antibody, but these techniques have been of limited commercial use. Commercial vaccines are available and have been effective in reducing the incidence of MD. Vaccination does not prevent infection or shedding of virulent MDV, but it does prevent tumor formation, particularly in visceral organs. Peripheral nerve lesions continue to occur, but at reduced rates. Turkey herpesvirus strains (HVT; MDV serotype 3) are antigenically related viruses that have been routinely used for vaccination, but the emergence of more virulent strains of GaHV2 has resulted in vaccine failures and the increased use of

avirulent Gallid herpesvirus 3 (MDV serotype 2) viruses or strains of low-pathogenicity GaHV2 for vaccination. Birds are vaccinated annually in the United States and the vast majority are vaccinated *in ovo*.

PsHV1

Pacheco's disease is caused by Psittacid herpesvirus 1 (Pacheco's disease virus), which is a member of subfamily *Alphaherpesvirinae*, genus *Iltovirus*. This is a disease of psittacine birds and is found in the United States and around the world, in countries that have imported parrots from Central and South America. Amazon parrots account for the majority of cases, followed by African grey parrots, macaws, cockatoos, and conures. Most birds die without any premonitory signs. When signs do occur, they tend to be nonspecific (depression, anorexia, diarrhea, tremors, and instability), and parrots with clinical signs rarely survive. There is evidence to suggest that this virus may cause mucosal papillomatosis of parrots, which is common in imported and captive-raised Amazon parrots and macaws. Clinically, this appears as wart-like lesions in the oral cavity, upper digestive tract, and the cloaca. Diagnosis of PsHV1 in live birds is made by detection of the virus in the feces by PCR or virus isolation. Gross necropsy findings are nonspecific. Histologically, there is marked acute hepatic necrosis with minimal inflammation, with, or without the presence of inclusion bodies. Most birds are viremic at death, so virus may be found in multiple tissues. Variable findings include splenic necrosis, enteritis, pancreatitis, tracheitis, and air sacculitis. Detection of the virus may be done by PCR, immunohistochemistry of affected tissues, or isolation of virus in chicken embryo fibroblasts. The epidemiology of the disease is not well understood. Four serologically distinct subtypes of this virus have been described, and disease may vary by virus type and species of bird. Subclinical and persistent infections have been described, with virus shedding that may be intermittent or continuous. There is a monovalent vaccine available in North America, but with the discovery of additional serotypes, it has been suggested that a polyvalent vaccine is needed to protect against all variants.

EEHV

Disease

Elephant endotheliotropic herpesvirus (EEHV) is a systemic infection that causes an acute onset of hemorrhagic disease with an 85% mortality rate, occurring predominantly in juvenile Asian elephants. The greatest incidence of death from EEHV occurs in calves from one to eight years of age. Disease onset is acute, with lethargy, edema of the head/neck and thoracic limbs, and cyanosis of the tongue, followed by death in most animals. Asian elephants are listed as endangered by the International Union for Conservation of Nature (IUCN). EEHV has resulted in the death of 20% of all captive Asian elephant calves born in zoos in the United States and Europe since 1980, making this disease a major threat to conservation efforts. A few cases of this disease have been reported in captive African elephants in the United States, but there are no recorded cases of EEHV-related deaths in African elephant range countries. Lung and skin nodules have been associated with EEHV in African elephants but not Asian elephants.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

EEHV hemorrhagic disease has been associated with infections with multiple different species of EEHVs that are likely to be endogenous to elephants. There are two chimeric variants of EEHV-1 (EEHV-1A and EEHV-1B), which cause the majority of lethal disease. A small number of lethal and nonlethal cases of EEHV in young Asian elephants involved two additional related but considerably diverged species: EEHV-4 and EEHV-5. EEHV-2 was found in the first African elephant zoo calf known to have died of this disease. Three species have been defined, Elephantid betaherpesvirus 1, 4, and 5, in the *Proboscivirus* genus in the subfamily *Betaherpesvirinae*. Sequencing data suggest that these viruses differ from other betaherpesviruses, and it has been proposed that they should be considered a new subfamily, *Deltaherpesvirinae*.

Resistance to Physical and Chemical Agents

Infectivity for Other Species and Culture Systems

Elephants appear to be the natural host for these viruses. All currently available data support the concept that EEHV-1A, EEHV-1B, EEHV-4, and EEHV-5 are endemic in Asian elephants, whereas EEHV-2, EEHV-3, EEHV-6, and EEHV-7 are endemic to and largely ubiquitous in African elephants. Currently, attempts to replicate these viruses in cell culture have failed.

Host–Virus Relationships

Distribution, Reservoir, and Transmission

This disease has been seen in Asian elephants in zoos in Europe and North America, as well as in both captive and wild populations in Asia. Although described in a few captive African elephants, this disease has not been reported in African elephant range countries. A high prevalence of EEHV antibodies has been detected in Asian elephants. This finding supports the hypothesis that subclinical EEHV infection is normal in Asian elephants, so elephant cows likely transmit the virus to calves via direct contact. Latently infected elephants are the reservoir for these viruses.

Pathogenesis and Pathology

A generalized hemorrhagic disease is seen with EEHV. On necropsy, multi-organ hemorrhages are prominent, involving the heart, liver, kidney, lungs, spleen, lymph node, and small intestine tissues. Edema is prominent in tissues and lymph nodes. On histopathology, blood vessels in multiple organs show varying degrees of endothelial cell hypertrophy, necrosis, and edema of the vascular walls. Various sizes (80–90 nm) of basophilic intranuclear inclusion bodies indicative of EEHV infection are observed in the endothelial cells of small blood vessels in multiple organs. EEHV is primarily recognized as an endotheliotropic virus. EEHV has also been demonstrated to replicate in the epithelial cells of the oropharynx, salivary glands, and intestines, which would allow the shedding of virus for horizontal transmission. The virus can be found in monocytes/macrophages. It is suggested that the virus can replicate in these cells and that viremia is monocyte associated. In addition, the virus infects bone marrow cells, which may serve as the reservoir site for latent infection.

Host Response to Infection

Immunologically naïve young Asian elephants one to eight years of age are susceptible to the most severe forms of hemorrhagic disease. It is believed that maternal antibodies are passed from mother to calf, which protects against disease until at least one year of age, when the calf develops its own immunity. For reasons that are not yet understood, approximately 20% of Asian elephant calves appear to be susceptible to the disease when primary infections are not controlled by normal innate cellular and humoral immune responses.

Laboratory Diagnosis

PCR can be used to detect viremia in whole blood (EDTA) or viral shedding by collecting nasal secretions via trunk wash, or saliva and mucosal cells via oral swabs. Unfortunately, these techniques require extensive training of elephants, and samples are not easily obtained from untrained or wild elephants. Noninvasive sampling methods have been initially evaluated, such as collection of feces. Further evaluation is required because fecal shedding, which has been shown to occur, may be intermittent.

Prevention and Control

Stress should be minimized in young elephant calves to prevent stress-related EEHV hemorrhagic disease. This includes limiting transportation and exposure to new environments. There are currently no vaccines for these viruses. There have been reports of juvenile Asian elephants with acute systemic disease symptoms surviving after treatment with the antiherpesvirus drugs famciclovir or ganciclovir, but similar treatment has not been successful in many other cases.

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53

Poxviridae

Gustavo Delhon

General Characteristics of Poxviruses

Virus Structure

Viruses of the *Poxviridae* family are among the largest and more complex viruses infecting animals. The enveloped, pleomorphic, roughly brick-shaped or oval poxviral virions are 220–380 nm long and 140–300 nm wide, just at the limit of the resolution power of light microscopes, and consist of copies of approximately 80–90 different viral proteins. Mature poxvirus virions lack the helical or icosahedral capsid architectures common to most viruses, and are described to have a complex structure. When using negative staining electron microscopy, characteristic tubular structures are seen on the virion surface (Figure 53.1A,B). The size and morphology of poxvirus particles are so distinctive that many laboratories use negative-stain electron microscopic examination of lesion material for rapid preliminary diagnosis of poxviral infection.

Viral Genes and Virus Replication

The poxvirus genome consists of a double-stranded linear DNA molecule with lengths ranging from 127 to 365 kilobase pairs, and encodes for 130 to more than 300 genes. Genes with roles in virus structure and morphogenesis, transcription, and DNA replication are clustered into a large central genomic region and are conserved among poxvirus genera. Regions flanking the central genomic region are less conserved within the family and are enriched in genes with roles in immune evasion, host range, and virulence, with some exhibiting homology to host genes that function in innate immunity. It is believed that early modulation of host antiviral responses is important for a successful poxviral infection. Exceptional for DNA viruses, poxviruses replicate in the cytoplasm of infected cells, implying that they can dispense with host nuclear functions. Accordingly, poxvirus genomes encode a large number of replicative enzymes and factors. Indeed, factors controlling early gene expression are generated late or early/late in infection and packaged into progeny virions for immediate use following virus entry. Cytoplasmic replication of poxviruses occurs in virus-infection-induced cytoplasmic compartments known as *virus factories* or *viroplasm*, where viral genome replication and virion assembly take place (Figure 53.1C).

Classification

Viruses of the family *Poxviridae* are classified into two subfamilies, *Chordopoxvirinae* (poxviruses that infect vertebrates) and *Entomopoxvirinae* (poxviruses that infect insects), with chordopoxviruses being currently grouped into 18 genera. Table 53.1 lists poxviruses of veterinary interest together with their genus affiliation, hosts, and geographical distribution. Some poxviruses are very host species-specific, whereas others infect multiple species. Several poxviruses maintained in animals cause zoonotic infections in humans.

Poxviruses and Skin Disease

A common feature of chordopoxviruses is that, at some stage during infection of the host, they colonize, replicate, and induce pathology in the skin and, to a lesser extent, in selected mucosae. Lesions in the skin may be localized or extend over a large surface. Frequently, skin lesions adopt the form of exanthema (pocks), evolving through stages of erythema, papule, and scabs, with, or without clear intermediate vesicular and pustular stages. For some diseases such as swinepox, skin lesions may be the only manifestation of disease. For others, skin lesions are accompanied by fever and other manifestations of systemic disease such as lesions in internal organs (e.g. sheeppox and mousepox) and generalized lymphadenopathy.

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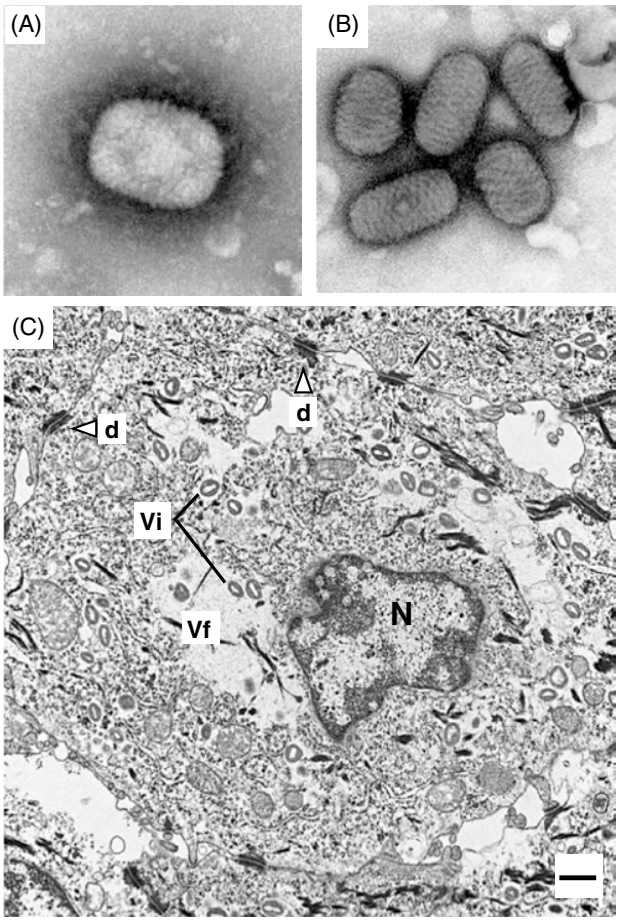


Figure 53.1 Transmission electron micrographs of (A) *Orthopoxvirus* and (B) *Parapoxvirus* particles. Note the differences in particle shape and arrangement of surface tubular structures. Negative stain; A, X 35 K; B, X 30 K; (C) Transmission electron micrograph of a capripoxvirus-infected stratum spinosum keratinocyte (sheep). N, nucleus; Vf, virus factory; Vi, intracellular virions; d, desmosomes; Bar = 500 nm. Source A and B: Courtesy of David Bhella, University of Glasgow.

The main skin target cells for chordopoxviruses are the keratinocytes, which populate the interfollicular and follicular epidermis. In the interfollicular epidermis, keratinocytes are found in different stages of differentiation, forming multiple strata from basal stem cells to apical corneocytes (i.e. keratin scales). Typical poxviral histopathology during the papular stage shows hyperplasia of the epidermis, ballooning degeneration, and intracytoplasmatic inclusion bodies in keratinocytes of the stratum spinosum, hyperkeratosis, and inflammatory cell infiltration of the dermis (Figure 53.2A). Immunohistochemistry at this stage demonstrates high expression of viral antigen in infected keratinocytes (Figure 53.2B).

Two types of poxviral inclusion bodies are known. Most poxviruses induce formation of Guarnieri's or type B inclusion bodies, the histopathological denominations for the viral factories (Figure 53.2A). In addition, some poxviruses (e.g. cowpox virus, ectromelia virus, and avipoxviruses) induce type A inclusion- or ATI bodies, which consist of aggregates of one type of viral protein with or without associated viral particles. The size and type of inclusion bodies as well as the time window during infection in which they are detected vary broadly among poxviruses and even among strains of the same virus. Detection of inclusion bodies in tissue sections from lesion material aids the diagnosis of some poxviral diseases.

Transmission

Contact of infectious material (e.g. scabs, secretions, and fomites) with broken or lacerated skin is a common route of poxvirus transmission. In addition, some poxviruses have adapted to aerosol transmission (e.g. sheeppox virus), whereas others are mechanically transmitted by biting arthropods (e.g. myxoma virus, lumpy skin disease virus, avipoxviruses).

Physical, Chemical, and Antigen Properties

Poxviruses are stable at pH values ranging from 4.5 to 10, and very resistant to drying, especially when embedded in skin crust or blood, an effect that is enhanced at low ambient temperatures. Indeed, dried vaccinia virus can be stored at 4 °C for months without significant loss of infectivity. Poxviruses are highly sensitive to chemical disinfectants, including those based on phenol, sodium hypochlorite, formaldehyde, or hydrogen peroxide. Heat resistance differences have been reported for viruses belonging to different genera.

Poxvirus species within genera are antigenically related. Infection with any orthopoxvirus, for example, may confer various degrees of protection against infection with other orthopoxviruses.

Orthopoxviruses

Members of the genus *Orthopoxvirus* are the best-known poxviruses. One reason for this is that variola virus, the human pathogen that causes smallpox, is an orthopoxvirus. Historically, smallpox was one of the most devastating human diseases until its eradication in 1980. With mortality rates approaching 35%, and transmission by the respiratory route, smallpox caused massive epidemics that impacted human demographics worldwide. In its most

Table 53.1 Poxviruses of veterinary interest.

Virus[a]	Genus	Infected host	Reservoir host	Geographic distribution
Cowpox-like viruses	*Orthopoxvirus*	Cats, dogs, cattle, humans, zoo animals	Wild rodents	Europe, Asia
Vaccinia virus		Cattle, horses, humans	Unknown	Worldwide
Camelpox virus		Camels	Nil	Africa, Asia
Horsepox virus		Horses	Unknown	Asia?[b]
Ectromelia virus		Laboratory mice	Unknown	Europe
Myxoma virus	*Leporipoxvirus*	Common domestic rabbit	Wild rabbits	Americas, Europe, Australia
Rabbit fibroma virus		Common domestic rabbit	Wild rabbits	Americas
Swinepox virus	*Suipoxvirus*	Swine	Nil	Worldwide
Sheeppox virus	*Capripoxvirus*	Sheep, goats	Nil	Africa, Asia
Goatpox virus		Goats, sheep	Nil	Africa, Asia
Lumpy skin disease virus		Cattle	Nil	Africa, Asia
Orf virus	*Parapoxvirus*	Sheep, goats, humans, camels, wild ungulates	Sheep, goats	Worldwide
Bovine papular stomatitis virus		Cattle, Humans	Cattle	Worldwide
Pseudocowpox virus		Cattle, Humans	Cattle	Worldwide
Salmon gill poxvirus	*Salmonpoxvirus*	Farmed Atlantic Salmon	Unknown	Norway, Scotland
Fowlpox virus	*Avipoxvirus*	Chicken	Unknown	Worldwide
Canarypox virus		Canary	Unknown	Worldwide

[a] Type virus species of genus is indicated in italics.
[b] Likely extinct.

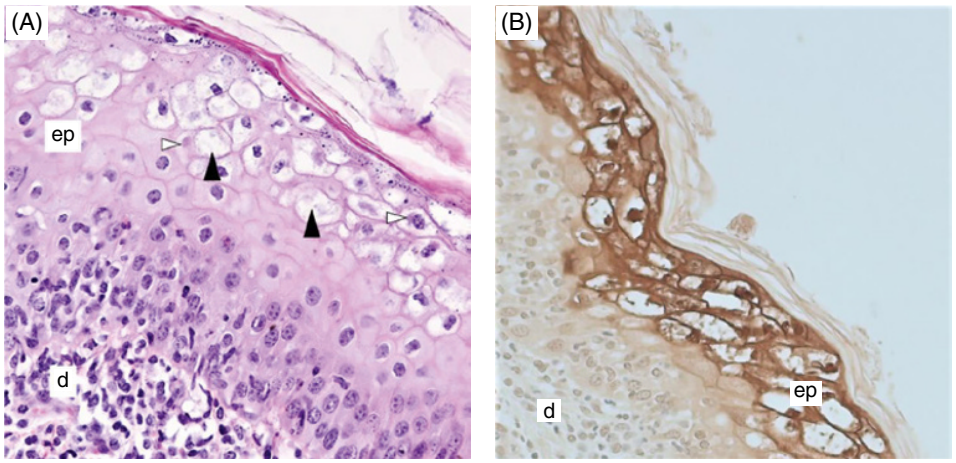


Figure 53.2 Histopathology of a skin lesion induced by poxvirus infection (contagious ecthyma). (A) The epidermis (ep) exhibits hyperplasia of keratinocytes, most notably in the stratum spinosum, with the upper cells in the stratum undergoing ballooning degeneration (black arrowheads). Pale cytoplasmic inclusion bodies are observed in a few keratinocytes (open arrowheads). The dermis (d) is infiltrated with neutrophils and mononuclear cells. H&E. (B) Immunoperoxidase staining of viral antigen in the infected epidermis X400.

frequent presentation (ordinary smallpox), the disease was characterized by fever, enanthema in the pharynx and oral cavity, and generalized exanthema. The most serious complication of the disease was bronchopneumonia, as a result of virus replication in the respiratory tract followed by secondary bacterial infection. Orthopoxviruses that cause disease in domestic animals include cowpox virus, vaccinia virus, camelpox virus, and ectromelia virus.

Cowpox

Cowpox was originally described as a disease characterized by teat lesions in milking cows caused by cowpox virus. As bovine cowpox became a rare condition, closely related viruses (cowpox-like viruses) have been isolated from cats, mice, dogs, humans, horses, farmed llamas, and a number of zoo animal species, including elephants, large felids, foxes, anteaters, and rhinoceros. The polyphyletic character of cowpox-like viruses has been confirmed by DNA sequencing and phylogenetic analysis, and it is accepted that at least five different lineages exist. Diverse as these viruses are, all are virulent in mice, produce hemorrhagic pocks in embryonated chicken eggs, and induce type A inclusion bodies.

Infections by cowpox-like viruses are endemic in Europe and Northern and Central Asia. The viruses are maintained in bank voles, wood mice, and other rodents, with spillover (i.e. infections with no onward transmission) reported in humans and other mammals, including cats. Cat-to-cat transmission has been reported, usually resulting in subclinical infection. Zoonotic transmission of cowpox virus to humans occurs via contact with infected animals. Transmission to humans has been reported primarily from cats, especially in children, but also from cattle, mice, pet rats, and zoo animals.

Cowpox viruses enter the host through broken skin. In most species, including humans, infection results in localized, self-limiting pustular, often erosive skin lesions that evolve to crust in a few days, a swelling draining lymph node and, occasionally in humans, mild fever. In domestic cats, cowpox virus infection produces a systemic infection. A primary infection occurs at the site of virus entry followed by viremia. 7–10 days later, multiple lesions develop, primarily on the skin of the head, neck, and limbs and, occasionally, in the oral cavity. Some cats exhibit conjunctivitis and become pyretic. In rare cases, the infection is fatal, with lesions manifested in internal organs such as the liver and lungs. Most cowpox virus infections in cats occur in late summer and autumn due to a higher density of potentially infected rodents.

Laboratory diagnosis is based on serology, polymerase chain reaction (PCR), and histopathology of skin lesions. The seroprevalence of cowpox in cats varies from 0 to 16%. There is no specific treatment for cowpox, but the disease is self-limiting. No commercial vaccines are available.

Vaccinia

The prototype orthopoxvirus, vaccinia virus, is antigenically related to variola, cowpox, and horsepox viruses. This antigenic relatedness was the basis for various vaccinia virus strains being successfully used as vaccines during the worldwide smallpox eradication campaign by the World Health Organization. The name "vaccinia" is derived from the Latin word *vacca* for cow, but the natural host for the virus remains unknown. Although some speculate that the vaccinia virus originates from a now extinct natural orthopoxvirus ancestor, others favor the hypothesis that the virus was unintentionally created during the poorly documented early production of smallpox vaccines from cowpox, horsepox, and even attenuated variola viruses.

Although smallpox vaccination was suspended 30 years ago, infections by vaccinia virus have been sporadically reported in multiple species. For example, infections by vaccinia virus strains have been reported in dairy cattle and milkers in Brazil. Disease in cows, known as bovine vaccinia, is indistinguishable from cowpox, with papules, vesicles, and crust in the udders and teats. Unprotected milkers become infected by contact and may spread the disease in cattle. Vaccinia virus strains have been isolated from wild rodents in bovine vaccinia areas as well as in Brazilian forests, providing a hypothesis for the maintenance of the virus in nature and outbreaks in cattle. However, the similarity of these viruses to vaccine viruses used against smallpox suggest that they represent escape viruses.

Similar to cowpox and bovine vaccinia viruses, buffalopox virus, a virus circulating in water buffalos in northern India and, to a lesser extent, in Pakistan, Egypt, and Indonesia, causes pustular lesions on the teats and udders of milking buffalos. Buffalopox can be enzootically transmitted to unprotected milkers, producing lesions on the hands and face. Buffalopox virus (and rabbitpox virus, the causative agent of a highly lethal airborne disease of laboratory rabbits) are considered vaccinia virus strains that have adapted to buffalos and rabbits, respectively.

Camelpox

Camelpox is an economically significant disease in countries of the Middle East, Asia, and Africa with indigenous camel populations. The causative agent, camelpox virus, is closely related to variola virus, and in the past, camels have been successfully protected against camelpox with vaccinia virus-based vaccines. Although New World camelids are susceptible to the virus, camelpox virus is considered to naturally infect only Old World camelids (the dromedary camel and the Bactrian camel). Seropositivity in enzootic areas ranges from 10% to 72%.

Camelpox virus entry into the host is thought to occur via the respiratory route and by contact with skin abrasions. Arthropod vectors, including ticks and biting flies, may also play a role in virus transmission. Camelpox may adopt several clinical forms, ranging from local mild skin

infections in old camels to moderate to severe systemic infections in younger animals. The incubation time for systemic forms is 8–13 days, which is followed by fever and various degrees of exanthema and lymphadenopathy. Skin rash appears one to three days after the onset of fever, following the progression macula-papule-vesicle-pustule-scab. Lesions are first observed in the head, and then in the neck and limbs. In the most severe infections (generalized camelpox), skin lesions spread to the entire body surface and the mucosa of the respiratory and gastrointestinal tracts. In uncomplicated cases, lesions resolve in four to six weeks, with virus remaining infectious in shed scabs for up to four months. Camels may exhibit lacrimation, salivation, and nasal discharge. In complicated cases, death occurs as a consequence of secondary bacterial infections. In epizootics involving young animals, the case-fatality rate may be as high as 25%. High rates of abortion have been described. Zoonotic transmission of camelpox to people in close contact with sick camels has been recently reported. Skin lesions, usually in the hands, are mild and transient.

Diagnosis of camelpox is based on clinical signs. Electron microscopic examination of lesion material, virus isolation, and serology confirm involvement of an orthopoxvirus. Various PCR assays have been reported that allow for specific orthopoxvirus identification. Mild forms of camelpox can be confounded with infections by Ausdyk virus, a poorly characterized parapoxvirus that causes localized skin lesions primarily in the face. Effective inactivated and live-attenuated virus-based vaccines can be used to protect camels from camelpox.

Mousepox

Mousepox is a rare but significant fatal disease of laboratory mice caused by mousepox (ectromelia) virus. Historically considered enzootic in Europe, Japan, and China, and infrequent in the United States, today mousepox is less common and is sporadically reported. Although natural infection by mousepox virus in wild mice has been documented, the natural reservoir for the virus is unknown.

Mousepox virus enters the body through skin breaks and causes an acute systemic disease, resulting in massive liver necrosis as well as in necrosis of the spleen, lymph nodes, and Peyer's patches. If mice survive acute disease, they develop a generalized skin rash together with necrosis and ulceration of distal parts of the feet, snout, and tail. Conjunctivitis is often associated with mousepox, with a large amount of virus being shed in ocular discharges.

Susceptibility to mousepox depends on the virus strain, mouse age, immune status, and genetic background (e.g. Balb/c and A/J mouse strains are highly susceptible, whereas AKR and C57BL6 mice resist severe disease). Resistance to mousepox is conferred by at least four genetic loci, which are associated with generation of robust innate and adaptive immunity. Highly susceptible mouse strains lack resistance alleles at these loci and the immune response against the virus is weak and delayed. As a consequence, susceptible mice die rapidly without shedding significant amount of virus. Little virus is also shed by resistant strains that exhibit limited infection.

Mice with intermediate susceptibility are, therefore, most important in spreading virus in a colony because they develop generalized disease and survive long enough to spread the virus to other animals. The persistence of ectromelia virus in inbred mice, recrudescence following immunosuppression, and transmission to naïve mice have been reported in experimental settings.

Mousepox outbreaks may significantly impact laboratory mouse colonies. Prevention is based on quarantine and regulation of mice traffic, and control of biologicals such as mouse serum. Serological tests such as enzyme-linked immunosorbent assay (ELISA), or immunofluorescence, and PCR are periodically performed on valuable mouse colonies to detect circulating virus. No vaccine specific for mousepox is commercially available. Immunization with vaccinia virus protects mice against mousepox, but vaccination is not sterilizing.

Leporipoxviruses

Myxomatosis and Rabbit Fibroma

Myxomatosis is a severe disease of domestic rabbits caused by myxoma virus, the type member of the genus *Leporipoxvirus*. The virus circulates in American wild rabbits *Sylvilagus brasiliensis* (South and Central America) and *Sylvilagus bachmani* (North America West coast), causing an innocuous localized cutaneous fibroma at virus entry sites. In European rabbits (*Oryctolagus cuniculus*), however, the virus causes myxomatosis, a disseminated disease approaching 100% mortality. Myxomatosis is characterized by fever, swelling of the head, eyelids, and anogenital region, mucopurulent conjunctivitis, and rhinitis, and multiple discrete cutaneous swellings (i.e. myxomas or tumors) ranging from a few millimeters to several centimeters in diameter. Most infected animals die between 8 and 12 days post infection. Myxoma virus is present in the secretions and skin lesions and is readily transmitted by direct contact and biting arthropods. Myxomatosis diagnosis is based on clinical signs. If classic symptoms are not seen before the animal dies, various virological, serological, and molecular laboratory tests are available.

Rabbit (Shope) fibroma is a disease of domestic rabbits caused by rabbit fibroma virus, which circulates in eastern cottontail rabbits (*Sylvilagus floridanus*) in the East and Central United States. The virus causes mild fibroma in cottontail and European rabbits. Because the immunologic response in the latter protects the rabbit against myxoma virus, rabbit fibroma virus has been used as a vaccine against myxomatosis. No vaccine against myxomatosis is approved in the United States.

Parapoxviruses

Viruses of the genus *Parapoxvirus* infect farm and wild animals worldwide, and cause mild zoonotic infections in humans. The genus includes orf virus, bovine papular stomatitis virus, pseudocowpox virus, red deerpox virus, and grey sealpox virus. Virus particles are ovoid, 260 nm long and 160 nm wide, and exhibit a regular array of surface tubular structures arranged in a characteristic crisscross pattern (Figure 53.1B). The size and shape of virus particles allows for rapid diagnosis of *Parapoxvirus* infection following electron microscopic examination of lesion material. In general, parapoxviruses cause self-limiting, nonsystemic, localized disease in the skin and oral mucosa, with a preference for mucocutaneous transitions.

Contagious Ecthyma (Orf)

Contagious ecthyma, orf, or contagious pustular dermatitis is a highly contagious ubiquitous disease of sheep and goats caused by orf virus. The disease is characterized by maculopapular, scabby, occasionally proliferative lesions affecting the skin around the mouth and nostrils, and less frequently the interdigital region and teats, and the oral mucosa (Figure 53.3). Subclinical infections have been reported. Spread in a flock is rapid and occurs by contact of broken tegument with affected animals or shed crust, implying that virus infection likely occurs in a tissue environment dominated by inflammatory and repair processes. Contagious ecthyma primarily affects animals less than 1 year old, with morbidities averaging 10–20%, although morbidities close to 90% have been reported. Mortality is usually low; however, lesions in lips and udders may prevent affected lambs and kids from suckling, which can result in rapid emaciation.

Orf virus is highly epitheliotropic, and epidermal keratinocytes and their counterparts in the oral mucosa are the most important if not the only cell type to support virus replication (Figure 53.2A,B). Inflammatory cell infiltration and active angiogenesis is seen in the underlying dermis. In severe forms, proliferative lesions characterized by papillomatous growths are seen in the mucosa lining the oral cavity (Figure 53.3B). In the absence of secondary infections, lesions are usually resolved in six to eight weeks; however, chronic infections have been reported.

In spite of a vigorous and typical antiviral T-helper type 1 (Th1) immune response, immunity elicited by contagious ecthyma virus is short-lived, and animals can be repeatedly infected, although lesions are smaller and resolve sooner than in primary infections. Contagious ecthyma is a

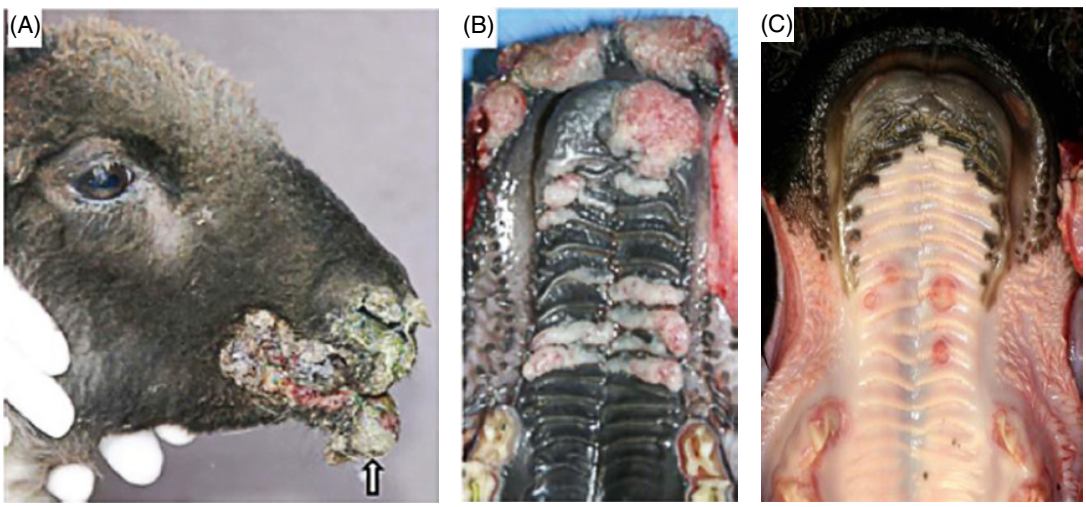


Figure 53.3 Infection of ruminants by parapoxviruses. (A) and (B) Ecthyma contagiosum (orf) in a lamb. Thick crust surrounds the mouth and nostrils. (A) The arrow indicates a proliferating mass originated from the inner side of the lower lip. (B) Papillomatous growths in the mucosa lining the hard palate and upper lip. (C) Bovine papular stomatitis. Erosive lesions in the hard palate and the alveolar mucosa of a calf. Source: Courtesy of Francisco A. Uzal, UC Davis.

zoonotic disease, affecting humans in close contact with infected animals. In general, lesions are solitary and nodular, usually on the hands. Contagious ecthyma virus occasionally infects camels and wild mammals, including chamois, ibex, musk ox, and the Japanese serow.

Although current vaccines cannot prevent orf, they can reduce pathology and disease duration. Pregnant ewes can be vaccinated using non-attenuated virus derived from crust material collected from sick animals. Although immunity achieved in this way is short-lived, vaccinated animals and their offspring are less prone to develop ecthyma.

Bovine Papular Stomatitis

Bovine papular stomatitis is a mild disease of cattle characterized by the presence of papules, often mildly erosive, on the muzzle, lips, nares, dental pad, palate, tongue, and udder and, rarely, in the esophagus and forestomach (Figure 53.3C). Histologicalldy, lesions are similar to but less extensive and proliferative than those in contagious ecthyma. The disease occurs worldwide, with the highest incidence in milking herds and animals less than one year old. Subclinical infections have been described. Similar to contagious ecthyma, reinfection with bovine papular stomatitis virus is commonly observed, suggesting that virus infection does not confer significant immunity. Because of its clinical resemblance to foot-and-mouth disease, the disease is reported to veterinary authorities for differential diagnosis. Like other *Parapoxvirus* infections, bovine papular stomatitis is an occupational zoonotic disease.

Pseudocowpox

Pseudocowpox is a frequent enzootic infection of cattle worldwide caused by pseudocowpox virus, previously known as paravaccinia virus. Infections by pseudocowpox virus are mild, affecting the teats and udder of milking cows, and occasionally the mucocutaneous transitions around the mouth and muzzle of nursing calves. Typical *Parapoxvirus* lesions develop at these sites. Manual removal or natural falling of crust leaves exposed granulomatous tissue that heals centrifugally, forming a characteristic scabby circular ring, a process that takes one to two weeks. Chronic infections have been reported. The infection spreads slowly in herds, likely by cross-suckling of calves and mechanical virus transmission by flies. Cattle may become reinfected in subsequent lactations. Electron microscopic examination of scab homogenates shows parapoxviral particles, thus excluding cowpox and vaccinia virus infections and mechanical injuries. Pseudocowpox can spread from cattle to man by contact, leading to painful nodular lesions, usually in the hands, known as milker's nodules.

Capripoxviruses

The three capripoxviruses, sheeppox virus, goatpox virus, and lumpy skin disease virus, are the causative agents of sheeppox, goatpox, and lumpy skin disease, respectively. The diseases, found in regions of Africa and Asia, are arguably among the most serious poxviral diseases affecting production animals. In addition to direct mortality, *Capripoxvirus* infections are associated with reduced milk production, increased abortion rates, decreased weight gain, and increased susceptibility to secondary bacterial infections. *Capripoxvirus* diseases can negatively impact international animal trade, and are on the World Organization for Animal Health list of important animal diseases that need to be reported. Capripoxviruses are not present in the Americas and Australasia.

Virus particles are ovoid, 300×270 nm in size, and morphologically indistinguishable between the three viruses. Comparison of the genomic DNA sequences of sheeppox, goatpox, and lumpy skin disease viruses has shown that they are closely related viruses. A reflection of this is that infection with any virus induces cross-protection to heterologous capripoxviruses. Although lumpy skin disease virus affects only cattle, some strains of sheeppox and goatpox viruses can infect both sheep and goats, with most virus strains causing more severe disease in the homologous host. Sheeppox and goatpox are clinically indistinguishable, and they are described as a single entity.

Sheeppox and Goatpox

Sheeppox and goatpox regularly occur in Central and North Africa, Middle East, Russia, Central and South Asia, and, more recently, Bulgaria and Greece. The diseases vary from inapparent to severe generalized disease depending on factors such as breed, age, and immune status of the animals, and virus strain. In the most frequent form, acute disease, the incubation time ranges from 7 to 12 days. Fever is the first clinical sign followed by skin rash 2–4 days later. Skin lesions start as erythema that develop into papules 0.5–1.0 cm in diameter (Figure 53.4A). Skin lesions initially localize to wool-less areas such as the axilla, groin and perineum, and the face, and they tend to become confluent in a few days, especially in the lips, eyelids, and around the nose. Eventually, skin lesions may cover the entire body surface. Coincident with the appearance of papules, animals show excessive salivation, and nasal and conjunctival discharge. As the rectal temperature drops, the superficial lymph

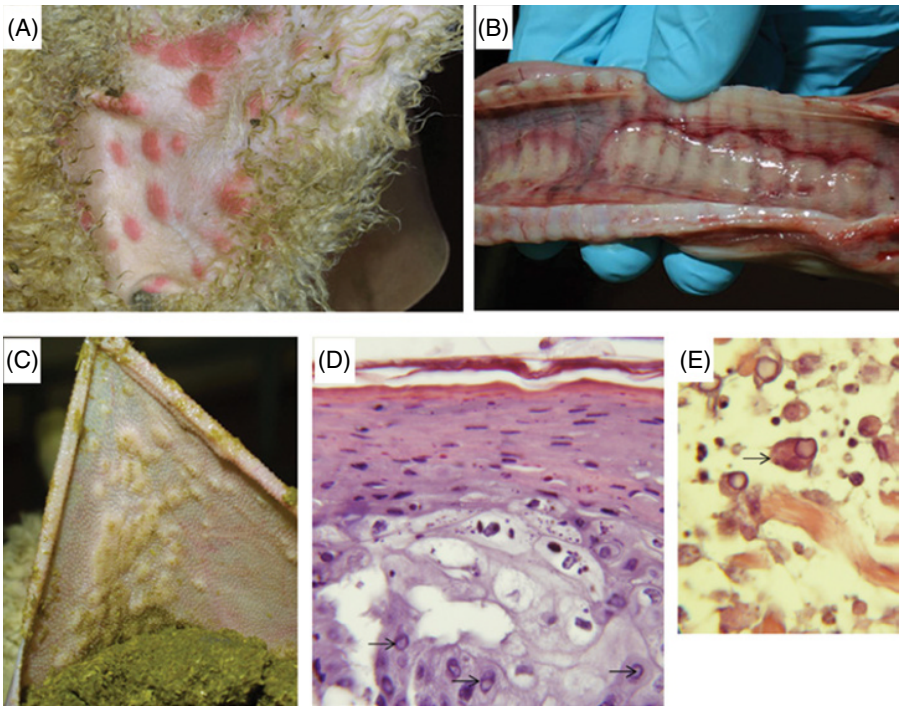


Figure 53.4 Sheeppox lesions in a lamb. (A) Papules in axillary skin. (B) Hemorrhagic ulcers in the tracheal mucosa. (C) Nodular lesions in the ruminal wall. (D) Histological section of a papular lesion of the skin showing parakeratosis, hyperplasia, and ballooning degeneration of keratinocytes. Keratinocytes with vacuolated nuclei are indicated by arrows, H&E, X380. (E) Macrophages with sheeppox cell phenotype in the dermis (arrow) H&E, X400.

nodes become enlarged. If animals survive this stage, papules become necrotic and develop into scabs, usually without intermediate vesicular and pustular stages. Scabs, which may persist for six to eight weeks, remain infectious for months after detaching from the skin. Bronchopneumonia is a frequent complication in fatal cases, and is usually associated with a second increase in rectal temperature. Although the virus causes large primary nodular lesions in the lungs, it is thought that secondary bacterial infection is responsible for bronchopneumonia. Nodular lesions are also observed in the gastrointestinal tract, including the tongue, palates, esophagus, and forestomach, whereas pale areas are seen on the surface of the liver and the kidneys (Figure 53.4C). Lesions in the upper respiratory tract and oral cavity are short-lived, and ulcerate, becoming a significant source of infectious virus (Figure 53.4B). Histologically, skin and lung lesions frequently contain cells with a distinctly vacuolated nucleus known as *sheeppox cells*, which represent virus-infected cells of phagocytic, fibroblastic, and/or epithelial origin (Figure 53.4D,E).

Mortality in young animals can be as high as 50–70%, and case-fatality rates may approach 100%. In adults, mortality rates range between 1% and 10%. In indigenous animals, generalized disease and high mortality are rare. Instead, high mortality is seen in unprotected imported breeds of sheep and goats. Sheeppox and goatpox viruses propagate mainly by aerosols, requiring close contact between animals. Experimental infections in sheep and goats suggest that extended excretion of virus in secretions may take place following clinical recovery. Mechanical transmission of virus by insect vectors has been suggested.

In endemic areas, clinical signs and necropsy findings are sufficient for a diagnosis. Several tests have been proposed for laboratory confirmation of sheeppox and goatpox, including ELISA and agar immunodiffusion tests to detect *Capripoxvirus* antigen, PCR, and real-time PCR. Virus isolation in primary sheep cells (e.g. lamb testis or kidney cells) can be achieved using skin samples or nasal and oral secretions collected within the first week of the occurrence of clinical signs. Virus neutralization is the most widely used assay for antibody detection.

Virus strains attenuated by serial passage in cell culture have been successfully used as vaccines, yet vaccine breakdown and short duration of protection have been reported. Field evidence suggests that some virus strains are quite host specific and are used only in sheep against sheeppox and only in goats against goatpox.

Lumpy Skin Disease

Although lumpy skin disease has been historically confined to sub-Saharan Africa, outbreaks have been

recently reported in the Middle East, Southern Russia, and parts of India, China, and Southeast Europe. It is essentially a cattle disease, with European breeds being more susceptible than Zebu, and with morbidity varying from 10% to 85%, and a mortality of 1%, although mortality rates over 75% have been recorded. Economic losses result from poor general condition, decreased milk production, abortions, infertility, and damaged hides. Although virus neutralization activity has been occasionally detected in selected wildlife species, there is no decisive support for a role of these species as *Capripoxvirus* reservoirs.

Lumpy skin disease is a subacute to acute generalized disease characterized by extensive cutaneous lesions. Following an incubation period of 4–14 days, fever of 40–41 °C develops, persisting for one to two weeks. At this time, animals show depression, salivation, nasal and ocular discharges, and enlarged superficial lymph nodes. Erythematous skin lesions appear in the first or second week after the onset of the disease, rapidly evolving to firm, well-circumscribed nodules up to 5 cm in diameter. The nodules are first seen in the head, neck, and perineum, but they can eventually cover the entire body surface. Some nodules may persist for weeks or even months before becoming hard and dry. Nodules finally detach, leaving ulcers that heal in a few weeks if not complicated with secondary bacterial infection or myasis.

Transmission of lumpy skin disease by direct contact between cattle is inefficient. The disease is prevalent in wet seasons when insects are abundant, and wanes with the onset of the dry season. Experimental transmission of the disease to susceptible cattle by the mosquito *Aedes aegypti* has been reported. Stable flies and hard ticks are likely mechanical vectors in certain regions. These observations suggest that insect-vectored transmission may be significant in epizootic outbreaks.

Clinical signs allow for lumpy skin disease diagnosis. For confirmation, several PCR-based and serological tests have been developed. Lumpy skin disease should be distinguished from pseudo-lumpy skin disease, a mild eruptive condition of cattle caused by bovine herpesvirus 2.

Live field strains of lumpy skin disease virus have been attenuated by serial passage in tissue culture or in the chorioallantoic membranes of embryonated hen's eggs and successfully used as vaccines. A goatpox virus-based vaccine (Gorgan strain) has also been used to protect cattle from lumpy skin disease. Annual vaccination is recommended. Recently, mass vaccination proved instrumental in containing incursions of lumpy skin disease into Southeast Europe.

Avipoxviruses

Avipoxviruses have been identified as the causative agents of disease in more than 200 bird species, including domestic, pet, and wild birds. They are classified on the basis of host species, disease characteristics, virus growth properties, and antigenicity. Genome analysis in a few avipoxviruses, including fowlpox virus, the type member of the genus, and canarypox virus, revealed that avipoxviruses are the most complex poxviruses currently known.

Fowlpox

Fowlpox is a serious slow-spreading, worldwide disease that affects chickens and turkeys. Transmission is by contact through abrasions in the skin and by biting insects. In its most frequent form, *cutaneous form* or dry fowlpox, the disease is characterized by proliferative lesions primarily confined to the skin of unfeathered body parts such as eyelids, comb, wattle, legs, feet, and the base of the beak. When lesions affect the eyelids, it is common to see complete closure of the eyes (Figure 53.5). Lesions range from small nodules to wart-like masses that evolve to scars before healing. Histologically, the hyperplastic epidermis shows degenerate keratinocytes containing large cytoplasmic eosinophilic (H&E) inclusion bodies known as Bollinger's bodies. In noncomplicated cases, lesions heal in three weeks.



Figure 53.5 Fowlpox, cutaneous form. Scab-stage lesions are seen in unfeathered parts of the head, with those in the eyelids completely obliterating the eyes. *Source:* Courtesy of Francisco A. Uzal, UC Davis.

Although mortality with cutaneous fowlpox is low, the transient drop in egg production in laying hens and decreased growth rates in young birds may be economically significant. A less common, severe form of the disease known as *diphtheric fowlpox* or wetpox is characterized by caseous plaques or necrotic pseudomembranes firmly attached to the mucosa lining the upper respiratory tract and esophagus. Cutaneous and diphtheric forms may coexist in the same animal. Aerosols have been suggested to play a role in transmission of fowl diphtheria.

Diagnosis of fowlpox is based on clinical signs and histopathology (inclusion bodies). The virus can be isolated by inoculation of lesion material into avian cell cultures for appearance of the cytopathic effect, or into the chorioallantoic membrane of chicken embryos for development of pock lesions. Although most strains induce pock lesions on the membrane by three to five days, not all isolates grow well in embryonated eggs. PCR allows for sensitive and specific detection of fowlpox virus DNA as well as distinction between field and vaccine virus strains.

Insect control programs and sanitation of housing are important to reduce the likelihood of fowlpox outbreaks. Attenuated live fowlpox and pigeonpox virus strains are used to protect poultry from fowlpox. The vaccines are usually applied by wing web inoculation, a laborious method for large facilities. Highly attenuated vaccines obtained by serial passage *in vitro* or by recombinant DNA technology exist for inoculation *in ovo*.

A distinctive feature of many field and vaccine fowlpox virus strains is the presence of reticuloendotheliosis proviral sequences integrated into the viral genome. Reticuloendotheliosis viruses are avian immunosuppressive retroviruses that cause lymphomas in chickens. Proviral sequences inserted in the fowlpox virus genome range from remnants of the long terminal repeats to nearly the full-length genome. Although the selective advantage for the viruses of this natural recombination is unknown, some speculate that reticuloendotheliosis virus-induced immunosuppression might favor fowlpox virus infection of the host and subsequent transmission.

Poxviral Diseases in Other Bird Species

Avipoxviruses infect a variety of wild and caged birds, including penguins, ostriches, canaries, parrots, and sparrows, with disease ranging from cutaneous lesions in the head and feet to severe diphtheric forms. Canarypox, a disease of birds both in the wild and in canaries, is usually systemic, with mortalities approaching 100%. Cutaneous and diphtheric forms have been described. In systemic forms, lesions are found in the skin, lungs, and liver. Vaccination is practiced by some canary breeders. With the exception of canarypox virus, little is known about the biology of causative viruses, strain variability, and their relationship to known avipoxviruses. Chickens are usually used to determine the pathogenicity of new isolates, but many viruses are not pathogenic or are marginally pathogenic in these species (e.g. pigeonpox virus), indicating host specificity of isolates.

Avipoxviruses as Vaccine Vectors in Veterinary Medicine

As an alternative to conventional vaccines, poxviruses have been engineered to express heterologous genes and are used as vaccine vectors. For this purpose, genes encoding immunogenic pathogen proteins are inserted into the genomes of attenuated poxviruses and expressed upon infection of vaccine recipients. Avipoxviruses such as fowlpox virus are used as vaccine vectors for avian influenza, Newcastle disease, and infectious laryngotracheitis. The important observation that avipoxviruses can enter mammalian cells and undergo a nonproductive infection (i.e. progeny virus is not produced) led to development of safe *Avipoxvirus* recombinant vaccines for mammals. Licensed canarypox virus–vectored vaccines exist for immunization against rabies, canine distemper, West Nile virus, feline leukemia virus, and equine influenza virus.

Swinepox

Swinepox is a mild acute disease of swine worldwide characterized by typical pocks in the skin. The disease is caused by swinepox virus, the sole member of the genus *Suipoxvirus* of the family *Poxviridae* (Table 53.1). Similar to vaccinia virus, swinepox virions are brick-like shaped and approximately 320 nm × 240 nm in horizontal section.

Swinepox morbidity rates can be as high as 100%, but mortality is generally negligible (<5%). Young animals are the most susceptible, with adults usually developing a mild, self-limiting form of the disease. Multiple cutaneous lesions are commonly found on the flanks, belly, inner side of the legs, ears, and less frequently on the faces of affected animals.

Swinepox is usually associated with poor sanitation conditions, which are rarely seen in modern production settings. Swinepox virus is mechanically transmitted by lice (*Haematopinus suis*), which affects the extent and distribution of cutaneous lesions, which often occur in less keratinized abdominal and inguinal regions. However, swinepox without evidence of louse involvement has been described, suggesting a role for other insect vectors or the possibility

of horizontal transmission. Vertical virus transmission is indicated by sporadic cases of congenital infection resulting in stillborn fetuses with generalized lesions.

Clinical signs and epidemiology are usually sufficient for a swinepox diagnosis. Given that the disease is of relatively low economic impact, no vaccine has been developed. Good animal husbandry, including ectoparasite control, should be practiced.

Poxvirus Infections of Horses

Although frequently reported in the nineteenth and early twentieth centuries in Europe, poxviral infections of horses are currently very rare. Several clinical forms of poxviral disease have been described in horses, but only in a few cases has the causative agent been characterized in any way. Horsepox virus, an orthopoxvirus, has been recovered from Mongolian horses with severe pustular stomatitis and dermatitis (horsepox) and found to be very similar to – but distinct from –vaccinia virus. The virus, considered extinct, has been recently constructed in the laboratory by assembling chemically synthesized subgenomic DNA fragments. Horsepox has also been associated with some form of equine pastern dermatitis (i.e. grease heel), but the identity of the involved orthopoxvirus remains unknown. Lesion material from at least some cases of equine pastern dermatitis has been used to immunize people against smallpox.

Severe cutaneous disease associated with vaccinia virus infection in horses in southern Brazil has been reported. The disease was characterized by papules and vesicles in the muzzle, external nares, and lips that evolved to scabs. Vaccinia virus infections were relatively frequent in horses by the times of the smallpox eradication campaigns.

A condition named Uasin Gishu, characterized by papillomatous skin lesions, affects horses in some regions of Africa, and is caused by a virus antigenically related to orthopoxviruses. Viruses morphologically similar to molluscum contagiosum virus, a poxvirus of the *Molluscipoxvirus* genus that affects humans, have been demonstrated in small persistent lesions affecting the skin of horses and donkeys.

Poxvirus Infections of Fish

Gill diseases associated with poxvirus infections have been described in ayu (proliferative bronchitis), common carp (carp edema), koi (sleepy disease) and, more recently, salmon. The latter is caused by salmon gill poxvirus, the most divergent chordopoxvirus and one of the multiple infectious agents associated with the gill disease complex in salmon. The disease is economically significant where salmon is intensively farmed, for example, Norway, and Scotland. When gills are infected with the virus, widespread apoptosis of epithelial cells and severe gill hyperplasia are observed. It has been shown that viral infection suppresses mucosal immunity, thus facilitating coinfection with other agents. Stressful farming conditions are thought to favor disease and mortality. Diagnosis is made by PCR. No treatment or vaccine is available.

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54

Picornaviridae

Luis L. Rodriguez and Jonathan Arzt

Introduction

The family *Picornaviridae* consists of small icosahedral RNA viruses. Recent metagenomic discoveries have led to a significant increase in the number of viruses associated with the family. Many of these novel picornaviruses have been discovered in stool samples from both healthy and clinically affected animals, have never been isolated in cell culture, and only their sequences are known. The family currently has 63 genera and 147 virus species, with many more viruses still pending classification. At least 11 of the genera contain members that cause diseases of veterinary importance (Table 54.1). Common characteristics of the family include a genome consisting of non-segmented single-stranded positive-sense RNA (ssRNA+) ranging in size from 6.7 to 10.1 kb and containing a poly A tail, resembling cellular mRNA except that it does not contain a 5′ cap. The genome is protected by a small (30–32 nm) non-enveloped icosahedral capsid. Although encompassing some of the smallest viruses, picornaviruses cause some of the biggest animal health problems, such as foot-and-mouth disease (FMD), one of the most contagious and economically devastating diseases of livestock. Picornaviruses are characterized by their rapid replication cycle, remarkable stability in the environment, and swift transmission among susceptible hosts. These three general features are the main reasons why some picornaviruses that affect animals are associated with high morbidity and extensive economic losses. This chapter will discuss the picornaviruses of veterinary importance from the perspective of general virology, pathogenesis, diagnosis, and control.

Virion Structure, Genome Characteristics, and Replication

Electron micrographs of picornaviruses demonstrate icosahedral viral particles with sphere-like morphology and no projections (Figure 54.1). The viral capsid is composed of 60 identical units (protomers), each consisting of three surface proteins (1B, 1C, and 1D, also referred to as VP3, VP2, and VP1, respectively) and an internal protein (1A or VP4).

Virion physicochemical properties vary among different genera, with some members showing instability below pH 7 (e.g. aphthoviruses) and others highly resistant to pH changes (e.g. enteroviruses). All virions are insensitive to organic solvents such as ether and chloroform.

Viral Replication

Entry into the cell occurs via interaction of the viral capsid with receptors on the susceptible cell. Receptors vary among genera, but include immunoglobulin-like family, the low density lipoprotein receptor (LDLR) family, the complement control family, the integrin family of cell adhesion molecules, and the T cell immunoglobulin domain mucin-like domain receptors. Following entry, the viral genome is released into the cytoplasm, where it functions both as mRNA and as a template for genome replication. Protein synthesis is initiated in a cap-independent manner, through recruitment of cellular ribosomal complexes by the viral internal ribosome entry site (IRES). One of the key mechanisms of pathogenesis in several picornaviruses is the interference with host protein translation by removal of ribosomal subunits that recognize the CAP in the cellular

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Table 54.1 Family Picornaviridae; genera with veterinary relevance and representative species.

Genus	Species with veterinary relevance	Associated major disease
Aphthovirus	Foot-and-mouth disease virus	Vesicular disease, fever, lameness
	Equine rhinitis virus A	Respiratory disease
	Bovine rhinitis virus A and B	Respiratory disease
Erbovirus	Equine rhinitis virus B	Respiratory disease
Enterovirus	Swine vesicular disease virus	Vesicular disease, CNS disease
Sapelovirus	Porcine sapelovirus	SMEDI, reproductive failure, gastroenteritis, respiratory disease
	Avian sapelovirus	Growth inhibition
Kobuvirus	Bovine kobuvirus	Enteritis
Teschovirus	Porcine teschovirus 1	Encephalomyelitis, SMEDI
	Porcine teschoviruses 2–11	SMEDI, Talfan disease
Tremovirus	Avian encephalomyelitis virus	Encephalomyelitis
Avihepatovirus	Duck hepatitis virus A (types 1, 2, 3)	CNS disease, hepatitis
	Turkey hepatitis virus	CNS disease, hepatitis
Cardiovirus	Encephalomyocarditis virus	Myocarditis
Senecavirus	Senecavirus A	Vesicular disease in pigs
Megrivirus	Megrivuris C	Turkey hepatitis

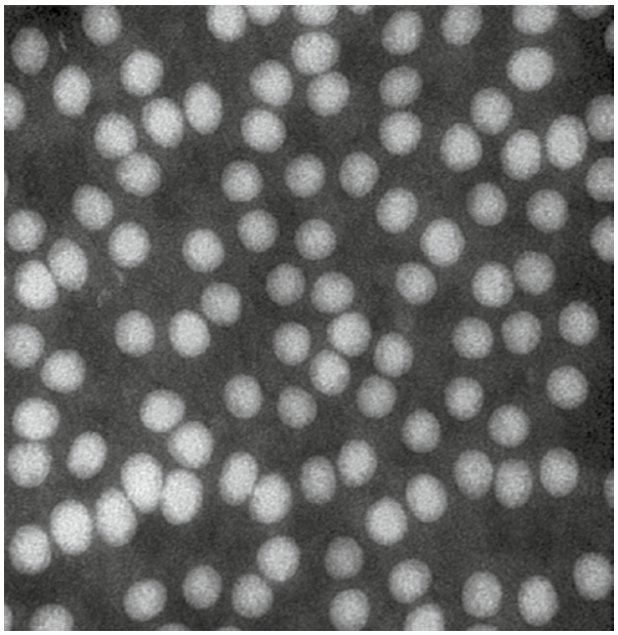


Figure 54.1 Electron micrograph of purified negatively stained foot-and-mouth disease (FMD) virus particles at a 50,000x magnification. *Source:* Benjamin A. Clark, 2014. Plum Island Animal Disease Center.

mRNA. Translation of the single open reading frame produces a 240–250 kDa polyprotein precursor that is cleaved by viral and host proteases into the viral structural proteins (SP) derived from the P1 region and the non-structural proteins (NSPs), derived from the P2 and P3 regions (Figure 54.2). Viral RNA replication in infected cells is carried out primarily by the viral RNA-dependent RNA polymerase (3Dpol), in conjunction with other viral or cellular proteins. At later stages during viral infection, the viral RNA is transcribed into a negative-strand RNA from which synthesis of the progeny plus strand RNA is copied in a multi-stranded replicative intermediate (RI) complex. The negative-sense RNA serves as a template for the synthesis of multiple copies of genomic RNA, some of which are translated, and others are packaged into viral particles. Due to the lack of proofreading activity of RNA-dependent RNA polymerase (3Dpol), errors are generated at a frequency of 1/10 000 bases incorporated during replication, with the result that every new genome contains at least one mutation. Therefore, the virus population consists of a collection of genetically diverse members (quasi-species) that give the virus the ability to rapidly respond to selective pressure exerted by host factors (e.g. immune response).

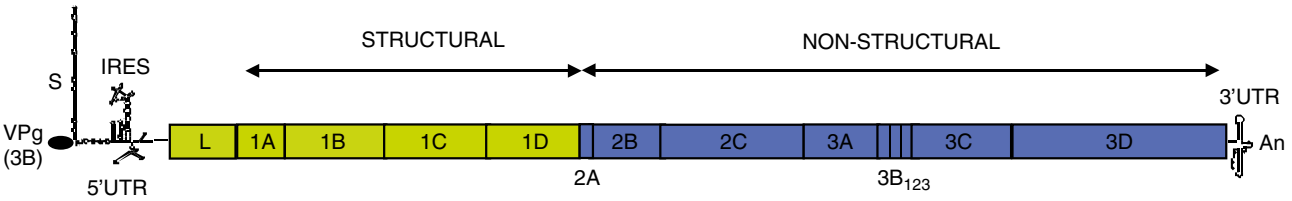


Figure 54.2 The genome consists of one molecule of positive-sense, single-stranded RNA, with a single open reading frame. A poly(A) tail of varying length is located at the 3′-terminus, and a small 2.2–3.9 kDa protein (3B or VPg) is linked covalently to the 5′ terminus. There is a highly structured 5′ untranslated region (UTR) containing signal sequences such as an IRES to initiate translation of the viral polyprotein and a clover-leaf "S" fragment essential for viral replication. In some picornaviruses (e.g. foot-and-mouth disease, encephalomyocarditis), there is also a poly (C) tract at the 5′UTR. *Source:* Grubman and Baxt (2004).

Genus *Aphthovirus*

Foot-and-Mouth Disease Virus

Disease

Foot-and-mouth disease virus (FMDV) causes a highly contagious disease, FMD, in domestic and wild cloven-hoofed animals including cattle, buffalo, pigs, sheep, goats, and deer. The clinical phase of disease is characterized by fever, lameness, and vesicular lesions in the oral cavity, feet, snout, and teats resulting in high morbidity but low mortality in adult animals (Figure 54.3). However, mortality can be high in younger animals, usually due to myocarditis. FMD is considered the most contagious diseases of animals, and outbreaks require immediate notification by member countries to the World Organisation for Animal Health (OIE). Outbreaks in FMD-free regions result in trade bans and restrictions of susceptible animals and their products, with devastating economic consequences for the affected countries.

Etiologic Agent

Physical, Chemical, and Antigen Properties

FMDV, the type species of the *Aphthovirus* genus, is an antigenically variable virus with seven serotypes: A, O, C, Asia-1, and South African Territories (SATs) 1, 2, and 3. Infection with one serotype generally does not provide cross-protection

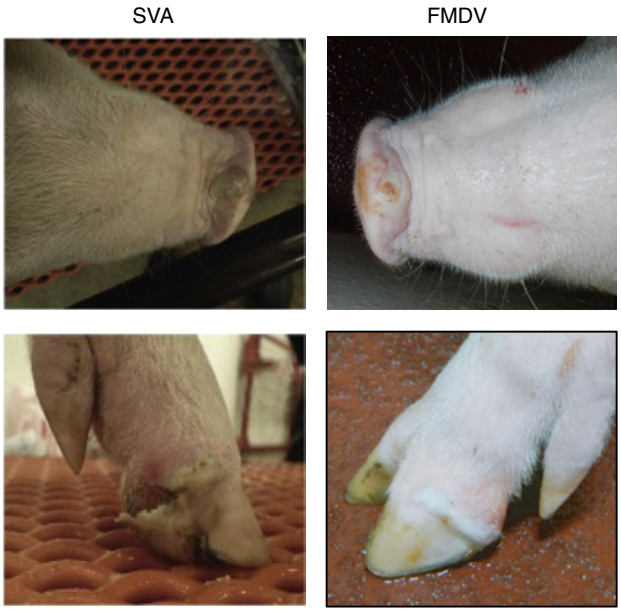


Figure 54.3 Snout and coronary band vesicles in pigs caused by foot-and-mouth disease (FMDV) and Seneca Valley virus (SVA). Generalized vesicular disease in pigs is a common pathway manifestation that occurs due to infection with various viruses including FMDV, swine vesicular disease virus (SVDV), vesicular stomatitis virus (VSV), SVA, and vesicular exanthema of swine virus (VESV). Clinical presentation is essentially indistinguishable across etiologies; differentiation may be supported by epidemiological characteristics, but must be confirmed by molecular techniques. The most common sites of vesicles are the oral cavity, snout, and coronary bands of the feet, with variable involvement of haired skin on the limbs and lips. Vesicles are typically filled with fluid, but rupture and slough rapidly to leave epithelial erosions. Vesicles often heal quickly, but may lead to sloughing of claws and chronic malformations. *Source:* Buckley et al. (2021)

against other serotypes and sometimes even among multiple subtypes within individual serotypes. Therefore, effective immunization requires careful consideration of geotemporally relevant serotype and subtype-specific vaccine strain selection.

Resistance to Physical and Chemical Agents

FMDV lacks an envelope, making it quite stable in the environment. However, FMDV is an acid-labile virus and begins to dissociate into its constituent pentamers at pH below 6.5 or over 7.5. This makes the use of acidic and basic disinfectants such as citric acid and sodium hypochlorite (bleach) effective. Recent studies have shown that FMDV is readily inactivated by sodium hypochlorite (1000 ppm), citric acid (1%), and sodium carbonate (4%) when dried on nonporous surfaces. However, on a porous surface (wood), citric acid (2%) was more effective in virus inactivation than sodium hypochlorite even when tested at 2500 ppm. Based on these studies, chemicals containing very low pH are preferred for FMDV disinfection.

Infectivity for Other Species and Culture Systems

The virus's typical host range includes domestic and wild cloven-hoofed animals including cattle, buffalo, pigs, sheep, goats, and deer; however various other species have been shown to become infected naturally and experimentally. Multiple cell lines may be used for isolating FMDV, including primary cells (e.g. bovine thyroid, lamb kidney) and immortalized cell lines (e.g. BHK-21, SK6, MDBK, LFBK). However, the most sensitive cell line reported for FMDV detection is LFBKavb6, a porcine cell line transfected with the bovine integrins alpha v beta 6.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

FMDV is still enzootic in much of Asia and Africa, and it has occurred in all continents except Australia and Antarctica. The disease was eradicated from Europe and North America. Large parts of South America control the disease through vaccination. However, disease outbreaks remain prevalent in endemic "pools" in South America; East, South East, and Central Asia; Southern Africa; Northern Africa; and the Middle East. From these pools, the virus sporadically makes incursions into FMDV-free countries, for example, in Taiwan in 1997, in the United Kingdom and continental Europe in 2001, Japan in 2001 and 2010, and multiple outbreaks in the Republic of Korea in 2010–2011 including an outbreak in 2011 that was the largest in that country's history. Some of these outbreaks were controlled without the use of vaccines, resulting in the slaughter of millions of animals at a cost of billions of US dollars, with the virus still not eradicated from Korea as of 2021. FMDV can be transmitted by direct or indirect contact with infected animals, their secretions, or contaminated food products, and it has been documented that the virus can travel over extensive distances to cause incursions at previously virus-free premises.

Pathogenesis and Pathology

Natural infection of cattle and sheep with FMDV occurs primarily via the upper respiratory tract (nasopharynx), whereas pigs are infected via the upper gastrointestinal tract (oropharynx). It has been shown that infected animals may transmit FMDV prior to clinical signs, which is referred to as preclinical or latent phase transmission. Similarly, FMDV may be shed in high quantities by animals that never develop clinical disease; this phenomenon, known as neoteric subclinical infection, may occur when viruses are highly host adapted and in animals that are vaccinated.

Recent studies have demonstrated that in most species primary replication in MALT epithelial cells precedes the onset of viremia. Viremia results in systemic dissemination throughout the body; but high-titer viral replication only occurs at lesion predilection sites including the interdigital epithelia, coronary bands of the hooves, the oral cavity, and less frequently the myocardium (Figure 54.3). The classical lesions of non-haired skin are often conspicuous and dramatic. However, some combinations of species and viral strain result in markedly diminished clinical and pathological manifestations. Lesions are typically conspicuous in pigs; but, it is noteworthy that the clinical syndrome in suids is largely indistinguishable across several other viral causes of vesicular disease, including Senecavirus A (SVA), vesicular stomatitis virus (VSV), swine vesicular disease (SVD), and vesicular exanthema of swine (VES). The specific factors determining tropism and high-titer replication at the lesion sites remain undetermined.

Following the acute phase, up to 90% of FMDV-infected ruminants, whether vaccinated or naïve animals, become chronic, asymptomatic carriers of virus. Carrier animals are those from which live virus can be isolated after 28 days post infection. During the carrier phase, infectious virus is limited to the same MALT tissues in which primary replication occurred, typically in the nasopharynx. The role of carrier animals in the epidemiology, ecology, and long-term maintenance of FMDV in animal populations remains unclear. The carrier state has been shown to last up to three years in domestic cattle and five years in African buffalo, with a small proportion of carriers clearing infection continuously. Pigs have been demonstrated to not be competent to become

carriers, but the mechanistic difference in pathogenesis has not been elucidated.

Molecular Pathogenesis

FMDV enters cells via specific cellular receptors (integrins αvβ1, αvβ3, αvβ6, and αvβ8). *In vitro*, FMDV binds to cellular integrins via a highly conserved Arg-Gly-Asp (RGD) sequence located on the G-H loop of capsid protein VP1. Studies *In vivo* suggest that the integrin αvβ6 is the major viral receptor because it is expressed constitutively at high levels on the surface of epithelial cells in tissues susceptible to FMDV, and it is also expressed on the surface of FMDV-infected epithelial cells. Upon binding to cells, virus entry occurs by clathrin-dependent endocytosis followed by acidification of endosomes, leading to capsid disruption and release of viral RNA.

Following uncoating, and release of viral RNA into the cytoplasm, translation is initiated internally in a cap-independent mechanism at the IRES located approximately 1500 bases downstream from the 5′ end of the genome. There are two functional in-frame start codons (AUGs), but translation is initiated mainly from the second initiation codon, resulting in a single polyprotein that is processed by viral encoded proteinases L^{pro} and $3C^{pro}$ and protein 2A to yield SP and NSP. Four structural proteins form the capsid (Vp1-4). and 10 NS proteins are necessary for viral replication and encapsidation. Once translated and processed, some NSPs interact with cellular factors to enhance viral replication. Viral proteins have diverse functions; for example, the RNA elongation polymerase activity is contained in 3D, 2C is an ATPase containing a nucleotide binding sequence motif, and 2B is essential in the association with cellular membranes and the formation of replication complexes. Protein, VPg (3B in aphthoviruses) acts as a transcription primer after being uridylated to form VPgpUpUOH by a cis-acting replication element (cre) in the viral genome and the viral 3D polymerase.

Viral RNA replication in infected cells is carried out primarily by the viral RNA-dependent RNA polymerase (3Dpol), in conjunction with other viral or cellular proteins. Virus assembly starts with the formation of a virus particle containing 3 surface proteins, VP1-3, and an internal protein, VP4. The final steps in infectious virus formation are the maturation cleavage of VP4 and VP2 (from VP0) and the encapsidation of plus-strand viral RNA.

Host Responses and Viral Interference with the Host Immune Response

Infection with FMDV induces a rapid innate response and robust humoral immune response. The innate response includes type I and type III interferons as well as numerous pro-inflammatory cytokines and chemokines. The humoral response may be detected as high levels of systemic immunoglobulin and mucosal secretion, the combined effect of which rapidly clears acute infection. The FMDV carrier state in ruminants represents a failure of host immunity that has been associated with decreased TH1 immunity and impaired apoptotic pathways and chemokine signaling in the sites of persistent infection in the nasopharynx.

FMDV has evolved a variety of strategies to interfere with the host response (defense) to viral infection. The leader protein (L^{pro}), in addition to cleaving the viral polyprotein, also cleaves the elongation factor eIF4G, which is involved in mRNA CAP recognition by the ribosome to initiate protein translation. This cleavage effectively prevents CAP-mediated translation of host mRNA. However, viral RNA translation continues, mediated by the IRES signal in the viral 5'UTR. In addition to this general mechanism of interfering with host protein translation, L^{pro} also causes specific interference with the host innate response by translocating to the nucleus during viral infection and interfering with the signaling of the interferon response by the degradation of NFkB, which regulates interferon beta transcription. Even though innate responses are inhibited, a solid antibody response develops quickly, within four to six days of infection, which is protective against reinfection by closely related strains of FMDV within the same serotype.

Another mechanism of interference with the host immune response used by FMDV is degradation of major histocompatibility complex (MHC) class I molecules, thereby impairing the cytotoxic T cell (CTL) response. In summary, FMDV infection has dramatic effects at the molecular, cellular, and host response levels, some of which are not yet fully understood. Understanding these viral mechanisms of pathogenesis will aid in the development of more effective vaccine, antiviral, and biotherapeutic strategies against this devastating disease.

Control and Recovery

Approaches to controlling FMD vary dramatically from country to country, and depend primarily on the disease status (endemic vs. free), international commerce of animals and animal products, and the political situation. The Food and Agriculture Organization (FAO) has identified guidelines with stages for countries to follow in the progressive control program (PCP) of FMD (for details, visit http://www.fao.org/ag/againfo/commissions/en/eufmd/pcp.html). Stage 0 countries do not monitor or control FMD, stages 1–3 include various levels of monitoring and control strategies including vaccination that result in no

circulation of virus (stage 4), whereas countries at stage 5 maintain no disease circulation in the absence of vaccination (i.e. free without vaccination). Outbreak response in countries in stages 2–3 might involve some level of animal movement control and targeted vaccination and in some cases slaughter of animals in affected premises. Outbreak response in countries free with vaccination might involve some animal depopulation and revaccination of at-risk animal populations. In FMD-free countries without vaccination, the main response is usually depopulation (stamping out), with minimal use of vaccination. This has had devastating consequences, with millions of animals destroyed, at a very high cost not only economically but also socially and ethically, considering animal welfare. In some cases, stamping-out efforts have not been successful, and countries have had to establish long-term mass vaccination campaigns in order to control the disease (e.g. Argentina and Uruguay in 2001; Republic of Korea 2011).

Vaccination

FMD vaccines have been available for decades and represent the largest share of the veterinary vaccine market worldwide in terms of sales. Commercial vaccines consist of killed whole-virus antigen preparations. Due to the high variability of FMDV serotypes and subtypes, the antigen composition of FMD vaccines is tailored for specific geographic regions. Effective use of vaccines in FMD-endemic regions requires an in-depth investigation of the epidemiology of disease and vaccine-matching studies to determine whether the selected vaccine will be effective against the strain(s) circulating in the target area. Some FMD-free countries have established vaccine antigen banks to strategically store a number of viral serotypes and strains that would be used to control an outbreak at least during the early stages. Strain selection for these vaccine banks is based on risk analysis and must be regularly updated to ensure that emerging FMDV strains are well covered.

Commercial inactivated FMD vaccines are usually formulated either as regular potency for routine control in endemic settings or high potency vaccines containing higher antigen payload for emergency use in non-endemic regions. These vaccines are effective in preventing clinical signs of disease and viral shedding, and have been effectively used in eradication programs in various parts of the world. However, they have some shortcomings, such as the fact that manufacturing requires the use of live virus, which poses a risk of escape from manufacturing facilities. Another problem is the relatively short duration of immunity, requiring semi-annual vaccinations in order to maintain protective immunity levels. Additional issues include the narrow antigenic coverage and the instability of the finished vaccines, particularly those of the O serotypes.

One of the important features of FMDV vaccines is the ability to differentiate infected from vaccinated animals (DIVA). For this purpose, it is very important that during vaccine manufacturing all NSPs are removed from the vaccine, thus allowing testing for antibodies for these proteins that would be present only in infected and not in vaccinated animals. Only high-quality FMD vaccines devoid of NSPs enable the differentiation of infected from vaccinated animals.

There is ongoing innovation in improved "next generation" FMDV vaccines. However, no such products are available commercially at this time. Recent accomplishments include the development and conditional licensure in the United States of a recombinant adenoviral-FMDV vaccine based on the human adenovirus 5 platform (HuAd5). More recently, inactivated vaccine platforms have been developed using attenuated leaderless FMD viruses that grow well *in vitro* but are harmless to animals, thereby decreasing the risk of release from vaccine production facilities. Another promising approach under development is the production of vaccines utilizing empty capsids produced by recombinant baculovirus in insect cells.

Other Members of Genus *Aphthovirus*

The dissolution of the rhinovirus genus was the result of sequence analysis comparing human rhinoviruses with viruses causing bovine and equine rhinitis. These viruses, bovine rhinitis virus A-1, bovine rhinitis virus A-2, bovine rhinitis virus B, and equine rhinitis virus A (ERAV) (formally bovine rhinoviruses type 1, 3, 2, and equine rhinovirus type 1, respectively) have been added to the *Aphthovirus* genus based on genomic organization and sequence similarity to FMDV. Specifically, the molecular evidence includes both genetic structural motif commonalities, substantial amino acid identity, as well as the presence of a functional L^{pro} (only found in aphthoviruses and erboviruses). The bovine rhinitis viruses are thought to cause only minor respiratory disease as they have been isolated from both healthy and symptomatic cattle. Experimental infection with these agents results in mild disease or asymptomatic infection in the natural host. The detection of widespread preexisting antibody in cattle populations further supports the notion that these viruses often move unnoticed among susceptible hosts. The lack of serious disease associated with the rhinitis-causing aphthoviruses in cattle has limited the need for any control measures. Conversely, ERAV has been associated with severe respiratory disease outbreaks in horses, with high fever and viremia. In addition, there is serological evidence for human infection by ERAV among workers with close contact to horses.

Genus *Erbovirus*

The genus *Erbovirus* includes the species Erbovirus A and three viruses: equine rhinitis virus B (ERVB) types 1, 2, and 3. These viruses were previously classified under the genus *Rhinovirus*. However, their increased acid stability distinguishes erboviruses from other rhinitis viruses. ERVB types 1, 2, and 3 are associated with upper respiratory disease and fever in horses similar to ERAV (classified as an aphthovirus). The virus is shed from nasopharyngeal tissues, in some cases for a month after symptoms have resolved. It is thought there may be significant subclinical infection with ERVB, and even in clinical infections the virus does not establish viremia in infected horses.

Genus *Enterovirus*

The enteroviruses are a large group of acid-resistant picornaviruses that mainly cause mild gastrointestinal disease or can be asymptomatic. They are transmitted via fomites, contaminated food products, the fecal–oral route, or direct contact, and are widely distributed among cattle and swine populations. Because each virus tends to have multiple serotypes, there is little attempt to control these viruses in a veterinary setting.

A notable exception to this is swine vesicular disease virus (SVDV). This virus, once epidemic worldwide, is mostly limited to occasional outbreaks in Europe. Genetic evidence suggests that SVDV may be a porcine variant of human coxsackie virus B5. It is transmitted via the fecal–oral and respiratory routes, and may have a carrier state. The most important feature of infection with this virus is that its clinical features in swine resemble FMDV, VSV, and viral exanthema of swine virus (VESV). The vesicles on the feet and mouth are indistinguishable between those caused by these other viruses. Besides vesicles, other symptoms include fever, lameness, and loss of appetite. Although mortality is low, young animals experience severe symptoms associated with high morbidity. In these cases, there are signs of neurological involvement such as ataxia and chorea. A rapid differential diagnosis to FMDV, VSV, and VESV is required because these agents are reportable. SVDV diagnosis is performed by virus isolation, antigen ELISA, or rRT-PCR. Serological tests are only useful for surveillance and are performed by virus neutralization or ELISA.

Genus *Sapelovirus*

The Sapelovirus (Simian, Avian, Porcine Entero-Like viruses) genus comprises new and reassigned viruses with genetic similarity outside the clades of other picornavirus genera. Reassigned viruses include duck picornavirus (now avian sapelovirus) and porcine enterovirus A (now porcine sapelovirus). Newly discovered viruses are classified as new sapeloviruses by sequence similarity, for example, sea lion sapelovirus types 1 and 2. Porcine sapelovirus has been isolated from swine with respiratory disease and gastroenteritis, and it causes the same symptoms in experimentally infected naïve pigs. Porcine sapelovirus has additionally been isolated from postmortem respiratory tissue of fetal piglets with the syndrome known as stillbirth, mummification, embryonic death and infertility (SMEDI). The symptoms are comparable to those caused by porcine parvovirus, the most common cause of SMEDI. Porcine sapelovirus has also been associated with cases of idiopathic vesicular disease, which is a differential diagnosis for FMD in swine.

Genus *Kobuvirus*

Kobuviruses are another genus organized by genomic similarity, members of which are found in human, bovine, and porcine hosts. These viruses are associated with gastroenteritis and have been shown to cause viremia in pigs. Bovine kobuvirus exhibits widespread prevalence in cattle feces. Transmission is thought to be via the fecal–oral route, based on studies on Aichi virus of humans. High levels of specific antibody are found in the serum of unaffected animals. Isolates of putative kobu-like viruses have been detected in bat and sheep feces based on the phylogenetic analysis of partial genomes.

Genus *Teschovirus*

Porcine teschovirus is a disease limited to swine hosts. There are 11 serotypes, but only serotype 1 (PTV-1) causes severe disease, known as Teschen disease or teschovirus encephalomyelitis, in adult pigs. The virus has worldwide distribution, with a recent outbreak in Haiti (discussed below). Like all picornaviruses, PTV is very stable in the environment; it is often introduced into swine populations through incompletely heat-treated swill or other food products. The virus enters the host by ingestion, replicates in the gastrointestinal tract, and is shed in the feces. Most infections with serotypes other than PTV-1 result in subclinical disease, except in very young piglets of naïve sows, which can sometimes display signs of neurological disease, but little mortality. Usually, virus access to the central nervous system (CNS) is limited by circulating maternal antibody, and the young pigs generate specific immunity subsequent to weaning. Mild pathogenesis is often referred to as Talfan disease. PTV serotypes 1, 3, and 6 infections have been associated with SMEDI.

On the other hand, teschovirus encephalomyelitis affects swine of all ages and has up to a 90% mortality rate. Symptoms

include fever, anorexia, depression, muscle tremors, encephalitis, and limb paralysis. Death can occur due to paralysis of the respiratory muscles as early as one week after the onset of symptoms. As the virus invades the CNS, histopathological lesions are found in the brain, spinal ganglia, and cranial nerves. Because of the CNS involvement, differential diagnoses include pseudorabies virus, Japanese encephalitis virus, rabies virus, and porcine respiratory and reproductive syndrome virus. PTV is confirmed by virus isolation, and the serotype determined by serological analysis. But as antibody is widespread, cases of encephalomyelitis must be confirmed by virus isolation or histopathology of the brain and spinal cord. There is no treatment for affected animals; in the past, PTV vaccines were available; however, they are no longer in use. Control is mediated by quarantine and disinfection.

A rapidly spreading outbreak of PTV-1 was reported in 2009 in Haiti, causing severe disease and devastating the pork economy, which was already reeling from endemic classical swine fever. By 2010, the virus was found in almost all areas of the country, and new cases were still developing in early 2011. Up to one third of all swine in the country were affected, and 25% mortality was observed in infected animals. Haiti's long eastern border with the Dominican Republic puts that country's swine population at high transmission risk.

In another recent report, a retrospective analysis of samples from 2002 suggested that PTV-1 caused swine polioencephalomyelitis on many farms in western Canada. Although fewer than 1% of swine were affected on each farm, most animals exhibiting symptoms were euthanized prior to death. Infected survivors had lingering neurological impairments. The significance of this report is difficult to assess because there was no mention of possible sources of the virus, and there was no evidence of spread beyond the initially affected sites.

Genus *Tremovirus*

The *Tremovirus* genus contains avian encephalomyelitis virus (AEV), which is the etiologic agent of epidemic tremor disease in chickens. This virus can also affect a wide range of other avian hosts, including partridge, turkey, quail, guinea fowl, and pheasants. Originally detected in the New England region of the United States in the 1930s, AEV has a worldwide distribution, causing a range of symptoms from decreased egg production to neurological disease in young chicks less than three weeks of age. Neurological signs include tremors, ataxia, weakness, wing drooping, and paralysis. Mortality can be as high as 25% with extensive morbidity. Chicks will sit and fall over due to paralysis, and the survivors often recover with associated blindness. Hens have a two-week temporary reduction in egg production. The virus is spread by the fecal–oral route. The virus replicates in epithelial cells of the digestive tract and is shed in feces three days after infection; shedding can continue for up to two weeks. In young chicks, the virus establishes a viremia, spreading the virus to the CNS and other organs. Diagnosis is by histopathology of brain lesions or by virus isolation on embryonated eggs; antibodies only indicate prior exposure to disease. Although there is no treatment for animals infected with AEV, a live attenuated vaccine can be given as eye drops or added to drinking water.

Genus *Avihepatovirus*

Another genus born of consolidation is *Avihepatovirus*, consisting of the duck hepatitis virus A (DHAV), previously classified as an enterovirus. DHAV has worldwide distribution and is a significant cause of high mortality in ducklings. DHAV has almost 100% mortality rate in ducklings aged less than one week; the mortality drops to less than 50% by three weeks of age. The clinical signs include paralysis, opisthotonus, paresis, enophthalmos, and sudden death. Clinical specimens show enlarged kidney, spleen, and liver, with the latter exhibiting massive hemorrhaging, necrosis, and a green color. The virus is diagnosed by inoculating embryonated duck and chicken eggs with liver homogenates or immunofluorescence of tissue sections. There is no treatment for this disease; although a vaccine is available that helps prevent spread, the best course of control for DHAV is to restrict contact of ducklings with adults until after the fourth week of life.

Related to the duck hepatitis A virus is the as yet unclassified turkey hepatopancreatitis virus, the cause of turkey viral hepatitis. It is found mostly in North America and Europe, affecting young turkeys under the age of six weeks. It is transmitted via the fecal–oral route. Clinical signs include weight loss, anorexia, and sudden death, with neurological signs seen in some cases. Adults are mostly asymptomatic with a temporary drop in egg production. The virus can be isolated by inoculating embryonated chicken eggs with tissue homogenates of liver, pancreas, spleen, or kidney. Histopathological observations include multifocal necrosis manifested as depressed gray lesions on the pancreas and liver. Fatal cases demonstrate massive hemorrhage in both liver and pancreas with vacuolation and monocyte infiltration, as well as pooled blood in the organs.

Genus *Cardiovirus*

Encephalomyocarditis virus (EMCV), the type species for the cardioviruses, is a virus of rodents that is transmissible to farm animals as well as humans. Outbreaks of EMCV

have also caused mortality in baboon colonies associated with infestation of their cages by wild rodents. It is primarily a disease associated with unsanitary conditions, as infection of animals, especially swine, is derived from contact with rodent feces. The virus replicates in the alimentary tract and establishes a viremia, providing access to the target organ, the heart. This disease manifests mostly as morbidity and mortality seen in young animals, but transplacental transmission can also occur, causing stillbirths. Young piglets die suddenly after primary symptoms of high fever, difficulty breathing, and a characteristic blue skin discoloration. Histopathological observations include edema in the lungs and abdomen as well as an enlarged liver. The heart becomes soft and pale with evident necrosis. Animals that survive infection have fibrosis. There is no treatment for infected animals; control is based on seclusion and disinfection of premises. An inactivated antigen vaccine is commercially available. Also, experimental inoculation with live Mengo virus (a cardiovirus of no veterinary relevance) can protect baboons, macaques, and swine against challenge with a lethal strain of EMCV.

Genus *Senecavirus*

Senecavirus A (SVA), Previously known as Seneca Valley virus (SVV), senecavirus A (SVA) is the sole member of the newly created *Senecavirus* genus, which has closest sequence homology with the *Cardiovirus* genus. SVA was discovered serendipitously in 2002 in the United States as a cell culture contaminant and remained relatively innocuous for over a decade, despite rare associations with cases of porcine idiopathic vesicular disease (PIVD) in the United States and Canada. Although cases of PIVD were rare, they were indistinguishable from other vesicular diseases of pigs including FMD, vesicular stomatitis (VS), and SVD, and therefore required reactive measures commensurate with FMD outbreaks. In 2014, SVA emerged as a cause of widespread PIVD and pre-weaning mortality in Brazil, which was followed by a similar emergence in 2015 in the United States. By 2017, this emergence erupted, with thousands of SVA-associated vesicular disease cases reported in the United States, which is believed to be only a fraction of the cases that actually occurred. Between 2015 and 2017, SVA was also discovered in China, Thailand, Colombia, and Vietnam, which likely represents only a fraction of the true global distribution.

The pathogenesis of SVA in pigs has varied across the relatively small quantity of studies that have been performed. The typical presentation in experimental and field observations is an acute febrile syndrome with vesicular lesions occurring at the same sites as other vesicular diseases of pigs (coronary band, snout), with relative sparing of the oral cavity in most studies (Figure 54.3). The clinical syndrome includes high morbidity and low mortality, except for neonatal pigs, which have a distinct syndrome with higher mortality. Viremia may last 1–10 days, and detection of shedding (RNA) may last up to 28 days in nasal secretions and 14 days in feces. In addition, serological studies have demonstrated that pigs may become infected without ever having signs of clinical disease.

Molecular epidemiological analysis has revealed that there are two distinct clades of SVA, with all isolates from pigs occupying a distinct clade from the original isolate discovered in cell culture in 2002. However, it is unclear if the specific genomic differences across strains are responsible for clinical or epidemiological distinctions. Also, it is still unclear if there are other environmental, physiological, or microbial cofactors that determine the divergence between clinical manifestations and subclinical infection.

Over the last decade, SVA has clearly emerged as a new and important pathogen of pigs. Although seropositivity against SVA has been detected in cattle and mice, there is no evidence that any species other than pigs have clinical disease or serve as a reservoir for the virus. It is likely that SVA will continue to be recognized in novel locations and scenarios, and many critical aspects still remain unknown. Substantial research is still required to better understand the epidemiological and virological aspects of this emergent pathogen, in order to implement appropriate control measures. Further research will ultimately elucidate less utilitarian aspects of the disease such as its original source and why it subsequently emerged as a global pathogen after years of relative latency.

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55

Caliciviridae

Mohamed A. Abouelkhair and Melissa Kennedy

Caliciviruses are small (27–40 nm in diameter), nonenveloped, icosahedral viruses with a genome of single-stranded, positive-sense RNA (Figure 55.1). The viral RNA serves as mRNA and is infectious. The name "calicivirus" is derived from the chalice-shaped spheres on the surface of the negatively stained viral particles. The family Caliciviridae currently comprises 11 genera (*Vesivirus, Lagovirus, Norovirus, Sapovirus, Salovirus, Minovirus, Nebovirus, Recovirus, Valovirus, Nacovirus,* and *Bavovirus*). Viruses infecting animals are found in all genera. Hepatitis E virus, which had initially been classified in Caliciviridae, has been reclassified as a Orthohepevirus A in the Hepeviridae family. Several members of Caliciviridae – specifically vesicular exanthema of swine (VES) virus, San Miguel sea lion virus (SMSV), feline calicivirus (FCV), European brown hare syndrome (EBHS), and rabbit hemorrhagic disease (RHD) viruses – are important animal pathogens.

General Properties

The genome of caliciviruses has only two or three open reading frames (Figure 55.2). Virions comprise a single major capsid protein (VP1; 55–70 kDa). The nonstructural proteins of caliciviruses share features with those of picornaviruses. Replication of caliciviruses occurs in the cytoplasm, although both cytoplasmic and intranuclear inclusions occur in infected cells.

Vesiviruses

VES and FCV are classified together in the genus *Vesivirus*. Viruses within this genus are readily propagated in cell culture, in distinct contrast to caliciviruses in the other genera.

Vesicular Exanthema of Swine

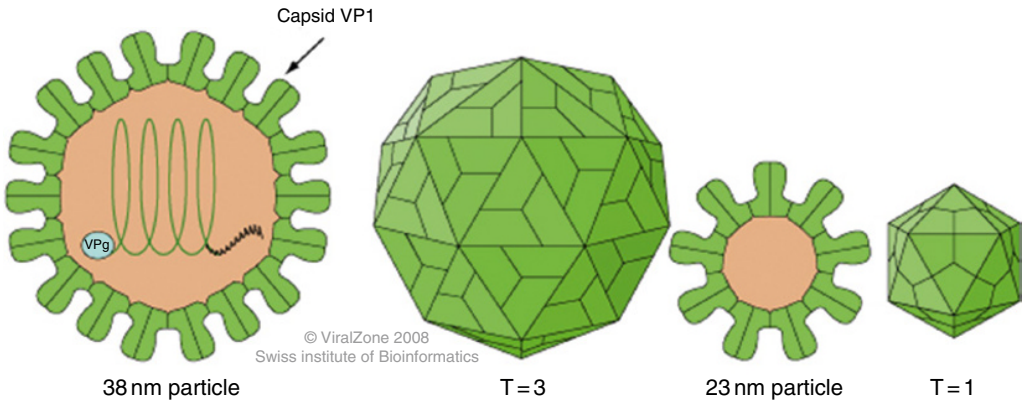
Disease

VES is an acute viral disease characterized by the formation of vesicles in the oral cavity, interdigital spaces, and coronary band of the foot. VES is clinically indistinguishable from foot-and-mouth disease (FMD), swine vesicular disease, Senecavirus A (which is endemic but is the main differential for vesicular disease in swine), and vesicular stomatitis. The incubation period of the VES disease is approximately 24–72 hours, and the course is about 1–2 weeks. The disease has a high morbidity but a low mortality. It is of largely historical importance; however, its main impact is that it mimics FMD, from which it must be distinguished. The disease has only occurred in the United States, and the last occurrence of this disease was in 1956. Subsequently, in 1959 the US Department of Agriculture declared VES an exotic disease. Suspected cases should be reported immediately to the proper authorities. Due to its historical presence only in the United States and its absence for half a century, VES is no longer included on the list of internationally reportable diseases by The World Organization for Animal Health (OIE) and is essentially considered extinct. VES is serotypically indistinguishable from a number of caliciviruses present in marine mammals and fish, known as the San Miguel sea lion viruses (SMS viruses), which are endemic in marine mammals. These viral infections also cause vesicular diseases and reproductive failure. A wide variety of seals, sea lions, walrus, and dolphins are infected with these viruses. It is thought that VES occurred when these marine mammal caliciviruses spread to swine, likely as a result of feeding of dead marine mammals to swine.

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Caliciviridae

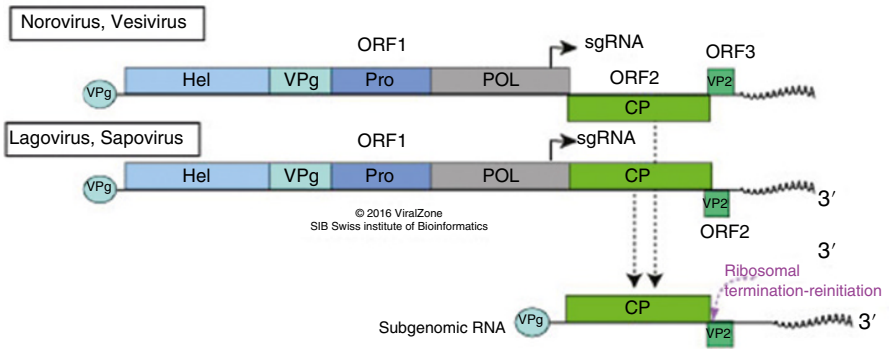
VIRION



Non-enveloped, capsid of about 27–40 nm in diameter, with T = 3 icosahedral symmetry. The capsid is composed of 180 capsid proteins.

Viper DB

GENOME



Monopartite, linear ssRNA(+) genome of 7.3 to 8.3 kb. The 5'-terminus is linked to a VPg protein and the 3'-terminus has a poly(A) tract.

Figure 55.1 Schematic diagram representing a T = 3 arrangement of a Caliciviridae capsid. *Source:* Courtesy of *ViralZone*.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

VES virus (VESV) is a typical calicivirus, and viral particles are associated with cytoplasmic cisternae in infected swine cells and in crystalline arrays in the cytoplasm. VESV is stable at low pH (pH 5). A large number of antigenically distinct types of VESV have been identified (at least 13). A number of antigenically distinct viruses that originally were isolated from species other than swine are capable of causing VES and so are classified as VES viruses, including bovine calicivirus Bos-1, bovine calicivirus Bos-2, Cetacean calicivirus, primate calicivirus, and a number of so-called San Miguel sea lion viruses (17 types). Similar viruses have been isolated from fish, birds, reptiles, and other mammals, including skunks. These viruses are distinguished by serological tests, usually serum neutralization, and the virulence of these viruses to pigs varies significantly.

Resistance to Physical and Chemical Agents

VESV can persist in the environment and in contaminated meat products for several weeks. The virus is completely inactivated by 2% sodium hydroxide or 0.1% sodium

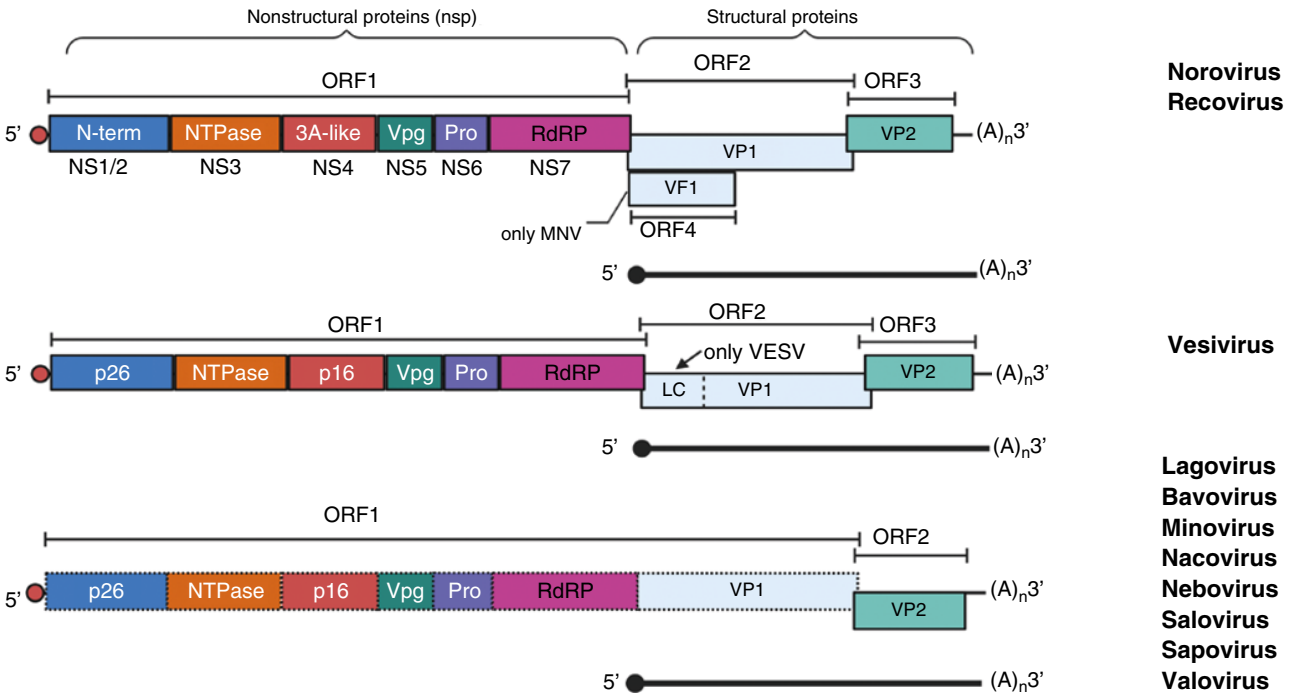


Figure 55.2 Schematic genome organization of members of genera in the family Caliciviridae. Protein VPg is covalently linked to the 5′-ends of both genomic and subgenomic RNAs and is depicted by a black circle. ORF1 encodes a polyprotein that is posttranslationally cleaved into individual nonstructural proteins. ORF2 (and for members of some genera, ORF3 and ORF4) are expressed from subgenomic mRNAs. Cleavage sites have been described for several genera, but there is considerable variation in their exact location. Murine norovirus (MNV) is the only norovirus thus far that possesses ORF4, which encodes VF1 at the 5′-end of ORF2, while feline calicivirus (FCV, Vesivirus) encodes a leader capsid (LC).

hypochlorite. Accelerated hydrogen peroxide has been proved effective against caliciviruses and has good activity in the face of organic matter.

Infectivity for Other Species or Culture Systems

Naturally occurring VES disease is confined only to domestic swine of all ages and breeds. Experimentally, VESV causes vesicles at inoculated sites in seals. Vesicles are also produced at the sites of inoculation in horses and hamsters. Virus is isolated in low titers from some sites of inoculation and draining lymph nodes. VESV can be propagated in cell lines of swine kidney or Vero monkey kidney.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

VES was first described in North America (California) in 1932, and outbreaks were reported every year in California between 1932 and 1951, with the exception of 1937–1938. The disease first appeared outside of California in 1951, and from 1952 to 1953 it spread to a total of 42 continental states in the United States. The disease had never been reported elsewhere in the world except in Iceland and Hawaii, and these two incidents resulted from shipping contaminated pork products from California.

Marine mammals serve as reservoirs for VESV infection. A calicivirus was first isolated in 1972 from sea lions on San Miguel Island off the coast of southern California. This calicivirus was named SMSV, which was indistinguishable from VESV by morphological, biophysical, and biochemical criteria. Experimental SMSV infection of swine produces a disease indistinguishable from VES. SMSV also has been isolated from asymptomatic domestic swine. Serum-neutralizing antibodies to several serotypes of SMSV and VESV have been demonstrated in marine mammals and in both wild and domestic swine in California. Earlier epidemiologic studies during the outbreaks of VES confirmed the relationship between feeding of raw garbage and outbreaks of the disease, and dead sea lions are known to have been utilized as a food source for swine.

Although outbreaks of VES likely originated from feeding SMSV-infected marine animal parts to swine, the infection subsequently spread rapidly within affected herds by direct contact.

Pathogenesis and Pathology

VES is characterized by the appearance of fluid-filled vesicles on the snout, coronary band, and tongue of infected swine. These same lesions develop in swine that are inoculated intradermally with either VESV or SMSV. Infected animals are febrile, and virus is present in blood and nasal-oral secretions for several days after infection. Vesicles appear on the coronary band and interdigital space of the feet at three to four days after infection. The vesicles rapidly rupture and healing takes place unless complicated by secondary bacterial infection. High titers of virus are present within the fluid in vesicles, which may also contaminate the environment. Mild encephalitis occurs in some swine infected with VESV, and virus also may be recovered from brain tissue of swine infected with SMSV.

Host Response to Infection

Neutralizing antibodies to VESV and SMSV appear in the sera of animals infected with viruses soon after inoculation, and titers peak within 7–10 days after infection.

Laboratory Diagnosis

In most countries, suspected cases of vesicular exanthema must be reported to regulatory authorities. VES must rapidly be differentiated from other vesicular diseases of swine, such as FMD, swine vesicular disease, and vesicular stomatitis. Laboratory diagnosis is accomplished by virus isolation in cell cultures, direct electron microscopic examination of vesicle fluid, or polymerase chain reaction. Although the vesicular diseases all produce similar signs in swine, there are major differences: whereas VES and swine vesicular disease are almost exclusively diseases of swine, vesicular stomatitis frequently affects horses as well as ruminants, and FMD also affects ruminants (Table 55.1).

Table 55.1 Susceptibility of domestic animals to four viruses that cause vesicles in Swine.

Animal species	SVD	VES	FMD	VSV
Cattle	–		++	++
Swine	++	++	++	+
Sheep	–	–	+	–[a]
Horse	–	–[a]	–	++
Guinea pig	–	–[a]	+	+
Suckling mice	+	–	+	+

[a] Occasional lesions produced by specific virus strains; SVD, Swine vesicular disease; VES, Vesicular exanthema of swine; FMD, Foot-and-mouth disease; VS, Vesicular stomatitis.

Treatment and Control

There is no treatment for VES, and there are no vaccines for control of the disease. It is now considered to be eradicated in the United States. Enforcement of laws requiring cooking of garbage before feeding it to swine was the most important factor in eliminating the disease.

Feline Calicivirus

Disease

FCV infects the oral cavity and upper respiratory tracts of cats to produce fever, sneezing, and nasal and ocular discharges. Clinical signs include rhinitis, conjunctivitis, oral, and skin ulcerations (Figure 55.3) and, in severe cases, pneumonia (Figure 55.4). Footpad lesions range



Figure 55.3 Feline calicivirus (FCV) in tissues. *Source:* Courtesy of Pesavento et al. (2004). With permission of Sage Publications. 10. Cat No. 1. Purified VS FCV. Particles are 33.5 nm in diameter and have the scalloped borders and surface indentations typical of Caliciviridae. Direct electron micrograph. Bar _ 64 nm.11. Skin; cat No. 1. A membrane-bound fragment contains paracrystalline arrays of virions. Transmission electron micrograph. Bar _ 332 nm.12. Skin; cat No. 3. Paracrystalline arrays of virus particles within the nucleus of an epithelial cell are associated with protofilaments. Asterisk indicates cytoplasmic cytokeratin bundles. Transmission electron micrograph. Bar _ 456 nm.

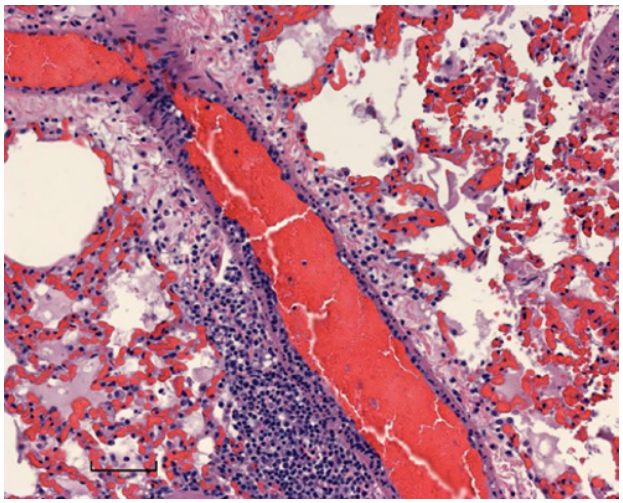


Figure 55.4 Marked facial edema, extensive hair loss, oozing, and ulceration of the skin in two cats affected by VS FCV. *Source:* Courtesy of FCV Disease Information Fact Sheet (2013). With permission of Sage Publications.

from mild hyperemia to sloughing of the entire pad (Figures 55.5 and 55.6). Joint or muscle soreness, hyperesthesia, and chronic oral ulceration also have been attributed to FCV infection, and a disseminated highly virulent and fatal systemic disease associated with specific strains of FCV has been described. The incubation period of the disease is 2–3 days, and infected cats usually recover in 7–10 days in the absence of secondary bacterial infections. Virulent systemic FCV infection is characterized by alopecia, cutaneous ulcers, subcutaneous edema, and high mortality.



Figure 55.5 Cat paw; the epithelial lining of the footpads of P2 and P5 have sloughed. There is peripheral ulceration at the pad-haired skin junction of P4. *Source:* Courtesy of Pesavento et al. (2004). With permission of Sage Publications.

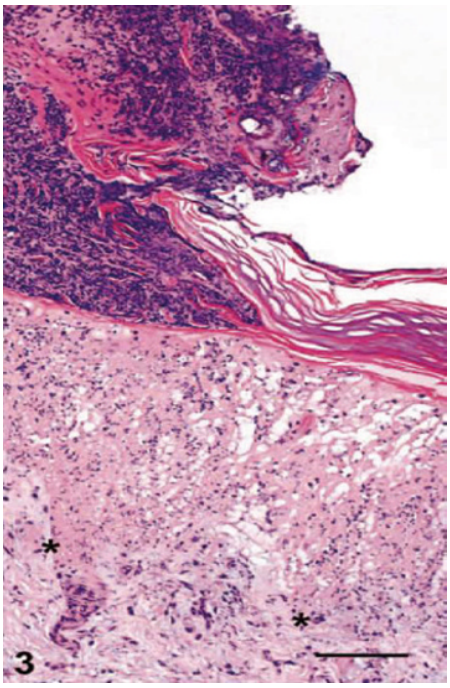


Figure 55.6 Cat pawpad, haired skin junction. Extensive infiltration of neutrophils with accompanying karyorrhexis and karyolysis of epithelial cells at the junction between haired skin (left) and footpad (right). Asterisks indicate dermalepidermal junction, which is obscured by necrosis of the epithelium. HE. Bar _ 140_m. *Source:* Courtesy of Pesavento et al. (2004). With permission of Sage Publications.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

FCV is classified into a single serotype, but there are multiple strains of FCV that vary in antigenicity, which impacts vaccine efficacy. The virulence of individual strains varies substantially as well, and is independent of antigenicity; that is, viruses that cause similar clinical syndromes are not necessarily similar in antigenicity.

Resistance to Physical and Chemical Agents

FCV is resistant to many common disinfectants. It is readily inactivated by a 0.175% sodium hypochlorite solution (Clorox® bleach), which is the disinfectant of choice. Quaternary ammonium compounds are generally ineffective against FCV. The virus is stable at a pH of 4–5 and is inactivated at 50 °C within 30 minutes.

Infectivity for Other Species and Culture Systems

FCV is a ubiquitous pathogen that has been isolated from cats all over the world. There is no evidence that FCV

produces disease in laboratory animals. The virus can readily be propagated in feline cell lines. Some strains have been grown in Vero monkey kidney and dolphin kidney cells.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

The disease occurs worldwide, and all felids are likely susceptible. Infection and disease are most common in young cats, as older cats are usually immune. Infected cats recovered from the disease may carry virus in their oropharynx for long periods and serve as reservoirs of infection. The virus is transmitted horizontally by aerosols and by contaminated fomites. The latter mode is of major importance because of the hardiness of the virus in the environment.

Pathogenesis and Pathology

Cats acquire FCV infection via the respiratory route, either by aerosol or from fomites. The primary sites of viral replication are epithelial cells of the oral cavity and respiratory tract and in the tonsils. Viremia occurs during acute infection.

The characteristic lesions in typical cases of FCV infection in susceptible kittens and young cats are the vesicles within the oral cavity (tongue, hard palate) and on the nares. The vesicles rapidly rupture, leaving erosions and ulcers. Highly virulent strains can cause pneumonia in kittens. Regeneration of the oral mucosa occurs rapidly in uncomplicated cases. FCV has also been associated with a limping syndrome characterized by synovitis and increased synovial fluid. The mechanism of lameness is unknown but may have an immune component.

Virulent systemic strains of FCV (VS FCV) cause epidemics of fatal disease in susceptible cats. VS FCV is characterized by vasculitis and multi-organ involvement. Affected animals may exhibit severe oral ulceration, extensive subcutaneous edema (especially of the head and limbs), and variable ulceration of the pinnae, paw pads, nares, and skin. Some affected cats also have pneumonia as well as liver and splenic necrosis. FCV antigen is detected by immunohistochemical staining in both epithelial and endothelial cells. Mortality is high, and disease has occurred in vaccinated cats. Most commonly, VS FCV arises in rescue facilities or other multi-cat settings where FCV is circulating.

Host Response to Infection

Cats infected with FCV or vaccinated with inactivated or modified-live FCV vaccines develop serum-neutralizing antibodies. Kittens born to cats that are immune to FCV acquire maternal serum-neutralizing antibody to FCV via colostrum.

Laboratory Diagnosis

Laboratory tests are required to distinguish FCV infection from other agents that produce similar respiratory signs in cats, particularly feline viral rhinotracheitis (herpesvirus). These include the isolation of FCV in feline cell cultures from nasal secretions, throat swabs, or conjunctival scrapings and the identification of FCV antigen in conjunctival scrapings of tonsillar biopsies by immunohistochemical staining. Genetic detection by reverse transcription/polymerase chain reaction may also be used. However, positive results must be interpreted carefully, as asymptomatic carriers occur. In addition, false negative results can occur due to the genetic variability of the virus. The characteristic appearance of the virus under the electron microscope can also be used for rapid diagnosis (Figure 55.7). VS FCV cannot be distinguished from classical FCV except by clinical manifestations.

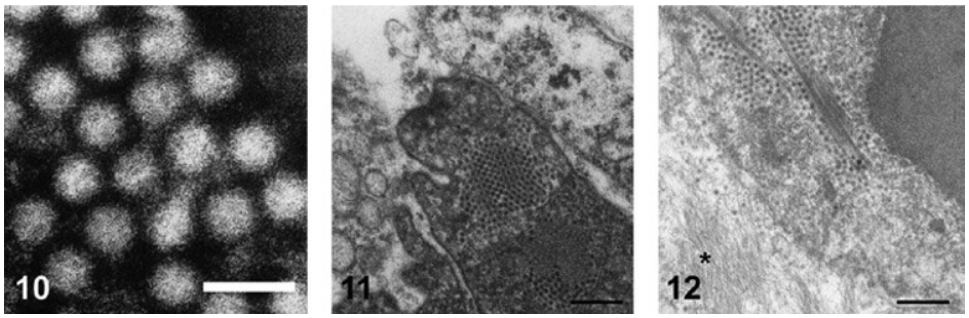


Figure 55.7 Pulmonary vasculitis caused by calicivirus infection of a cat. *Source:* Courtesy of Dr. David Steffen, Nebraska Veterinary Diagnostic Center, University of Nebraska, Lincoln.

Treatment and Control

Treatment for FCV infection in cats is mainly supportive and symptomatic. Both inactivated and modified-live FCV vaccines are commercially available. Currently, the majority of modified-live vaccines contain the FCV-F9 strain. However, due to the genetic diversity of caliciviruses, F9 vaccines may vary in their ability to protect against heterologous strains and may be ineffective against newly emerging systemic virulent strains such as FCV-Ari. Recently, multi-strain vaccines have been developed. Inactivated vaccines provide immunity for at least four years, and modified-live virus (MLV) vaccines provide immunity for approximately seven years. Inactivated vaccines may contain adjuvant and other excipient proteins to promote an adequate immune response, which have been linked to adverse reactions in cats and are thus not recommended.

The use of viral neutralization assays in vaccine development is the best method for evaluating cross-protection of vaccines. The FCV vaccines are usually combined with feline rhinotracheitis (a herpesvirus) and feline panleukopenia (a parvovirus) and administered either intranasally or intramuscularly.

FCV infection is controlled primarily by isolating cats that show respiratory signs and disinfecting cages and premises with appropriate disinfection before susceptible animals are introduced.

Lagoviruses

Rabbit Hemorrhagic Disease and European Brown Hare Syndrome

Disease

RHD is an acute highly contagious disease of European rabbits and frequently has a very high mortality rate in susceptible rabbit populations. A novel feature of RHD is that the disease is usually only fatal to rabbits over two months of age. The disease is characterized by a short incubation period, followed by fever, disseminated hemorrhage in all body tissues, and rapid death. Phylogenetic studies revealed three distinct groups: "classic RHDV," the antigenic variant RHDVa/G6 identified in 1996, and RHDV2 discovered in France in 2010. RHDV and RHDVa are phylogenetically related, and their capsid protein shares 85% nucleotide similarities with RHDV2. RHDV2, unlike other RHD viruses, affects both European rabbits and some species of hares (*Lepus* spp.); also, it affects young rabbits as well as older animals. Clinical cases have been reported in rabbits as young as 11 days of age. The clinical signs are indistinguishable from the illness caused by RHDV. RHDV and EBHS are similar diseases that are caused by related but antigenically distinct caliciviruses. EBHS occurs in the European hare, *Lepus europaeus* and some additional *Lepus* species.

Etiologic Agent

Physical, Chemical and Antigenic Properties

Various strains of RHD and EBHS viruses are recognized and distinguished serologically.

Infectivity for Other Species and Culture Systems

Neither RHD nor EBHS viruses are readily propagated in cell culture, which means that the viruses have been largely characterized using homogenates of the livers of affected animals. The viruses appear to be highly species specific.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

An outbreak of RHDV in China in 1984 resulted in extensive spread of the virus, and classical RHDV and RHDVa have become endemic in a number of locations where European rabbits exist in the wild. As of 2020, RHDV2 has become established in wild lagomorphs in a number of European countries, Australia, and parts of Africa. In some locations, RHDV2 has largely replaced classical RHDV or RHDVa. Outbreaks have also been reported in New Zealand and some countries in the Middle East, Asia, and North America, but whether the virus will persist in some of these locations is not yet certain. RHDV2 was first detected in the United States in 2018 and was reintroduced in 2020. RHDV2 has continued to spread across multiple states in the United States, including Arizona, California, Colorado, Nevada, New Mexico, and Texas. In North America, this virus was found in both domestic and feral rabbits. EBHS has been documented in Europe. Transmission occurs through direct contact or exposure to an infected rabbit's excretions or blood (mainly fecal–oral transmission). The virus can also survive and spread from carcasses, food, water, and any contaminated materials. It is most likely transmitted mechanically in the wild by flies, mosquitos, and fleas. RHD is considered a foreign animal disease in the United States, and suspected cases should be immediately reported to the USDA. This disease must also be reported to The World Organization for Animal Health.

Pathogenesis and Pathology

RHDV infections are usually subclinical in rabbits under the age of four to eight weeks. In older rabbits, the most common syndromes are acute and subacute disease. RHDV2 can infect both young and old rabbits. Rabbits with RHD have an enlarged spleen, swollen liver, and disseminated hemorrhages. Extensive liver necrosis is highly characteristic and potentially explains the disseminated intravascular coagulation (DIC) that occurs in affected animals. Lesions may also be found in other organs such as the kidneys, lungs, and heart. The DIC induced by RHDV is not characteristic of other calicivirus infections but does occur in diseases such as flavivirus-induced diseases as yellow fever and dengue in humans. The reported morbidity and mortality rates in RHDV outbreaks are in the range of 30–100% and 40–100%, respectively, with adult rabbits from naïve populations suffering the most fatalities. Mortality rates tend to be lower for RHDV2, which has been considered less virulent. Some recent isolates have been more pathogenic.

Laboratory Diagnosis

Immunofluorescence and ELISA tests have been developed for the rapid diagnosis of RHD. The genome of RHDV has been completely sequenced, so reverse transcription polymerase chain reaction (RT-PCR) can readily be used for rapid diagnosis of the infection. The liver is the tissue of choice for viral detection. This disease is reportable in the United States.

Treatment and Control

There is no treatment for the acute disease. Control can be achieved through strict quarantine and isolation to prevent transportation of RHDV-contaminated materials into commercial rabbitries. RHD is controlled in countries where it is endemic through good biosecurity and variant-specific vaccination. As cross-protection between RHDV-1 and -2 vaccines is variable, vaccination should be directed against both variants if the virus type is unknown. Monovalent and bivalent inactivated adjuvanted RHDV vaccines are available. Most are RHDV2 specific, but a bivalent product is preferable. An RHDV2 vaccine is now available in the United States with emergency use authorization from the USDA Center for Veterinary Biologics. The vaccine (an inactivated product) is given subcutaneously in two doses, 21 days apart. Full immunity develops 14 days after administration of the second dose.

Contaminated pelts and meat can serve as sources of the virus in nonendemic areas. It is interesting to note that although most countries have focused on the control and prevention of RHD, RHDV has been used as a biological weapon to control rabbit numbers in other countries.

Noroviruses

The first norovirus was identified in a human outbreak of gastroenteritis in Norwalk, Ohio, in 1968. Noroviruses have since been recognized in many species of animals, including mice, cattle, swine, sheep, and a lion cub, where they have been associated with diarrhea in young animals. Several genotypes of noroviruses have been identified, most of which cannot be grown *in vitro*.

Transmission is fecal–oral, and the virus is stable in the environment after shedding. The virus appears to target enterocytes, but the disease is generally mild and self-limiting. Diagnosis can be done by electron microscopy or RT-PCR. The zoonotic potential for animal noroviruses is unknown, though human and animal isolates are closely related.

Unassigned Caliciviruses

Enteric caliciviruses have been described in cattle, dogs, chickens, and pigs, and other terrestrial species. At least some of these appear to cause clinical signs of intestinal disease analogous to those caused by the Norwalk-like viruses in humans.

Acknowledgment

Thanks to Kathryn Kauffman, VT, for technical review of this chapter.

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Togaviridae and Flaviviridae

Christopher C.L. Chase

Introduction

Togaviridae and Flaviviridae are two similar positive-sense single-stranded enveloped RNA virus families that are characterized by a single mRNA that results in a polyprotein that is posttranslationally modified. Both families contain several arboviruses. Originally the genera *Flavivirus* and *Pestivirus* were included in the family Togaviridae but based on differences in genomic organization and glycoproteins, a new family, Flaviridae, was established in 1984 that included viruses in the genera *Flavivirus* and *Pestivirus*.

Togaviridae

The family Togaviridae derives its name from "toga," the Latin word for gown or cloak, which refers to the envelope possessed by all members of the family. The family includes two genera, *Alphavirus* and *Rubivirus* (which has a single member, rubella virus, the cause of human rubella [German measles]). Viruses in the *Alphavirus* genus are predominantly arboviruses that are transmitted by mosquitoes; thus, they have the capacity to replicate in insects and vertebrates. Although chikungunya virus (CV), an alphavirus, emerged in 2013 as an important human disease, the veterinary alphaviruses have not seen any new "emerging viruses." There are also seven non-encephalitic alphaviruses that will be discussed: six salmonid alphaviruses (SAV) and an elephant seal virus (Table 56.1).

Alphavirus

Sindbis virus is the prototype of the genus *Alphavirus*. Alphaviruses of veterinary significance include eastern equine encephalitis (EEE), western equine encephalitis (WEE), and Venezuelan equine encephalitis (VEE, including subtype II Everglades) viruses, along with several other viruses (Fort Morgan, Highlands J, and Semliki Forest). The three equine encephalitis viruses (EEVs) are also zoonoses. The six closely related SAVs are salmon pancreas disease (PD), sleeping disease virus (SDV), and Norwegian salmonid alphavirus (NSAV). Another alphavirus, Southern elephant seal virus (SESV), has been identified, but its role in disease is unknown.

Physical, Chemical, and Antigenic Properties

Alphavirus virions are spherical, enveloped, and approximately 70 nm in diameter (T = 4) (Figure 56.1a). Alphaviruses are sensitive to lipid solvents, chlorine, phenol, acid pH, and heating to 60 °C for 30 minutes.

The envelope is derived from the plasma membranes of the host cells through which virions bud as they mature. The envelope encloses an icosahedral nucleocapsid (T = 4) (Figure 56.1c) that is approximately 40 nm in diameter and consists of a single capsid protein as well as the genome of linear single-stranded positive-sense RNA (Figure 56.1b–d). The envelope contains a heterodimer of two viral glycoproteins (E1 and E2) (Figure 56.1a–b), and some alphaviruses (Semliki Forest virus) have a third glycoprotein (E3). At least four different nonstructural viral proteins are produced in infected cells (Figure 56.1d). The alphaviruses are all antigenically related as determined by serological assays, and are grouped into distinct antigenic complexes with numerous subtypes or strains within each. The extensive genetic and antigenic variation that occurs within the various subtypes and variants of each antigenic complex is reflected by differences in their virulence, biochemical characteristics such as electrophoresis mobility of protein and RNA digests, physicochemical characteristics, host range, geographic distribution, and vector/host tropism. WEE virus is the result of recombination between EEE virus and a Sindbis-like virus in an event that has been estimated to have occurred thousands of years ago (Figure 56.2).

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Table 56.1 Viruses of veterinary importance in the Togaviridae family.

Genus	Serogroup/agent	Vector	Affected species	Distribution
Alphavirus	Family alphavirus			
	Equine Encephalitis Group Eastern equine encephalitis virus	Mosquitoes – *Culex (Melanoconion)* *Culiseta melanura*	Horses, humans, pheasants, turkeys, emus, pigs, deer, dogs, sheep, penguins, cranes	Eastern N. America, Caribbean Basin, C. America, S. America
	Venezuelan equine encephalitis virus	Mosquitoes –enzootic *Culex (Melanoconion)* -epizootic *Ades* and *Psorophora* spp.	Horses, humans	C. America, S. America
	Western equine encephalitis virus	Mosquitoes – *Culex tarsalis*	Horses, humans, emus	Western N. America, S. America
	Salmonid alphavirus Group Salmonid alphavirus 1 and 3, 4–6 (Pancreatic Disease Virus)	None	Salmon	N. Europe
	Salmonid alphavirus 2 (sleeping disease virus)	None	Rainbow trout	Western Europe
	Getah virus (Sagiyama virus)	Mosquitoes – *Culex* spp.	Horse, pigs	Southeast Asia (India and Japan)
	Southern elephant seal virus	Lice – *Lepidophthirus macrorhini*	Elephant seal	Australasia

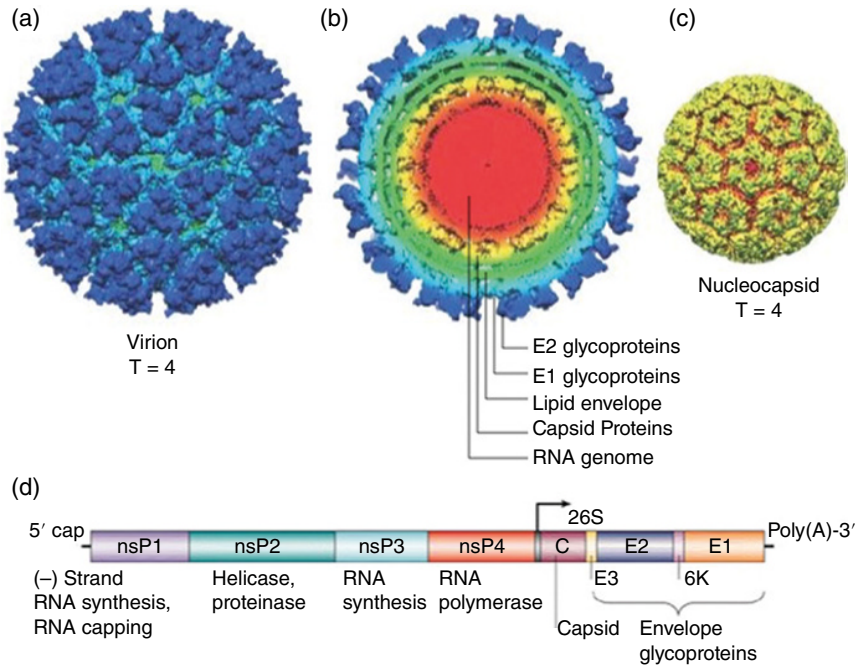


Figure 56.1 Virion structure and genomic organization of an alphavirus. (a) Surface-shaded view of Sindbis virus, the prototype togavirus. The flower-like trimeric spikes of E1–E2 heterodimers are seen in blue and turquoise, and small portions of the lipid bilayer are seen in green. (b) A central cross-section of the virus particle showing the organization of the particle with the E2 glycoprotein (blue), the E1 glycoprotein (turquoise), the lipid bilayer (green) penetrated by the transmembrane helices of glycoproteins, protease domain of the capsid protein (yellow), protein–RNA region (orange) and RNA region (red). (c) The nucleocapsid core showing the pentameric and hexameric capsomeres viewed at the icosahedral twofold axis. (d) The genome of Venezuelan equine encephalitis virus (VEEV). The single positive-strand RNA genome encodes four non-structural proteins (nsPs) and three main structural proteins. During infection, two mRNAs are synthesized: a full-length mRNA that forms the viral genome and a smaller mRNA that is used to produce virion proteins. The three structural proteins are translated from a subgenomic 26S message and combine with the genomic RNA to form virions. *Source:* Panels A, B and C adapted from Jose et al. (2009) and Panel D from Weaver and Barrett (2004).

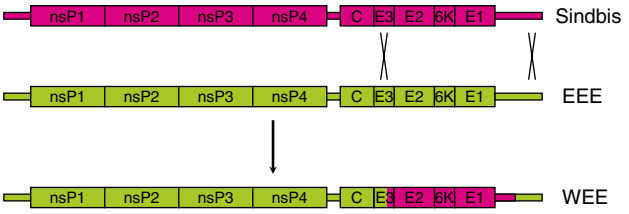


Figure 56.2 Diagram of the recombination event that led to WEE. *Source:* Adapted from Weaver et al. (1997).

Infectivity for Other Species and Culture Systems

The EEV can infect a wide host range, including humans, horses, rodents, reptiles, amphibians, monkeys, dogs, cats, foxes, skunks, cattle, pigs, birds, and mosquitoes. Alphaviruses can be propagated in a variety of cell cultures, including chick and duck fibroblasts, Vero, L cells, and mosquito cells; cytopathology is often absent in the latter. A variety of laboratory animals can be experimentally infected, with suckling mice being the most common. Embryonated chicken eggs and young chicks may also be susceptible to infection.

Veterinary and Zoonotic Alphaviruses

Equine Encephalitis Viruses

Disease EEE, WEE, and VEE all cause encephalitis in horses, but the signs can vary from inapparent to fatal disease. Mild and/or inapparent infections are more common with WEE, whereas EEE and VEE are typically more virulent. Death from VEE may occur in the absence of neurological signs. Central nervous system (CNS) involvement of horses following encephalitis virus infection is characterized by aimless walking, followed by severe depression and behavioral changes (dummy), central blindness, paralysis, and death soon after the onset of clinical signs. Mortality can be very high (up to 90%) with VEE and EEE. Young horses are more susceptible to severe disease. Other members EEE and WEE may also cause significant disease in domestic birds; EEE is more common and mortality can be very high.

Clinical disease is especially common in pheasants and ratites (emus, etc.). EEE also occurs in swine and cattle. EEE disease in birds is characterized by encephalitis with leg paralysis, torticollis, and tremors. Wild birds may also be infected but rarely experience disease, and they serve as vertebrate reservoirs of virus.

Epidemiology The EEV occur in the western hemisphere, although related viruses occur elsewhere in the world (Figure 56.3a–b). EEE occurs in eastern North America (predominantly east of the Mississippi River and the Atlantic Seaboard region in particular), the Caribbean Basin, and Central and South America (Figure 56.3a). EEE is the most common EEV, with an average of 138 cases/year (range from 70 to 209 cases/year) from 2012 to 2020 (Table 57.2), which is about an 80% decrease over the previous 10-year period. Often there are localized outbreaks in the United States (Figure 56.3a). VEE is confined to South and Central America (Figure 56.3b), although incursions into North America have occurred periodically, with the last outbreak in the United States being in 1971. The United States considers VEE to be a foreign animal disease; however, an avirulent VEE virus (Type II, Everglades) is endemic in portions of Florida. WEE occurs throughout much of North America, particularly in areas west of the Mississippi River, and South America (Figure 56.3b). Unlike EEE, WEE infection rates have declined dramatically, with no cases diagnosed in the United States from 2012 to 2020 (Table 56.2) and the last case diagnosed in the United States in 2009. There was a WEE outbreak in horses in Mexico in 2019 (Figure 56.3a). Other encephalitic alphaviruses sporadically occur in the United States but at much lower rates, the most prominent being Highland J virus, which causes encephalitis in horses, especially in Florida, and is also pathogenic to turkeys and partridges.

Mosquitoes transmit the EEV and, despite their names, horses and humans are dead-end hosts that are unimportant to the natural cycle of EEE and WEE virus infections (Figure 56.4). These viruses were the first described arthropod-borne viruses (arboviruses). The viruses all persist in similar but distinct natural cycles of infection that include mosquitoes and birds or rodents, which function as the vertebrate reservoirs of each virus. Except in tropical areas, where infection occurs year-round, the peak incidence of these diseases typically is in late summer and declines when climatic conditions are less favorable for the mosquito vectors. Mosquitoes are the biological vectors of these viruses, which requires that the mosquito actually become infected with the virus rather than simply transmitting it mechanically. For a biological vector to become infected, it must obtain a blood meal from a viremic vertebrate host (Figure 56.5). The level of viremia required to infect the vector is dictated by viral strain and/or species of mosquito vector. Upon ingestion, the virus infects the insect gut and then spreads to the salivary glands, where replication provides a ready source of virus to infect additional vertebrate hosts during insect feeding. The time required for this process is the extrinsic incubation period (EIP), which varies with type of virus and species of mosquito. The EIP for EEE virus is quite short at two to three days (Figure 56.5). Once infected, the vector remains infected for life.

EEE virus exists in two distinct ecosystems: (i) North American (eastern United States and Canada) and the Caribbean and (ii) Central and South America. *Culiseta*

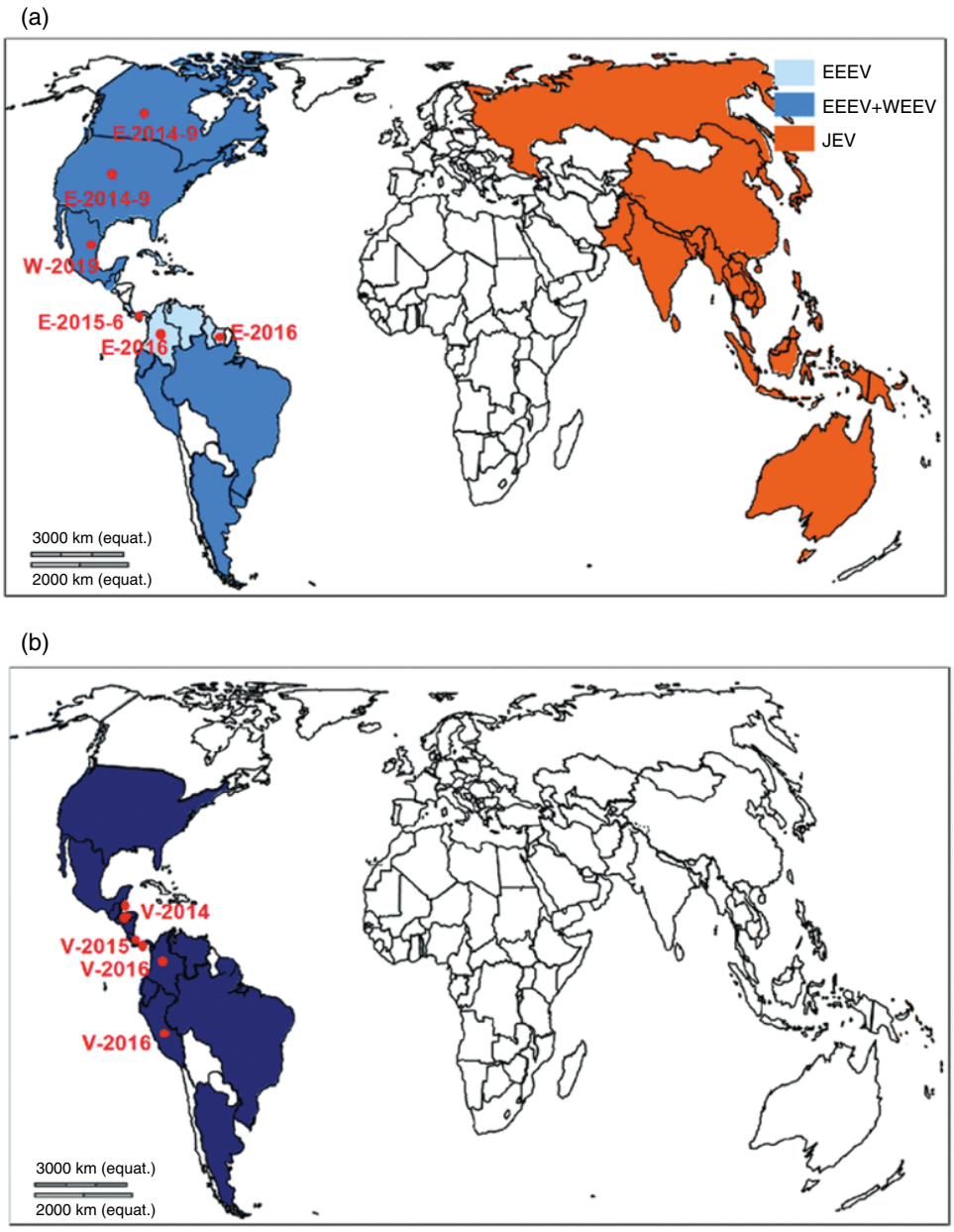


Figure 56.3 Range and distribution of outbreaks of eastern (a, EEEV), western (a, WEEV), and Venezuelan equine encephalitis viruses (b, VEEV) and Japanese encephalitis virus (a, JEV) in horses. Recent outbreaks in horses reported to the OIE World Animal Health Information System (OIE WAHIS). See Figure 56.11. Distribution of WNV. Recent outbreaks in horses reported to the OIE WAHIS interface, ProMED Alerts, or in the scientific literature are depicted by dots (2014–2019). *Source:* From Lecollinet et al. (2019).

melanura mosquitoes transmit the North American strains of the virus. The virus is maintained in an enzootic cycle of infection that includes these ornithophilic (bird-feeding) mosquitoes and the passerine and wading birds that serve as vertebrate virus reservoirs in coastal and inland swamp environments (Figure 56.4). Periodic spillover of the virus occurs in adjacent horses, humans, birds, and other animals (Figure 56.4). A variety of mosquito species can transmit the virus during epidemics, and direct horizontal spread occurs among birds when they peck viremic birds. Another mosquito (*Culex* (*Melanoconion*)) is responsible for transmission of EEE in Central and South America, where small mammals and birds serve as the vertebrate reservoirs of the virus.

The host–virus relationship of WEE is similar to that of EEE, with the virus being maintained in a transmission cycle between mosquitoes (*Culex tarsalis*) and domestic and passerine birds, with periodic spillover into humans, horses, and domestic birds. Other species of mosquitoes can transmit the virus during outbreaks.

Table 56.2 Viruses of veterinary importance in the Flaviviridae family.

Genus	Serogroup/agent	Vector	Affected species	Distribution
Flavivirus				
	Japanese Encephalitis Group Japanese encephalitis virus	Mosquitoes-*Culex* spp.	Horses, humans, swine, poultry	Southeastern Asia
	West Nile virus	Mosquitoes –*Culex* and *Aedes* spp.	Horses, humans, geese, raptors, corvids	Worldwide
	Yellow Fever Group Wesselsbron virus	Mosquitoes –*Aedes* spp.	Sheep	Sub-Saharan Africa
	Tick-borne encephalitis virus Louping ill virus	Tick – *Ixodes ricinus*	Sheep	British Isles, Southern Europe
Pestivirus				
	Bovine viral diarrhea virus	None	Cattle, sheep, wild ruminants	Worldwide
	Border disease virus	None	Sheep	Worldwide
	Classic swine fever virus	None	Swine	Asia, Africa, Europe, and South and Central America

The lifecycle of VEE is more complex. There are several distinct clusters of VEE viruses (Types I–VI), most of which do not cause disease in horses (Type 1, varieties D through F, Types II–VI). These viruses are endemic throughout the tropical and subtropical regions of the Americas (including Florida, where VEE Type II [Everglades] occurs). These endemic VEE viruses are maintained in a natural cycle of infection between *Culex* mosquitoes and small rodents in tropical swamps. Only VEE Types 1AB and 1C are virulent to horses, and these are only isolated during the epidemics of VEE that regularly occur in northern South America. It is believed that these epidemic strains emerge after mutation of the E2 envelope glycoprotein of endemic Type 1D strains of

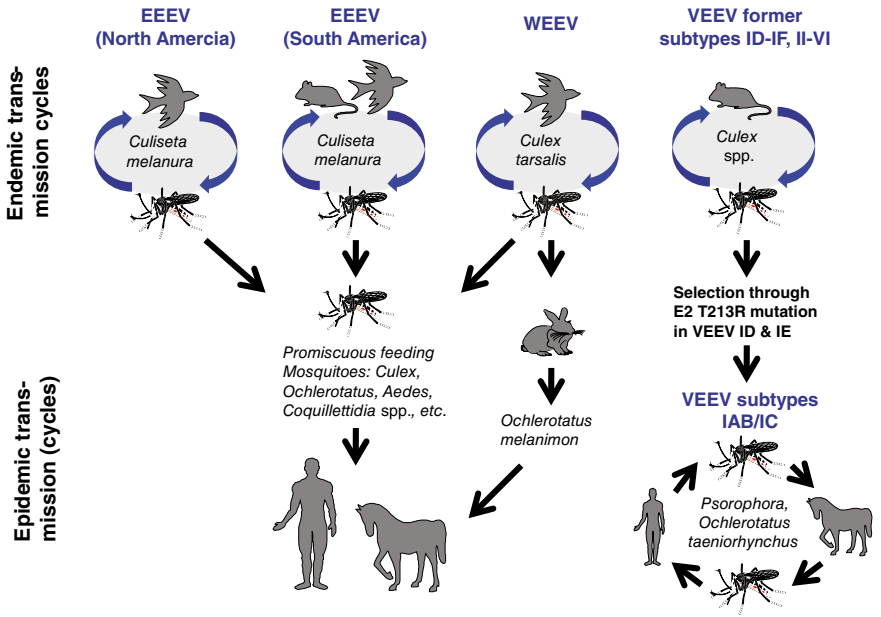


Figure 56.4 The endemic and epidemic transmission cycles of eastern (EEEV), western (WEEV), and Venezuelan equine encephalitis viruses (VEEV). *Source:* From Pfeffer and Dobler (2010).

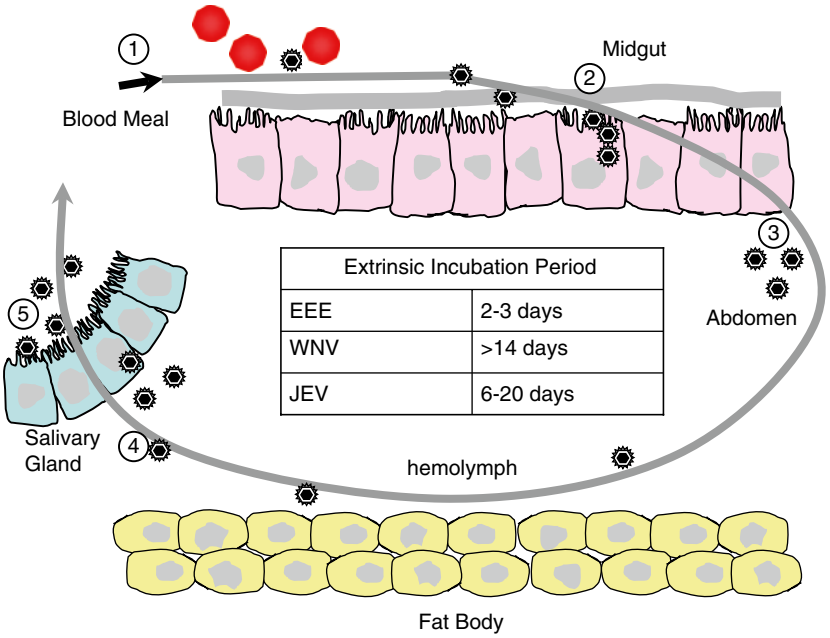


Figure 56.5 Arbovirus replication in female mosquito following blood meal. (1) Ingests infected blood; (2) virus infects and multiples in mesenteronal epithelial cells; (3) virus is released across the basal membrane of the epithelial cells and replicates in other tissues; (4) virus infects salivary glands; and (5) virus is released from cells of salivary glands and transmitted in saliva. The extrinsic incubation periods for equine encephalitis virus (EEE), Japanese encephalitis virus (JEV), and West Nile virus (WNV) are listed.

VEE that constantly are circulating in endemic areas but are not pathogenic to horses (Figure 56.4). Once strains of VEE emerge that are virulent to horses and humans, infected horses are a major source of virus both via vector and horizontal spread because, unlike EEE and WEE, VEE replicates to high titers in horses. Thus, the endemic and epidemic (epizootic) VEE viruses have very different cycles of infection (Figure 56.4).

Pathogenesis and Pathology Equine encephalitis alphavirus infections can range from inapparent to severe, fatal disease. Infection follows the bite of an infected mosquito. Primary viral replication occurs in the regional lymph nodes adjacent to the site of the bite and is followed by generalized infection and viremia in which the virus replicates in the lymphoid tissues throughout the body, bone marrow, and certain other tissues. The encephalitis viruses likely gain entry into the CNS after replication in the endothelial cells lining the blood vessels in the brain, and passage of virus to the brain occurs after the acute phase of infection and viremia. Lesions occur throughout the gray matter of the brain and include perivascular cuffing with inflammatory cells, infiltration of neutrophils into the gray matter with neuronal and parenchymal necrosis, and vasculitis, thrombosis, and hemorrhage. Neutrophils are especially characteristic of the early cerebral lesions of EEE and VEE.

Host Response to Infection The development of neutralizing antibody to EEV appears to be important in limiting viral replication and spread, and is clearly important in preventing reinfection. Antibodies develop to all viral proteins, and peak titers of neutralizing antibody typically develop within two weeks of infection in animals that survive. This antibody may be effective in neutralizing virus, enhancing viral clearance, and lysing infected cells via complement or natural killer cells. Cell-mediated immunity also appears to contribute to viral clearance and protective immunity. Cytotoxic T lymphocytes can be identified as early as three to four days post infection.

Laboratory Diagnosis Equine encephalitis alphavirus infection may be suspected based on the occurrence of neurological disease among susceptible animal species in endemic areas; thus, prior history and seasonal occurrence can be important. Definitive diagnosis requires laboratory confirmation, which is usually done by serology with an IgM-capture enzyme-linked immunosorbent assay (ELISA) to detect virus-specific antibodies in the serum of acutely affected animals. The diagnosis is unequivocally confirmed by virus isolation from blood or CNS tissue, the latter being preferable due to the variable occurrence of viremia by the time signs of encephalitis are manifest in infected animals. Antigen capture ELISA for EEE may be used to help identify the virus in CNS tissue. Immunohistochemistry and

polymerase chain reaction (PCR) may also be used to identify virus in neurological tissue. Virus may be isolated in a variety of systems, including cell cultures, chick embryos, and suckling mice.

Treatment and Control There is no treatment for clinically ill animals. Control of equine encephalitis virus infections can be achieved through vaccination and pest management programs. Vector control can be approached by eliminating mosquito-breeding sites by water control or spraying programs. In the case of domestic bird farms, using tightly screened (insect-proof) rearing pens and locating such pens away from freshwater swamps is also a possibility. Effective inactivated (EEE, WEE, VEE) and attenuated (VEE) vaccines have been developed for the EEV. Regular annual vaccination of susceptible animals is required to maintain immunity if inactivated vaccines are used.

Salmonid Alphaviruses

Disease SAVs are recognized as serious pathogens of farmed Atlantic salmon, *Salmo salar L.*, and rainbow trout, *Oncorhynchus mykiss*, in Europe. There are six alphavirus salmonid viral diseases. Salmon pancreas disease virus (SPDV or SAV subtype 1 [SAV 1], subtype 3 [SAV 3] and SAV 4–6) is the causative agent of pancreatic disease (PD). The disease occurs in farmed Atlantic salmon smolts during their first year at sea with peak prevalence occurring from late July to early September, but outbreaks have occurred at all stages of Atlantic salmon production. Clinical signs associated with PD in order of their appearance may be sudden inappetence, lethargy, and an increased number of fecal casts in the cages, increased mortality and ill-thrift. Affected fish may appear to be unable to maintain their position in the water column/currents within the cages due to muscle damage, predisposing them to erosion and ulceration of the skin and fins. Apparently healthy fish may also die suddenly due to cardiac and skeletal muscle damage and exhaustion. Fish with normal or good-condition factor may sometimes be seen swimming in a spiraling or circular motion or appear dead at the bottom of the cage (similar to sleeping disease [SD] signs), but swim away if handled. Mortality rates in PD outbreaks have ranged from 1% to 48%. High mortality tends to occur in higher-energy-density feeding sites and when the fish are returning to feeding after a period of inappetence. Up to 15% of survivors fail to grow and become runts.

SD is an infectious disease of rainbow trout reared in fresh water, and it was confirmed by the isolation of SDV in France and is now SDV (SAV 2). SDV affects rainbow trout at all stages of production. The characteristic clinical presentation is of affected fish lying on their side on the bottom of the tank, hence the name "sleeping disease." These clinical signs are primarily due to extensive necrosis of skeletal red muscle. Reported mortality levels have been variable, from negligible to over 22% in affected populations.

Epidemiology The SAV have been found almost exclusively in farmed Atlantic salmon and rainbow trout in Europe. SPDV [or SAV subtype 1, 4–6 (SAV 1, 4–6)] was found in farmed Atlantic salmon in Ireland and Scotland. SDV [or SAV subtype 2 (SAV 2)], which was first confirmed in France and is now SAV 2, has been isolated from diseased rainbow trout in England, Scotland, Italy, and Spain. SPDV (SAV 1) and SDV (SAV 2) are closely related subtypes of the same virus species with the proposed name salmonid alphavirus. NSAV (SAV 3) was isolated from farmed Atlantic salmon with PD and has been detected only in Norway. The genomic organization of SAV 3 is identical to that of SAV 1 and SAV 2, and the nucleotide sequence similarity to the other two alphaviruses is 91.6% and 92.9%, respectively. PD was described only once in North America, in 1987, but no virus was detected, and this remains the only report of a PD-like condition outside Europe. A dual infection of infectious salmon anemia virus (a member of the Orthomyxoviridae family) and a toga-like virus was described in New Brunswick, Canada, in 2000.

Pathogenesis and Pathology The principal gross findings at necropsy in the early stages of a PD outbreak are the absence of food in the gut and the presence of fecal casts. The significant lesions in naturally occurring PD occur sequentially in the pancreas, heart, and skeletal muscle of affected fish. Pancreatic necrosis, cardiomyopathy, and skeletal myopathy, including esophageal muscle lesions, are the most significant PD lesions. Approximately three out of four cases of PD have cardiac and/or skeletal myopathies. These myopathies are significant in the pathogenesis of PD outbreaks. Relatively small numbers of fish have acute pancreatic lesions (<2% of fish).

The acute infection phase of PD is relatively short-lived with rapid destruction of the majority of pancreatic acinar tissue and a variable inflammatory response. As skeletal muscle lesions tend to first appear three to four weeks after the appearance of pancreatic and heart lesions, fish sampled in late phase disease may only have skeletal muscle lesions.

SD has the same sequential histological lesions: exocrine pancreas, heart, and skeletal muscle as those described for PD. The major histological lesion sign is extensive necrosis of skeletal red muscle.

Host Response to Infection Very few studies have been carried out on the specific immune responses to SAV infections in fish. There is cross-protection between SAV 1(SPD) and SAV 2 (SD) in both Atlantic salmon and rainbow trout. The specific virus neutralization (VN) antibody response occurred in 60% of fish by 14–16 dpi with all the fish seroconverting by 21 dpi. There have been no reported cases of recurrence of PD or SD in previously infected populations, indicating that protection following natural infection is adequate for a complete production cycle.

Laboratory Diagnosis Early acute SAV infections can be diagnosed using histology (pancreas, heart, and muscle), immunohistochemistry (IHC-pancreas), detection of viremia, and detection of virus RNA in serum and heart tissue (real-time PCR [RT-PCR]). Chronic-stage SAV infections could be diagnosed by histology, serology (VN antibody detection), and virus RNA detection.

Treatment and Control Vaccines have been developed for SAV 1 and SAV 2. Four commercial PD vaccines are available. Although it has proved to be very effective in some field trials, there are concerns about the duration of immunity. Sea vaccination or revaccination is being currently investigated for sites where PD occurs in Atlantic salmon in the second year at sea. A recombinant SAV 2 live vaccine protected rainbow trout from wild type SAV 2 infection for five months.

SAV 1 can survive for more than two months in sterile seawater at low temperatures, so endemic SAV 1 impact must be minimized by reducing stress through careful management and good hygiene methods. Some risk factors that have been identified include sea-site-to-sea-site movements and sea-site-to-harvest movements by well boat. Biosecure slaughter methods and safe disposal of offal and effluent are also important to minimize the risk from these processes. Good sea lice control is desirable, not only for the health and welfare of the fish, but because sea lice may act as reservoirs or vectors of infection. Because most alphaviruses are arboviruses, the possibility of a biological transmission cycle between susceptible vertebrate hosts and blood-feeding arthropods is possible. Although SAV infections can be transmitted without an insect vector, the potential role of sea and freshwater lice in SAV infections needs to be investigated. There is some evidence that SAV 2 can be transmitted vertically from brood stock to eggs and fry, but further work is required to confirm vertical transmission.

Other Medically Important Alphaviruses

The major medically important alphavirus is CV, which was introduced into the Americas in 2013. CV is most often spread to people by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Most people infected with CV will develop some symptoms three to seven days after being bitten by an infected mosquito. The most common symptoms are fever and joint pain. Other symptoms may include headache, muscle pain, joint swelling, or rash. Chikungunya disease does not often result in death, but the symptoms can be severe and disabling. Most patients feel better within a week. In some people, the joint pain may persist for months. People at risk for more severe disease include newborns infected around the time of birth, older adults (≥65 years), and people with medical conditions such as high blood pressure, diabetes, or heart disease. Infection is thought to lead to lifelong immunity. There is no vaccine. A number of other alphaviruses can infect animals, including Sindbis, Semliki Forest, Highlands J, and Getah viruses. Highlands J virus has a similar distribution in North America as EEE virus, but only rarely causes encephalitis in horses (Figure 56.6). It is, however, an important pathogen of turkeys, pheasants, chukar partridges, ducks, emus, and whooping cranes. Getah virus infects horses and swine in Asia and Southeast Asia. Infection in horses is sometimes characterized by fever, rash, and limb edema (but not encephalitis), and Getah virus also can cause abortion in pregnant swine. Getah virus is included within the Semliki Forest virus complex of alphaviruses, and Semliki Forest virus also can cause a febrile disease of horses in Africa. The SESV, an alphavirus, was isolated from the elephant seal louse, *Lepidophthirus macrorhini*, on Macquarie Island in Australasia, and the high SES virus seroprevalence in the seal population suggests that it is transmitted by the lice. The southern elephant seal population has decreased by 50% in the last 50 years, but not because of the SESV.

Flaviviridae

The family Flaviviridae contains a large number of viruses within three antigenically distinct genera (Table 56.2): Flavivirus, Pestivirus, and Hepacivirus (human hepatitis C virus). The family Flaviviridae includes a number of important human pathogens. Although Zika, a flavivirus, emerged in 2015 as an important human arbovirus, the veterinary flaviviruses have not seen any new "emerging viruses."

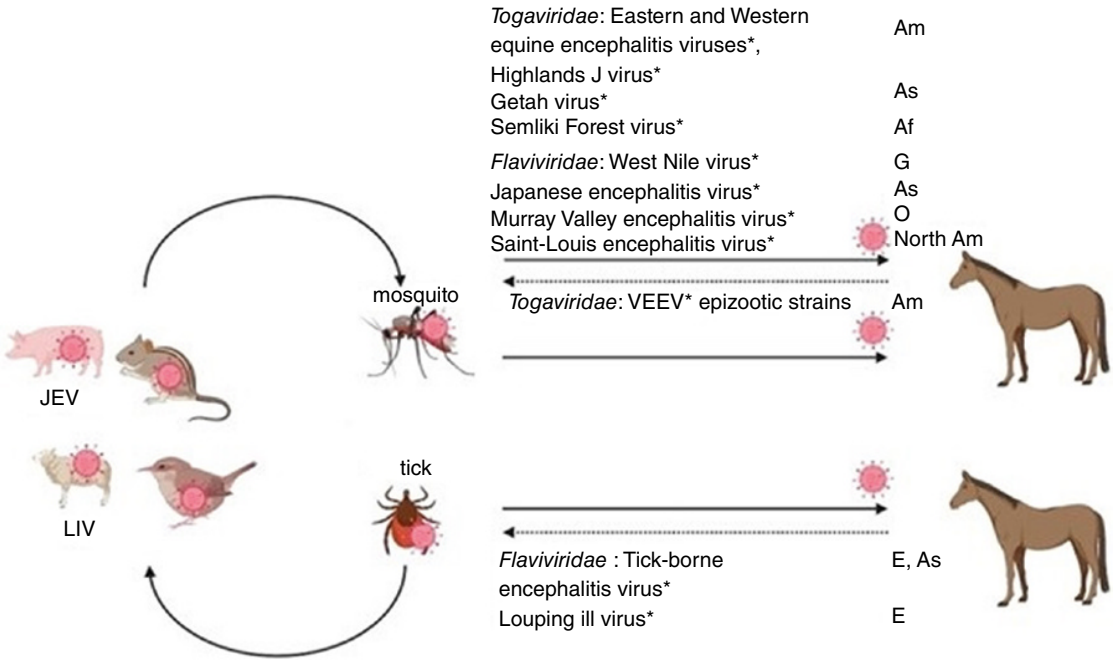


Figure 56.6 Arthropod-borne transmission of encephalitis viruses in horses. Zoonotic viruses are marked with an asterisk and geographical distribution (Af for Africa, Am for America, As for Asia, E for Europe, ME for Middle East, O for Oceania, and G for global) are presented. VEEV: Venezuelan equine encephalitis virus. Black arrows represent established virus transmission between the two partners. Dotted arrows indicate limited virus transmission possibility from the infected horse to its reservoir, with the exception of the mosquito-borne VEEV epizootic variants for which horses serve as amplifier hosts. *Source:* Adapted from Lecollinet et al. (2019).

Flavivirus

Members of the genus *Flavivirus* include arboviruses that are transmitted by either mosquitoes or ticks. Those that are mosquito transmitted include the Japanese encephalitis virus (JEV) group (including Japanese encephalitis, Murray Valley encephalitis, St. Louis encephalitis, and West Nile and Kunjin viruses), the Yellow Fever (YF) virus group (including YF and Wesselsbron viruses) and dengue virus (DV) group (the cause of human dengue hemorrhagic fever). The tick-borne flaviviruses include tick-borne encephalitis (TBE) (European, Far Eastern, and Siberian), Powassan, and Louping ill viruses. These viruses cause either encephalitis or systemic hemorrhagic septicemia in animals and/or humans.

Physical, Chemical, and Antigenic Properties

The name *Flavivirus* is derived from the Latin word "flavus," which means yellow, since YF virus is the family prototype. Members of the genus consist of spherical (50 nm in diameter) enveloped particles with small surface projection (peplomers) (Figure 56.7). Virions are stable at a pH of 7–9, but are inactivated by acid pH, temperatures above 40 °C, lipid solvents, ultraviolet light, ionic and nonionic detergents, and trypsin. A single nucleocapsid protein encapsulates the genome, and the envelope contains two viral membrane proteins (E and M). Several nonstructural viral proteins are also produced in virus-infected cells (NS1-5). The viral genome is a single linear strand of positive-sense RNA. The genomic RNA is infectious and encodes a single large polyprotein that is co- and posttranslationally cleaved into the various viral structural and nonstructural proteins. The flaviviruses are all serologically related as determined by group-specific assays such as ELISA and hemagglutination inhibition. They are distinguished by neutralization assays, although there is considerable cross-reactivity among viruses within the same serogroup (sero complex). The envelope E protein contains the major determinants of VN.

Veterinary and Zoonotic Flaviviruses

Japanese Encephalitis Group

JEV is the prototype of the JE antigenic complex within the *Flavivirus* genus. They are all mosquito-transmitted flaviviruses that cause encephalitis. The JE complex includes St. Louis encephalitis virus, Murray Valley encephalitis virus, and West Nile virus (WNV) (and the related Kunjin virus), in addition to JEV itself. These are all human pathogens, but JEV and WNV are important veterinary pathogens as well.

Japanese Encephalitis Virus

Disease JEV infection is typically inapparent but can cause clinical disease in humans, horses, and swine. Infection of dogs and domestic poultry also occurs. JEV

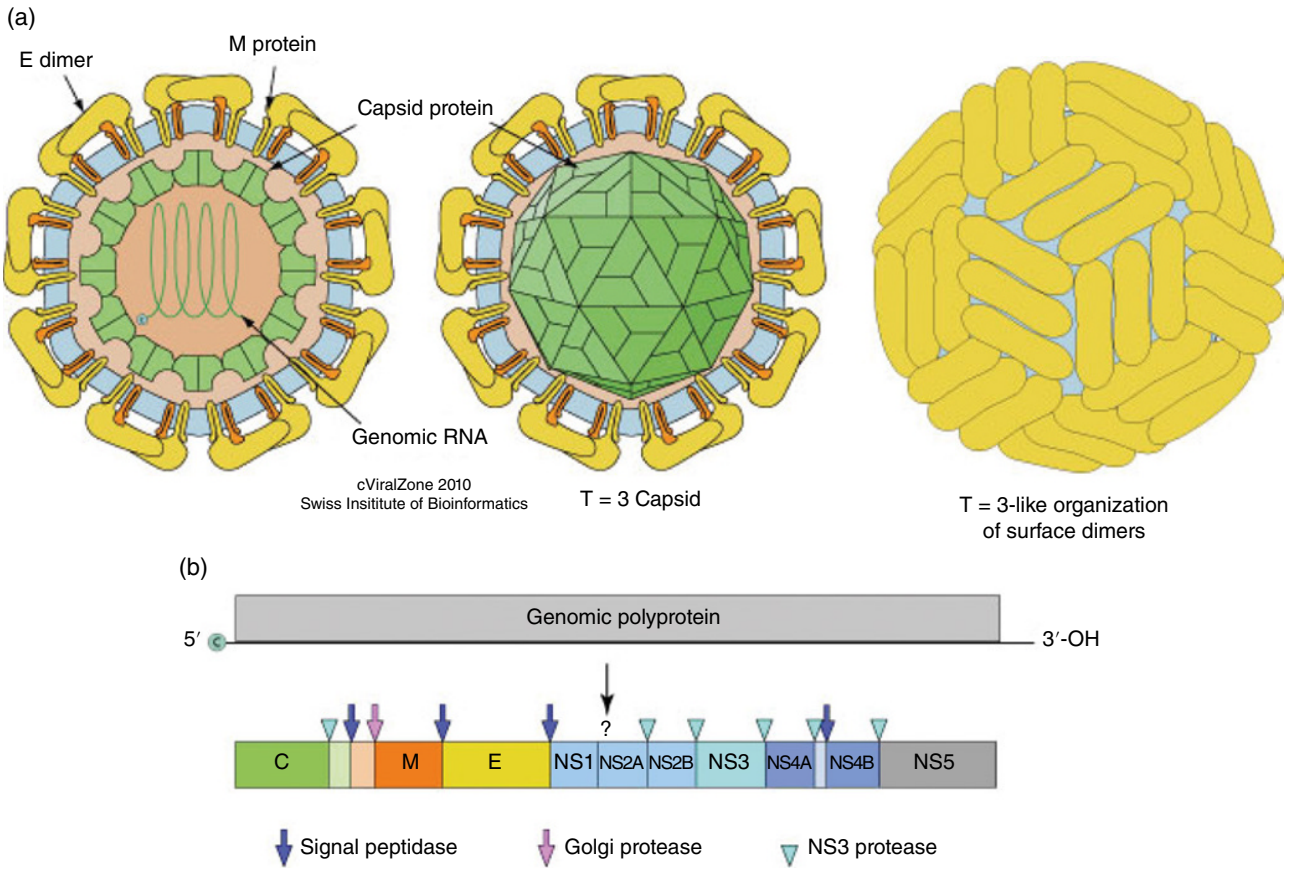


Figure 56.7 Virion structure and genomic organization of a flavivirus. (A) A flavivirus. Enveloped, spherical, about 50 nm in diameter. The surface proteins are arranged in an icosahedral-like symmetry. (B) Monopartite, linear, ssRNA(+) genome of about 9.7–12 kb. The genome 3′ terminus is not polyadenylated but forms a loop structure. The 5′ end has a methylated nucleotide cap (allows for translation) or a genome-linked protein (VPg).

infection of swine is generally inapparent in swine but results in abortions and stillbirths of naive pregnant sows. Infected horses can develop severe neurological disease that resembles EEE, but mortality is lower.

Epidemiology JEV infection currently is confined to the temperate and tropical areas of Asia, where serological surveys indicate that infection of horses, cattle, and swine is widespread. The virus occurs throughout much of Asia, from the Indian Subcontinent to the west to the Pacific Islands in the east (Figure 56.3). Most infections are inapparent or mild. Horses and humans are unimportant to the epidemiology of JEV infection because viremias are at very low titers in these species, insufficient to serve as a virus reservoir for susceptible mosquito vectors (Figure 56.8). In contrast, infected swine and birds serve as amplifying hosts for the virus because they have high-titer viremias (Figure 56.8). *Culex* spp. mosquitoes are the principal vectors of JEV, and the EIP is moderate at 6–20 days (Figure 56.5). In swine, virus may also be transmitted from the infected dam to the fetus and from boar to sow via insemination of viral-contaminated semen. The virus is maintained in tropical regions, probably by continual transmission between mosquitoes, birds, and swine.

Pathogenesis and Pathology The pathogenesis and lesion of the disease in horses are similar to that described above for the EEV.

Host Response to Infection Both humoral and cell-mediated immune responses develop in animals following JEV infection. Antibodies (HI and viral-neutralizing) develop within a few days after infection. Neutralizing antibodies largely are directed at the E envelope protein, whereas cytotoxic T lymphocyte responses are directed at the nonstructural viral proteins. Humoral responses appear to play an important role in both recovery and long-term protection against reinfection. Cell-mediated immune responses (cytotoxic T lymphocytes) likely contribute to viral clearance. Immunity is lifelong after natural infection.

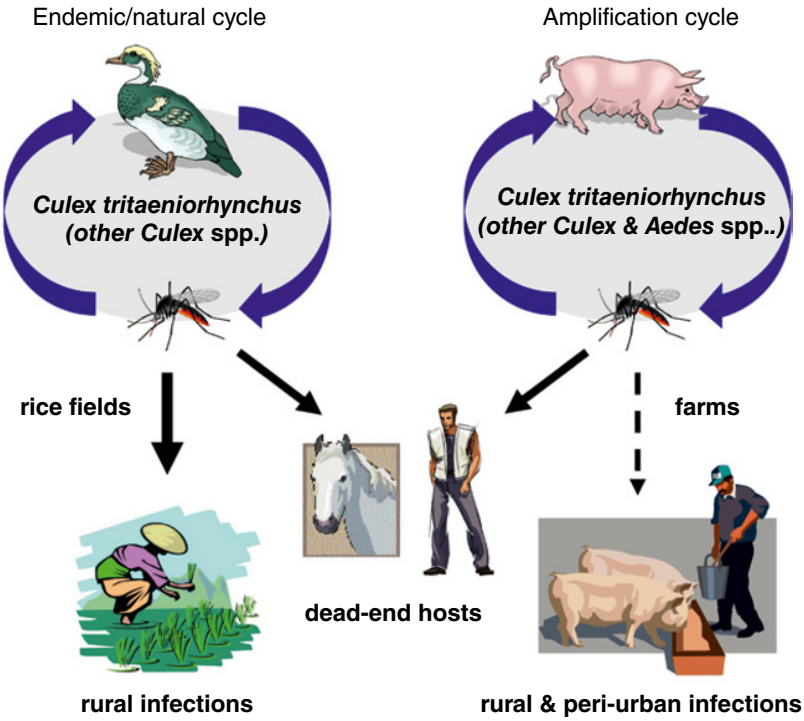


Figure 56.8 Transmission cycles for Japanese encephalitis virus. *Source:* From Pfeffer and Dobler (2010).

Laboratory Diagnosis Diagnosis of JEV is accomplished by isolating virus from the tissues or blood of affected animals using cell cultures (vertebrate or insect) or suckling mice. Viral RNA can be identified by PCR from any sample. Viral antigen may also be demonstrated directly in brain tissue sections by immunohistochemical staining. A variety of serological assays also are available, but demonstration of virus-specific IgM (indicative of recent infection) is generally required as IgG assays often detect antibodies to related flaviviruses, which complicates interpretation.

Treatment and Control Vaccination is used for control of JEV infection in endemic areas, and both attenuated and killed vaccines are available. Vector control measures are also an important approach to control.

West Nile and Kunjin Viruses

Disease WNV, a member of the JEV antigenic complex, recently emerged in the western hemisphere, precipitating a massive epidemic of disease in humans, horses, birds, and a variety of other animals (alligators, squirrels, mountain goats, llamas, sheep, cats, dogs, etc.). Although most horses are asymptomatic (>90%), the neurological disease that occurs in WNV-infected horses is characterized by facial nerve paralysis, ataxia, weakness, recumbency, muscle fasciculations, and high fatality rates. Some species of birds suffer high mortality similar to horses, including geese (particularly goslings), raptors, and corvids (crows).

Epidemiology Prior to 1999, WNV occurred throughout Africa as well as in parts of Europe, the Middle East, Asia, and Australia (where it is called the Kunjin virus). However, in 1999, the virus entered New York City and the northeast United States and swept across 2/3 of the country by 2002, reaching the West Coast by 2003 (Figure 56.9). Horse cases peaked in the United States in 2002 and declined since the introduction of an inactivated WNV in 2003 (Table 57.4). Human cases peaked in 2004, but WNV is the leading cause of arbovirus infections in the United States (Table 57.4). WNV equine outbreaks have occurred in Europe, North Africa, and Middle East along with the Americas in the last seven years (Figure 56.10) The virus is maintained in a mosquito-bird cycle of infection, and humans and horses are "dead-end" hosts because viremia is not sufficient to infect susceptible mosquitoes that feed on infected individuals (Figure 56.11). There are two distinct genetic lineages of WNV, "lineage 1" and "lineage 2." Lineage 2 viruses are endemic south of the equator in Africa, where they cause little if any disease in horses. In distinct contrast, lineage 1 strains of WNV have been associated with outbreaks of disease in Mediterranean and Eastern Europe, North Africa, Asia, and North America. Many bird species undergo subclinical or asymptomatic

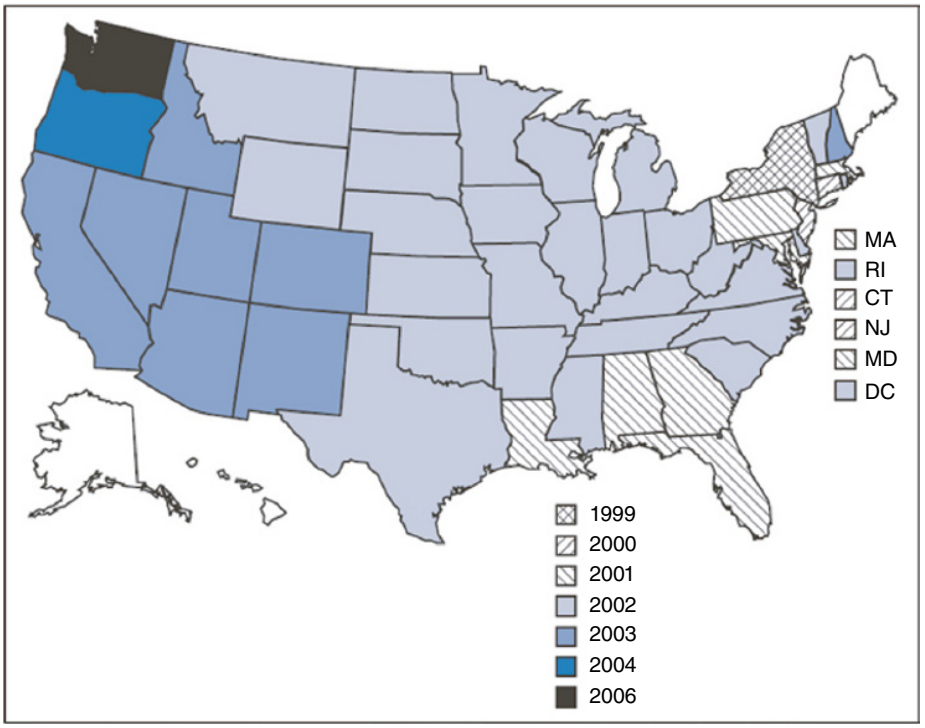


Figure 56.9 Year of first reported human West Nile virus disease case, by state – United States 1999–2008. *Source:* From Lindsey et al. (2010).

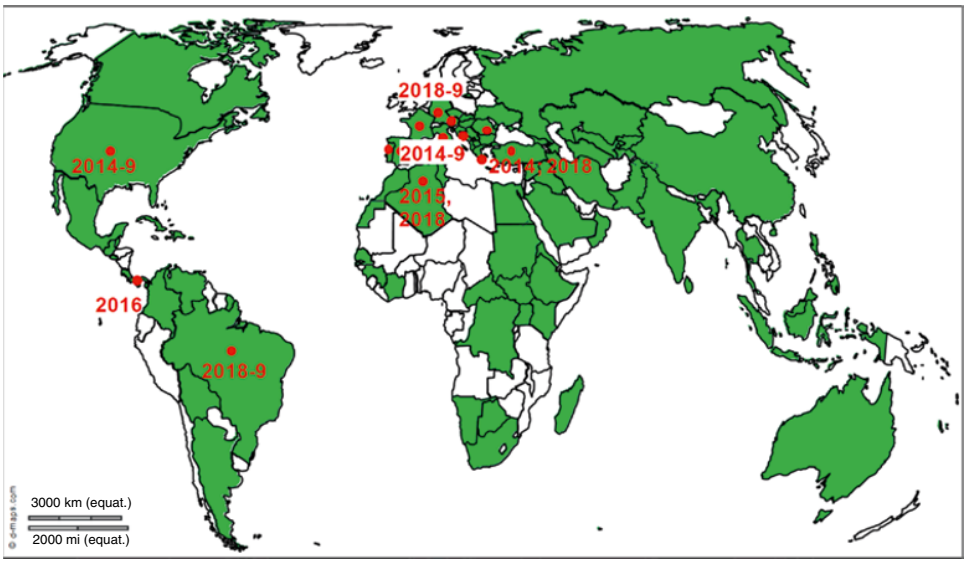


Figure 56.10 Transmission cycles for West Nile virus. *Source:* From Pfeffer and Dobler (2010).

infection and have viremias of sufficient magnitude to serve as amplifying reservoir hosts of the virus. Specific species of mosquitoes transmit WNV in endemic areas, whereas a wide variety of mosquito species have been incriminated in the transmission of WNV infection in North America (Figure 56.11). The EIP for WNV is quite long (>14 days) compared to the other encephalitic arboviruses (Figure 56.5).

Pathogenesis and Pathology The pathogenesis and lesion of the disease in horses are similar to that described above for the EEV.

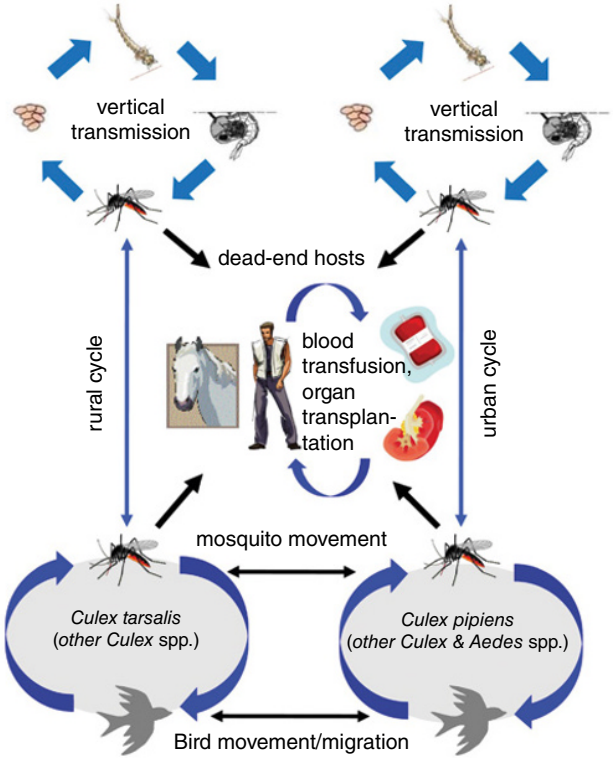


Figure 56.11 Range and distribution of outbreaks of West Nile virus (WNV) in horses. Recent WNV outbreaks in horses reported to the OIE World Animal Health Information System (OIE WAHIS) interface, ProMED Alerts, or in the scientific literature are depicted by dots (2014–2019). *Source:* From Lecollinet et al. (2019).

Host Response to Infection The host immune response is similar to that described above for JEV.

Laboratory Diagnosis Diagnosis of WNV is best done by PCR. Virus isolation in appropriate cell cultures also is possible. WNV-specific IgM-capture ELISA is very useful for the serological identification of animals that are acutely infected with WNV.

Treatment and Control Four different WNV vaccines were developed for horses: an inactivated WNV vaccine, a canarypox recombinant WNV vaccine, a YF virus-WNV chimera, and a WNV DNA vaccine. The inactivated WNV vaccine is the more frequently used as it is often combined with EEE, VEE, and/or WEE. Annual revaccination is highly recommended. Vector control measures are also an important approach to control.

Other Medically Important Members of the Flavivirus-Japanese Encephalitis Group

Murray Valley encephalitis virus, another member of the JE mosquito-borne encephalitis complex, is enzootic in New Guinea and Australia and causes sporadic encephalitis in humans (Figure 56.6). St. Louis encephalitis virus, another member of the JE complex, is enzootic in North, Central, and South America and also causes sporadic encephalitis in humans.

Yellow Fever Virus Group

Yellow Fever virus group are a small group of mosquito-borne flaviviruses that cause hemorrhagic fevers in humans. Yellow fever virus is the prototype virus, and this group includes Wesselsbron virus.

Wesselsbron Virus

Disease and Epidemiology Wesselsbron virus causes disease of sheep in sub-Saharan Africa. Subclinical infection of cattle, horses, and swine also occur, and the virus is a zoonotic pathogen causing febrile disease in humans with headache, myalgia, and arthralgia. The virus causes extensive liver necrosis, jaundice, subcutaneous edema, gastrointestinal hemorrhage, and fever in infected sheep; mortality in lambs can be high, and abortion is common in pregnant ewes. *Aedes* mosquitoes transmit Wesselsbron virus.

Pathogenesis and Pathology The virus infects and causes necrosis of the liver, resulting in hemorrhage and systemic organ failure.

Host Response to Infection All immune cells are depleted following infection.

Laboratory Diagnosis Diagnosis may be based upon viral isolation using a variety of cell cultures (BHK and lamb kidney), chick embryos, or suckling mice. Viral RNA can be identified by PCR from any sample. Vaccination and/or prior exposure to other flaviviruses complicate the interpretation of serological assays.

Treatment and Control Attenuated virus vaccines have been used to prevent the disease in endemic areas; however, the vaccines are reported to no longer be available. Vector control measures are also an important approach to control.

Other Medically Important Mosquito-Borne Flaviviruses

YF virus is one of the most severe hemorrhagic viral fevers of humans. It occurs predominantly in areas with jungle canopies in Central and South America and Africa. It involves an interaction between canopy mosquitoes, monkeys, and vector mosquitoes. *A. aegypti* is the predominant vector in the western hemisphere. YF occurred in the southern United States until the 1890s, when vector

control measures eliminated *A. aegypti*. There is an effective YF vaccine. DV is responsible for dengue hemorrhagic fever, one of the greatest human arbovirus threats. DV occurs in a wide belt across Central and South America, Africa, and Southeast Asia and has occurred in the Caribbean and the extreme southern United States. *A. aegypti* is also predominant vector of DV. Reinfection with DV results in an antibody-dependent enhancement (ADE) that causes the subsequent DV infection to infect macrophages and cause severe disseminated intravascular coagulation and hemorrhagic fever. DV has four different serotypes that do not cross-protect, and there is no vaccine. Zika virus (ZV) outbreaks occurred in humans in Polynesia in 2014 and Brazil in 2015. It is transmitted by *Aedes* species mosquito (*A. aegypti* and *A. albopictus*). ZV disease is generally asymptomatic and usually mild, rarely causing hospitalization or mortality. Zika infection during pregnancy can cause serious birth defects including microencephaly and is associated with other pregnancy problems. ZV infection has also been occasionally associated with Guillain-Barré syndrome. Following the initial outbreak in Brazil, there were few cases in subsequent years. There is no vaccine.

Tick-Borne Encephalitis Group

This group of viruses includes a number of human pathogens including prototype virus, TBE virus with its three geographical subtypes (European [Western], Siberian, Far Eastern [Russian spring summer encephalitis virus]), Kyasanur forest disease virus, Omsk hemorrhagic fever virus, Powassan virus, and the important veterinary pathogen, Louping ill virus. The viruses in this group are all transmitted by ticks, making their epidemiology more complex as the ticks serve as both a reservoir and as a virus vector. Unlike mosquitoes, ticks can live for several years, making them an effective reservoir. In addition, these tick-borne viruses are transmitted from one developmental stage to another (transstadial transmission) and vertically from one generation to another (transovarial transmission). The tick larvae and nymphs feed on birds or small rodents, whereas adult ticks prefer larger animals (Figure 56.11).

Louping Ill Virus

Disease Louping ill virus is a tick-transmitted flavivirus that naturally infects many animal species, including humans, sheep, horses, deer, and birds (Figure 56.6). The virus causes encephalomyelitis in sheep, and high mortality may result when susceptible sheep are introduced into an endemic area. Louping ill is characterized by neurological signs such as hyperexcitability, cerebellar ataxia, and progressive paralysis. The disease derives its name from the leaping gait sometimes observed in ataxic animals. Louping ill is a zoonotic disease causing influenza-like syndromes followed by meningoencephalitis in humans that resolves in 4–10 days. The disease also sometimes occurs in cattle, horses, and goats.

Epidemiology Louping ill is confined to the British Isles and portions of continental Europe (Spain, Greece, and Turkey). *Ixodes ricinus* ticks are the principal vector.

Laboratory Diagnosis Diagnosis of Louping ill virus is done by viral isolation, immunohistochemical staining of CNS tissue sections from affected animals, or serology. Viral RNA can be identified by PCR from any sample. Virus can be isolated using vertebrate or insect cell cultures and intracerebral inoculation of suckling mice. Propagated virus can be definitively identified by viral neutralization.

Treatment and Control Louping ill can be controlled through tick control by dipping and spraying of sheep. A formalin-inactivated cell culture-origin vaccine is available and effective. Lambs born to immune dams are protected by colostral antibody for approximately four months.

Other Medically Important Tick-Borne Encephalitides

Powassan virus is the cause of a tick-transmitted encephalitis of humans and horses in North America. The virus infects a wide variety of animal species, both wild and domestic, as well as several species of ticks. It causes encephalomyelitis in horses that mimics that caused by other flaviviruses like WNV, from which it must be distinguished by either PCR assay or by virus isolation. Similar tick-borne encephalidities of humans are caused by related viruses in Europe and Asia, including Omsk hemorrhagic fever virus, TBE viruses (Europe, the Far East, and Siberia), and Kyasanur forest disease virus. TBE encephalitis results in a high proportion (up to 50%) of the patients having permanent neurological disorders. These TBE viruses are maintained in a cycle of infection that includes ticks and vertebrates (livestock and dogs) that serve as amplifying hosts of the virus. The virus can be transmitted to humans in the milk or dairy products of TBE-infected cattle sheep and/or goats (Figure 56.11). Encephalitis occurs sporadically among animals in areas where these viruses are endemic.

Pestivirus

The genus *Pestivirus* includes bovine viral diarrhea virus (BVDV, Types 1 and 2), border disease virus (BDV), and classical swine fever virus (CSFV, previously referred to as hog cholera virus), all of which are important pathogens of

livestock. In contrast to the genus *Flavivirus*, members of the genus *Pestivirus* are not arthropod borne.

Physical, Chemical, Biotype and Antigenic Properties

Pestiviruses are readily inactivated by low pH, heat, organic solvents, and detergents. Virions are spherical to pleomorphic (40–60 nm in diameter) and enveloped with small surface projections (spikes) emanating from the viral envelope. The virions consist of an envelope and nucleocapsid, and include four structural proteins: a nucleocapsid protein (C) and three envelope glycoproteins (Erns, E1, and E2) (Figure 56.12). Some seven or eight viral nonstructural proteins also are produced in infected cells. The genome is a single molecule of positive-sense, single-stranded RNA that contains a single, large open reading frame that encodes a large polyprotein that is co- and posttranslationally cleaved into the various structural and nonstructural viral proteins (Figure 56.12).

Pestivirus infection of cell culture can results in one of two different phenotypes: cytopathic (CP), where the cells die, or noncytopathic (NCP), where the cells appear normal. Although both BDV and CSFV CP isolates have been identified, BVDV is most frequently associated with CP viruses. NCP virus are the predominant biotype in nature. All CP viruses arise from mutations to NCP, usually from recombination events that include point single nucleotide, cellular genes, or viral gene insertions or deletions (Figure 56.13) in or near the NS2-3 gene resulting in the production of the NS3 gene product in CP viruses. These cell culture phenotypic differences do not reflect pathogenesis or severity of disease, as the most pathogenic viruses are NCP.

The pestiviruses are very closely related and can only be distinguished by application of monoclonal antibodies and/or molecular biology techniques; however, they tend to be host specific. The pestiviruses are all antigenically related and include cross-reactive epitopes. Several other pestiviruses have been isolated that are genetically distinct from BVDV (Types 1 and 2), BDV, and CSFV, and it is proposed that pestiviruses isolated from a giraffe, for example, be identified as a new species. Typing of pestiviruses is based on genetic sequence analysis of 5′ untranslated region (Figure 56.12). Neutralizing antibodies are directed against the Erns and E2 envelope glycoproteins. Animals infected with CP BVDV also mount a strong immune response against the NS3 protein, whereas the antibody response to other viral proteins is generally weak.

Infectivity for Other Species and Culture Systems

BVDV infects cattle, sheep, goats, pigs, and wild ruminants, but principally it is an infectious pathogen of cattle and sometimes sheep. Isolates of BVDV, whether CP or NCP, can be grown in cell culture systems, including various bovine embryonic cell cultures. Immunofluorescence or other immunohistochemical staining methods are used to detect NCP strains of BVDV, which contaminate many commonly used cell lines. CP strains produce plaques and can be used for accurate viral titrations.

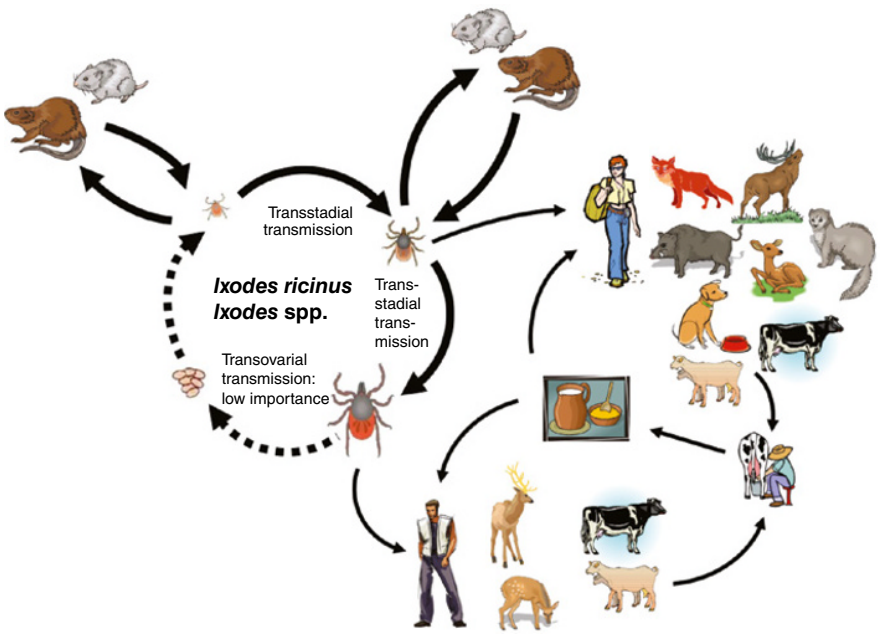


Figure 56.12 Transmission cycle of tick-borne encephalitis virus. *Source:* From Pfeffer and Dobler (2010).

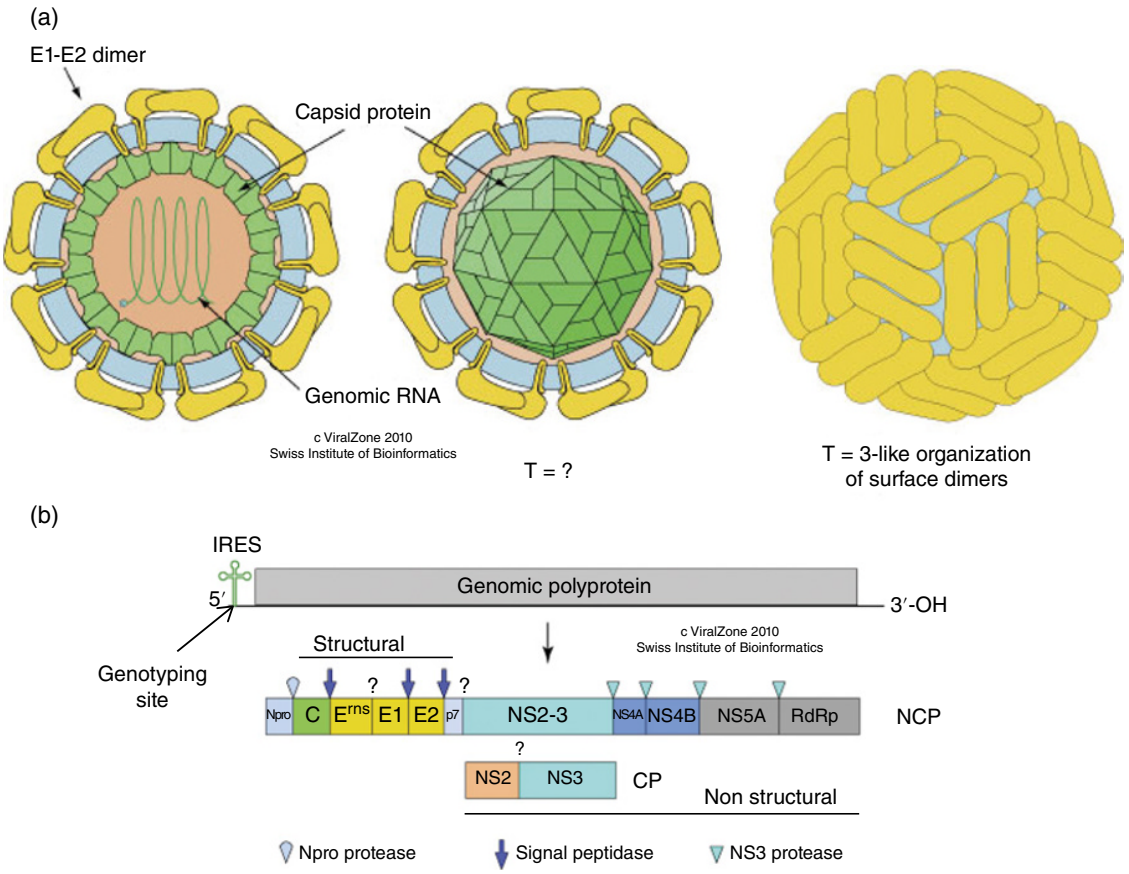


Figure 56.13 Virion structure and genomic organization of a pestivirus. (a) BVDV virion. Enveloped, spherical, about 50 nm in diameter. Mature virions contain three viruses: encoded membrane proteins (Erns, E1, and E2) in addition to the capsid protein. (b) BVDV genome. Monopartite, linear, ssRNA(+) genome of about 12 kb. The genome 3′ terminus is not polyadenylated, but terminates with a short poly-(C) tract. There is an internal ribosome entry site (IRES) at the 5′ end that mediates translation initiation. Duplications, deletions, and other rearrangements are found in most cytopathic (CP) pestiviruses isolates.

Infectivity for Host Species and Culture Systems.

Although BDV is classically associated with infection of sheep, it also can infect cattle and goats, and pregnant sows that were experimentally infected with BDV gave birth to piglets with cerebellar hypoplasia. The virus can be propagated in primary and secondary cell cultures of bovine and ovine origin and in established cell lines, including pig kidney, fetal lamb muscle, and bovine turbinate.

Classical swine fever (CFSV) only infects domestic swine and wild hogs. It replicates in cultures of porcine cells such as spleen, kidney, testicle, and peripheral blood leukocytes. Most strains of the virus are non-cytopathogenic and may persist in culture for many cell passages. The presence of HCV in infected cell cultures is readily demonstrated by immunofluorescent or immunohistochemical staining techniques. Some cytopathogenic strains have been reported.

Bovine Viral Diarrhea Virus

Disease

BVDV is responsible for a variety of respiratory, digestive tract, and reproductive syndromes. This disease complex actually includes three distinct disease syndromes:

1) The consequences of infection of cattle with BVDV vary from an inapparent infection to severe fatal disease. Immunodysfunction may be the most important consequence of postnatal infection of nonpregnant cattle. Acute BVDV infection is usually a mild disease of young calves characterized by leukopenia, fever, and erosions and ulcers of the upper gastrointestinal tract (particularly the hard palate and gums). The infection is typically characterized by high morbidity and low mortality if uncomplicated. Frequently BVDV infection is the viral component of the bovine respiratory disease complex, a disease complex that predisposes cattle to secondary bacterial and/or mycoplasma

respiratory infections that result in severe bronchopneumonia with high morbidity and mortality. Occasionally, acute BVDV infection can caused severe, fatal disease with high mortality in both calves and adult animals often accompanied by widespread hemorrhages as a consequence of thrombocytopenia. Since the initial emergence of this disease syndrome in the 1990s, Type II strains of BVDV have become increasingly associated with mild disease in calves, similar to that produced by most Type I strains.

2) Reproductive BVDV infection results in a variety of syndromes depending on the stage of development of the fetal calf. Infections prior to 40 days of gestation will result in fetal death. BVDV infection of bovine fetuses with an NCP BVD virus prior to mid-gestation (40–150 days of gestation) often leads to the birth of calves that are persistently infected (PI) with BVDV (Figure 56.14). Usually, other animals are the source, but a PI cow will always produce a PI calf (Figure 56.14) These animals subsequently serve as a reservoir of virus in the herd, which they transmit both horizontally and vertically (to their own progeny) (Figure 56.15); furthermore, they develop weak or no obvious humoral immune response to the virus with which they are PI. PI animals have a high mortality rate in the first year of life. Calves infected with NCP BVDV after 150 days of gestation (after the immune response develops) are congenitally infected. Although they clear the BVDV infection, lifelong damage can occur to their immune and reproductive systems. Both PI and congenitally

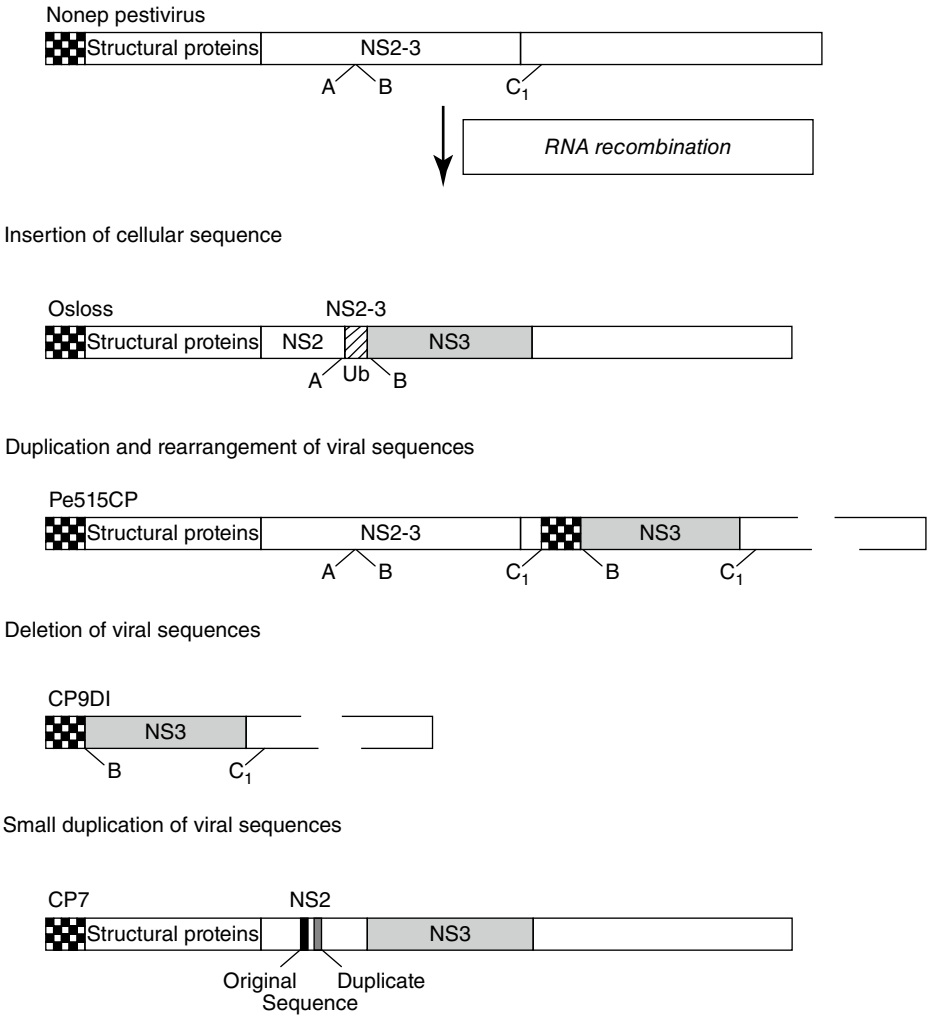


Figure 56.14 Recombinations that generated four CP BVDV strains. (1) Insertions of cellular sequences coding for ubiquitin. (2) Duplications and rearrangement of viral sequences including the NS3 gene (>2 kb). (3) Deletions that led to generation of cp defective interfering particles (DIs). (4) A small duplication of a viral sequence (27 nucleotides); the insertion is identical with a sequence located about 300 nucleotides upstream. *Source:* From figure 3 of Kümmerer et al. (2000).

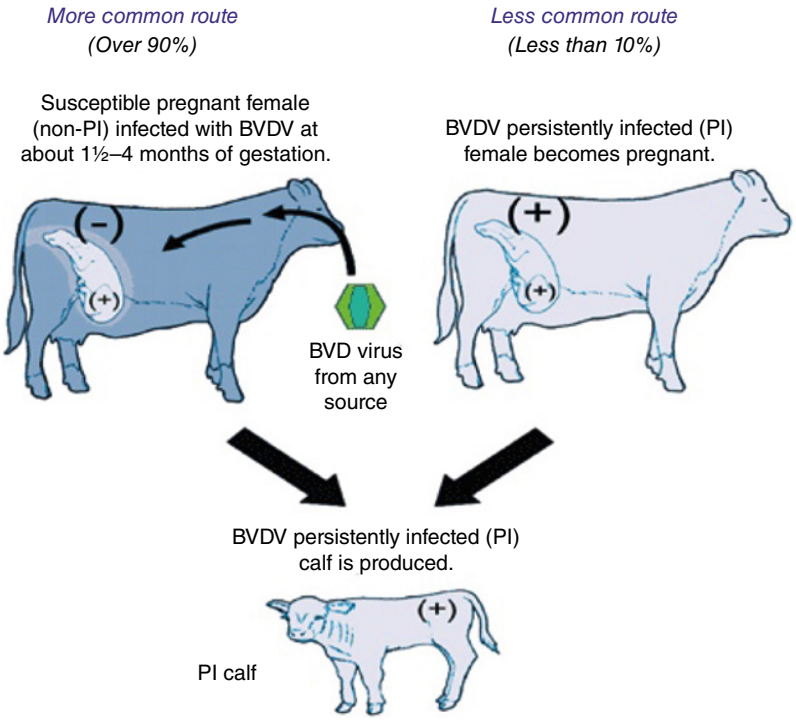


Figure 56.15 The generation of bovine viral diarrhea virus (BVDV) persistent infection. *Source:* From NCBA Publication (2009).

infected calves can exhibit "weak calf syndrome" where the calves fail to thrive following birth. Congenital abnormalities can also occur. BVDV can also cause abortion throughout pregnancy and is the most frequent diagnosed viral pathogen from abortions.

3) Mucosal disease (MD) is characterized by low morbidity but high mortality in cattle of several months to several years of age. It is characterized by severe, fatal disease with extensive ulceration of the upper gastrointestinal tract, diarrhea, and lymphopenia. MD occurs only in PI animals.

Epidemiology

BVDV has a worldwide distribution, and infection of cattle is very common with high seroprevalence in most countries of the world. Transmission is by contact and horizontal transfer of virus, particularly from PI carriers of the virus that shed high titers of virus in all their body secretions and excretions for horizontal spread; they also transmit the virus vertically to their progeny (Figures 56.15 and 56.16).

Pathogenesis and Pathology

The pathogenesis and consequences of BVDV infection of cattle are dependent on the age and immune status of cattle at infection, as well as on the biological properties of the infecting virus strain. BVDV infection in calves results in systemic infection of variable severity. Lesions in affected calves range from mild erosion and ulceration of the upper gastrointestinal tract to severe ulceration throughout the gastrointestinal tract and disseminated hemorrhages. Highly virulent strains of BVDV can produce severe lesions in susceptible calves that mimic MD (see below).

The lesions associated with congenital BVDV infection include ocular lesions (retinal degeneration and hypoplasia, optic neuritis) and cerebellar atrophy/hypoplasia. PI animals have BVDV virus in all tissues, but the hair follicles have high levels of BVDV virus and can be used for diagnostic samples.

MD manifests when a CP strain of BVD virus emerges in the PI animal. The CP strain usually is a mutant of the NCP PI strain, and these mutations arise as described above. These NCP and CP viruses in MD are referred to as a pair. MD is characterized by the presence of erosions and ulcers on the muzzle, throughout the oral cavity, esophagus, and small intestine (ulcers over Peyer's patches are characteristic). There is also widespread necrosis of lymphocytes. The mechanism of tissue destruction in MD currently is uncharacterized but might involve apoptosis of cells.

Host Responses to Infection

BVDV infection of cattle usually results in the production of high titers of neutralizing antibodies in serum, and

cattle that recover from infection have long-lasting immunity. BVDV infection of animals *in utero* prior to 150 days of gestation can result in immunotolerance to BVDV, resulting in persistent postnatal infection and no or very low titers of virus-specific antibodies.

Laboratory Diagnosis

Clinical diagnosis may be difficult. MD must be differentiated from other diseases that cause ulceration of the gastrointestinal tract, including malignant catarrhal fever and rinderpest. Rapid diagnosis is best accomplished by antigen capture ELISA or immunohistochemical staining of the tissues of affected animals with BVDV-specific antibodies. PCR can be used to detect viral nucleic acid from any sample. An ear biopsy has been shown to be an effective sample to use to identify PI animals. Virus isolation also can be used. The widespread use of vaccines complicates serological diagnosis of BVDV infection, as does the low or no obvious titers to BVDV in PI animals. BVDV infections maybe reportable in some US states.

Treatment and Control Control of BVDV infection of cattle involves good biosecurity that includes quarantine and testing of new introductions to the herd for PI. Purchased bred animals are a high risk of bringing BVDV into the herd. Within the herd, all PI carriers need to be identified and removed. Vaccines are only an adjunct to control and will fail if PI animals are in the herd. Both attenuated (live) or killed BVDV vaccines are used, and programs have been developed that use live BVDV vaccines in young stock and killed vaccines in the older animals to control PI. Concerns have been raised about the use of live attenuated BVDV vaccines, including their potential reversion to virulence, dissemination in cattle populations, and potential to cause immunosuppression as well as fetal infections. In the past, inadvertent contamination of bovine cell cultures used for vaccine production with virulent but NCP BVDV strains has also occurred. Vaccination of PI cattle with attenuated vaccines can sometimes produce MD. Vaccination prior to four months of age in calves may not be effective due to maternal antibody interference, and repeated revaccination may be necessary.

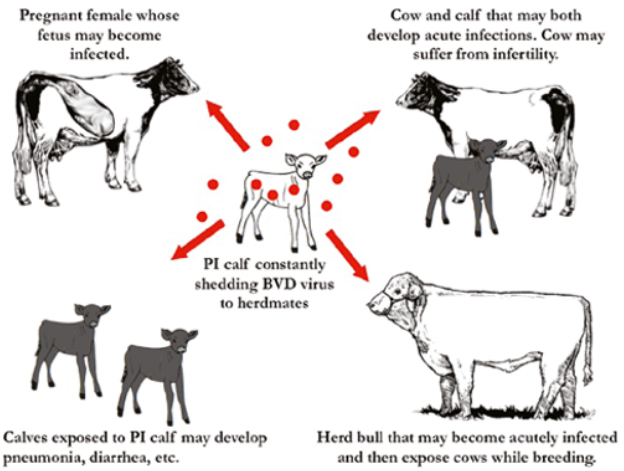


Figure 56.16 The threat of bovine viral diarrhea virus (BVDV) persistent infection to a herd. *Source:* From Gulf Coast Cattlemen April 2011 (Dan Givens).

Border Disease Virus

Disease

Border disease (BD) is a congenital *Pestivirus* disease of sheep characterized by the birth of lambs with abnormal ("hairy") wool and tremors that reflect abnormal myelination in the CNS (so-called "hairy-shaker" lambs). Expression of disease varies depending on gestational age of the lamb at infection as well as on the infecting viral strain. Infection of the developing fetus may result in fetal death, mummification, abortion, stillbirth, or birth of abnormal lambs, whereas infection of adult sheep is subclinical.

Epidemiology

BDV was initially described in the late 1940s as a congenital disease of sheep in England and Wales. The disease has since been recognized in many European countries, Australia, New Zealand, Canada, and the United States. The virus persists in sheep, likely in a manner similar to that of BVDV in cattle, and extensive outbreaks of BDV can occur when the virus is introduced into immunologically naive flocks. Transmission of BDV is probably most common by the oral and intranasal routes, from infected animals or infected fetuses.

Pathogenesis and Pathology

BDV infection of immunologically naive sheep results in viremia, with subsequent spread of the virus to the fetus in pregnant animals. Generalized infection of the fetus can lead to fetal death, with or without expulsion, or teratogenesis. The consequences of fetal infection are inversely related to fetal age; thus, infection during the first trimester is relatively more severe than infection later in gestation. Infection after 80 days' gestation often results in viral clearance without disease. The teratogenic effect of BDV infection is also dependent on fetal age. Infection of the developing CNS leads to reduced or altered myelination and demyelinization, resulting in the characteristic congenital tremors of the affected newborn lambs. Necrosis of the developing brain can cause more serious developmental injury, resulting in hydranencephaly, porencephaly, and/or cerebellar dysplasia.

Host Response to Infection

BDV infection of adult sheep usually results in a prompt humoral immune response characterized by the appearance of long-lived neutralizing antibody in serum. The fetal immune response reflects its gestational age at the time of infection. Infection during the first half of gestation usually leads to persistent postnatal infection and a minimal, inadequate immune response. Such animals may remain PI and seronegative to BDV for the remainder of their lives. Infection during the second half of gestation, at a time when the fetus is gaining immunologic competence, usually results in immune responses that resolve the infection.

Laboratory Diagnosis

Detailed necropsy and histological evaluation is often diagnostic, especially when immunohistochemical staining is used to identify viral antigens in the tissues of affected lambs. PCR can be used to detect viral nucleic acid from any sample.

Treatment and Control

Control of BDV is difficult. Losses can be high during initial infection of naive animals, whereas losses are relatively minimal when the virus is endemic on a farm. BVDV vaccines are sometimes used.

Classic Swine Fever Virus

Disease

CSFV is an important disease of swine worldwide; it has been eliminated in many intensive swine-producing countries but often reemerges to cause serious, economically devastating outbreaks. CSFV is characterized by fever (104 °F or higher), leukopenia, and loss of appetite. Affected animals may appear dull and drowsy and crowd together as if chilled. Vomiting and diarrhea are common, as is conjunctivitis, erythema of the skin, and neurological signs such as paralysis and locomotory disturbances. Infection of pregnant sows can result in small litters, fetal death, premature births, stillbirths, and the birth of piglets with cerebellar ataxia or congenital tremors. Morbidity and mortality are both high during epidemics caused by virulent strains of the virus in fully susceptible swine, whereas disease is less apparent when the virus is endemic, making detection and eradication more difficult.

Epidemiology

HCV occurs worldwide, but it has been eradicated from North America, Great Britain and Ireland, Scandinavia, Australia and New Zealand, and parts of Europe. It is endemic in extensive areas of Asia, Africa, Europe, and South and Central America. Domestic swine and wild hogs serve as reservoir hosts, often as inapparent carriers. Pigs infected in utero may become PI carriers of the virus, analogous to the reservoirs of other pestiviruses such as BVDV and BDV. Transmission is by droplet, fomites, and ingestion of infected materials, particularly uncooked garbage.

Pathogenesis and Pathology

CFSV is an acute, highly contagious disease that is characterized by disseminated intravascular coagulation leading to hemorrhage and infarction in many tissues. The incubation period is short (three to eight days), and the virus first replicates in the lymphoid tissues of the upper respiratory tract or tonsils. The virus then spreads widely and replicates in endothelial cells and mononuclear inflammatory cells throughout the body. The characteristic lesions are petechial hemorrhages on all serous surfaces, lymph nodes (hemorrhagic lymphadenitis), and kidney, and the presence of infarcts in the spleen. More chronic forms of CSFV occur in some endemic areas. Affected pigs may exhibit growth retardation (stunting), chronic diarrhea, and secondary bacterial pneumonia.

Host Response to Infection

Animals that recover from hog cholera have a long-lasting immunity. Neutralizing antibody titers correlate with protection against CFSV infection. Suckling pigs acquire colostral antibodies from the immune dam. Pigs infected *in utero* with the virus are often PI carriers, whether or not they are healthy at birth.

Laboratory Diagnosis

Diagnosis of CFSV can be suspected in free areas by explosive outbreaks of severe disease in pigs, but the diagnosis always requires laboratory confirmation to distinguish it from other septicemias. The virus is identified in the tissues of affected pigs by immunohistochemical staining or by virus isolation from the spleen, tonsils, lymph nodes, and blood. Because many strains are non-cytopathogenic in cell culture, the fluorescent antibody method is required for the detection of CFSV. PCR can be used to detect viral nucleic acid from any sample. The diagnosis of chronic forms of CFSV is more difficult and requires careful laboratory investigation. CFSV is a reportable disease in the United States and is important to differentiate from African Swine Fever virus.

Treatment and Control

Control of CFSV depends on whether the virus is endemic in a particular country or region. In free areas, exclusion of the virus is accomplished by regulating the movement (importation) of swine from endemic areas and by prohibiting the feeding of garbage and/or food scraps containing

pork products to swine. In endemic areas, vaccination and/or eradication are used. Vaccines for CFSV are attenuated and effective in preventing disease. DIVA (differentiating infected from vaccinated animals) vaccines have been introduced and have shown promise as a tool for eradicating CFSV from a region or country.

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Orthomyxoviridae

Wenjun Ma

Orthomyxoviridae is a family of RNA viruses, which includes seven genera: *Alphainfluenzavirus Betainfluenzavirus, Deltainfluenzavirus*, *Gammainfluenzavirus, Isavirus*, *Thogotovirus, and Quaranjavirus*. Viruses in the first four genera, which are identified by antigenic differences in their nucleoprotein (NP) and matrix (M) proteins, cause influenza disease in vertebrates, including birds, humans, and other mammalian species. The genus *Isavirus* has a single member, infectious salmon anemia virus (ISAV), which causes a highly fatal disease of marine-farmed Atlantic salmon. Thogotoviruses infect both vertebrates and invertebrates such as mosquitoes and sea lice. Quaranjaviruses are arboviruses and can infect both vertebrates and invertebrates (arthropods).

Influenza viruses

Influenza viruses are named according to their type, species where isolated (except humans), location where isolated, the successive isolate number from that location, and year of isolation. Influenza viruses are divided into four types, including influenza A, B, C, and D viruses, based on antigenic differences in their NP and M proteins. All pandemics in human history were caused by the influenza A virus (Spanish flu H1N1, 1918–1919; Asian flu H2N2, 1957–1958; Hongkong flu H3N2, 1968–1969; and swine flu H1N1 2009) due to the emergence of new subtypes of this virus; the Spanish influenza pandemic in 1918–1919 was the most devastating one, resulting in the deaths of up to 50 million people worldwide.

Classification

Influenza A viruses are further divided into different subtypes based on the antigenic nature of the surface hemagglutinin (HA) and neuraminidase (NA). To date, there are 18 HA and 11 NA subtypes of influenza A virus, all of which except H17, H18, N10, and N11 subtypes have been isolated from wild aquatic birds, including waterfowl and shore birds that are the natural reservoir of influenza A viruses (Figure 57.1). Influenza A viruses are also able to infect a large variety of mammalian species including human, horse, swine, dog, cat, bat, and marine mammals. Influenza B viruses infect humans and have only two unique genetic and antigenic lineages (Yamagata-like and Victoria-like lineages), which are different from influenza A viruses with different subtypes. Influenza C viruses also infect humans and are associated with common cold-like illness, principally in children. Although influenza B and C viruses are predominantly human pathogens that have been sporadically isolated from other species such as influenza B virus from pigs and seals, and influenza C virus from pigs, only influenza A and B viruses cause major outbreaks and severe disease in humans. Influenza D virus has been isolated from cattle and swine, and it is likely to contribute to a respiratory disease complex in each species. Antibodies to influenza D viruses have also been detected in multiple species including sheep, goats, and humans. The nomenclature system for different subtypes of influenza A viruses includes the host of origin, geographic origin, strain number, and year of isolation. A description of the two major surface antigens, the HA and the NA, is given in parentheses, for example, A/swine/Kansas/8/2007 (H1N1). By convention, the host of origin for human strains is now omitted.

Influenza A virus antigenic subtypes are distinguished by double immunodiffusion assays (HA inhibition and NA inhibition) with hyperimmune animal sera because such tests reveal the antigenic relationships among influenza A virus isolates that are not apparent with other methods.

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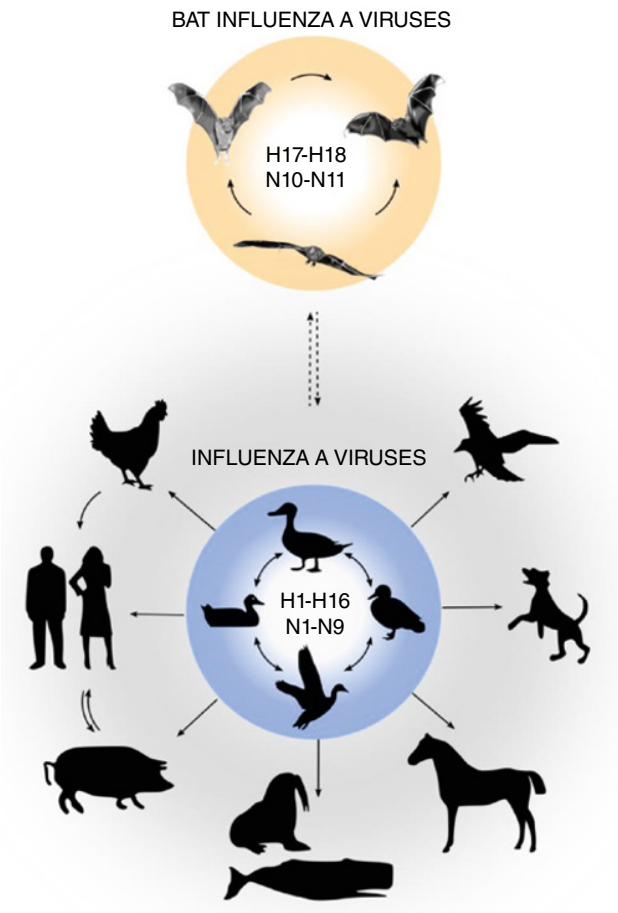


Figure 57.1 Reservoirs of influenza A viruses and bat influenza viruses. Natural reservoirs of classical influenza A viruses are wild water birds, from which they can be transmitted to a wide variety of other species. Bat influenza A viruses are likely to circulate in various bat species in Central and South America and possibly originate from classical influenza A viruses (indicated by dashed arrow). *Source:* Ma et al. (2015). Licensed Under CC BY 4.0.

Morphology

Influenza virus particles are pleomorphic: their envelope can have spherical and filamentous forms, and the virion envelope is derived from the membranes of host cells. Normally, the virion morphology is irregularly shaped spherical particles 80–120 nm in diameter (Figure 57.2) or filamentous virions 20 nm in diameter and 200–300 nm long (Figure 57.2). The genome of influenza A and B viruses contains eight negative-sense RNA segments, whereas the genome of influenza C and D viruses each has seven negative-sense RNA segments. Each of the eight genomic RNA segments of influenza A and B viruses encodes for one or two proteins. There are two distinct types of surface spikes (peplomers) attached to the viral envelope in influenza A and B viruses: one is the

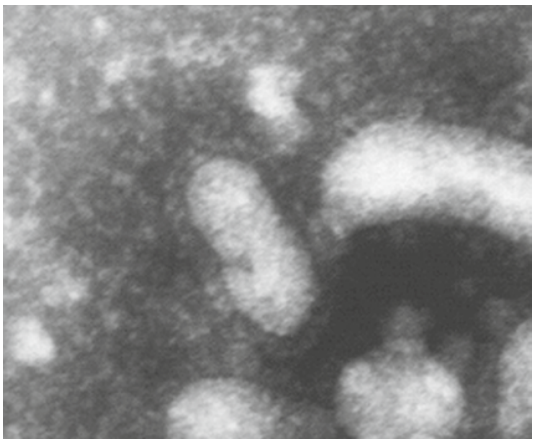


Figure 57.2 Electron micrograph demonstrating irregularly shaped spherical particles (80–120 nm in diameter) of an avian influenza virus. *Source:* Courtesy of Dr. David Swayne, Southeast Poultry Research Laboratory, USDA/Agricultural Research Service, Athens, Georgia.

HA with a rod-shaped form that is important for virus attachment and envelope fusion. The other surface protein is the NA with a mushroom-shaped form and possessing NA activity. The NA from influenza A and B viruses is responsible for the cleavage of the sialic acid residues from the virus and the infected cells during both entry and release from the cells. Both the HA and the NA are viral glycoproteins that attach to the viral envelope by short sequences of hydrophobic amino acids (Figure 57.3). Influenza C and D viruses have only one spike protein, the hemagglutinin-esterase-fusion (HEF) glycoprotein (Figure 57.3), which is similar to HA as a typical type I transmembrane protein that is located on the surface of the virus. HEF possesses receptor-binding, receptor-destroying, and membrane fusion activities, thereby combining the functions of HA and NA of influenza A and B viruses.

The viral envelope surrounds a M protein shell. The M protein (M1), the most abundant protein in virus particles, lies inside the lipid envelope, whereas the M2 protein encoded by RNA transcript splicing acts as an ion channel. The M protein shell surrounds the viral genome, which along with the NP and three large proteins – polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA) – forms the ribonucleoprotein (RNP) complex that is responsible for RNA replication and transcription (Figure 57.2).

Viral Genome

The eight segments of influenza A and B viruses, as well as the seven segment of influenza C and D, are numbered based on decreasing length (Figure 57.3). In influenza A and B viruses, the segments 4 and 5 encode one protein per

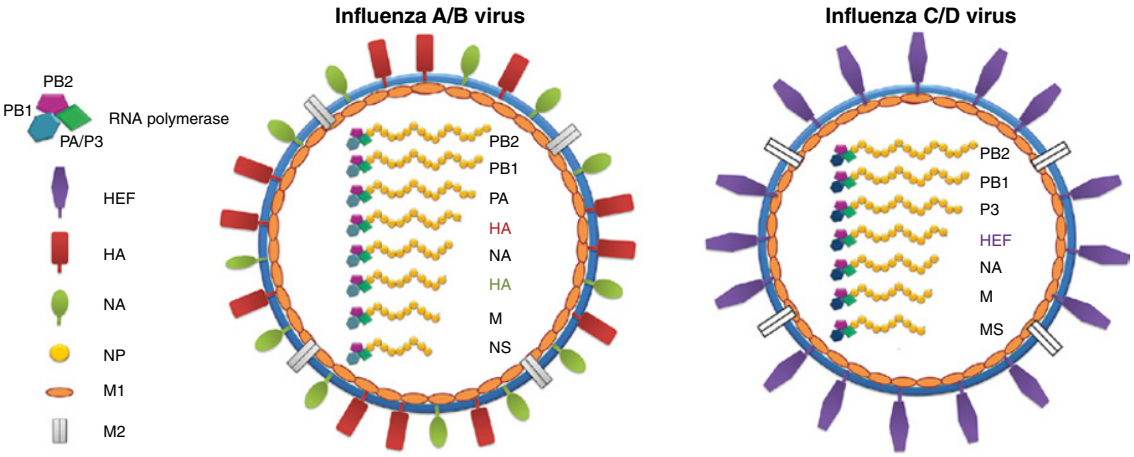


Figure 57.3 Diagram of genomes and viral particles of influenza A, B, C, and D viruses. Influenza A and B virus particles possess eight genomic segments, whereas influenza C and D virus particles contain seven genomic segments. The envelope and membrane proteins HEF (ICV/IDV), HA and NA (IAV/IBV), and M2 project from the virion surface with the M1 protein just underneath the inner leaflet of the membrane, which encases the virion core containing the virus genome. Each vRNA segment forms a vRNP together with the NP and RNA polymerase complex (PB2, PB1, and PA/P3). *Source:* Yu et al. (2021).

gene segment: the HA and NP. All influenza viruses encode the polymerase subunits PB2 and PB1 by segments 1 and 2, respectively; in some influenza A virus strains, segment 2 also encodes a second short protein PB1-F2 using an additional open reading frame. No analogue for PB1-F2 has been identified in influenza B, C, or D viruses. Segment 3 of both influenza A and B viruses encodes the polymerase subunit PA, whereas that of influenza C and D viruses encodes the polymerase subunit P3. In some influenza A virus strains, this segment also encodes a second short protein PA-X through a +1 ribosomal frameshifting event, whereas no analogue for PA-X has been identified in influenza B, C, or D viruses. The segment 6 of influenza A virus only encodes the surface glycoprotein NA, whereas that of influenza B virus encodes both the NA protein and NB matrix protein using a −1 alternate reading frame, which is an integral membrane protein corresponding to the influenza A virus M2 protein. Segment 7 of both influenza A and B viruses encodes for the M1 matrix protein; this segment in the influenza A genome also encodes the M2 ion channel by RNA splicing, whereas that of influenza B genome encodes its BM2 membrane protein in a +2 alternate reading frame. Segment 8 of both influenza A and B viruses encodes the interferon-antagonist nonstructural protein 1 (NS1) protein and nuclear export protein (NEP)/NS2 by RNA splicing. The genomic structure of influenza C and D viruses is mostly similar to that of influenza A and B viruses; however, the HEF protein of influenza C and D replaces the HA and NA proteins. Therefore, the genome of influenza C and D viruses has seven segments in contrast of that of influenza A or B viruses. Influenza viral variation is frequent, and involves two major mechanisms: antigenic drift and shift. Antigenic drift is the accumulation of random mutations in viral genes because of low fidelity of the viral RNA polymerase. Among the four types of influenza viruses, influenza A virus mutates the most rapidly, and the mutation rate and speed of influenza B virus is in the second place. Antigenic drift can occur in each type of influenza virus, and it is responsible for the variability of the human seasonal influenza viruses caused by influenza A viruses. The segmented nature of the influenza genome contributes to antigenic shift or reassortment that occurs when a cell is infected with different influenza viruses, resulting in exchange of viral RNA segments and the generation of new viruses with a novel combination of genes. Reassortment can occur within each type of influenza viruses but is not possible between different types of influenzas viruses such as between influenza A and B viruses, or influenza B and C viruses. Reassortment among influenza A viruses has resulted in three of four pandemics in human history, including the 1957 Asian flu pandemic and the 1968 Hong Kong flu pandemic as well as the 2009 pandemic.

Viral Proteins

Hemagglutinin

Influenza A and B viruses have two major surface glycoprotein antigens: HA (or H) and NA (or N). For influenza A virus, 18 antigenically distinct HAs have been recognized, all of which except the H17 and H18 can be found circulating in aquatic birds, where they are generally carried

asymptomatically. Both H17 and H18 subtypes were only detected in specific bat species that do not show any clinical signs. The HA protein is a type I transmembrane glycoprotein, which is initially synthesized as a single polypeptide precursor (HA0) with a molecular weight of approximately 76 kDa. The mature HA forms homotrimers, and each monomer is generated by cleavage of HA0 into HA1 and HA2 subunits by trypsin-like or furin-like proteases. The HA has two functions during the early steps of the replication of the virus in host cells: receptor binding and membrane fusion. First, it attaches the virus to the cell surface by binding to the cellular receptors that are sialic acids of cell surface glycoproteins and glycolipids for classical H1–H16 subtypes and are major histocompatibility complex (MHC) II molecules for bat H17 and H18 subtypes. Second, once bound it promotes viral penetration by mediating fusion of the endosomal and viral membranes to release the viral genome into the cell cytoplasm of the target cells. The conserved sialic acid receptor-binding pocket or MHC II-binding pocket is located on the HA1 subunit at the distal end of the molecule. There are five major antigenic epitopes A–E on the HA1 subunits of the HA of influenza A viruses. All strains of influenza A (H1–H16) and B viruses are capable of agglutinating erythrocytes from humans, guinea pigs, and chickens as well as many other species. Antibodies to the HA prevent infection of host cells by neutralizing the virus and are important to host immunity. In fact, it is the variation of this molecule that is primarily responsible for emergence of new strains of the virus leading to new outbreaks of influenza, and the failure to control them by vaccination.

Neuraminidase

Influenza A and B viruses encodes NA, which is a type II membrane glycoprotein located on the surface of the virus. The NA protein comprises mushroom-shaped tetramers with an average molecular weight of approximately 220 kDa. Each tetramer is composed of four identical monomers. The ectodomain of NA consists of a stalk and a globular head. The stalk and the transmembrane domain sequences are highly variable among the 11 recognized NA subtypes. The NA stalk separates the head region with the enzymatic center from the transmembrane and cytoplasmic domains. The NA from influenza B and influenza A viruses – except for bat H17N10 and H18N11 strains – is responsible for the cleavage of the sialic acid residues from the virus and the infected cells during both entry and release from the cells. In addition, it appears that NA helps the virus penetrate the mucin layer in the respiratory tract to reach the epithelial cells, which are the target cells of the virus. The enzyme activity of the NA prevents self-aggregation and promotes release of the newly made virus from the infected cells. The antibody against the NA does not protect against infection but does confer protection against disease and reduces transmissibility. The NA of bat influenza viruses does not exhibit sialidase activity, as some important conserved amino acids at active and framework sites have changed in N10 and N11, respectively. The function of the NA from bat H17N10 and H18N11 viruses remains unclear; it likely promote virus release by downregulating the expression of MHC II molecules.

Segment 6 of influenza B virus also encodes the NB matrix protein in addition to NA by using a −1 alternate reading frame, which is highly conserved in all influenza B viruses isolated from clinical cases and even in strains such as influenza B/Lee/40 that have been passaged in eggs and cell culture for more than 80 years. It is highly likely that this protein does serve a role for the virus. *In vitro* and *in vivo* studies have revealed that the NB is not required for virus replication and/or transmission of influenza B viruses.

Hemagglutinin-Esterase Fusion

The HEFs of ICV and IDV share approximately 53% amino acid homology and have an almost identical structure and mode of ligand binding. Like HA, HEF is synthesized as a precursor (HEF0) composed of two subunits: the globular head subunit HEF1 and the membrane-near stalk subunit HEF2. IAV and IBV recognize α2,3- or α2,6-linked sialic acid moieties as receptors. In contrast, ICV uses N-acetyl-9-O-acetylneuraminic acid ($Neu5,9Ac_2$), which has an additional O-acetyl group as the receptor, which is independent of α2,3 or α2,6 linkage to the adjacent galactosyl residue. Studies on the IDV HEF have revealed that IDV can utilize both 9-O-acetylated Neu5Ac ($Neu5,9Ac_2$) and Neu5Gc (Neu5Gc9Ac) as its receptor for entry into the cell. Similarly, antibodies to the HEF are able to prevent infection of host cells by neutralizing the virus and are important to host immunity.

Nucleoprotein

The NP is a structural protein with a molecular weight of approximately 56 kDa. It encapsidates vRNA and interacts with PB1, PB2, and PA subunits in the vRNA polymerase forming the RNP, which is involved in viral genome transcription and replication. NP also forms homooligomers to maintain the structure of RNP. It is also thought to be the major switching factor that determines whether genomic vRNA is transcribed into mRNA or used as a template to synthesize complementary RNA (cRNA) for genome replication.

The NP is one of the type-specific antigens used to distinguish genera of influenza viruses and can be identified by enzyme-linked immunosorbent assay (ELISA), double immunodiffusion, complement fixation, single radial diffusion, and agar gel precipitation. Antibodies to the NP do not provide passive protection. However, the NP of influenza A viruses is a major protein recognized by cytotoxic lymphocytes (CTLs) and plays a critical role in inducing cell-mediated immunity.

Matrix Protein

RNA segment 7 of both influenza A and B viruses encodes the M1, which is a non-glycosylated and type-specific antigen. The functions of the M1 include (i) interaction with vRNP and NEP and regulation of vRNP transport between the cytoplasm and the nucleus; (ii) regulation of vRNP transcription and replication; (iii) interaction with viral envelope proteins including HA, NA, and M2; (iv) recruitment of viral components at the assembly site and initiation of budding; and (v) recruitment of host components for bud completion and virus release. Segment 7 of influenza A viruses also encodes the M2 protein by RNA transcript splicing, which acts as an ion channel and plays a role in triggering the viral uncoating in the endosome. It has been well studied because the virus ion channel is an ideal antiviral drug target. Amantadine and rimantadine have been developed and are used clinically as an anti-influenza drug. However, amantadine-resistant strains have been detected widely and contain mutations in the transmembrane domain of the M2 protein. Antibodies against M1 protein provide little, if any, protection against infection. The M2 protein is one of important proteins of influenza A viruses recognized by CTLs.

Segment 7 of influenza B viruses encodes the BM2 protein by using a bicistronic mRNA in an open reading frame that is +2 nucleotides with respect to the matrix (M1) protein. The BM2, similar to the influenza A virus M2, functions as a proton channel essential for viral replication, and it can functionally replace the M2 of the influenza A viruses. Segment 6 of both influenza C and D viruses also encodes the M1, which lies inside the lipid envelope, is associated with both the RNP and the viral envelope, and the M2 that as a proton channel is essential for viral replication.

Nonstructural Protein and Nuclear Export Protein

The RNA segment 8 of influenza A and B viruses encodes two proteins by mRNA splicing: NS1 and NEP, formerly referred to as the NS2 protein. The NS1 protein is not a structural component of the virion but is expressed at very high levels in infected cells. It is a multifunctional protein with a molecular weight of 26 kDa, and is an antagonist of the innate host type I interferon-mediated antiviral response and the antiviral effects of interferon-induced proteins, such as dsRNA-dependent protein kinase R and 2′ -5′ oligoadenylate synthetase/RNase L. NS1 also acts directly to modulate other important aspects of the virus replication cycle including (i) temporal regulation of vRNA synthesis; (ii) enhancement of viral mRNA translation; (iii) regulation of virus particle morphogenesis; (iv) suppression of host immune/apoptotic responses; and (v) activation of phosphoinositide 3-kinase pathway.

The NEP was thought to be a nonstructural protein, but is present in small amounts in the virions in association with the RNP through interaction with the M1 protein. The NEP functions to mediate the export of newly synthesized RNPs from the nucleus.

Polymerase Proteins

The three largest vRNAs in influenza viruses encode the three subunits of the RNA-dependent RNA polymerase. Segments 1 and 2 of influenza A, B, C, and D viruses encode the PB1 and PB2, respectively, while segment 3 of influenza A and B viruses encodes the PA, and that of influenza C and D encodes the P3 protein. The polymerase subunits PB2, PB1, and PA (or P3) together with the NP and vRNAs form the RNP complex, which is responsible for viral transcription and replication. In the nucleus, influenza polymerase employs a "cap-snatching" mechanism to initiate vRNA replication. The PB2 binds cap-containing cellular mRNA in order to produce primers for RNA synthesis. An endonuclease from the PA (or P3) subunit cuts the capped oligonucleotide pre-mRNA as primers for viral mRNA synthesis. The polymerase then transcribes vRNA by elongation, leading to the formation of viral mRNA. The PB1 subunit is thought to be involved in the catalytic activity of nucleotide elongation. During the viral genome replication, the vRNAs are used as templates to synthesize cRNAs that are positive-sense antigenomic RNAs, and then the cRNAs are transcribed into vRNAs that are incorporated into progeny viruses. The influenza polymerase has no proofreading activity, resulting in a high gene mutation rate of approximately one error per replicated genome.

PB1-F2, a short polypeptide expressed from an alternative −1 open reading frame of the PB1 in some influenza A stains, is believed to be an important determinant of influenza virus virulence. However, the PB1 segment of many influenza A viruses encodes a truncated PB1-F2.

PA-X, a short polypeptide expressed in an open reading frame of the PA in some influenza A stains by ribosomal

frameshifting, is found to play a major role in influenza virus-induced host shutoff. Further studies have demonstrated that PA-X has a strong impact on viral replication and pathogenicity and also reduces the host innate immune response. However, the PA segment of many influenza A viruses encodes a truncated PA-X. To date no analogues of PB1-F2 and PA-X have been detected in influenza B, C, and D viruses.

Avian Influenza

Disease

The first description of avian influenza, a contagious disease of poultry associated with high mortality, was reported in northern Italy in 1878 and was termed "fowl plague" initially. Influenza A virus was found to be the pathogeny of fowl plague until 1955. The term *fowl plague* was replaced by "highly pathogenic avian influenza (HPAI)" at the First International Symposium on Avian Influenza in 1981. To date, 16 different HA and 9 different NA subtypes of influenza A viruses have been isolated from waterfowl and shorebirds. There are 144 theoretical combinations (e.g. H5N1, H9N2, and H6N1) between 16 HA and 9 NA that make up different subtypes of avian influenza viruses. Avian influenza is further classified as HPAI and low pathogenic avian influenza (LPAI) on the basis of specific molecular genetic and pathogenic criteria. HPAI is an extremely contagious, multiorgan systemic disease of poultry resulting in high mortality. Up to now, all HPAI outbreaks have been caused by the H5 or H7 subtype of viruses in which their HA proteins possess multiple basic amino acids at the cleavage site (see Section 57.6.2.2). Avian influenza of H5 and H7 subtypes are known as the Notifiable Avian Influenza (NAI). The United States Department of Agriculture (USDA) works to prevent NAI from becoming established in US poultry populations. Most avian influenza viruses are the LPAI viruses, which are usually associated with mild disease in poultry, and they only replicate in the respiratory and intestinal tracts. The HAs of LPAI viruses normally have a single basic amino acid at the cleavage site.

Infection of domestic poultry with avian influenza viruses including the LPAI or HPAI virus has caused significant losses for the industry worldwide. Waterfowl and shorebirds, the natural reservoir of influenza A viruses, normally do not show any clinical signs with infection of different subtypes of viruses. In wild ducks, influenza virus replicates in intestinal mucosal cells and is excreted in high concentrations. The virus has been isolated from lake and pond water, and surveys have demonstrated that as many as 60% of juvenile birds may be infected as they congregate prior to migration. In 2005, over 6000 wild migratory birds died from HPAI H5N1 infection in Qinghai Lake in China. From then on, the HPAI H5N1 virus has spread to Europe, the Middle East, and Africa, most likely through migratory birds. In 2014, an HPAI H5N8 virus was first reported in Korea, and subsequently the virus has spread globally. The HPAI H5N8, and reassortant H5N2 and H5N1 viruses generated by the HPAI H5N8 reassorting with local avian influenza viruses (altogether called H5Nx viruses) were subsequently detected in 21 states in the United States, where 15 states reported outbreaks in domestic poultry and 6 states only in wild birds. The control of this 2015 HPAI outbreak in the United States resulted in the euthanasia of approximately 49 million domestic birds, causing significant economic losses for the poultry industry (estimated to be $4 billion) and international trade issues. Currently, HPAI H5N1, H5N4, H5N6, and H5N8 viruses have been often detected in wild birds worldwide, and they are posing potential threat for the poultry industry.

Host–Virus Relationship

Geographic Distribution, Reservoir, and Mode of Transmission

Although LPAI viruses exist in wild birds and poultry worldwide, HPAI is a major threat to the world's poultry industry. Before 2002, only a few HPAI outbreaks in poultry were documented. Large-scale outbreaks of HPAI H5N1 occurred in domestic poultry in several countries in Southeast Asia from 2003, including China, Indonesia, Thailand, and Vietnam. Subsequently, the HPAI H5N1 spread to Europe, the Middle East, and Africa after wild birds were killed by the H5N1 virus at Qinghai Lake in China in 2005, resulting in HPAI outbreaks worldwide. This phenomenon caused influenza experts to believe that the H5N1 virus would cause the next pandemic. Luckily, field H5N1 isolates still cannot transmit from human to human. The disease is transmitted through poultry flocks mainly through ingestion of the virus, but it may also be transmitted by inhalation and by mechanical means involving movement of personnel throughout flocks or between premises. In birds, avian influenza viruses are shed in the feces as well as in saliva and nasal secretions. The feces contain large amounts of virus, and fecal–water–oral transmission is a major route in waterfowl, but the fecal–water–cloacal route of transmission is also possible. Furthermore, avian influenza may be transmitted on objects such as shoes, clothing, and crates that come in

contact with infected birds or premises. Although the possibility of vertical transmission of avian influenza through infected eggs exists, no evidence demonstrates its spread by this means. The transmission mode of HPAI H5N1 viruses is intensely debated, and migratory birds and poultry trade have been discussed as potential factors.

Waterfowl have been implicated as the major natural reservoir for influenza A viruses. Infected ducks can shed the virus for prolonged periods without showing clinical signs or producing a detectable antibody response. There is evidence that influenza virus can persist in some birds for several months after infection. Various factors affect the viral life cycle, including cell surface receptors, host body temperature, and immune responses; thus, different species-specific virus lineages are formed after adaptation in different animal species. Avian influenza viruses have been found to be capable of infecting a wide range of mammalian species in addition to birds, including humans, pigs, horses, dogs, cats, and terrestrial birds, exhibiting their interspecies transmissibility (see Figure 57.1).

Pathogenesis and Pathology

The pathogenesis of avian influenza varies widely depending on strains of the virus, age, and species infected, concurrent infections, and husbandry. It appears that the HA gene is important in pathogenicity. The HAs of highly pathogenic H5 and H7 viruses contain multiple basic amino acids at the cleavage site that are recognized by ubiquitous proteases, such as furin and PC6, resulting in a systemic infection. In contrast, the HAs of LPAI viruses lack this series of basic residues at the cleavage site and thus are cleaved by proteases in a limited number of organs, restricting viral replication to the respiratory system. In addition to the HA gene, other genes such as PB2 and NS1 have also been identified to affect viral pathogenicity.

Sudden deaths without any clinical signs may occur in poultry infected with an HPAI virus; at necropsy, no obvious lesion exists in the dead avian body. Chickens without immediate death may exhibit listless, edema, and cyanosis of the comb, wattles, and legs. At necropsy, lesions include foci of necrosis of various sites including the skin, comb, wattles, spleen, liver, lung, kidney, intestine, and pancreas. There may be fibrinous exudates in the air sacs, oviduct, pericardial sac, or peritoneum. Other lesions include petechiation of the heart muscle, abdominal fat, and the mucosa of the proventriculus, nonsuppurative encephalitis, and serofibrinous pericarditis (Figure 57.4). Studies have shown that chickens infected by the LPAI virus have histological lesions limited to the respiratory system or lack histological lesions. Other poultry species, such as ducks, geese, ratites, and pigeons, are less vulnerable to avian influenza; however, infection with HPAI viruses may cause nervous symptoms including ataxia, torticollis, and seizures.

Laboratory Diagnosis

Avian influenza viruses can be identified by RT-PCR assay, antigen detection, and virus isolation. Influenza A subtype-specific RT-PCR or real-time RT-PCR tests have been developed and utilized widely; these kinds of assays are usually used for the primary and rapid detection of avian influenza with high sensitivity. However, these assays need to target genes that are highly conserved among different subtypes to avoid false negative results. If an HPAI is suspected, the state authorities should be notified in the United States. This testing is regulated by the USDA. Antigen-capture ELISA tests have been used for rapid viral detection on-farm. However, the main limitation of antigen-capture tests is the low sensitivity, which makes this assay unsuitable for monitoring and early detection of avian influenza. In addition, false positive results are possible, so positive results should be further confirmed by molecular testing. Virus isolation is a traditional method for the detection and identification of avian influenza viruses. The viruses can be cultivated in chick embryos or in duckling, calf, and monkey kidney cell cultures. Viruses can be isolated from samples of trachea, lung, air sac, sinus exudate, or cloacal swabs. The presence of influenza A virus in allantoic fluids or cell culture can be confirmed by HA assay, antigen-capture ELISA, or sequencing. For further subtyping of the virus, HA and NA inhibition tests should be conducted by using a battery of antisera against each of the 16 hemagglutinin (H1–H16) and 9 neuraminidase (N1–N9) subtypes. Alternatively, the HA and NA genes of the virus can be sequenced for subtyping.

Treatment and Control

Recovered birds remain immune to subsequent challenge by a homologous strain for at least several months. It has been demonstrated that the anti-HA antibody is important for protection against infection, whereas the anti-NA antibody protects against disease and reduces virus shedding but does not prevent infection. Different avian influenza vaccines, such as inactivated whole virus vaccine, Newcastle disease virus vectored, or fowlpox virus vectored vaccine expressing the HA from avian influenza viruses, have been developed, are commercially available, and are used to control avian influenza. Normally, they have been effective in protection against virus infection. However,

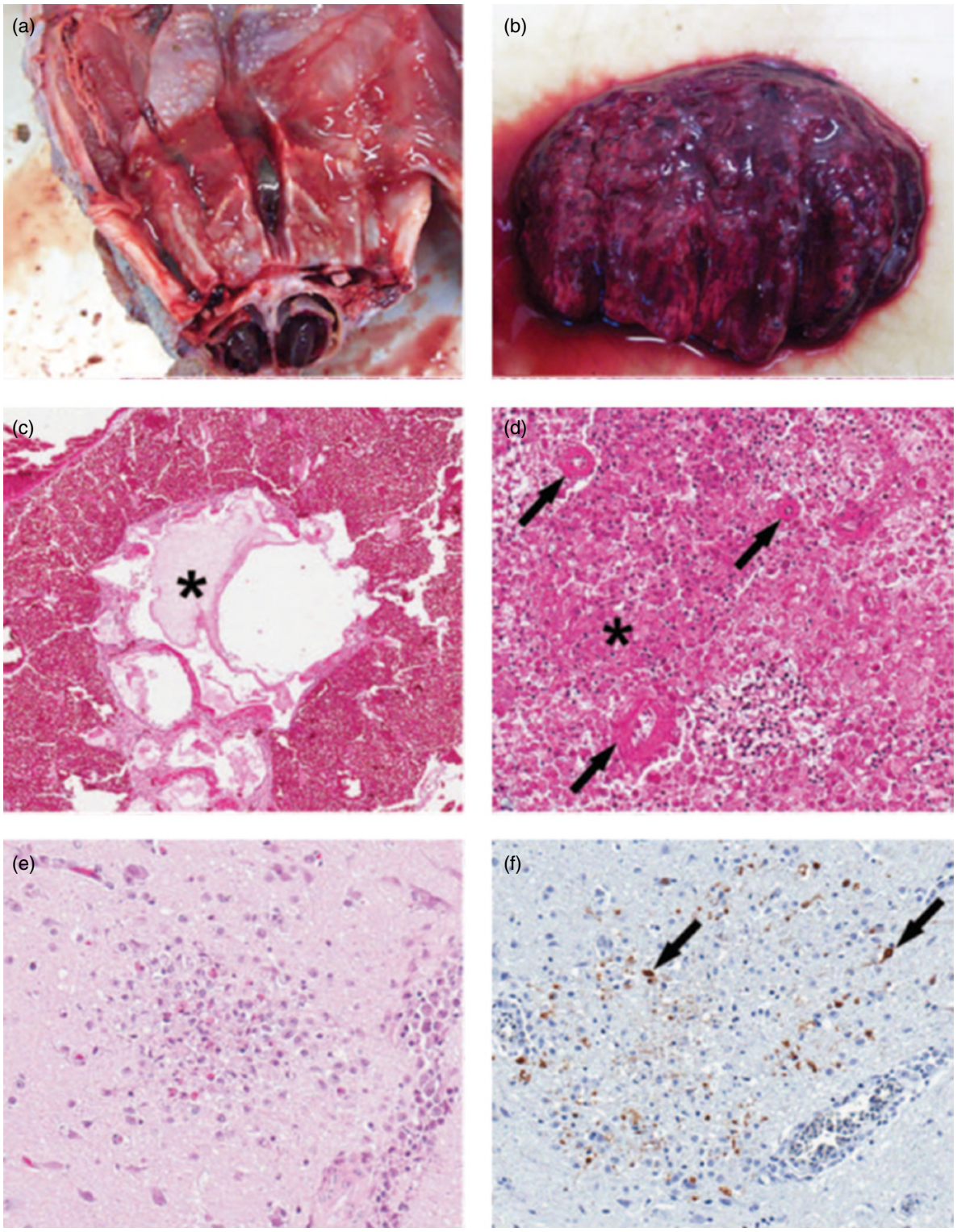


Figure 57.4 Pathologic characterization of dead commercial turkeys infected with a highly pathogenic avian influenza H7N8 virus. (a) Red-tinged mucous is present in the choana and oral cavity. (b) Severe pulmonary congestion and edema. (c) Photomicrograph of the lung. Capillaries are congested. Airways are filled with edema (*) H&E. (d) Photomicrograph of spleen. Foci of necrosis are present (*), and blood vessel walls show fibrinoid degeneration (arrows). H&E. (e) Photomicrograph of cerebrum depicting a focus of necrosis infiltrated with leukocytes and glial cells. H&E. (f) Positive immunohistochemical labeling of cerebral lesions with an anti-influenza antibody. Glial cells, leukocytes, and neurons (arrows) show positive labeling. DAB and hematoxylin counterstain. *Source:* Burcham et al. (2017).

control of avian influenza faces many challenges owing to the great genetic and antigenic diversity among avian viruses and the rapid evolution of influenza A virus. Therefore, relying on vaccination alone is not enough to control avian influenza; careful husbandry and strict biosecurity are also necessary. Careful husbandry to prevent the introduction of the virus into the flock is important. New birds should not be introduced into a started flock, and careful precautions should be taken to prevent either direct or indirect contact with wild, migratory, or exotic birds. As turkeys have also been found to be susceptible to a swine influenza virus, it is good management practice not to have pigs on the same farm as turkeys. Eggs for hatching should come from flocks demonstrated to be free of avian influenza viruses. The virus has been demonstrated to persist for 105 days in liquid manure following depopulation. Strict measures should be employed to eliminate movement of personnel and equipment, potentially contaminated by manure, between flocks and premises. During an outbreak, isolation of a flock along with orderly marketing of the flock should be considered. Treatment of infected flocks with broad-spectrum antibiotics is useful in controlling secondary bacterial infections, and proper nutrition and husbandry may help reduce mortality. To effectively control HPAI outbreaks, depopulation of infected flocks is employed worldwide, and HPAI cases are required to be reported to the World Organization for Animal Health, formerly the Office International des Epizooties (OIE).

Swine Influenza

Disease

Swine influenza is an important respiratory disease of pigs. The natural disease commonly affects large numbers of animals in the herd with high morbidity (approaching 100%) and generally low mortality (<1%) rates. Swine influenza occurs more frequently during colder months. Disease caused by swine influenza alone is usually mild; however, disease may be severe when secondary infections occur. The incubation period is short, between one and three days, with rapid recovery beginning four to seven days after onset. Disease symptoms include fever, anorexia, weakness, dyspnea, sneezing, coughing, and nasal discharge. Some animals develop conjunctivitis, pulmonary edema, or bronchopneumonia.

Swine influenza was first recognized during the massive human influenza pandemic in 1918, and subsequent studies confirm that a similar H1N1 virus caused disease in both humans and swine. Swine influenza virus can spread from pigs to humans causing illness, and there is concern that swine may serve as a reservoir from which influenza virus can emerge to precipitate human epidemics and pandemics. Although 16 HA and 9 NA subtypes have been isolated from birds, only the H1N1, H3N2, and H1N2 subtypes have established in swine and are most commonly responsible for disease outbreaks in pigs worldwide. With introductions of human seasonal H1N1, H3N2, and the 2009 pandemic H1N1 viruses into swine herds, different reassortant H1 and H3 viruses have been globally detected in pigs. Genetic and antigenic differences among strains of swine influenza viruses have been observed, and different genotypes of swine influenza viruses exist and circulate in swine globally. For example, at least seven genetically and antigenically distinct clades of H1 viruses and four antigenically different clusters of H3 viruses have presented and co-circulate in swine herds in North America.

Pathogenesis and Pathology

On postmortem examination of swine with influenza, the mucosa of the upper respiratory tract is congested and the bronchial and mediastinal lymph nodes are enlarged and edematous. The affected lungs display a purple-red, multifocal to coalescing consolidation of predominantly cranioventral portions of the lung (Figure 57.5a). Microscopic lesions usually consist of airways filled with exudate, widespread alveolar atelectasis, interstitial pneumonia, and emphysema. Peribronchial and perivascular cellular infiltration is also seen (Figure 57.5b), and viral antigen can be detected (Figure 57.5c).

Swine influenza virus can be transmitted by droplet and aerosol infection, and also by direct contact between infected and uninfected animals. Infection with aerosols and droplets produced by pigs coughing and sneezing is an important transmission mechanism in swine influenza. The virus can spread rapidly through a herd, resulting in infection of all the pigs in a few days. Direct contact is the main route of transmission of swine influenza, especially by pig touching noses. The virus is shed by nasal secretions, which are laden with viruses during the acute febrile stage. If the pigs are raised in the concentrated feeding operations, it will increase the risk of transmission of swine influenza virus. Transmission can also occur through humans and wild animals, which can spread disease to uninfected farms from infected farms.

Laboratory Diagnosis

Swine influenza is suspected whenever there is an "explosive" appearance of respiratory disease involving many pigs, particularly during the fall or winter months. A

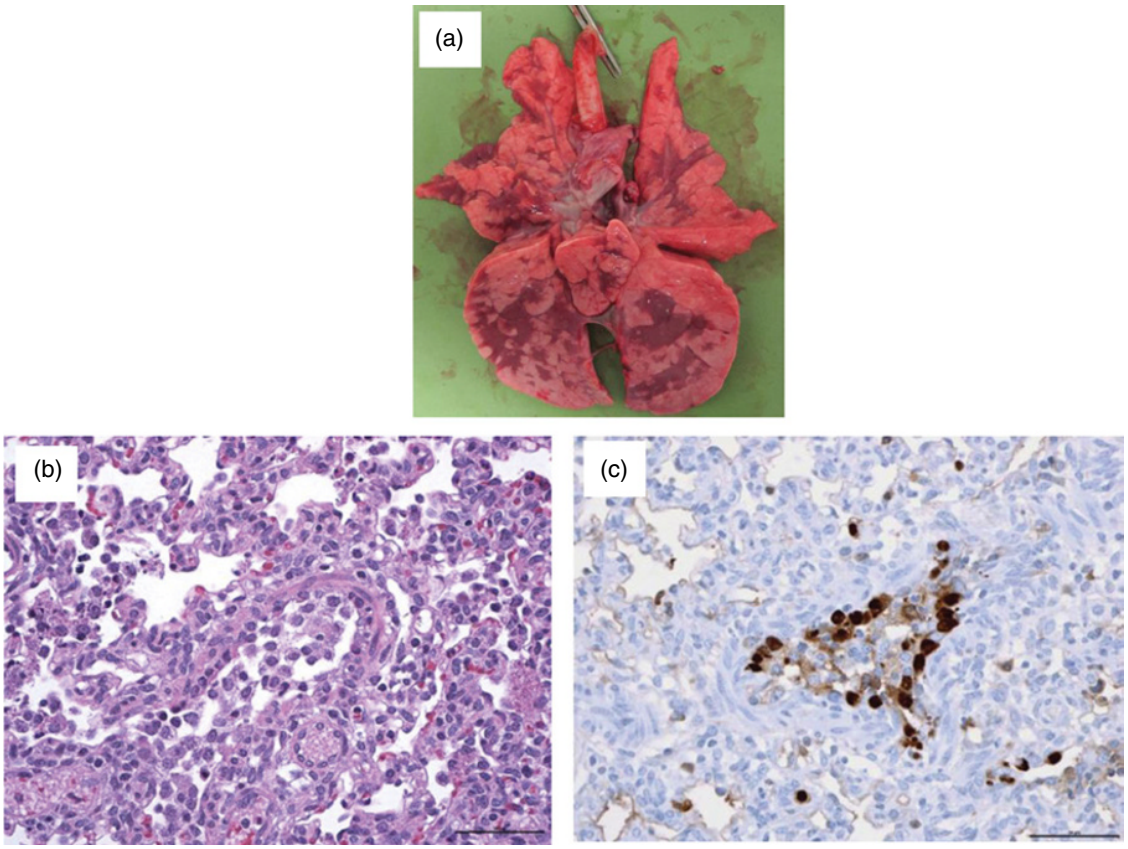


Figure 57.5 Macroscopic and microscopic pneumonia associated with an H1N1 swine influenza virus infection of pigs at day 3 post infection. (a) Macroscopic pneumonia characterized as purple-red consolidation located in each lung lobes. (b) Acute bronchiolitis and alveolar atelectasis in a pig at day 3 post inoculation. There is attenuation of the mucosa with degeneration and necrosis of airway epithelium with sloughed epithelial cells within the airway lumen. Associated parenchyma is atelectatic. (c) IHC staining shows viral antigens within bronchiolar epithelium in lungs of a pig infected with an H1N1 virus. *Source:* Ma et al. (2011).

definitive diagnosis requires either viral isolation from nasal secretions or the lung of dead pigs or demonstration of a rising titer between acute and convalescent sera or vRNA and antigen detection. Portions of the viral matrix sequence are conserved among all influenza A viruses, so a reverse-transcription PCR (RT-PCR) assay targeting the M gene can be used for virus detection. The HA and NA genes can be sequenced for virus typing. Swine influenza virus can be cultivated in 10- to 12-day-old embryonated chicken eggs and various tissue culture monolayer systems involving primary or stable cell cultures, such as Madin–Darby canine kidney, PK15 (porcine kidney), and swine testicular cells.

Treatment and Control

Swine that have recovered from influenza develop neutralizing antibodies, which normally can protect animals with infection of homologous viruses. Therefore, vaccination is one of efficient measures of controlling swine influenza. Swine flu vaccines have been developed, are commercially available, and are widely used in European countries and the United States. Commercial swine influenza inactivated vaccines contain H1N1 and H3N2 subtypes of circulating viruses and are effective when the virus strains used in the vaccines match the epidemic viruses. The first commercial swine live attenuated influenza vaccine (LAIV), Ingelvac Provenza (Boehringer Ingelheim), a bivalent H3N2 and H1N1 intranasal LAIV, was approved for use in pigs in the United States in 2017. Prevention and control of swine influenza by vaccination has become very difficult in recent years because the rapid evolution of the virus and emergence of novel viruses resulted in evasion of immune responses to the traditional vaccines. As much of the illness and death associated with swine flu involves secondary infection by other pathogens, control strategies that rely on vaccination may be insufficient. The best way to deal with swine influenza is to prevent the occurrence and spread of the disease. Therefore, facility and herd management is also essential to control swine influenza. Facility management includes using standard sanitary measures to

control viruses in the environment. Influenza A viruses are easily inactivated by disinfectants, heat, and formalin. Be sure to clean and disinfect trucks, trailers, and any equipment that may be contaminated. If a group of finishing pigs had swine influenza, it is necessary to clean and disinfect the house completely before the next group enters. Pigs carrying or exposed to influenza virus are normally responsible for the introduction of viruses to uninfected herds; therefore, new animals should be quarantined before they are added into herds. Treatment entails supportive measures including a draft-free environment with clean, dry, dust-free bedding, fresh clean water, and a good source of feed. Antibiotics given on a herd basis may help prevent secondary bacterial infection.

Equine Influenza

Disease

Influenza is an acute respiratory disease of horses. Horses of all ages are affected, while those between two and six months of age are at greatest risk. The mortality rate of equine influenza is low, whereas the morbidity rate can approach 100%. The incubation period lasts one to five days. Disease is manifested by a high fever, up to 106 °F, which lasts about three days. Other clinical signs include a frequent, strong, dry cough lasting one to three weeks, a nasal discharge that is initially serous in character but that later becomes mucoid, anorexia, depression, photophobia, lacrimation with a mucopurulent ocular discharge, and corneal opacity (sometimes with loss of sight). Limb edema and muscle soreness can occur, and in some severe outbreaks, acute deaths due to fulminant pneumonia have occurred. Enteritis was described in a 1989 outbreak in northern China due to a new equine influenza virus, A/equine/2/Jilin (H3N8), which is considered to have emerged from birds and moved into horses.

Equine influenza is caused by two subtypes of influenza A viruses: A/equine/1 (H7N7) and A/equine/2 (H3N8) based on antigenic differences in their HA and NA. There is no immunologic cross-reaction between these two subtypes. A/equine/1 (H7N7) has one prototype, A/equine/1/Prague, which was first isolated in 1956. Antigenic drift has occurred among A/equine/1 viruses with the subsequent designation of two subgroups that do not appear to differ significantly in terms of their immunity after vaccination. In recent years, the H7N7 subtype has not been isolated from horses and is believed to be extinct or to be persisting at a very low level in some regions. Strains of A/equine/2 (H3N8) virus have been responsible for all known outbreaks of the disease since 1980. Significant drift has occurred among A/equine/2 viruses from the original prototype A/equine/2/Miami/63 that was first isolated from a severe outbreak in Florida horses in 1963, resulting in the evolution of two distinct lineages that have been designated the American and Eurasian lineages based on the initial geographical distribution of the viruses. The American lineage has evolved into three sublineages including Argentinian, Kentucky, and Florida, based on HA phylogenic analysis. Notably, two distinct clades have emerged in the Florida sublineage due to fast evolution, and they are circulating worldwide and causing outbreaks. The Clade 1 viruses caused outbreaks in Japan and Australia in 2007–2008, whereas the Clade 2 viruses were responsible for major outbreaks in China, India, and Mongolia. Emergence of variant strains has shown that considerable antigenic drift occurs in the A/equine/2 viruses, which has implications for efficacious vaccination because outbreaks in 2018–2019 in both the United States and Europe have been involved in vaccinated horses. Influenza occurs in horses worldwide and is a common and troublesome problem when horses congregate at shows, sales, stables, and racetracks.

Etiologic Agent

Resistance to Physical and Chemical Agents

Equine influenza virus is usually inactivated at 56 °C in 30 minutes. Like all influenza A viruses, the virus is inactivated by phenol, lipid solvents, detergent, formalin, and oxidizing agents such as ozone.

Infectivity for Other Species and Culture Systems

Equine influenza virus normally infects horses, asses, and mules, and also is able to cross the species barrier to infect dogs. The first cross-species transmission of the equine influenza H3N8 virus to racing greyhounds was reported in the United States in 2004, resulting in respiratory disease and death of the infected dogs. Retrospective studies in the United Kingdom have shown that the equine influenza H3N8 virus has been responsible for severe outbreaks of respiratory disease in dogs. Equine influenza virus can be experimentally adapted to infect mice when introduced intranasally. There is molecular, serological, and virological evidence from Mongolia that the equine influenza virus is able to infect humans. Most people infected by this virus do not have any clinical signs, but some might show influenza-like diseases. All orthomyxoviruses, including equine influenza virus, can be propagated in the allantoic cavity of

embryonated chicken eggs. Equine influenza virus can also be grown in chick embryo kidney, bovine kidney, rhesus monkey kidney, and human embryo kidney cells.

Pathogenesis and Pathology

Equine influenza virus infects both the upper and lower respiratory tracts. Virus antigen has been detected in samples collected from the nasopharynx, trachea, bronchus, and alveoli by bronchoalveolar lavage. There is an early lymphopenia with accompanying enlargement of the lymph nodes of the head. Initially there may only be a slight serous nasal discharge, which later becomes mucoid. Fatal pneumonia can occur in foals and on occasion in older animals. Occasionally, there is ventral edema of the trunk and lower limbs. A/equine/2 has been known to cause a postinfection encephalopathy in foals. Catarrhal and even hemorrhagic enteritis may occur. Necrotizing bronchiolitis in which the bronchioles are progressively obstructed is characteristic of equine influenza. Severe necrotizing myositis with elevated serum enzyme levels has been observed with A/equine/2 infections. Most animals that do not develop chronic obstructive pulmonary disease recover within two to three weeks. Prolonged recovery and severity of disease appear to be related to the level of stress that an affected horse undergoes, and thus adequate rest is important for recovery.

Equine influenza virus is most commonly transmitted via aerosol and can spread extremely rapidly due to the frequent, violent cough of infected horses. Infected animals continue to shed the virus for about five days after the first signs appear. The virus can also be spread by fomites such as contaminated vehicles.

Laboratory Diagnosis

A tentative clinical diagnosis of equine influenza can be made from the characteristic rapid spread of the disease, especially among stabled horses, and the frequent dry cough of affected horses. A definitive diagnosis requires isolation of the virus, demonstration of viral antigen, or demonstration of rising antibody titers between acute and convalescent sera by complement fixation and hemagglutination inhibition tests. During outbreaks it is important to isolate and type the causative strains of the influenza virus for the success of future vaccination programs.

The clinical samples are taken by nasal or nasopharyngeal swabs or by nasal or tracheal washings. The washings are usually taken by endoscopy. The virus can be isolated in embryonated chicken eggs or cell cultures, and it can take 5–10 days to complete. Once isolated, the virus can be sequenced to determine its subtype and phylogeny. There are three tests for detecting the presence of antibodies to equine influenza viruses in the horse blood: the hemagglutination inhibition, single radial hemolysis, and competitive ELISA. Other assays have been developed that rely on detecting vRNA (real-time polymerase chain reaction test, qPCR), viral proteins (antigen-capture ELISA test), or various commercially available rapid assay kits (such as Directigen Flu-A, Directigen EZ Flu A + B tests). Equine influenza virus is commonly tested in combination with other causes of equine respiratory diseases (e.g. Equine herpesviruses 1 and 4 and strangles) in a PCR panel.

Treatment and Control

Vaccination is somewhat effective in preventing influenza in horses. Protection, however, depends on the mode of vaccination and the quality of the vaccine, with particular emphasis placed on proper selection of vaccine strains. Available vaccines contain the inactivated virus of both A/equine/1 and A/equine/2 subtypes. Classically, A/equine/Prague/56 (H7N7) and A/equine/Miami/63 (H3N8) have been used as the prototype A/equine/1 and A/equine/2 strains, respectively. There is increasing evidence of antigenic diversity among contemporary equine influenza viruses that circulate in nature, suggesting that the effectiveness of the conventional vaccine strains in providing protection will become limited with time. For this reason, current vaccines include some of the newer variant A/equine/2 viruses. However, the level of protection has varied because of the higher rate of antigenic drift of H3N8 strains. The various inactivated vaccines available incorporate adjuvants that have proved to significantly augment their immunogenic potential. Initial immunization requires two doses of vaccine two to four weeks apart. These should be followed by a single booster when the horse is one year of age and then repeated every six months until the horse is about three years of age, at which time the booster interval may be increased to not more than one year. Due to the limitations of inactivated vaccines, live attenuated equine influenza vaccines have been developed and are available in some countries. The live attenuated equine influenza vaccine has been shown to be effective for the vaccination of healthy horses 11 months of age or older against disease caused by the equine influenza virus. Duration of immunity has been shown to be at least six months.

In horses, a relationship has been shown between titers of the nasal antibody to the influenza virus and resistance to infection. The presence of prechallenge serum antibody has also been shown to shorten the duration of viral

excretion and febrile response. In addition to vaccination, isolation, and quarantine measures are advised during outbreaks to reduce the spread of disease, and disinfection of affected premises, equipment, and clothing is essential to prevent mechanical transmission of the virus.

Zoonotic Significance of Animal Influenzas

A growing body of evidence suggests that pandemic strains of human influenza arise because of reassortments between different (human and animal, or animal and animal) strains of influenza A virus. For example, both the 1957 H2N2 Asian flu and the 1968 H3N2 Hong Kong flu viruses were reassortants between human and avian influenza viruses, and the 2009 pandemic H1N1 virus was generated by reassortment between North American and Eurasian swine influenza viruses. Waterfowl appear to be of particular significance in the origin of new human isolates. Ducks appear to act as a "melting pot" where various strains of influenza A viruses can come together and undergo genetic reassortment, resulting in the generation of new strains of influenza viruses. Although little is known about whether the past pandemic viruses were produced in pigs, swine have been considered as the "mixing vessels" of "avian-like" and "human-like" influenza viruses. The supportive evidence includes the fact that pigs have receptors for both avian and human influenza viruses; and that triple-reassortant swine influenza viruses carrying the gene from swine, avian, and human influenza viruses have been circulating in swine herds in North America for more than 12 years and sporadically transmitted to and infected humans. As the internal and HA genes are critical for host range, and the HA and NA are important for host immunity, reassortment events in an intermediate host, such as swine or quail, can result in the formation of new virus strains. They contain the same or similar internal genes but possess very different HA and NA proteins, and this HA is able to bind to the mammalian receptors. The novel viruses generated in this manner may still be infective to humans but possess surface antigens that are very different from those to which the human population previously was exposed (and immune to). The result could be a substantial influenza pandemic as the new strain rapidly spreads through a susceptible population.

Normally avian influenza viruses do not cross the species barrier to infect humans and other mammalian hosts directly. However, the accumulated evidence demonstrates that avian influenza viruses can directly infect human beings. The first outbreak of H5N1 avian influenza in Hong Kong in 1997 caused 6 deaths of 18 infected people. Since 2005 the H5N1 HPAIV has caused an outbreak in wild birds in Qinghai Lake in China, and the H5N1 HPAIV has spread to Europe, the Middle East, and Africa from southeast Asian countries. The H5N1 HPAI has been endemic in poultry in several countries, for example, Egypt and Vietnam, and human infection with this virus continues to be reported. As of February 24, 2012, 586 confirmed human cases for H5N1 HPAIV have been reported to the World Health Organization, and 346 of them died. Since 2013, an LPAI H7N9 virus has been circulating in domestic poultry in China and caused five waves of human infections. As of 25 July 2017, 1557 human cases and 605 deaths have been confirmed in 24 provinces in China. Notably, the LAPI H7N9 has surpassed H5N1 infected laboratory-confirmed human cases despite its limited dissemination outside of China. The HPAI H5N1 and LPAI H7N9 viruses were considered candidates to trigger the next pandemic. Furthermore, other subtypes of avian influenza viruses, such as H9N2, H7N7, H10N8, and H10N3, have also been found to infect humans directly. Fortunately, all of these avian influenza viruses, including H9N2, H5N1, and H7N9, do not exhibit efficient human-to-human transmission. Obviously, avian influenza is an important zoonotic disease for humans.

Swine are susceptible to infection with human, avian, and swine influenza viruses, suggesting that novel viruses may be generated by reassortment in this host to cause epidemics and pandemics as humans lack immunity to such viruses. In particular, the H5N1 and H9N2 avian influenza viruses have been isolated from pigs in Southeast Asian countries. Southeast Asia has been considered an "influenza hotbed" because of the proximity of human habitations to farms, and the close association between swine, poultry, and humans on family farms. It has been implicated as an ideal situation for establishing new antigenic strains and introducing these viruses to the human population. This emphasizes importance of surveillance of animal and human influenza viruses in this area in order to prevent next pandemic. However, the H1N1 virus caused the 2009 pandemic in the twenty-first century, which first occurred in Mexico. Lessons we learned from the 2009 pandemic are that the pandemic virus could also be generated in other locations, including the highly industrialized countries in North America or Western Europe with modern swine and poultry facilities.

Bat Influenza

Sequences of two novel H17N10 and H18N11 influenza viruses have been found in New World bats, which has expanded the influenza A virus natural host species. Although neither of these viruses have been isolated, infectious clone technology has permitted significant progress in understanding their biology, which has unique

features compared to all other known influenza A viruses. Both H17N10 and H18N11 viruses have been shown to use MHC II molecules as receptors to mediate cell entry. However, it remains unclear whether MHC II molecules are the only binding receptors or serve as necessary entry cofactors. So far, normal cell lines typically used for influenza A viruses do not support replication of bat H17N10 and H18N11 viruses; only several cell lines such as MDCK II and RIE1495 support these two unique viruses' replication. These two novel viruses can infect animals such as mice, ferrets, and bats; however, infected mice and ferrets do not show any clinical signs, and very limited replication was detected in these species. Studies using Neotropical Jamaican fruit bats, a putative reservoir host of the H18N11 virus, show that the wild type H18N11 can infect and transmit among bats. To date, available evidence shows no reassortment between bat and conventional influenza A viruses, and bat influenza viruses pose low risk for human populations. Therefore, its zoonotic potential remains very low. In addition, an H9N2-like influenza A virus was isolated from Old World bats that showed characteristics similar to normal influenza A viruses. In contrast to both bat H17N10 and H18N11 viruses, the novel H9N2-like bat virus may pose a greater threat to humans and other species because it binds to sialic acid receptors used by conventional IAVs and can infect and replicate in mice very effectively. More research is needed to understand the biology of novel H17N10 and H18N11 as well as H9N2-like bat influenza viruses.

Isavirus

The genus *Isavirus* has a single member: the ISAV. It is able to infect farmed and wild salmons, resulting in a highly fatal disease, infectious salmon anemia (ISA). This disease was officially discovered among salmon parr in a hatchery in Western Norway in 1984, where it caused approximately 80% mortality. This virus has caused outbreaks in fish farms in Canada, Norway, Scotland, and Chile, resulting in severe economic losses to infected farms. The pathology of ISA was first described in 1991. ISAV infection causes severe anemia in infected fish and can be horizontally and vertically transmitted among salmon. ISAVs can be divided into two major genotypes based on phylogenic analysis of their genomes: European and North American genotypes. The European genotype ISAVs contain several distinct clades based on analysis of segments 5 (fusion protein gene) and 6 (hemagglutinin-esterase gene). Not all ISAVs are virulent, and some strains are not pathogenic. The ISAV genome has eight segments of single-stranded RNA, like influenza A and B viruses, which encode 11 viral proteins. Due to the limited research on the ISAVs, whether virus infection and replication, genome structure, functions of each protein is similar to those of influenza A and B viruses needs to be investigated.

Infected fish may develop clinical signs including pale gills, raised scales, pinpoint areas of bleeding, a bloated abdomen, or bulging eyes. Some infected fish may not show any external signs of disease but suddenly die. Pathological findings in the terminal stage of this disease are severe anemia with hematocrit below 10%, exophthalmia, pale gills, ascites, and hemorrhages in several organs including the liver, kidneys, spleen, and intestine. Multiple experimental studies by infecting Atlantic salmon reveal liver lesions detected in 90.6% of the infected individuals, and hemorrhagic kidney syndrome in 78.1% of the infected fish, and both types in 65.6% of the infected fish. It is difficult to distinguish ISA from other fish diseases because the clinical signs of ISA are not unique. Therefore, testing for virus presence is the only way to determine if a fish has ISA. Several diagnostic tests including RT-PCR, indirect fluorescent antibody test, and virus isolation and sequencing, have been used to detect ISAV. ISAV can be infectious for 20 hours in sea water and for 6 days in blood and liver if the samples are kept at 6 °C. Thus, horizontal transmission is the major infection route among salmon. To prevent and control ISA, enhanced biosecurity in fish farms is important and necessary. In addition, several commercial inactivated vaccines against ISA are available and have been shown to be effective to increase the survival of vaccinated salmon.

Thogotovirus

The genus *Thogotovirus* has multiple species, including Araguari, Aransas Bay, Bourbon, Dhori, Jos, Thogoto, and Upolu viruses. Of these viruses, only two have been identified in the United States: Aransas Bay virus from soft ticks found in the nests of seabirds, and Bourbon virus (BRBV) from infected humans in Kansas and Missouri. Most thogotoviruses have been shown to infect arthropods that are generally hard or soft ticks. Members of the *Thogotovirus* can also infect birds and wild and domestic mammals such as hares, marsupials, rodents, mongoose, camels, goats, sheep, horses, and cattle. In the genus *Thogotovirus*, Thogoto, Dhori, and BRBVs have been reported to be associated with human disease and are transmitted by ticks.

Thogoto virus. Thogoto virus (THOV) was first identified in the early 1960s in Kenya. Subsequently, two human infection cases associated with THOV were reported in 1966. THOV is able to infect both humans and domestic animals such as sheep, cattle, and camels, resulting in

severe clinical consequences such as febrile symptoms to meningoencephalitis. THOV is an important veterinary pathogen, for example, causing a febrile illness and abortion in sheep.

Dhori virus. Dhori virus (DHOV) was first isolated from *Hyalomma dromedarii* hard-bodied ticks that infest in camels in India in 1961. Subsequently, the virus has been isolated from *Hyalomma marginatum* ticks on livestock in Russia, Pakistan, Egypt, Saudi Arabia, Kenya, and Portugal. The virus has been also isolated from a wild hare and a cormorant, indicating that it has a broad range of host species. Antibodies to the virus have been detected in livestock including camels, goats, horses, and cattle as well as in humans from areas where DHOV is widespread. DHOV can infect humans, resulting in a febrile illness and encephalitis. DHOV is highly pathogenic in mice under experimental infection and has been considered as a model system for highly pathogenic influenza virus.

Bourbon virus. BRBV was first isolated from a blood sample of a patient who had fever and a history of tick bites in Bourbon County, Kansas, United States, in 2014 and died later from severe illness. Subsequently, several human BRBV infections and deaths have been reported in the midwestern and southern United States. The human BRBV and the virus strain isolated from tick pools show approximately 99.0% sequence similarity at the amino acid level and 95.0% identity at the RNA sequence level. Available studies show high seroprevalence in wildlife, including raccoons and white-tailed deer, and low seroprevalence in domestic animals such as the dog and the horse. BRBV is likely transmitted to vertebrates including humans though lone star ticks, which are an abundant arthropod in the south-central United States and widely carry BRBV.

Although these three viruses (THOV, DHOV, and BRBV) are important zoonotic pathogens, no specific treatment or vaccine is available. Antiviral drugs such as ribavirin and favipiravir, which have a broad spectrum of activity that includes some other orthomyxoviruses, have been shown to inhibit DHOV and BRBV replication. Supportive therapy is used for *Thogotovirus*-related diseases and has been recommended by the US Centers for Disease Control and Prevention for infection with BRBV.

Quaranjavirus

The genus *Quaranjavirus* includes Quaranfil, Johnston Atoll, and Lake Chad viruses, which are arboviruses and can infect vertebrates and invertebrates. Quaranfil virus (QRFV) was first isolated in 1953 from ticks collected near Cairo, Egypt. Later, multiple QRFV strains were isolated from ticks and seabirds in several countries such as Egypt, India, South Africa, Afghanistan, Nigeria, Kuwait, Iraq, Yemen, and Iran. In addition to seabirds, the vertebrate hosts of QRFV include mice and humans. QRFV was isolated from the blood samples of two children during a febrile illness in Quaranfil, Egypt. Further serological studies in Egypt in the 1960s have shown that approximately 8% of the local human population had neutralizing antibodies against this virus, indicating that human infection had occurred and raising the question of whether QRFV infection might cause an unrecognized viral disease in humans. To date, the clinical diseases in humans related to QRFV infection remain unclear.

Johnston Atoll virus (JAV) was isolated from ticks (*Ornithodoros capensis*) in Johnston Atoll in the central Pacific in 1964. Several strains were subsequently isolated from this kind of tick in Hawaii, eastern Australia, and New Zealand. Experimental infection studies of younger animals with JAV such as newborn and weanling mice and 1- to 2-day-old chicks result in severe disease and mortality. The QRFV and JAV have been shown to be transmitted between vertebrates by ticks.

The Lake Chad virus (LKCV) was first isolated from a vitelline masked weaver bird at Lake Chad, Nigeria, in 1969. Studies have shown that LKCV is lethal to newborn mice, and it is antigenically related to QRFV. So far, no human disease has been reported to be associated with JAV and LKCV.

Due to limited research on the quaranjaviruses, whether they can infect other host species such as domestic and wild animals, the clinical consequences of infections, their virus gene and protein functions, and so on, remain poorly understood. More research is needed in order to understand the biology of these viruses.

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Bunyavirales

William C. Wilson, Dana Mitzel, Lee W. Cohnstaedt, Leela Noronha, Barbara S. Drolet, and D. Scott McVey

Bunyavirales

In 2016, the International Committee on Taxonomy of Viruses elevated the family *Bunyaviridae* to the order *Bunyavirales* to incorporate an increasing number of identified related viruses with new family classifications. The order *Bunyavirales* includes the families *Arenaviridae, Cruliviridae*, *Fimoviridae, Hantaviridae, Leishbuviridae, Mypoviridae, Nairoviridae, Peribunyaviridae, Phasmaviridae, Phenuiviridae, Tospoviridae,* and *Wupedeviridae*. There are over 400 viruses in this order but only a few have veterinary disease significance These include (i) Black Creek Canal, Hantaan, Prospect Hill, Seoul, and Sin Nombre orthohantaviruses belonging to subfamily *Mammantavirinae* within the *Hantaviridae* family; (ii) *Nairoviridae* including Nairobi sheep disease (NSD) orthonairovirus, (iii) members of *Peribunyaviridae*, including Aino, Akabane, Cache Valley, Schmallenberg virus (SBV), Shuni, and Simbu orthobuyaviruses; and (iv) members of *Phenuiviridae*, including Rift Valley fever (RVF), Sicilian, and Toscana phleboviruses. Many of the viruses in the *Bunyavirales* order are arboviruses that are transmitted by arthropods such as mosquitoes, ticks, and biting flies, and thus have the capacity to alternately replicate in vertebrate animals and insects. Table 58.1 presents significant bunyavirus veterinary pathogens.

Several bunyaviruses are important zoonotic pathogens, including hantaviruses (the cause of hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome in humans). These viruses are carried by rodents, and humans get infected by inhalation of virus-contaminated rodent excreta and detritus. Other zoonotic bunyavirus pathogens include Rift Valley fever virus (RVFV), Crimean-Congo hemorrhagic fever virus (CCHFV), and NSD.

General Order Properties

Bunyaviruses are enveloped, pleomorphic viruses that are 80–120 nm in diameter with surface projections (spikes) emanating from the envelope surface of mature virions (Figure 58.1). The virions consist of four structural proteins, including two external glycoproteins in the envelope, a nucleocapsid protein that encapsulates the genome, and a polymerase protein (L). The envelope glycoproteins are responsible for neutralization and hemagglutination. A variety of non-structural proteins also are encoded by the viral genome. The nucleic acid is helical and is included in three distinct segments (large [L], medium [M], and small [S]), each comprising single-stranded, negative-sense RNA. There is a very large number of bunyaviruses. The organization and structure of the genomes determine the genera of these viruses. In addition, serological methods are used to provide further classification. However, many epitopes on envelope and capsid proteins are highly conserved, creating unique taxonomic challenges. There is considerable genetic diversity and serological cross-reactivity among viruses within the various genera of *Bunyavirales*. Taxonomic classification is further complicated by the genetic reassortment that may occur when cell cultures or insects are simultaneously infected with multiple, but closely related, bunyaviruses. Furthermore, bunyaviruses undergo genetic drift and natural selection within an established geographic range, especially in arthropod hosts. Nevertheless, it is unusual for new, serologically distinct strains to emerge. The bunyaviruses are generally susceptible to drying, heat, acids, bleaches, detergents, and most common disinfectants.

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Table 58.1 Oder of *Bunyavirales*, diseases, hosts, principal vectors, and zoonotic status.

Virus	Distribution	Arthropod vector	Target species	Disease in animals	Zoonotic?
Orthobunyavirus					
Akabane virus	Australia, Asia, Africa, Middle East	Mosquitoes, *Culicoides*	Cattle, sheep, goats	Abortion, congenital defects	No
Cache valley virus	United States	Mosquitoes	Sheep, cattle	Congenital defects	Rarely
La Crosse virus, California encephalitis group	North America	Mosquitoes	Rodents, humans	None	Yes, encephalitis
Schmallenberg virus	France, Germany, Netherlands	*Culicoides*, probably	Sheep, cattle, goats	Febrile illness, abortion, congenital defects	None described
Phlebovirus					
Rift Valley fever virus	Africa	Mosquitoes	Sheep, cattle, wild ruminants, camels, humans	Systemic disease, hepatitis, encephalitis, abortions	Yes, hepatitis, encephalitis, hemorrhagic disease
Orthonairovirus					
Crimean-Congo hemorrhagic fever virus	Africa, Asia, Europe	Ticks	Sheep, cattle, goats, humans	Subclinical or nonspecific	Yes, hemorrhagic fever, multi-organ disease
Nairobi sheep disease virus	Eastern Africa	Ticks	Sheep, goats	Hemorrhagic enteritis, death	Mild febrile illness
Orthohantavirus					
Hantaan virus	China, Russia, Korea	None	Striped field mouse	None documented	Hemorrhagic fever and renal failure
New World hantavirus	Western hemisphere	None	Multiple rodent species	None documented	Pulmonary syndrome

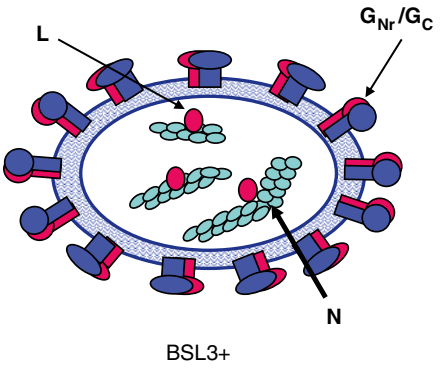


Figure 58.1 Representations of bunyavirus structure and genomic organization. *Source:* from https://viralzone.expasy.org. ViralZone, SIB Swiss Institute of Bioinformatics.

Family *Peribunyaviridae*, Genus *Orthobunyavirus* – Akabane, Aino, Schmallenberg, and Cache Valley Viruses

The genus *Orthobunyavirus* contains several viruses of veterinary importance known to have teratogenic effects on the developing offspring of immunologically naive ruminants infected during early pregnancy. Notable among these are the viruses of the Simbu serogroup, which includes Akabane virus (AKAV), Aino virus (AINOV), and SBV, among others. AKAV is the cause of periodic cases of fetal malformation in ruminants, especially cattle, in Asia, Australia, the Middle East, and Africa. The virus is transmitted by mosquitoes and *Culicoides* midges and causes distinctive teratogenic defects of the musculoskeletal and nervous systems (arthrogryposis–hydranencephaly [AGH] syndrome) in fetuses that are born to dams that become infected during pregnancy. The gestational age of the fetus at infection determines the type of lesions that will be present at birth; fetuses infected prior to mid-gestation are typically born with their limbs held in rigid flexion (arthrogryposis), with variable deviation of their vertebral columns. The brains of affected fetuses may lack cerebral hemispheres, or these may be represented only as fluid-filled sacs as a consequence of virus-mediated destruction of the developing cerebrum during gestation (hydranencephaly). An inactivated vaccine is used to protectively immunize ruminants prior to conception. AINOV causes a similar disease syndrome as AKAV and has much the same global distribution. SBV, which was the most recent of these viruses to emerge, was first identified in Germany and the Netherlands in 2011 and subsequently spread throughout Europe within two years. SBV was initially isolated from a dairy cow but is more commonly a disease of small ruminants. SBV causes abortion, stillbirth, and congenital malformations, including AGH syndrome, because of transplacental infections during the first and early-second trimester of gestation. Clinical disease is not typically observed in adult sheep and goats, but adult cattle may experience mild febrile illness with nonspecific signs such as anorexia, diarrhea, and decreased milk production. Inactivated commercial SBV vaccines are available in endemic areas. There is serological evidence that some viruses of the Simbu serogroup, such as AINOV, can infect humans; however, AKAV, AINOV, and SBV currently are not considered to be significant public health threats.

Cache Valley virus (CVV), a member the *Bunyamwera* serogroup, is a New World virus. CVV has been isolated from at least 44 species of mosquitoes from multiple genera; however, the principal vector is not currently known. Sheep, and to a lesser extent, goats, are the species most affected by CVV, but the virus has shown the ability to infect a wide variety of vertebrate species. Infections in non-pregnant animals are typically subclinical, but like AKAV, AINOV, and SBV, CVV can cause abortion, stillbirth, and fetal malformations depending on the gestational age at the time of infection. Outbreaks of AGH have been attributed to CVV in naive sheep herds in the western and southwestern United States. Humans can be infected through the bites of mosquitoes, and seroprevalence may be as high has 19% in endemic areas; however, documented cases of clinical disease are rare, with only six cases described, three of which were fatal in conjunction with other immunocompromising comorbidities. No vaccine is commercially available for CVV.

When vaccines are not available to reduce viral transmission and infection of insect-transmitted viruses, population management of the vector species is the best method to reduce the size of viral outbreaks. The most effective management method is aerial spraying of chemical pesticides. This method requires the applicator to treat the area when the vector species are flying or host seeking, typically at dawn or dusk. Other biting midge population management methods such as larval habitat removal or on-animal treatments are generally less effective.

The *Orthobunyavirus* genus also includes the California encephalitis serogroup viruses that are related (serologically cross-reactive) mosquito-transmitted viruses which include La Crosse, snowshoe hare, and Jamestown Canyon viruses. These viruses occur in endemic cycles of infection in different regions of North America, and although infection occurs in a variety of mammalian species, encephalitis is rarely documented even in humans. Other members of the genus *Orthobunyavirus* have been sporadically identified as the cause of encephalitis in animals – Main Drain virus in horses, for example.

Family *Phenuiviridae*, Genus *Phlebovirus* – Rift Valley Fever

RVFV is a zoonotic, mosquito-transmitted virus that causes epidemics of severe, frequently fatal systemic disease of ruminants, especially sheep and goats. Mortality is highest in young animals, and pregnant ruminants often abort following infection. RVFV causes extensive liver necrosis in affected sheep and goats, and widespread hemorrhages are common. Encephalitis with neuronal necrosis also occurs in some affected animals.

RVF is endemic in sub-Saharan Africa and periodically spreads to adjacent northern regions such as Egypt (Figure 58.2). Climatic conditions influence the occurrence of epidemics, particularly high rainfall that results in rapid expansion of the populations of *Aedes* mosquitoes that harbor the virus. RVF is zoonotic, and human mortality can be very high during epidemics.

RVFV is widely feared because of its virulence to humans and multiple species of animals, as well as its capacity for rapid spread by a variety of species of mosquito during epidemics. Thus, rapid diagnosis is imperative, usually by virus isolation genetic detection, or serology. No human vaccine is available; thus, veterinarians, and laboratory staff working with RVFV-infected material should follow biosafety guidelines to prevent exposure.

Physical, Chemical, and Antigenic Properties

The RVFV is a spherical, enveloped virus 80–90 nm diameter. The viruses have glycoprotein spikes (Gn/Gc) but do not have a matrix protein. The genome is composed of three RNA segments: (i) an ambisense S segment that encodes N and NSs non-structural proteins, (ii) an M segment that encodes the Gn and Gc spike proteins, and (iii) an L segment that encodes the transcriptase. After the

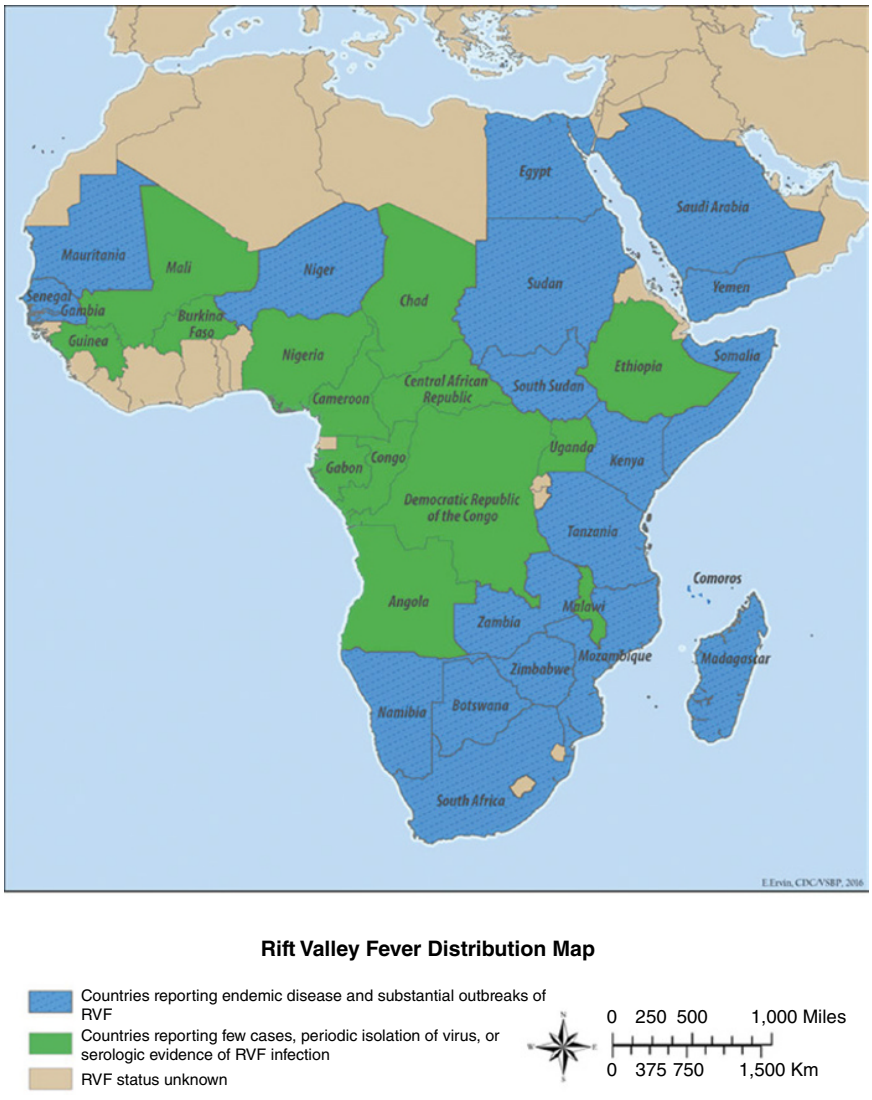


Figure 58.2 Distribution of recent Rift Valley fever disease in Africa and Asia (as of February 2021). *Source:* CDC / Rift Valley Fever / Public domain, https://www.cdc.gov/vhf/rvf/outbreaks/distribution-map.html.

RVFV attaches to target cells through multiple receptors by endocytosis, replication then occurs in cellular cytoplasm. The virus is uncoated, and the RNA-dependent polymerase (transcriptase) begins initial transcription of non-structural proteins critical for viral replication. Subsequent rounds of replication result in synthesis of the structural proteins essential for virus assembly and release by budding from intracytoplasmic vesicles from the Golgi apparatus. Virus particles are then released by exocytosis from apical or basolateral plasma membranes.

Distribution, Reservoirs, and Transmission

The RVF virus survives in multiple enzootic regions of Africa, primarily Kenya and South Africa (Figure 58.2). It is not completely understood how the virus survives the inter-epizootic periods, but mosquito and wild ruminant cycles are thought to be important. During periods of disease emergence, associated with heavy rainfall, infected mosquitoes spread virus to wild and domestic ruminants, where expansion occurs. From this point, expansion and spread occur through multiple species of *Aedes*, *Culex*, *Anopheles,* and other genera of mosquitoes. The virus may also be contracted by direct contact with infected tissues, especially aborted fetuses, milk, or by aerosols from these sources. In the United States and other non-endemic countries, RVF is a reportable foreign animal disease.

Pathogenesis

Incubation periods for this disease are relatively short at three to five days. Sheep, cattle, humans, and camels are frequently affected. Clinical signs include fever, loss of appetite, purulent nasal discharge, and diarrhea. Almost all pregnant ewes will abort. Mortality may approach 100% in lambs and up to 60% in adults. In cattle, the disease is usually less severe as mortality is usually under 30%. Infection of susceptible species with RVFV can result in hepatitis an encephalitis. However, abortion in pregnant animals may approach 100%. The RVFV replicates efficiently in maternal placental epithelial cells and subsequently infects fetal trophoblasts. Horses, camels, cats, and dogs may also be infected during epizootic periods, but only very young animals will develop clinically significant disease. North American whitetail deer are experimentally profoundly susceptible to RVF. Humans with significant exposure to infected livestock are at most risk for infection. Exposure to virus may come through infected mosquitoes that feed on livestock and humans as well as direct contact with infected animals (aerosols), meat, milk, or aborted tissues. The disease is an influenza-like illness in humans. Most cases are usually relatively mild with a less than 2% mortality rate. However, up to 10% of cases may be associated with hepatitis or neurological complications (blindness and encephalitis).

As the virus spreads rapidly to hepatic and primary lymphoid tissues, liver necrosis, splenomegaly, and subserosal hemorrhages are observed (Figures 58.3 and 58.4). Fever, as well as local and systemic inflammation, is associated with the tissue necrosis. These tissue changes are seen within three to five days. In the more severe cases, liver, and kidney failure, hemorrhagic fever syndrome, and encephalitis all may occur.

Host Response

Animals usually respond by producing detectable IgM antibody by day 5 and IgG antibody by days 10–14. This immune response is associated with recovery, and immunity is generally long-lasting.

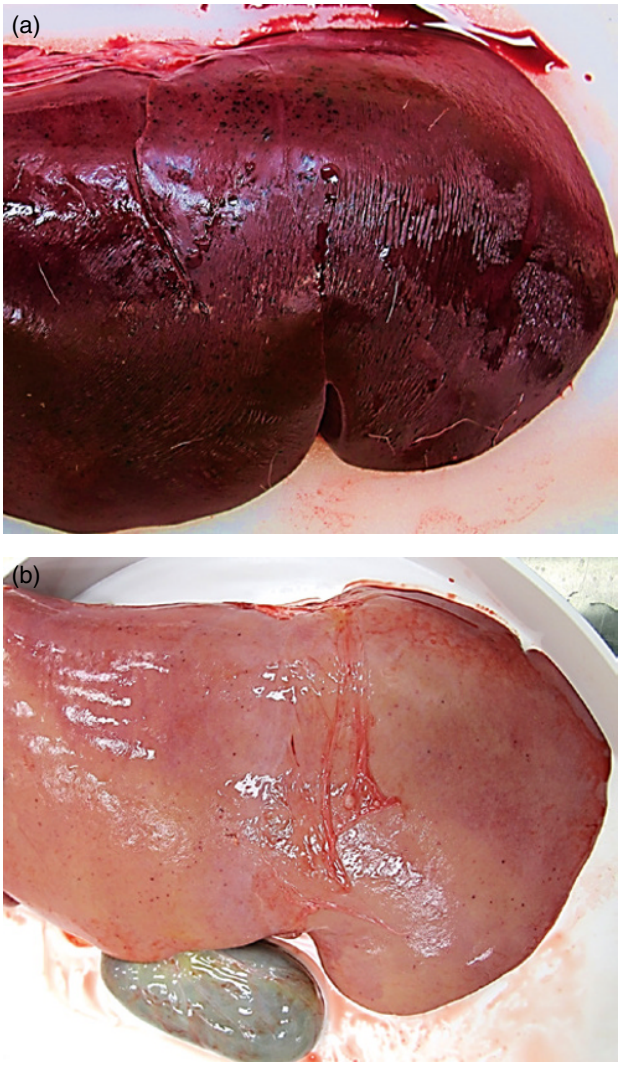


Figure 58.3 Rift Valley Fever in white tail deer (a) in domestic cattle (b). In deer, there is marked multifocal hemorrhage and necrosis throughout the liver while in cattle the hemorrhage is petechial with hepatic lipidosis (Photographs and descriptions courtesy of Dr. Jesse Trujillo, Kansas State University.

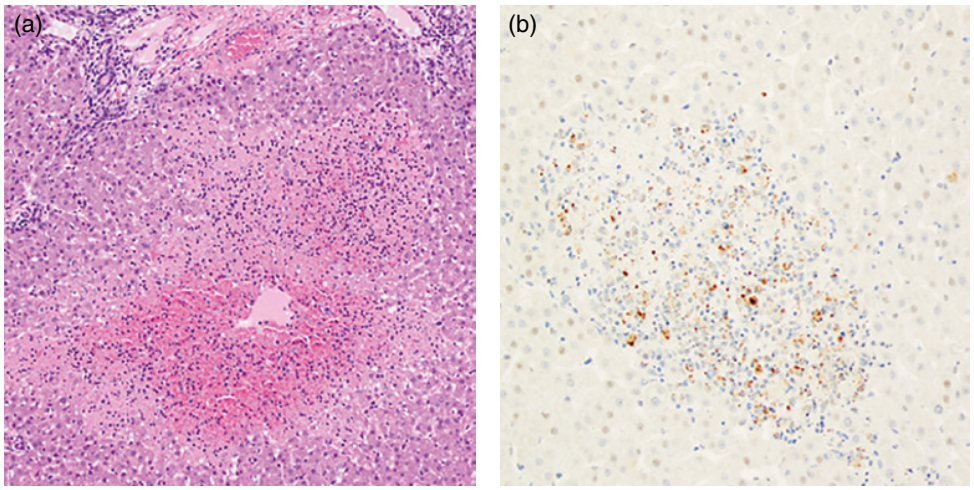


Figure 58.4 Rift Valley Fever in domestic sheep (a) H&E with centrilobular (*) to mid-zonal (**) hepatocyte necrosis and inflammation (b). IHC staining (brown) of RVFV antigen in necrotic foci (Photomicrographs and descriptions courtesy of Dr. Jesse Trujillo, Kansas State University). *Source:* Drolet et al. (2012)

Diagnosis

Virus isolation in cell culture is the gold standard test; however, this requires direct laboratory exposure of technicians to a virulent, zoonotic virus. The rapid inactivation for viral RNA isolation prior to polymerase chain reaction (PCR) analysis improves safety, and multiple PCR assays are available. These assays are very specific and sensitive, and have proved useful in detecting viral RNA from blood or other tissues. Also, IgM-capture assays and direct antigen detection dip-sticks are under development and/or are available for detecting recent RVF virus infection or virus antigen detection. These assays can be performed using recombinant protein antigen and are therefore safe to produce, and with heat inactivation of the sera, safer for laboratory technicians performing the analysis.

Control

Vaccination of susceptible livestock in endemic regions is the primary method of control. Both inactivated virus and attenuated virus vaccines have been used with reasonable success. In addition, attenuated strains of virus (MP-12 and Clone 13) are efficacious in cattle and sheep and licensed in some countries in Africa. MP-12 is conditionally licensed in the United States. Several new vaccines are in development, some of which can distinguish vaccinated from infected animals.

Control of vectors in endemic zones as well as restricting animal movement and minimizing human exposure are also moderately effective methods of control. Mosquito population management and reducing the contact between host-seeking mosquitoes and animals are the most effective methods to stop the spread of pathogens during an outbreak. Larval habitat removal such as draining ponds and removing containers that may collect rainwater will reduce the available larval habitat for mosquito egg laying and subsequent development. If pathogen or insect surveillance can identify the potential for an outbreak early, mosquito larval habitat treatments with insect growth regulators (such as methoprene, pyriproxyfen, or diflubenzuron) or biorational products (such as spinosad or *Bacillus thuringiensis* israelensis) are highly effective in reducing mosquito populations. During an outbreak, aerial space spraying is the best option to kill adult mosquitoes, but these treatments must be applied when the mosquitoes are in flight to maximize their effectiveness. Next generation management methods such as sterile insect technique and population replacement are effective but are currently available only for a few species of mosquito vectors.

Family *Nairoviridae*, Genus *Orthonairovirus* – Crimean-Congo Hemorrhagic Fever and Nairobi Sheep Disease

Crimean-Congo hemorrhagic fever (CCHF) virus is perhaps the best-known virus of this genus. This widely distributed tick-borne virus is found in Africa through the Middle East into northwestern China and north into Eastern Europe. CCHF is a disease of humans; numerous vertebrate species including livestock such as cattle, sheep, goats, and horses, are susceptible to infection but usually show mild or no clinical symptoms. Asymptomatic animals can act as important virus reservoirs, and people who work with livestock have an increased risk of exposure though contact with the tissues of infected animals, or indirectly though *Hyalomma* spp. ticks. No vaccines are available for use in animals. Effective tick treatments

include ear tags, topical application products, and feed through medications that take advantage of the long attachment time while the tick is feeding on the host animal.

NSD and related viruses cause severe disease in ruminants, principally sheep and goats, in Africa and Asia. The virus is transmitted by ixodid ticks such as the brown ear tick. NSD is characterized by high fever, intestinal hemorrhage, and death of susceptible ruminants with mortality rates that can exceed 90%. Pregnant animals frequently abort. NSD is a zoonotic pathogen but only causes mild influenza-like symptoms.

Family *Hantaviridae*, Genus *Orthohantavirus*

There are at least 20 members of the genus *Orthohantavirus*. These viruses are not maintained through or transmitted by arthropod vectors. This is a unique characteristic of the *Orthohantavirus* genus. The hantaviruses are generally human pathogens but are maintained in rodents, so these viruses are important from a veterinary perspective because of their zoonotic potential. The Old World hantaviruses usually cause a hemorrhagic fever and renal syndrome disease, whereas the New World hantaviruses cause pulmonary disease (Table 58.1). A major feature associated with hantaviruses is that they are difficult to isolate and maintain in cell culture. Thus, PCR assays are critically important for diagnosis.

The vast majority of the Old World hantaviruses infections occur in Korea, China, eastern Russia, and the Balkans. Human disease usually occurs after direct contact with rodent feces. Viremia is transient and is suppressed by the appearance of neutralizing antibody. However, the virus persists in the kidneys and lungs, where the presence of necrosis and inflammation causes disease. Diagnosis is usually by demonstration of virus in tissue by immunologic or molecular (PCR) methods. Inactivated vaccines have been used in Korea and China. But the most effective means of reducing pathogen transmission is though rodent control and personal hygiene.

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Paramyxoviridae, Pneumoviridae, Filoviridae, and Bornaviridae

Stefan Niewiesk and Michael Oglesbee

The order Mononegavirales includes viruses of veterinary importance within the families Paramyxoviridae, Pneumoviridae, Filoviridae, Bornaviridae, and Rhabdoviridae (Chapter 60). These viruses are ancestrally related as reflected by their common characteristics such as a single-stranded genome of negative-sense RNA, similar replication strategy and gene order, and virion morphology that includes a helical capsid and an envelope.

Resistance to Physical and Chemical Agents

Mononegavirales can be inactivated by standard physical measures and chemical agents effective against enveloped viruses. These measures include treatment with heat, ultraviolet light, acidic or alkaline solutions, or chemical agents such as lipid solvents, Lysol, phenol, its derivative butylated hydroxytoluene, and quaternary ammonium compounds. Disinfection is most effective after thorough cleaning of materials. The rate of viral inactivation varies with the strain of virus, quantity of virus initially exposed, structure of the environment, and presence of organic matter. Infectious virus can survive for long periods in organic and frozen matter.

Paramyxoviridae

The Paramyxoviridae family is divided into four subfamilies (Avulavirinae,Metaparamyxovirinae,Orthoparamyxovirinae, and Rubulavirinae; see Table 59.1). Viruses in this family cause a number of serious respiratory and/or systemic diseases of humans, animals, and birds.

Paramyxoviruses are characterized by virions that are enveloped, pleomorphic (filamentous or spherical; approximately 150 nm or more in diameter), and contain a genome of linear, negative-sense, single-stranded RNA. The viral nucleocapsid has helical symmetry and is approximately 13–18 nm in diameter (Figure 59.1). At least three proteins are associated with the nucleocapsid, including an RNA-binding protein (N or NP), a phosphoprotein (P) that enables transcription, and the viral polymerase (L = large protein). The nucleocapsid is enclosed by a bilayered lipoprotein envelope derived from the host cell plasma membrane, and contains two or three different transmembrane viral glycoproteins that form spikes (8–12 nm) projecting from the surface. The spikes are formed by the receptor-binding attachment protein (hemagglutinin (H) or hemagglutinin-neuraminidase (HN) in the Paramyxoviridae; G protein in the Pneumoviridae) and the fusion protein (F) that is essential for virus infectivity and for cell-to-cell spread. Another protein, the matrix (M) protein, lines the inner surface of the envelope. Various nonstructural viral proteins are also formed in infected cells that usually regulate virus replication.

Vaccination

All vaccines developed against infection with paramyxoviruses are based on live tissue culture attenuated viruses that do not cause disease but induce a protective immune response. In some cases, inactivated virus vaccines are being used, which in general are not as efficient as the live virus vaccine. For canine distemper (CD) virus, an additional vaccine has been developed based on a canarypox virus vector. Paramyxoviridae are monotypic; for example, antibodies against one virus strain can neutralize all strains of the same species.

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Table 59.1 Order Mononegavirales.

Family	Genus	Species
Bornaviridae	*Bornavirus*	Borna disease virus
Filoviridae	*Ebolavirus*	Zaire ebolavirus
	Marburgvirus	Lake Victoria marburgvirus
Paramyxoviridae		
Avulavirinae	*Orthoavulavirus*	Newcastle disease virus
Orthoparamyxovirinae	*Henipavirus*	Hendra virus
		Nipah virus
	Morbillivirus	Canine distemper virus
		Measles virus
		Feline morbillivirus
		Peste des petits ruminants virus
		Phocine distemper virus
		Cetacean morbillivirus
		Rinderpest virus
	Respirovirus	Bovine parainfluenza virus 3
		Human parainfluenza virus 3
Rubulavirinae	*Orthorubulavirus*	Mumps virus
		Parainfluenza virus 5 (Canine)
Pneumoviridae	*Metapneumovirus*	Avian metapneumovirus
		Human metapneumovirus
	Orthopneumovirus	Bovine respiratory syncytial virus
		Human respiratory syncytial virus
		Murine pneumonia virus

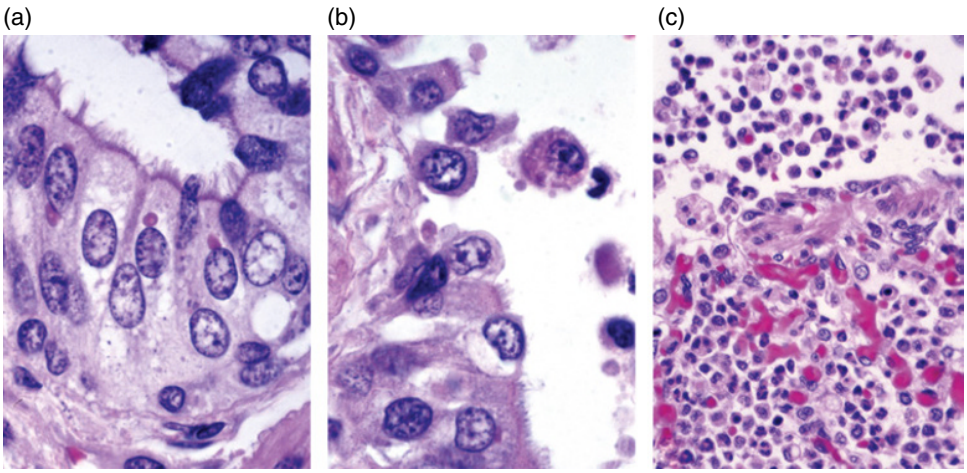


Figure 59.1 Canine distemper virus (CDV) infection of dog lung. (a) The initial infection of airway epithelium is noncytopathic and results in extensive viral shedding into the airway lumens. Numerous eosinophilic (pink) cytoplasmic inclusions are present within the infected cells. (b) Cytopathic effects are observed as the infection progresses, resulting in loss of cell differentiation, rounding, and sloughing, resulting in a denuded mucosal surface. (c) Secondary bacterial bronchopneumonia is a sequel to the mucosal injury and virus-induced immune suppression. Airway lumens (top) are filled with sloughed epithelial cells and neutrophils, and alveolar spaces (bottom) in the vicinity of these airways are filled with neutrophils and alveolar macrophages. Hematoxylin and eosin-stained tissue sections. *Source:* Michael Oglesbee, The Ohio State University.

Avulavirinae

Newcastle Disease

Disease

Newcastle disease (ND) is caused by avian paramyxovirus 1, a member of the genus *Orthoavulavirus,* subfamily *Avulavirinae*. ND is a highly contagious disease of chickens that is characterized by respiratory distress, diarrhea, and neurological signs. ND should be considered as an important rule-out for poultry that experience an unexplained increase in mortality, respiratory disease, and/or drops in egg production. The severity of the disease is dependent upon the age and immune status of the birds, and on the virulence of the strain of ND virus that is responsible for the infection. The most virulent strains are designated velogenic and produce mortality rates in affected birds as high as 90% or more. The disease caused by mesogenic strains is less severe, and the mortality rate is often less than 25%. Infection with both velogenic and mesogenic Newcastle disease virus (NDVs) is reportable. The lentogenic strains are relatively avirulent and are often used as vaccines.

Host–Virus Relationship

Geographic Distribution and Transmission

NDV infects chickens, guinea fowls, turkeys, and a large number of species of domestic and wild birds. Sea birds are less susceptible but may act as carriers. A self-limiting conjunctivitis develops in humans accidentally infected with NDV through infected birds or live viral vaccines.

ND occurs worldwide, and domestic birds are the major reservoir for NDV. Virulent NDV strains are endemic in poultry in most of Asia, Africa, and some countries of North and South America. Other countries, including the United States and Canada, are free of those strains in poultry and maintain that status with import restrictions and eradication by destroying infected poultry. Although NDV has been isolated from a large number of wild birds such as sparrows, crows, ducks, and geese, they appear to play a minimal role in transmitting this disease. Exceptions seem to be pigeons, doves, double-crested cormorants, and imported psittacine species, which may be infected with and transmit NDV variants that are highly pathogenic for poultry. Aerosol respiratory infection is the most common route for transmission of NDV. Infected birds begin to shed virus two to three days after exposure from their respiratory tracts and continue to shed virus for several weeks. The virus is also shed in feces and is readily spread by fomites, with movement of contaminated people, equipment, or litter.

Pathogenesis and Pathology

Initial replication of NDV occurs in the mucosa of the upper respiratory tract following aerosol infection. Viremia then disseminates the virus throughout the body, and widespread multiplication of virus in cells of parenchymal organs leads to a secondary viremia, which in some instances leads to the infection of the cells of the central nervous system (CNS). Disease takes several different forms in chickens, depending on the virulence of the strain involved. Differences in the virulence of individual strains of NDV are due to differences in the proteases used to cleave the fusion protein. Less virulent strains use trypsin-like proteases that are present only in the respiratory and gastrointestinal tract, whereas more virulent strains use the more ubiquitously expressed furin-like proteases. The very virulent (velogenic) strains cause very rapidly fatal infections involving the visceral organs and/or the CNS. Mesogenic strains of NDV cause respiratory and, occasionally, neurological disease in infected chickens with low mortality, whereas lentogenic strains produce a mild or often inapparent disease.

Lesions in ND vary greatly. Inapparent infections cause few, if any, lesions, whereas hemorrhagic necrosis that affects the intestinal tract, respiratory tract, and visceral organs is characteristic of more severe forms. In chickens with CNS involvement, necrosis of glial cells, neuronal degeneration, perivascular cuffing of lymphocytes, and monocytes, and hypertrophy of endothelial cells are often present.

Host Response to Infection

Chickens infected with NDV produce antibodies 6–10 days after infection. Antibodies to the envelope glycoprotein, HN, exhibit viral neutralizing and hemagglutination inhibition activities and are responsible for host immunity to the disease.

Laboratory Diagnosis

As the clinical signs and pathological lesions of ND are variable and nonspecific, definitive diagnosis of the disease must depend on laboratory methods of identification. This is best accomplished by using PCR to detect the virus and testing for virulent strains by evaluating the fusion cleavage site. NDV is a reportable disease, and testing is controlled through the USDA. NDV can be isolated by inoculating embryonated eggs or cell cultures with respiratory exudate or tissue suspensions (spleen, lung, or brain), but virulent strains require Biosafety Level 3 precautions. NDV antigen in affected tissues or cell cultures can be identified by immunofluorescence or immunohistochemical staining.

Serological diagnosis requires demonstration of rising NDV antibody titers by the hemagglutination inhibition or neutralization assay, or enzyme-linked immunosorbent assay (ELISA).

Control and Prevention

Sanitary management to prevent exposure of susceptible chickens to NDV is an important aspect of control against the disease. As there is only one serotype of NDV, vaccination with either inactivated or live virus vaccines is also used to prevent ND. The majority of live vaccines incorporate lentogenic strains of NDV administered in drinking water or applied as aerosols. Vaccination will prevent disease but not infection of flocks.

Orthoparamyxovirinae

Henipaviruses

Hendra Virus

Hendra virus disease is unique to Australia, where it was first recognized in 1994 as the cause of an outbreak of severe disease with respiratory and neurological symptoms that killed a number of horses and their trainer. The virus was named after the suburb of Brisbane in which the disease was first described. Affected horses develop severe interstitial pneumonia and pulmonary edema as well as neurological symptoms that rapidly prove fatal. The virus is harbored asymptomatically by several species of fruit bat (flying foxes). Although fruit bats are the reservoir of Hendra virus, the virus is contagious between horses and to humans by direct contact with nasal secretions or fomites that contain the virus.

Nipah Virus

Nipah viruses are closely related to Hendra virus. It has been found in several outbreaks in Malaysia, Singapore, Bangladesh, the Philippines, and India since 1998. The virus reservoirs are bats of (mostly) the genus *Pteropus* that are subclinically infected and shed virus in urine. These bats have a worldwide distribution, making dissemination to new areas likely to occur. When pigs become infected (presumably through infected feed), the illness presents with pneumonia and encephalitis. Other animals, including dogs, can also be infected. Close contact with infected pigs and foodborne (contaminated palm sap) transmission have been shown to lead to human illnesses with the same clinical symptoms observed in infected pigs. Human to human transmission also occurs. The case fatality rate in humans is high due to encephalitis. In humans, ribavirin treatment has been demonstrated to reduce fatalities.

Morbilliviruses

Although the various morbilliviruses are all closely related as evidenced by extensive serological cross-reactivity between them, they are distinguished on the basis of their individual host range, genome sequence, and antigenic differences. These viruses all have a hemagglutinin (H) surface protein, but only rinderpest virus (RPV) has a neuraminidase (HN). The principal receptor for wild-type morbilliviruses is the CD150 molecule (signaling lymphocytic activation molecule [SLAM]) of the respective species.

Canine Distemper Virus

Disease

CD is an important viral disease of dogs (Figures 59.2–59.4). The clinical presentation and pathogenesis of acute CD is highly similar to measles virus infection in humans and is characterized by any combination of diphasic fever, ocular, and nasal discharges, anorexia, depression, vomiting, diarrhea, dehydration, leukopenia, and pneumonia. The severity of clinical signs exhibited by individual animals can vary markedly. The disease has an incubation period of three to five days. The mortality rate depends largely on the immune status and age of the infected dog and is highest among puppies. Very young dogs may exhibit neurological disease during this acute phase. Animals that survive the acute disease may develop other signs, including hyperkeratosis of the footpads (hard pad disease) and neurological disease that is characterized by any combination of convulsions, tremor, myoclonus, locomotor disturbances, paralysis, and blindness. The onset of neurological signs frequently is delayed until several weeks after the systemic signs of acute CD manifest. Old dog encephalitis is a rare form of chronic neurological disease caused by defective CD virus (CDV).

Host–Virus Relationship

Host Range, Geographic Distribution, and Transmission

CDVs infect a wide range of animals. In addition to dogs, other members of the Canidae (e.g. fox, coyote, wolf, and wild dogs), Ailuridae (red panda), Hyaenidae (hyena), Mustelidae (e.g. ferret, mink, skunk, and badger), Tayassuidae (javelinas (collared peccaries), Ursidae (bears), Viverridae (civet, mongoose), and Procyonidae (e.g. raccoon and panda) are susceptible. Some members of the Felidae (lion, tiger, and leopard) also are susceptible,

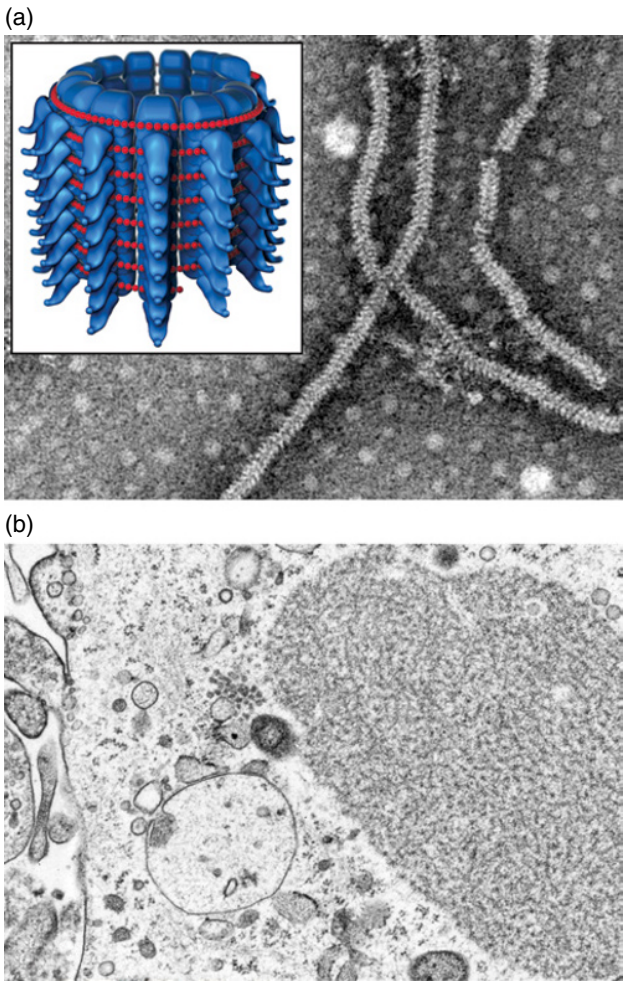


Figure 59.2 Canine distemper virus (CDV) cytoplasmic inclusions are the result of accumulations of virus core particles (nucleocapsids) during replication. (a) Nucleocapsids are formed by a helical arrangement of N protein (inset, blue) that packages the virus's single-stranded genomic RNA (inset, red). Negative stain transmission electron microscopy illustrates the length of these particles, and the superimposition of N proteins on the sides of the nucleocapsids creates a herringbone pattern that is typical of paramyxoviruses. (b) Transmission electron microscopy of an infected cell cytoplasm shows that nucleocapsids tend to form large aggregates that are the basis for the light microscopic inclusions. Osmium tetroxide-stained section. *Source:* Michael Oglesbee, The Ohio State University.

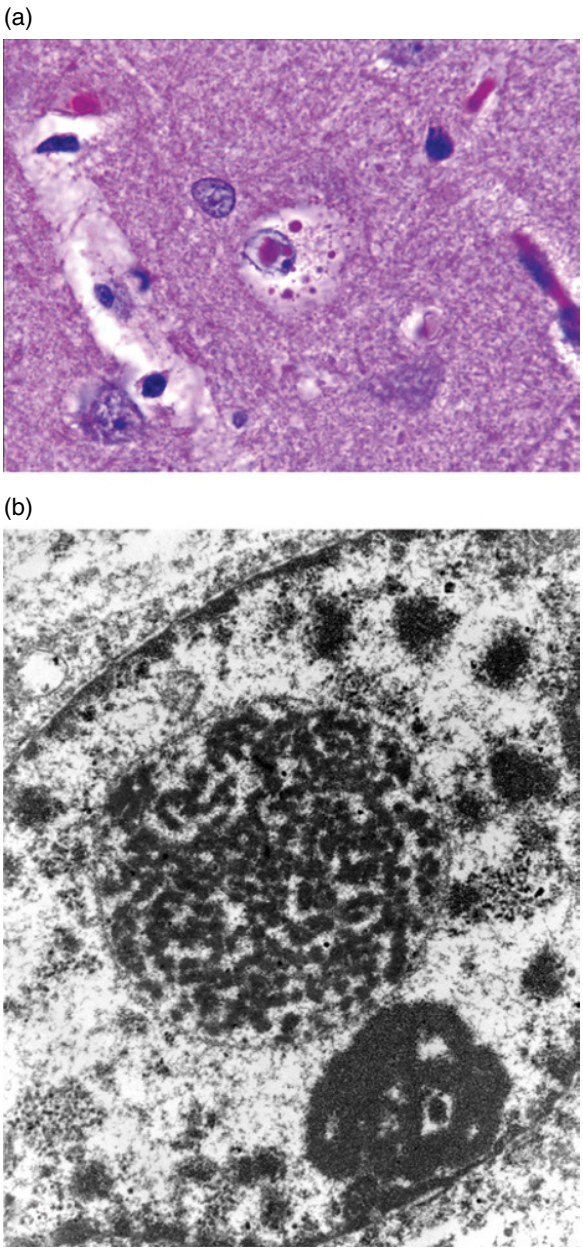
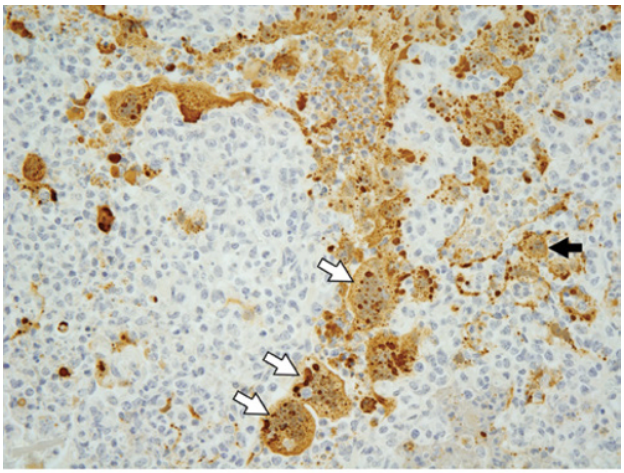


Figure 59.3 Canine distemper virus (CDV) forms both intranuclear and cytoplasmic inclusion bodies, a feature unique to morbilliviruses. (a) Eosinophilic (pink) intranuclear and intracytoplasmic inclusions are present in an astrocyte of an infected raccoon brain (H&E stain). The intranuclear inclusion is contrasted with the basophilic (blue) nucleolus. Cytoplasmic inclusions are aggregates of viral nucleocapsid, often referred to as a viral "factories." (b) Intranuclear inclusions are a unique cell structure known as complex nuclear bodies, shown here by transmission electron microscopy (osmium tetroxide stain). The nuclear bodies contain coarse filaments surrounded by a fine fibrillar capsule (top) and are readily distinguished from the compact reticulate nucleolus (bottom). The structures are induced by intranuclear trafficking and aggregation of the viral N protein and are thought to influence cellular RNA metabolism in a manner that supports viral replication. *Source:* Michael Oglesbee, The Ohio State University.

and devastating outbreaks of distemper have occurred among lions in Africa and tigers in Russia and India.

CD occurs worldwide and remains endemic in many areas despite the widespread use of vaccines that are highly effective in preventing the disease. Infected dogs shed virus in their nasal and ocular secretions, and CDV is present in the urine of experimentally infected dogs 6–22 days after infection. Feces of infected dogs may also contain CDV. Infection usually is transmitted by aerosol or by direct contact, leading to respiratory infection of susceptible animals.

(a)



(b)

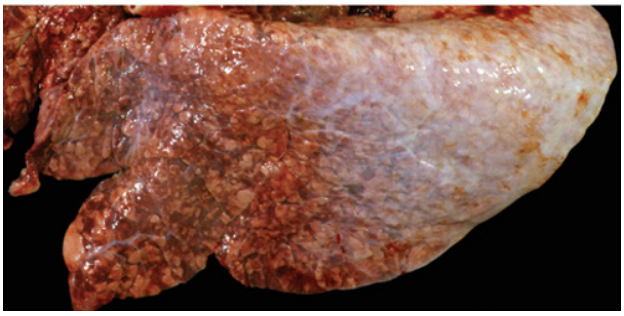


Figure 59.4 Bovine parainfluenza virus type 3 (BPIV3) infection of a seronegative calf, seven days post challenge. (a) Immunoperoxidase staining of viral antigen in lung (brown) shows extensive infection of epithelium in an airway (center). Discrete aggregates of viral antigen in the cytoplasm are the basis for eosinophilic (pink) inclusion bodies that are readily identified by routine hematoxylin and eosin staining, and represent aggregates of virus core particles. Viral envelop proteins mediate fusion of infected epithelial cells to form syncytia (white arrows). Viral antigen positive pulmonary alveolar macrophages are also observed (black arrow). (b) Virus infection precipitates a secondary bacterial bronchopneumonia, and also collapse of lobules associated with airway obstruction by inflammatory cells (atelectasis), illustrated by the brown discoloration of lobules in the cranial and ventral aspects of the lung lobe. *Source:* Illustrations courtesy of John Ellis, University of Saskatchewan.

Pathogenesis and Pathology

CDV is pantropic and has an especially strong tropism for epithelium and lymphoid tissues. The lungs are also central to the pathogenesis of CDV infection. Initial replication of CDV occurs in macrophages in the bronchial lymph nodes and tonsils immediately following respiratory infection, resulting in a primary viremia. The virus then infects other lymphoid tissues (lymph nodes, spleen, thymus, and bone marrow), where further replication occurs, resulting in a secondary viremia that disseminates CDV infection to virtually all the organs of the body, including epithelium of the alimentary, respiratory, and urogenital tracts; the skin and mucous membranes; endocrine glands and the CNS, the latter occurring approximately eight or nine days after infection and only in dogs that fail to develop sufficient titers of neutralizing antibodies to CDV by that time.

Virus-induced cytopathic effects are prominent in the respiratory and alimentary tracts, these being the organs primarily involved in viral shedding and transmission. The lungs of dogs with acute CD have diffuse bronchointerstitial pneumonia, reflecting viral replication in airway epithelium and alveolar macrophages. Secondary bacterial bronchopneumonia is common in dogs as a consequence of virus-induced mucosal damage and immune suppression (Figure 59.1). Characteristic eosinophilic intranuclear and intracytoplasmic inclusion bodies are often present within infected tissues, especially within respiratory epithelium and in the epithelium lining the stomach, urinary bladder, and renal pelvis. Cytoplasmic inclusions are formed by aggregates of virus nucleocapsid (Figures 59.1 and 59.2), whereas intranuclear inclusions are the result of intranuclear transport of the viral RNA-binding protein (N) (Figure 59.3). Recognition of these structures facilitates diagnosis by light microscopy. Extensive necrosis of lymphocytes is characteristic of acute CD, leading to lymphoid depletion that is one basis for immune suppression. Immune suppression may assist the virus in gaining access to the nervous system.

Dogs that survive acute CD may subsequently develop neurological signs that are associated with virus-induced demyelination. The lesions within the CNS of these dogs begin as focal areas of demyelination that are accompanied by lymphocytic inflammation and accumulation of macrophages. Lesions occur within the cerebellum, brain stem, and cerebrum, and CDV inclusion bodies may occur in astrocytes and neurons. Although CDV predominantly replicates in astrocytes and microglial cells within the brains of infected dogs, virus-mediated injury to oligodendrocytes is proposed to be responsible for much of the initial virus-induced demyelination.

Old dog encephalitis is a rare disease of mature dogs that results from very long-term persistent CDV infection of the CNS, leading to dementia. The disease is characterized by severe non-suppurative encephalitis with neuronal degeneration and, often, the presence of CDV inclusion bodies in neurons and astrocytes. Demyelination is not a typical feature of this disease.

Host Response to Infection

CD virus infection induces long-lasting immunity in dogs that recover from natural infection. Protective neutralizing antibodies first appear in serum of infected dogs eight to nine days after infection and persist for years

thereafter. Cell-mediated immune responses are also generated by dogs infected with CDV. Virus-induced immune suppression contributes to the pathogenesis of CDV infection by predisposing affected dogs to secondary bacterial infections and by facilitating spread of the virus to the CNS.

Laboratory Diagnosis

As the clinical signs of CD are variable and sometimes nonspecific, definitive diagnosis requires identification of CDV by PCR. Serological diagnosis can be performed by demonstrating rising IgG titers in paired sera by ELISA. Routine histological evaluation of postmortem cases allows a presumptive diagnosis based upon distribution of lesions and the occurrence of cytopathic effects that include formation of intranuclear and intracytoplasmic inclusion bodies and formation of virus-induced syncytia and may be confirmed by immunohistochemistry

Control and Prevention

Treatment for CD is supportive, such as the use of antibiotics to control secondary bacterial infections and electrolyte solutions to restore fluids and electrolytes. Vaccination against CD is the best means of preventing the disease. Both modified-live and inactivated CD viral vaccines are available, and vaccination has greatly reduced the incidence of this disease in dogs. Maternally derived antibody to CDV can interfere with the successful vaccination of puppies against CDV. Puppies should be vaccinated for CD at an age when maternally derived antibody has been metabolized. Although modified-live virus CDV vaccines safely have been used to immunize a variety of wild animal species, including foxes, ferrets, mink, bush dogs, and maned wolves, there have been reports of vaccine-induced CD in a variety of species, including mink, kinkajous, and lesser pandas after immunization with modified-live CDV vaccine. Similarly, instances of vaccine-induced CD have been described in dogs. In order to provide a safer vaccine, a canarypox virus vector vaccine has been developed that expresses both the hemagglutinin and the fusion protein of CDV. Versions of this vaccine are marketed for dogs and ferrets. Ferrets are highly susceptible to CDV (almost always a fatal infection), and use of a modified-live vaccine in ferrets can lead to CD.

Feline Morbillivirus

Feline morbillivirus was identified in 2012 in stray cats in Hong Kong. It has subsequently been detected worldwide. The pathogenesis of virus infection is still not well understood, but a connection to chronic kidney disease is suspected.

Morbilliviruses of Aquatic Mammals (Phocine Distemper and Cetacean Morbilliviruses)

A number of epidemics in pinnipeds and cetaceans have been determined to be due to morbillivirus infections. Phocine distemper is an infectious disease of seals that resembles CD. The disease first was recognized in the late 1980s when massive die-offs of seals occurred in the Baltic and North seas. The lesions and clinical signs in affected seals were remarkably similar to those of CD (i.e. pneumonia and sometimes evidence of CNS infection), and the disease was subsequently shown to be caused by Phocine distemper virus (PDV), a morbillivirus that is closely related to CD virus. A similar disease caused extensive mortality among dolphins and porpoises in the early 1990s, and this was attributed to two distinct viral strains (dolphin and porpoise morbilliviruses) that are collective known as cetacean morbilliviruses. The cetacean morbilliviruses and PDV are closely related. In addition, seals have been found to be infected with CD virus, leading to speculation that morbilliviruses infecting aquatic mammals might be derived from CDV.

Rinderpest

RPV was declared eradicated on May 25, 2011. After human poxvirus (declared eradicated in 1979), RPV is the second virus to have ever been eradicated worldwide and the first virus recognized to be of veterinary importance.

Disease

Rinderpest (also known as cattle plague) was a devastating pandemic viral disease of domestic cattle, buffalo, and some wild ruminant species. RPV infects a number of species of the order Artiodactyla, which includes cattle, water buffalo, pigs, warthogs, and many species of African antelope. The reported infection of sheep and goats is now thought to be due to peste de petits ruminants (PPR) virus. Rinderpest is characterized by fever, lymphopenia, nasal, and lacrimal discharges, diarrhea, and erosions and ulcers throughout the oral cavity (ulcerative stomatitis). The disease has an incubation period of three to eight days and the virus is extremely contagious, so morbidity rates are very high. The mortality rate also can be high (up to 100%) in cattle that have no immunity, but varies depending on the virulence of the infecting virus strain.

Host–Virus Relationship

Geographic Distribution and Transmission

Rinderpest historically occurred throughout Europe, Africa, and Asia but never became established either in the Americas or in Australasia. Before eradication, the virus was enzootic in certain regions of Asia, the Middle East, and Africa.

RPV is present in high titers in the nasal discharges, saliva, ocular secretions, and excretions of infected animals, and transmission requires direct contact of susceptible animals with the secretions and excretions of infected animals.

Pathogenesis and Pathology

RPV infection normally occurs through the upper respiratory tract, although cattle can be experimentally infected by any parenteral route of inoculation. The virus first replicates in the tonsils and regional lymph nodes, and a predominantly lymphocyte-associated viremia then disseminates the virus throughout the body. The virus replicates in lymphoid tissues (spleen, bone marrow, lymph nodes), and the mucosa of the alimentary and upper respiratory tracts. Nasal and ocular secretions contain high titers of virus at this time. Fever (up to 107 °F) and leukopenia occur prior to the appearance of oral ulcers and onset of diarrhea that may contain blood (dysentery). Titers of virus in the tissues and secretions of infected cattle decline after the appearance of neutralizing antibodies in serum. The convalescent phase begins with the healing of mouth lesions, and complete recovery from the disease may take four to five weeks.

Lesions of rinderpest occur in the tissues in which the virus replicates. Oral ulcers are characteristic of severe cases of rinderpest in cattle, and these begin as areas of necrosis in the basal cell layer of the epithelium. Necrosis also occurs in the mucosa of the abomasum and small intestine. The presence of syncytial cells within the affected epithelium is highly characteristic of rinderpest, but not of other ulcerative diseases like malignant catarrhal fever or bovine viral diarrhea. There is also extensive necrosis of gut-associated lymphoid tissues as a result of virus-mediated destruction of lymphocytes, and similar lympholysis occurs in lymph nodes, spleen, and bone marrow.

Eradication

The concerted efforts of a number of international organizations led to the eradication of rinderpest, which over the last few years resided in pockets in east Africa. The following aspects of the eradication program were seen as essential for eradication: a short infectious period of infected animals, no persistent infection and no viral reservoirs, transmission only by close contacts, a safe and reliable vaccine protecting against all viral strains, easy and reliable diagnosis, and an economic incentive to comply with the program.

Peste des Petits Ruminants

Disease

PPR viruses infect goats and sheep. Clinical symptoms are similar to rinderpest in cattle, although both viruses are clearly distinct. Cattle and pigs can be infected and develop antibodies in the absence of clinical symptoms. The infection leads to high fever in goats and sheep after an incubation period of three to nine days with subsequent pneumonia and diarrhea. The severity of symptoms depends on species, breed, age, and the presence of secondary infections.

Host–Virus Relationship

Geographic Distribution and Transmission

The PPR virus is found in both Africa and Asia. It has recently been found in China and African countries bordering Europe. It is considered a foreign animal disease in the United States, and suspected cases are reportable. The World Organization for Animal Health (OIE) in conjunction with other agencies has set a goal to eradicate this disease by 2030. Its economic impact is particularly important in lower-income communities that depend on small ruminants for their livelihoods. The virus induces an acute infection so that there is no persistently infected animal reservoir. It is passed through direct contact between animals or contact with respiratory secretions or feces of infected animals.

Pathogenesis and Pathology

PPR virus infection occurs through the upper respiratory tract. The virus first replicates in the tonsils and regional lymph nodes, and a predominantly lymphocyte-associated viremia then disseminates the virus throughout the body. With the onset of fever, respiratory symptoms such as congestion of the mucous membranes of the eyes, nose and mouth, and ocular and nasal discharge are seen. Many animals show labored breathing and a productive cough. Necrotic lesions begin to appear as white/gray spots that spread throughout the mucosa of the mouth, and these eventually turn yellow and become encrusted with exudate. Animals continue to drink but stop eating. Some animals develop diarrhea one to

three days later due to infection of the gastrointestinal mucosa. In fatal cases, animals will die 8–12 days after the onset of disease. During infection and recovery, animals are immunosuppressed as indicated by low white blood cell counts and susceptibility to secondary infections. At postmortem examination, congestion of lung tissue is seen with marked infiltration of macrophages and proliferation of pneumocytes. In contrast to RPV infection, syncytia are seen in lung epithelium.

Laboratory Diagnosis

Clinically, PPR virus infection can often not be easily distinguished from other infectious diseases of goats and sheep. The laboratory diagnosis of acute infection is based on the presence of virus as detected by PCR or capture ELISA, with prior infection diagnosed based on detection of antiviral antibody by ELISA.

Control and Prevention

The best way to prevent PPR virus infection is to separate infected and uninfected animals. As such, the movement of livestock from endemic areas should be banned. If that is not feasible, movement must be controlled by quarantine and surveillance. As another preventive measure, vaccination with a modified-live virus should be performed. Vaccination induces immunity for at least three years and has been used to control outbreaks.

Respiroviruses

Bovine Parainfluenza Virus Type 3

Disease

Clinical disease due to infection with bovine parainfluenza virus type 3 (BPIV3) is most common in calves with poor passive transfer of, or decayed, maternal antibodies. It is usually a mild disease with fever, nasal discharge, and dry cough. Disease can be more severe in seronegative calves, with high fever for up to seven days and clinical signs ranging from tracheitis to pneumonia. BPIV3 infection is often part of the bovine respiratory disease complex (see Section 59.19).

Host–Virus Relationship

Geographic Distribution and Transmission

BPIV3 was first isolated in the United States but has been found to be endemic in cattle populations worldwide. It is transmitted through the respiratory route via nasal mucus and ocular secretions. The severity of disease is promoted by poor ventilation, close contact due to overcrowding, and stressed feedlots. Although antibodies reactive with BPIV3 have been found in a number of species (mostly ungulate), the importance of cross-species transmission is not resolved.

Pathogenesis and Pathology

After gaining access to the respiratory system, BPIV3 infects epithelia cells. This leads to loss of ciliary function of epithelia cells. In addition, the virus infects pulmonary macrophages, leading to a weakened immune response. The inhibitory effects on the clearance function of the mucociliary apparatus and on the local and systemic immune responses contribute to the establishment of secondary bacterial infections that are commonly associated with the bovine respiratory disease complex. The histological hallmarks of BPIV3 infection are bronchitis, bronchiolitis, and alveolitis. During acute infection, typical eosinophilic inclusion bodies are seen in epithelium, and infected epithelial cells may form syncytia (Figure 59.4) During the repair phase beginning 14 days after infection, hyperplasia of airways and alveolar epithelium is seen.

Laboratory Diagnosis

During the acute infection, virus can be cultivated from nasal secretions or be detected by PCR. To detect antibodies, the hemagglutination inhibition assay, the neutralization assay, and (less often) ELISA are used. Antibody diagnosis may be complicated by the presence of maternal antibodies or antibodies due to the endemic nature of the virus. To demonstrate acute infection, an increase in antibody must be observed in paired serum samples from acutely infected and convalescent animals.

Control and Prevention

Good husbandry practices that avoid overcrowding, stress, and poor ventilation help to control the transmission and severity of disease. Inactivated and modified-live PI-3 viral vaccines are available, usually combined with other viral or bacterial vaccines.

Rubulavirinae

Parainfluenza Virus Type 5 (Canine Parainfluenza Virus)

Canine parainfluenza virus type 5 has been implicated as a cause of infectious tracheobronchitis (kennel cough) in dogs, along with canine adenovirus 2, CD virus, canine herpesvirus, canine influenza virus, canine respiratory

coronavirus, and *Bordetella bronchiseptica*. Spread is by short-distance aerosols or fomites; thus, this virus is most frequently seen in dogs in kennels, shelters, or boarding/daycare facilities. The disease is characterized by sudden onset, mild fever, slight to copious nasal discharge, and a harsh, nonproductive cough. Canine parainfluenza virus is antigenically related to the Simian virus-5 virus of monkeys, and it appears that the same or very similar viruses infect a wide variety of animal species. Detection is by PCR from a nasal or oropharyngeal swab. Modified-live viral vaccines are available, usually combined with other canine viral vaccines such as CD and canine adenovirus or with a *B. bronchiseptica* vaccine.

Pneumoviridae

This taxon was formally classified as a subfamily within *Paramyxoviridae*, but was reclassified as a family in 2016. Members of the genus *Orthopneumovirus* infect mammals, whereas members of *Metapneumovirus* are specific for mammals or birds. There are no known vectors for these viruses, and transmission is thought to be primarily by aerosol droplets and contact.

Metapneumoviruses

Metapneumoviruses are distinguished from orthopneumoviruses on the basis of their gene order and virion protein composition. Avian and human metapneumoviruses are assigned to this group.

Avian Metapneumoviruses

Disease

Clinical disease due to infection with avian metapneumoviruses (aMPV) occurs worldwide in poultry. It causes respiratory disease in turkeys (turkey rhinotracheitis) and is associated with swollen head syndrome in chickens. Both are characterized by tracheal rales, sneezing, swollen sinuses, swollen head, and nasal and ocular discharge. In ducks and chickens, aMVP causes a significant drop in egg production. The incubation period is 2–7 days. The mortality varies from 1% to 50% depending on the age of the animals and the occurrence of secondary infections.

Host–Virus Relationship

Geographic Distribution and Transmission

aMVP was first isolated in the 1970s in turkeys in South Africa. Since then the virus has been found in all major egg-producing countries with the exception of Australia.

Pathogenesis and Pathology

After gaining access to the respiratory system, aMVP infects epithelia cells. This leads to loss of ciliary function of epithelia cells. In addition, the virus may also infect pulmonary macrophages. The inhibitory effects on the clearance function of the mucociliary apparatus and on the local and systemic immune responses contribute to the establishment of secondary bacterial infections. The histological hallmarks of aMVP infection are rhinitis, tracheitis, sinusitis, and airsacculitis.

Laboratory Diagnosis

During the acute infection, virus can be cultivated from nasal secretions or be detected by PCR. To detect antibodies, ELISA, virus neutralization assay, and immunohistochemistry are used. Both acute and convalescent (paired) serum samples should be submitted for analysis.

Control and Prevention

Good management practices with optimal stocking density, good ventilation, temperature control, and biosecurity help to control the transmission and severity of disease. Inactivated and modified-live aMVP viral vaccines are available.

Orthopneumoviruses

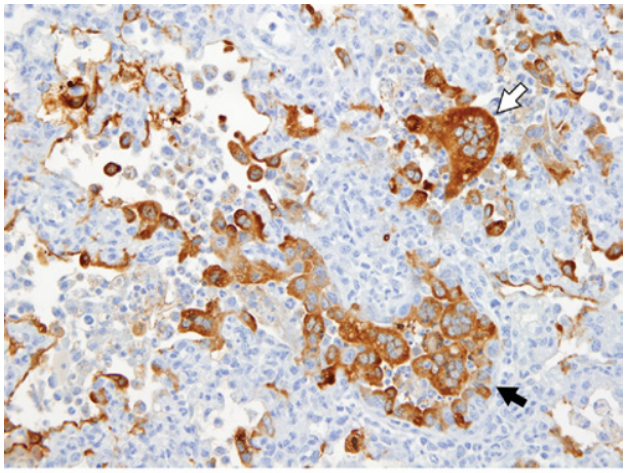
Viruses within the genus *Orthopneumovirus* include respiratory syncytial viruses that infect humans, cattle, and mice, as well as those that infect sheep and goats, which are closely related to bovine respiratory syncytial virus (BRSV). The viruses within this genus are distinguished on the basis of their host range and lack of cross-neutralization. The members of this group lack neuraminidase, and the nucleocapsid has a diameter of 13–14 nm, which is smaller than the nucleocapsid of the paramyxoviruses (18 nm). All members of the genus *Orthopneumovirus* are antigenically related but are antigenically distinct from other paramyxoviruses and pneumoviruses. Their receptor-binding protein is designated as G protein.

Bovine Respiratory Syncytial Virus

Disease

The BRSV is a major cause of respiratory disease in cattle (Figure 59.5). It causes an acute pneumonia in calves, which show clinical signs such as coughing, fever, anorexia, nasal discharge, and respiratory distress. Infection of cattle with BRSV leads to major economic losses.

(a)



(b)

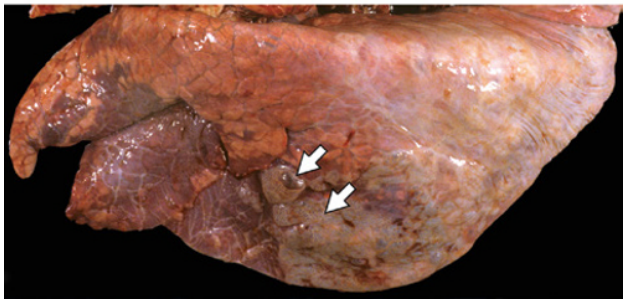


Figure 59.5 Bovine respiratory syncytial virus (BRSV) infection of a seronegative calf, eight days post challenge. (a) Immunoperoxidase staining of viral antigen in lung (brown) shows extensive infection of airway epithelium. Expression of viral membrane proteins causes extensive fusion of epithelial cells to form large syncytia (white arrow), and syncytia are widespread in an airway present in the lower portion of this field (black arrow). The more linear pattern of immunoperoxidase staining reflects viral infection of alveolar epithelium. Cytoplasmic viral inclusion bodies are not conspicuous. (b) Virus infection precipitates a secondary bacterial bronchopneumonia, and also collapse of lobules associated with airway obstruction by inflammatory cells (atelectasis), illustrated by the brown discoloration of lobules in the cranial and ventral aspects of the lung lobe (lower left). Airway obstruction leads to rupture of air spaces during labored breathing, giving rise to emphysematous change (large pockets of trapped air; white arrows). *Source:* Illustrations courtesy of John Ellis, University of Saskatchewan.

In addition, BRSV is a major contributor to the bovine respiratory disease complex, which has a multifactorial etiology and may be associated with multiple organisms including bovine parainfluenza virus (BPIV), bovine herpesvirus 1, bovine coronavirus, bovine adenovirus 3, bovine viral diarrhea virus, *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and/or *Mycoplasma* species. The resulting clinical syndrome has been labeled bovine respiratory disease complex or "shipping fever." The disease is characterized by high fever, conjunctivitis, respiratory distress, mucopurulent rhinitis, and pneumonia, and occurs after cattle congregate on feedlots. The disease is widespread in the United States and remains one of the major causes of economic losses in the cattle industry.

Host–Virus Relationship

Geographic Distribution and Transmission

BRSV was first identified in Europe in 1970 but has since been shown to be present worldwide in cattle populations, causing respiratory diseases. The virus is transmitted through nasal secretions and close contact.

Pathogenesis and Pathology

After infection with the virus through the respiratory route, epithelial cells of the respiratory tract are infected. Animals develop fever, coughing, low nasal discharge, and tachypnea between three and nine days after infection. By gross pathology, lungs are red, depressed, and firm after uncomplicated BRSV infection. Histologically, bronchial and bronchiolar epithelial necrosis and multinucleated syncytial cells are found. Sometimes, intraepithelial inclusion bodies are detected in airways, although their formation is less conspicuous compared to that associated with BPIV type 3. Younger animals are more severely infected than older ones, and the complication by secondary infections (see Section 59.19 on the bovine respiratory disease complex) leads to more severe disease and economic losses.

Laboratory Diagnosis

Detection of BRSV in nasal secretion or lung by immunofluorescence and quantitative RT-PCR assays is being used to diagnose BRSV. Paired serum samples (with a 10–14 days interval) can be used to determine infection through an increase in antibody titers by neutralization assays.

Control and Prevention

Given the high prevalence of BRSV worldwide, control of the virus must be attempted by vaccination. Experimental vaccination using formalin-inactivated virus did not protect against infection but instead lead to an enhanced disease after infection with wild-type virus. This is very similar to findings with formalin-inactivated vaccine against human RSV in people. Currently, inactivated and modified-live vaccines are being used, usually in combination with other viral and bacterial vaccines.

Filoviridae

Viruses within this family are characterized by enveloped, pleomorphic (filamentous) virions and a genome of single-stranded negative-sense RNA. The family includes six genera, but there are there are two major groups of filoviruses: Ebola and related viruses, and Marburg and related viruses, that are pathogenic for humans and highly virulent. Viruses within these groups cause severe, fatal diseases of humans that are characterized as "hemorrhagic fevers" because of the spectacular diseases they cause in affected humans. These viruses infect primates and can be adapted to laboratory animals. Outbreaks in human populations are rare and geographically restricted to certain parts of Africa. It is hypothesized that contact between humans and a rodent virus reservoir is the cause of these outbreaks.

Bornaviridae

Viruses within this family are characterized by enveloped spherical virions that are 80–140 nm in diameter, and a genome of single-stranded, negative-sense RNA. In contrast to other viruses of the Mononegavirales family, bornaviridae use the nucleus of host cells for transcription and replication. Avian bornavirus (ABV) and Borna disease virus (BDV) are members of this family.

Avian Bornavirus

An ABV is the causative agent of proventricular dilatation disease (PDD) in psittacine birds. The disease is also known as macaw wasting syndrome, proventricular dilatation syndrome, neuropathic gastric dilatation of psittaciforms, or myenteric ganglioneuritis. Not all birds have proventricular dilation. Birds affected by PDD frequently show weight loss with reduced appetite and various degrees of gastrointestinal dysfunction. In a number of cases, central nervous disease symptoms that include seizures, ataxia, and motor deficits can be observed. The disease is progressive and ultimately fatal. ABV infection in psittacine birds has been documented worldwide. In addition, case reports of ABV infection in other species of bird suggest that the host spectrum of this virus is larger than currently defined, and that persistent infection without clinical symptoms occurs often. Most positive birds do not exhibit clinical signs of disease. There are different genotypes (producing several distinct viral species), and virulence varies with the genotype and species of bird infected. Diagnosis is complicated by intermittent shedding of the virus in droppings and unpredictable seroconversion. Multiple negative tests (including PCR and serology) are required to determine that a bird is negative.

Borna Disease Virus

Originally, Borna disease was described as a neurological disease of horses in Germany, but since then, BDV infections have been discovered worldwide. The precise significance of BDV as a veterinary pathogen remains to be adequately defined. Natural infections with BDV are most frequently seen in sheep and horses. The natural reservoir for BDV has not been defined. Cases are more frequent in spring and early summer, suggesting arthropods as potential vectors, but the virus has not been isolated from insects. BDV is neurotropic and persistently infects neurons in the brain that may result in clinical symptoms. Cattle, rabbits, goats, dogs, cats, and rodents can be experimentally infected with BDV. Whether BDV is the cause of neuropsychiatric disorders in humans is fiercely debated. BDV infection can be determined by the presence of antiviral antibodies. ABV infection is currently diagnosed by PCR. No vaccine is available to prevent infection with either virus. To prevent infection, infected animals must be kept separated from uninfected animals. Bornaviruses are susceptible to common disinfectants against enveloped viruses.

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60

Rhabdoviridae

Susan M. Moore and D. Scott McVey

Rhabodviruses infect a wide variety of hosts including vertebrates, invertebrates, and plants. The genomes, typically non-segmented, single-stranded RNA, produce enveloped bullet-shaped virions. Plant rhabdoviruses are bacilliform, and some are reported to be non-enveloped and filamentous.

Next generation sequencing techniques combined with increased research into possible emerging infectious diseases of zoonotic potential has led to the expansion of assigned genera and species within the Rhabdoviridae family. Currently, viruses in this family are grouped into 30 assigned genera, 20 of which have the known or potential ability to infect vertebrates (Table 60.1). Most of the assigned rhabdovirus 191 species are known primarily from meta sequencing and are largely uncharacterized. Included in the 30 genera are viruses of public health and economic importance causing severe infection and even death in mammals, fish, and plants. In mammals, rabies virus infection is almost invariably fatal in both humans and animals once clinical signs are evident, vesicular stomatitis virus (VSV) causes a disease similar to foot-and-mouth disease (FMD), Chandipura-virus-caused encephalitis is fatal in 55–77% of cases, and bovine ephemeral fever (BEF) virus causes a disabling disease in cattle and water buffalo. Three of the Rhabdoviridae genera contain fish pathogens of significance to farming operations. Those in *Novirhabdovirus* genus are infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV). Sea trout and perch rhabdovirus are in the *Perhabdovirus* genus. Spring viremia of carp virus (SVCV) in the *Sprivivirus* genus causes a severe hemorrhagic disease of cyprinids. Plant rhabdoviruses can cause plant diseases that are economically devastating to food crops including maize mosaic, rice transitory yellowing, and potato yellow dwarf disease. The best-studied viruses in the Rhabdoviridae family are rabies virus (RABV) and VSV. Most rhabdoviruses contain five structural genes coding for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large subunit protein (L). The L gene is broadly conserved; thus, L sequences are the basis for genera and species assignment along with genome architecture, and serological and ecological properties. The G protein is the target of neutralizing antibodies. Some low-level serological cross-reactivity has been reported to occur between specific rhabdoviruses.

Rabies

Disease

Rabies is a progressive encephalitic disease that almost always results in the death of the victim although rare cases of prolonged life and the survival of one human without the administration of vaccine has been reported using the Milwaukee protocol or other supportive care approaches. In many of the reported "survivors" in recent years, the resulting sequelae is severe. In humans, early symptoms of rabies are indeterminate, flu-like, and may include general malaise, headache, fever, and weakness, or discomfort. These signs progress into more specific symptoms and may include insomnia, anxiety, confusion, partial paralysis, hypersalivation, hydrophobia, hallucination, and painful spasms of the pharyngeal muscles. Pain or tingling at the sight of the initial exposure wound (paresthesia) may also occur. Death usually occurs within a few days of the onset of these symptoms; with intensive medical support the period between symptoms and death may be prolonged. In animals, as in humans, the first signs of illness may be nonspecific and even overlooked. Early signs of rabies may include one or more of the following: lethargy, fever, vomiting, and anorexia. These signs progress rapidly to cerebral dysfunction, ataxia, weakness,

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Table 60.1 *Rhabdoviridae* genus with virus species in warm-blooded animals and fish.

Genus	Virus name	Biology
	Vertebrate host	
Lyssavirus	**Rabies lyssavirus**[a]	Reservoirs include carnivores and bats with specific lyssaviruses associated with specific mammals. RABV is most broadly distributed in species and geography. Spillover infections occur in vertebrates of other species.
	Khujand lyssavirus	
	Lagos bat lyssavirus	
	Aravan lyssavirus	
	Australian bat lyssavirus	
	Bokeloh bat lyssavirus	
	Duvenhage lyssavirus	
	European bat 1 lyssavirus	
	European bat 2 lyssavirus	
	Gannoruwa bat lyssavirus	
	Ikoma lyssavirus	
	Irkut lyssavirus	
	Lleida bat lyssavirus	
	Mokola lyssavirus	
	Shimoni bat lyssavirus	
	Taiwan bat lyssavirus	
	West Caucasian bat lyssavirus	
Novirhabdovirus	Hirame novirhabdovirus	Reservoir fish; host range size varies largely per virus species; broad geographic range
	Piscine novirhabdovirus[a]	
	Salmonid novirhabdovirus[a]	
	Snakehead novirhabdovirus	
Perhabdovirus	Anguillid perhabdovirus	Reservoir fish, including eel, perch, trout; broad geographic range
	Perch perhabdovirus	
	Sea trout perhabdovirus	
Sprivivirus	**Carp sprivivirus**[a]	Reservoir fish, primarily cypriniform fish; broad geographic range
	Pike fry sprivivirus	
	Vertebrate host, arthropod vector	
Ephemerovirus	Adelaide river ephemerovirus	Disease caused by BEFV and Kotonkan virus (KOTV) in cattle and water buffalo; Only BEFV has worldwide distribution; Isolates from cattle, mosquitoes, midges
	Berrimah ephemerovirus	
	Bovine fever ephemerovirus[a]	
	Kimberley ephemerovirus	
	Koolpinyah ephemerovirus	
	Kotonkan ephemerovirus	
	Obodhiang ephemerovirus	
	Yata ephemerovirus	
Vesiculovirus	Alagoas vesiculovirus	Wide range including humans and livestock/ insects
	American bat vesiculovirus	
	Carajas vesiculovirus	
	Chandipura vesiculovirus	
	Cocal vesiculovirus	
	Indiana vesiculovirus	
	Isfahan vesiculovirus	
	Jurona vesiculovirus	
	Malpais Spring vesiculovirus	
	Maraba vesiculovirus	
	Morreton vesiculovirus	
	New Jersey vesiculovirus	
	Perinet vesiculovirus	
	Piry vesiculovirus	
	Radi vesiculovirus	
	Yug Bogdanovac vesiculovirus	

Table 60.1 (Continued)

Genus	Virus name	Biology
Arurhavirus	Aruac arurhavirus	Isolated from mosquitoes, neutralizing antibodies (NA) found in rodents and birds
	Inhangapi arurhavirus	
	Santabarbara arurhavirus	
	Xiburema arurhavirus	
Barhavirus	Bahia barhavirus	Isolated from mosquitoes, NA found in humans, cattle, sheep, rats, and opposums
	Muir barhavirus	
Curiovirus	Curionopolis curiovirus	Isolates from midges, mosquitoes, sandflies; NA found in South American coati and capuchin monkey
	Iriri curiovirus	
	Itacaiunas curiovirus	
	Rochambeau curiovirus	
Hapavirus	Flanders hapavirus	Isolates from birds, reptiles (Marco only), midges, mosquitoes; NA found in sea birds and marsupials
	Gray Lodge hapavirus	
	Hart park hapavirus	
	Holmes hapavirus	
	Joinjakaka hapavirus	
	Kamese hapavirus	
	Lo Joya hapavirus	
	Landjia hapavirus	
	Manitoba hapavirus	
	Marco hapavirus	
	Mosqueiro hapavirus	
	Mossuril hapavirus	
	Ngaingan hapavirus	
	Ord River hapavirus	
	Parry Creek hapavirus	
	Wongabel hapavirus	
Ledantevirus	Barur ledantevirus	Infect mammals/ isolated from arthropods, bats, humans, rodents, ungulates
	Fikirini ledantevirus	
	Fukuoka ledantevirus	
	Kanyawara ledantevirus	
	Kern Canyon ledantevirus	
	Keuraliba ledantevirus	
	Kolente ledantevirus	
	Kumasi ledantevirus	
	Le Dantec ledantevirus	
	Mount Egon bat ledantevirus	
	Nishimuro ledantevirus	
	Nkolbisson ledantevirus	
	Oita ledantevirus	
	Vaprio ledantevirus	
	Wuhan ledantevirus	
	Yongjia ledantevirus	
Lostrhavirus	Lonestar zarhavirus (LSTRV)	Disease in humans, isolated from ticks
Mousrhavirus	Moussa mousrhavirus	Isolated from mosquitoes
Ohlsrhavirus	Culex ohlsrhavirus	Isolated from mosquitoes
	Northcreek ohlsrhavirus	
	Ohlsdorf ohlsrhavirus	
	Riverside ohlsrhavirus	
	Tongilchon ohlsrhavirus	
Sawgrhavirus	Connecticut sawgrhavirus	Isolated from ticks; NA found in eastern cottontail rabbits
	Island sawgrhavirus	
	Minto sawgrhavirus	
	Sawgrass sawgrhavirus	
Sripuvirus	Almpiwar sripuvirus	Infect reptiles; isolated from sandflies
	Chaco sripuvirus	
	Charleville sripuvirus	
	Cuiaba sripuvirus	
	Hainan sripuvirus	
	Niakha sripuvirus	
	Sena Madureira sripuvirus	
	Sripur sripuvirus	

(*Continued*)

Table 60.1 (Continued)

Genus	Virus name	Biology
Sunrhavirus	Garba sunrhavirus	Isolated from birds, midges, mosquitoes; NA horses and crocodiles, feral pigs
	Harrison sunrhavirus	
	Kwatta sunrhavirus	
	Oakvale sunrhavirus	
	Sunguru sunrhavirus	
	Walkabout sunrhavirus	
Tibrovirus	Bas-Congo tibrovirus	Infect cattle, water buffalo, humans; Isolated from midges
	Beatrice Hill tibrovirus	
	Coastal Plains tibrovirus	
	Ekpoma 1 tibrovirus	
	Ekpoma 2 tibrovirus	
	Sweetwater Branch tibrovirus	
	Tibrogargan tibrovirus	
Tupavirus	Durham tupavirus	Fish
	Klamath tupavirus	
	Tupaia tupavirus	
Zarhavirus	Zahedan zarhavirus	Isolated from ticks

Genera with pathogenic rhabdoviruses species are in italic.
[a]Virus species in bold are described in the chapter text.

paralysis, seizures, difficulty breathing, and or swallowing, hypersalivation, abnormal behavior, aggression, and/or self-mutilation. In dogs, rabies has a relatively short course, and they may exhibit one or more of the following signs: drooping jaw and or tongue, abnormal barking, vomiting, biting and eating unusual objects, aggression, biting without provocation, restlessness, or stiff gait. In cats, rabies also causes dramatic changes in behavior. Cats can become very aggressive and exhibit one or more of the following conditions: poor body condition, ruffled, and dirty coat, high fever, restlessness, and pupil dilation. Approximately 90% of rabid cats exhibit aggressive behavior. Unexpected aggressive or abnormal behavior should be considered suspicious in cats that may have experienced an exposure to a potentially rabid animal. In horses, clinical signs of rabies include behavior changes ranging from aggression, ataxia, paresis, hyperesthesia, fever, colic, lameness, and recumbency. The disease generally results in death in four to five days once clinical signs are evident. In cattle, initial clinical signs may include depression, lack of eating, and seeking isolation. As the disease progresses, cattle exhibit weakness in legs, straining, bellowing, and inability to swallow resulting in excessive salivation.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

The rabies virion, 100–300 nm in length and 75–80 nm in diameter, contains a single-stranded negative-sense RNA genome that is compactly encapsidated with the nucleocapsid (N) protein, the RNA polymerase (L), and polymerase cofactor phosphoprotein (P). This RNP core, surrounded by the matrix protein (M), is condensed into the typical bullet-shaped particle characteristic of rhabdoviruses. Glycoprotein (G) spikes, obtained from the host cell membrane upon budding, are anchored into the RNP-M structure and protrude through the virus envelope, approximately 10 nm, and contribute to (RABV) neurotropism and pathogenicity. Antibodies produced against the G protein after vaccination with cell culture rabies vaccines (CCV) induce the production of protective antibodies and function to neutralize the virus.

The N protein is the most highly conserved of all of the viral proteins but actually has a high degree of diversity within short segments. As a result, molecular analyses of the N protein have provided information on identifying RABV genotypes that can be used as a molecular epidemiological surveillance tool to explain the evolution of rabies viruses, identify the transmitting species, and to track movement of rabies over time. For instance, when rabies is detected in animals in a previously rabies-free zone, the virus can often be traced back to the point of origination. See Figure 60.1a,b for the rhabdovirus structure and genome.

Resistance to Physical and Chemical Agents

RABV is readily destroyed through exposure to soap and other disinfectants, desiccants, and sunlight. The virus is inactivated by heating to 56 °C for 30 minutes or by various diluted chemicals and disinfectants including a 0.1% bleach solution and a 1% formalin solution. Saliva from a rabid animal is no longer considered infective in the environment or on intact skin after it has dried. In the case of human exposure, all wounds should be washed vigorously with soap and water for at least 15 minutes in order to inactivate virus.

Infectivity to Other Species or Culture Systems

RABV can theoretically infect all mammals, but each RABV variant tends to circulate in specific species of animals in each region with occasional spillover into animal species

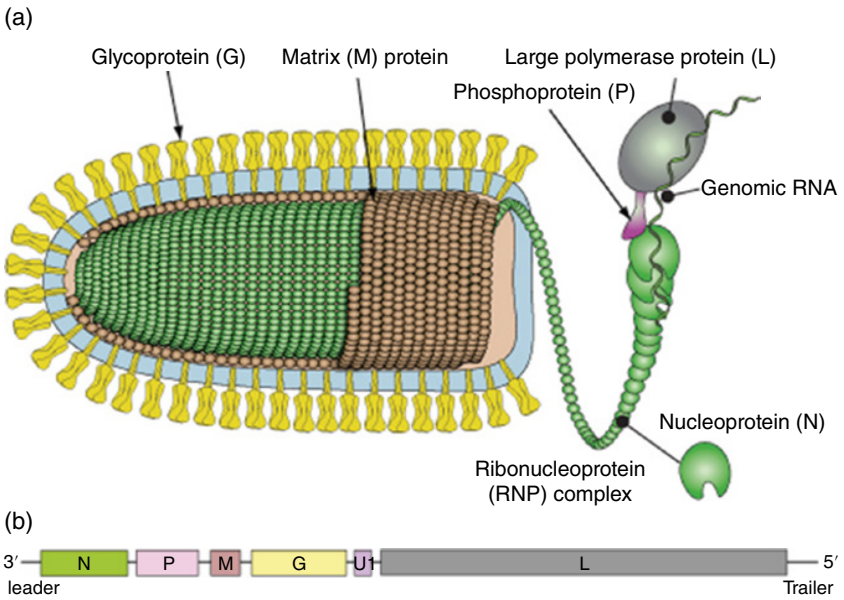


Figure 60.1 (a) Structure of rhabdoviruses. (b) Structure of rhabdoviral single-stranded, negative-sense RNA genome. *Source:* From https://viralzone.expasy.org. ViralZone, SIB Swiss Institute of Bioinformatics.

that co-inhabit the same region. Cross-species transmission presents a risk of variant adaption for sustained transmission into new species. Over 98% of all human rabies deaths occur in Africa and Asia and are caused by exposure to an infected dog. In the Americas, most human deaths result from exposure to bat RABV variants, although there are additional RABV variants circulating in other species of wildlife including raccoons, skunks, and foxes. RABV can be propagated in many different mammalian and plant cell lines for research, diagnostic, and vaccine production purposes. Virus strains specifically used for diagnostic testing and vaccine production have been stabilized or "fixed" to have a predictable incubation period as opposed to "street" virus strains isolated directly from infected animals that may have varying incubation periods. There are several cell lines that are commonly used for diagnostic purposes including neuroblastoma cells (CCL-131) and baby hamster kidney cells (BHK-21). Several cell lines have been used to produce human and animal vaccines. The most commonly used types include embryonated egg cells, Vero cells, human diploid cells, and baby hamster kidney cells.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Rabies is an underreported disease that is present on every continent except Antarctica. Human rabies, especially paralytic rabies, may represent as much as 30% of total clinical rabies presentations and is often misdiagnosed as other encephalitic diseases such as malaria or Guillain-Barré syndrome, thus masking the true global burden of the disease. Although all mammals are susceptible to rabies, the primary reservoirs of the disease belong to the orders *Carnivora* and *Chiroptera* (dogs, foxes, jackals, coyotes, raccoon dogs, skunks, raccoons, mongoose, and bats). Maintaining rabies in a susceptible animal population is dependent upon several factors including the population and ecological characteristics of the host as well as susceptibility to infection and contact rate. Transmission of rabies from one mammal to another generally occurs through the contamination of tissue with virus-laden saliva inflicted during a bite wound. Non-bite transmission with virus-infected saliva or body fluids occurs via scratches, abrasions, or direct contamination of mucous membranes or open wounds, with greatly decreased probability of infection. RABV cannot penetrate intact skin and therefore, even if infected saliva or body tissues come in contact with intact skin, it should not be considered an exposure. Aerosol transmission has been reported, but it is extremely rare. Transplantation of RABV-infected organs or corneas has resulted in rabies deaths as well.

Pathogenesis and Pathology

RABV infection begins with an exposure after which the virus attaches to the cell surface and penetration occurs. A specific RABV receptor site has not been reported, but various lipids, gangliosides, carbohydrates, and proteins have been implicated. At least four RABV receptors have been proposed: nicotinic acetylcholine receptor, neural cell adhesion molecule receptor, low affinity p75 neurotrophin receptor, and metabotropic glutamate receptor subtype 2. Strong evidence suggests that RABV, after entering the peripheral nerves, travel centripetally to the central

nervous system (CNS) by retrograde fast axonal transport at 50–100 mm/day. The motor neuronal pathway appears to be more important than the sensory pathway; however, differences in the virus pathway spread between dog and bat RABV variants have been proposed to explain the contrast in the typical manifestations of each. The virus crosses the synapse of one dendritic process to another, eventually reaching the CNS and continually moving toward the brain. Once the virus reaches and infects the brain, it moves centrifugally back through the peripheral nerves to various body organs including the salivary glands. In spite of the severe clinical neurological signs, the neuropathological findings in rabies-infected humans and animals are relatively mild, suggesting that neuronal dysfunction occurs in rabies without detectable morphological changes. There may be some congestion of leptomeningeal and parenchymal blood vessels, perivascular cuffing, microglial activation with formation of "Babes" nodules and neuronophagia, but subarachnoid or parenchymal hemorrhage is not a reported feature of rabies. Negri bodies, identified as neuronal intracytoplasmic bodies, may be found in brain tissue from animals or humans infected with "street" RABV strains as opposed to "fixed" viruses. However, Negri bodies are infrequently seen with "fixed" virus strains. Negri bodies are identified as dense, well-defined, oval, or round, eosinophilic cytoplasmic inclusions that are typically 2–10 μm in diameter (Figure 60.2). Pathological studies to explain severe clinical disease and fatal outcome tend to be fewer but have indicated degenerative structural changes in neuronal processes such as loss of dendric spines and mitochondrial swelling in axons and dendrites in experimental rabies. Immunostaining of the RABV best defines the extent of CNS infection (Figure 60.3).

Host Response to Infection

RABV synchronously infects the limbic system, causing organically induced fury while the virus is being secreted into the saliva for transmission to the next victim through an inflicted bite. Human rabies patients experience episodes of arousal or hyperexcitability, aggression, confusion, and hallucinations interrupted by periods of lucidity when they are able to understand their condition. After an exposure occurs, the highly neurotropic virus initially replicates at the entry site, enters, and moves along the peripheral nervous system to the spinal cord, eventually reaching the brain. Once RABV reaches the brain, the virus continues to replicate and is disseminated centrifugally through the nerves to the body organs including the salivary glands, where it is emitted into the saliva and is passed on to the next victim.

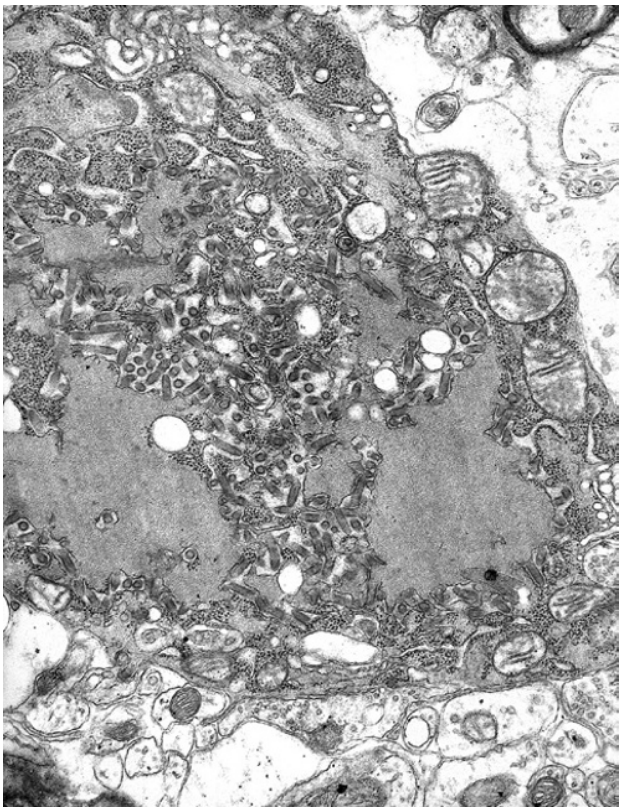


Figure 60.2 This transmission electron microscopic (TEM) image reveals some of the ultrastructural features exhibited by numerous bullet-shaped, rabies virus particles, as well as cellular inclusions known as Negri bodies. *Source:* Courtesy of Dr. Fred Murphy, Centers for Disease Control and Prevention; located in public health image library domain, https://phil.cdc.gov, accessed July 14, 2021; photo ID 1876.

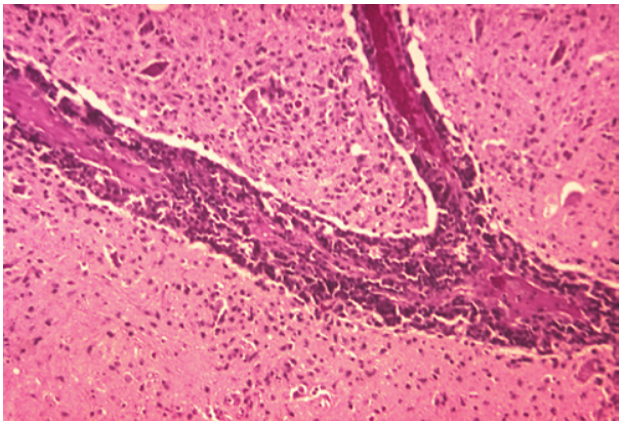


Figure 60.3 This photomicrograph of a hematoxylin–eosin (H&E) stained brain tissue specimen, revealed some of the histopathologic changes that had manifested due to a case of rabies encephalitis. Note the perivascular cuffing due to the accumulation of inflammatory cell infiltrates, including lymphocytes and polymorphonuclear leukocytes (PMNs). *Source:* Courtesy of Dr. Daniel P. Perl, Centers for Disease Control and Prevention: location in public health image library domain http://phil.cdc.gov, accessed July 14, 2021; photo ID # 4069.

Hydrophobia, or "fear of water," is present only in humans. As with humans, initial signs of rabies in animals are nonspecific and may include anorexia, lethargy, fever, dysphagia, vomiting, stranguria, straining to defecate, and diarrhea. As the disease progresses, the behavior of infected animals change, and normally friendly animals may become more aggressive and vice versa. Wildlife may lose their fear of humans, and normally nocturnal animals may be active in the daytime. Pressing or "head butting" may be observed. Clinical rabies in humans may include parasthesias, and a similar clinical sign may be observed in animals as they bite or scratch at the wound site where virus entered. Infected animals may also attack inanimate objects for no reason. The clinical representation of rabies is often categorized as being either "furious" or "dumb" rabies, but it is more likely that clinical signs such as these are likely to be a continuum of expressive behavior as the disease progresses to coma and death. Recovery from rabies is extremely rare, although at least one human has been confirmed to have survived rabies without post-exposure prophylaxis reported after administration of the Milwaukee protocol. Subsequent to this successful outcome, increased attempts of the protocol or other experimental therapeutic approaches have resulted in various degrees of survival in several cases worldwide.

Laboratory Diagnosis

Bites or other exposures from suspected rabid animals and the disposition and testing of the animals in question should involve contacting and working with the appropriate public health departments. The decision as to whether animals that expose humans or other animals should be isolated and observed, or humanely euthanized and submitted for testing is ultimately the decision of professionals in the health department. If an animal is submitted for testing, it should be humanely euthanized and the head removed without damage. Tissue should be kept refrigerated to retard decomposition and provide reliable results because autolyzed samples can reduce the sensitivity and specificity of the test. Multiple regions of the brain are examined to ensure that if virus is present, it will be detected. The fluorescent antibody test (FAT) is the "gold standard" assay by which to confirm the presence of RABV in animal tissues. The FAT detects virus in tissues through the binding of rabies-virus-specific antibodies labeled with a fluorescent dye to RABV. Confirmatory testing on negative tissue samples often includes the use of the mouse inoculation test (MIT) or the rabies tissue culture infection test (RTCIT). The use of the direct rapid immunohistochemical test (dRIT) can be used as an alternative to the FAT with similar sensitivity and specificity. The dRIT is less expensive to use than the FAT and can be used in field conditions. Increased usage of the dRIT in resource-poor countries is a valuable tool to help increase surveillance and better evaluate the global epidemiology of rabies. Increasingly, reverse transcription polymerase chain reaction (RT-PCR) assays are under evaluation as a primary diagnostic assay, in lieu of the FAT, to detect lyssaviruses in CNS tissues. Currently, RT-PCR assays are used as a confirmatory test or for samples unsuitable for the direct fluorescent antibody (DFA) assay. Molecular methods provide identification of specific lyssaviruses, and thus are useful tools to elucidate the global epidemiology of specific virus variants. Because they require expensive equipment, cost analysis within the laboratory is a consideration. Implementation of rapid laboratory diagnoses by a reliable laboratory after a bite incident from a suspected rabid animal can aid in significantly reducing the cost of postexposure prophylaxis (PEP) for humans. If the suspected animal is confirmed to be uninfected with rabies, PEP can be avoided or halted if it has already been initiated.

Treatment and Control

Rabies is virtually 100% fatal, and there are no treatment protocols for animals once clinical signs are evident. However, rabies can be prevented in humans and domestic animals through effective vaccination. Animal rabies vaccines that are currently available include inactivated and recombinant vaccines. Inactivated rabies vaccines that are produced according to the OIE requirements protect against all strains of rabies genotype 1 isolated to date and are administered parenterally initially at three months of age, and then according to the recommendations of the manufacturer. Local and state ordinances may also regulate the timing of rabies vaccination. Modified-live vaccines are not licensed for use in the United States, but may be produced and utilized in other countries. Recombinant vaccines are built on the backbone of another vector virus, for example vaccinia or canary pox viruses, by incorporating the glycoprotein of the RABV into the carrier virus. The recombinant rabies vaccine produced from the canary pox vector is currently licensed for cats in the United States and is administered parenterally. Globally, rabies control and prevention are achieved by adequate vaccination of the free-ranging target reservoir species, which for most rabies endemic regions is the dog. The concept of developing oral rabies vaccines (ORV) to vaccinate wildlife to overcome the need of catching and vaccinating individual animals involved years of research. ORV was initially utilized in 1969 as a strategy in Switzerland to successfully eliminate rabies in red foxes and was a modified-live vaccine (SAD Berne strain) imbedded in chicken heads and

distributed throughout fox habitat. Newer, and safer, ORVs are manufactured as recombinant vaccines that have incorporated the rabies glycoprotein into vaccinia virus vector or the adenovirus vectors. These ORVs have successfully eliminated rabies in foxes in Western Europe and Eastern Canada and in coyotes in southern United States and continue to be an important component of the national strategy in both Europe and the United States. Control of rabies requires coordination of human and animal health branches of government. For the first time in 2015, the World Organization for Animal Health (OIE) and the World Health Organization (WHO) joined together to declare their cooperation in eliminating dog-mediated human rabies by 2030.

Vesicular Stomatitis

Disease

Infection of VSV causes an acute febrile disease of horses, cattle, swine, and deer that is currently limited to the Americas. In cattle, vesicular stomatitis (VS) closely resembles FMD, causing vesicles in the mucosal lining of the mouth and tongue (Figures 60.4 and 60.5). Vesicles can also be found on the teats, the sink of the coronary band, and in the interdigital spaces of the hoof. In swine, the disease is similar to vesicular exanthema (VES) or swine vesicular disease (SVD). Neither VES nor SVD occurs in horses. Therefore, horses can serve as a sentinel species for VS when there is an outbreak of disease. Confirmation of VS is critical in order to prevent the potential spread of one of the three exotic diseases mentioned above and to limit spread within the Americas. VS is a self-limiting disease, with infected animals generally recovering without residual disease impact. During outbreaks, milk production can be reduced in dairy cattle, and the painful lesions can cause infected animals to reduce feed uptake or stop eating altogether, thus impacting production.



Figure 60.4 Horse with vesicular stomatitis. *Source:* Used by permission from Dr. Ashley Salinas, Texas, and Dr. Jason Lombard, Colorado.

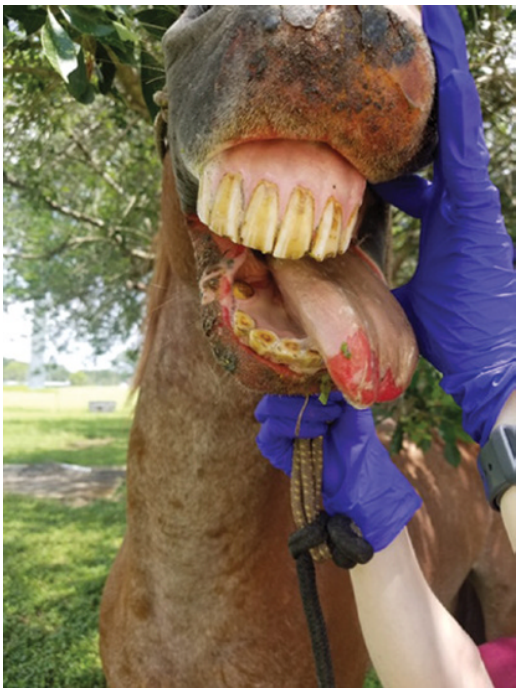


Figure 60.5 Horse with vesicular stomatitis. *Source:* Used by permission from Dr. Ashley Salinas, Texas, and Dr. Jason Lombard, Colorado.

In humans, VS causes flu-like symptoms and can cause severe conjunctivitis when VSV is directly deposited into the mucous membrane of the eye. Infections are generally related to exposure to infected animals.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

VSV closely resembles RABV in morphology, genome, and protein structure. There are two major serotypes of VSV currently identified including: New Jersey (NJ) and Indiana (IND). In addition, the IND serogroup is divided into three subtypes: IND-1 (classical IND), IND-2 (Cocal virus), and IND-3 (Alagoas virus) (ICTV, *2012*).

Resistance to Physical and Chemical Agents

VSV can be inactivated using an effective disinfectant including formaldehyde, either, and other organic solvents, chlorine dioxide, 1% formalin, 1% sodium hypochlorite,

70% ethanol, 2% glutaraldehyde, 2% sodium carbonate, 4% sodium hydroxide, and 2% iodophor disinfectants. Although the virus can survive for long periods at low temperatures, it is readily inactivated by direct sunlight or heating to 58 °C for 30 minutes. VSV can remain stable between pH 4.0 and 10.0 and is resistant to lye (2–3% NaOH will not fully inactivate VSV).

Infectivity to Other Species or Culture Systems

VSV can infect domestic animals including cattle, swine, horses, donkeys, and mules. Natural infection in sheep and goats is rarely reported, but both species can be infected experimentally. Wild animal hosts include white-tailed deer and many species of small mammals. Humans can be infected from handling infected livestock. In the laboratory, experimental infection has been confirmed in mice, rats, guineapigs, deer, raccoons, bobcats, and monkeys. VSV is used as a laboratory model for studies in viral morphology, replication, and genetics.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Although VSV was historically described in France in 1915 and in South Africa in the late 1800s, its current distribution appears to be limited to the Americas. The NJ and subtype IND-1 are endemic, causing more than 80% of all infections in regions of southern Mexico, Central America, Venezuela, Colombia, Ecuador, and Peru. Sporadic outbreaks of NJ and IND-1 VSV have been reported in northern Mexico and western United States. IND-2 is currently reported only in horses in Argentina and Brazil. IND-3 subtype has also been identified sporadically in Brazil mostly in horses but to a lesser degree in cattle. Virus transmission is not fully understood, but VSV has been isolated from sandflies, mosquitoes, and other insects, indicating that they may be involved in virus transmission although disease transmission through direct contact has also been confirmed.

In North America, the disease has often emerged and reemerged along the eastern slopes of the Rocky Mountain region in the early spring in horses, although multiple species of livestock are susceptible. It is not clear why the spread of infection accelerates in some years, but available moisture, vegetation density, proximity of water, seasonal weather patterns, competent vector density, and concentrations of susceptible hosts are all important factors. It is also not clear if the virus persists in a local biological niche through the winter (within vectors potentially).

Pathogenesis and Pathology

The pathogenesis of VS is not entirely clear, but clinically it is indistinguishable from FMD, SVD, and VES. Other diseases to be considered in the differential diagnosis include infectious bovine rhinotracheitis, bovine viral diarrhea, malignant catarrhal fever, bovine popular stomatitis, rinderpest (eradicated in 2011), bluetongue, epizootic hemorrhagic disease, foot rot, and chemical or thermal burns.

The incubation period for VS is two to eight days after exposure with the first sign usually presenting as fever and excessive salivation followed by blisters and eruption of vesicles, ulcerous erosions, and crusting of muzzle and lips. The disease is limited to the epithelial tissues of the mouth, nostrils, teats, and feet. The size of the vesicles is highly variable and can be as small as the size of a pea or large enough to cover the entire surface area of the tongue. In cattle, vesicles are usually found on the hard palate, lips, and gums, and may extend to the nostrils, and muzzle, whereas in horses, the first vesicles may go unnoticed until crusting scabs appear on the muzzle lips or ventral abdomen. In pigs, vesicles generally appear on the feet and frequently on the muzzle. Vesicles on the feet often cause lameness.

Dairy cattle with lesions on the teats may develop mastitis from secondary infection. VS occurs throughout the year in Latin America but is particularly common at the end of the rainy season. In the southwestern United States, outbreaks of VS are common in the warmer months and are identified along rivers and in valleys.

Host Response to Infection

It has been reported that humoral specific antibodies do not always prevent infection. The morbidity rate is 5–90%, with deaths being rare in cattle and horses, but higher mortality rates are reported in some pigs infected with the NJ strain. Sick animals recover in approximately two weeks, and the most common complications during infection are mastitis and loss of production in dairy cattle. Disease incidence varies according to the serotype and species involved. Usually, 10–15% of the adult animals in infected herds present with clinical signs. Cattle and horses less than one year of age are rarely reported affected, and mortality is close to 0% in horses and cattle.

Laboratory Diagnosis

If VS infection is suspected, the proper authorities should be immediately notified. Sample collection should also comply with the required samples for FMD, VES, and SVD in order to facilitate rule-out of these diseases. To prevent spreading of VSV, all samples should be collected and sent under secured conditions to an authorized laboratory. Due to the

painful nature of the vesicles and animal welfare concerns, sedation of the animal is highly recommended. Samples to be submitted should include vesicular fluid, the epithelium covering of unruptured vesicles or the epithelial flaps of ruptured vesicles from mouth, feet, and other sites of vesicle eruption. If epithelial tissue is not available, samples esophageal/pharyngeal liquid should be collected. Samples from pigs should include throat swabs. All samples should be sent under refrigeration or frozen if the transport will take longer than two days. Paired serum samples taken two weeks apart may also be collected. In the United States, paired serum samples are only required for the index case. Once an outbreak has been confirmed, a single serum sample is all that is required to confirm infection. Assays to detect antigen include virus isolation, ELISA, complement fixation, and PCR. The indirect ELISA is the diagnostic method of choice to identify viral serotypes. Serological tests to confirm antibodies to VSV for international trade concerns include liquid-phase blocking ELISA or competitive ELISA, virus neutralization, and complement fixation for detection of IgM.

Treatment and Control

There is no treatment for VS, but antibiotics may prevent secondary bacterial infection in abraded tissues. Suspicion of VS includes quarantine of infected herds pending confirmatory testing. Vehicles and fomites should be disinfected, and subclinically infected animals kept in isolation indoors. Humans should use precautions when handling infected or suspected infected animals including gloves to prevent contamination and spreading of the disease. Cattle should not be moved from infected areas before 21 days after all lesions are healed with the exception of animals sent to slaughter. Insect control may help to prevent disease spread, and all breeding areas should be eliminated. VSV killed vaccines are currently sold in Venezuela and Columbia.

Bovine Ephemeral Disease

Disease

BEF is a noncontagious epizootic arthropod-borne disease of cattle and water buffaloes commonly known as "3-day sickness," "3-day stiff sickness," or "Dragon boat disease." It is an economically important disease in cattle due to the impact that it has on milk production, abortion, temporary infertility in bulls, and prolonged recovery in some animals. Mortality rates are low in cattle in good condition but can be more severe, with rates as high as 30%, in fat cattle. Rule-out diseases include Rift Valley fever, heartwater, bluetongue, botulism, babesiosis, and blackleg. Excess salivation may resemble FMD, but no vesicles are observed.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

BEF is caused by bovine ephemeral fever virus (BEFV), a rhabdovirus that is antigenically related to several other nonpathogenic viruses, for example Adelaide River virus, as well as rhabdoviruses that cause similar diseases to BEF including Kotonkan and Puchong viruses.

Resistance to Physical and Chemical Agents

BEFV is destroyed by disinfectants like sodium hypochlorite.

Infectivity to Other Species or Culture Systems

BEFV is not spread by casual contact but is spread intravenously generally through the bite of an infected insect vector. Symptomatic infections have been reported in hartebeest, waterbuck, wildebeest, and goats. In addition, antibodies to BEFV have been detected in Cape buffalo, deer, and antelope in Africa and deer in Australia. BEF impacts trade in live animals and semen to disease-free zones.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

BEF is endemic in tropical and subtropical zones in Africa, Asia, and Australia. Geographically, this includes all countries south of a line that also includes Israel, Iraq, Iran, Syria, India, Pakistan, Bangladesh, southern and central China, and southern Japan that extends through Southeast Asia to Australia. In Australia, the estimated economic impact of BEF is estimated to exceed $100 million. The disease is spread by arthropods and has been isolated from the mosquito species of *Culex* and *Anopheles* and from *Culicoides* biting midges in Africa and Australia. The disease can also be spread through intravenous inoculation of blood from an infected animal. BEFV is not spread by close contact, body secretions including semen, or aerosol droplets. It is rapidly inactivated in meat and does not appear to be spread by consumption.

Pathogenesis and Pathology

Experimentally, the average incubation period is two to four days although as much as nine days has been reported. Clinical signs of BEFV infection occur as a result of a vascular inflammatory response. The onset of fever and other clinical signs is accompanied by a marked leucopoenia,

relative neutrophilia, elevated plasma fibrinogen, biochemical imbalance including hypocalcemia, and elevated levels of cytokines. There have been reports of prolonged paralysis or ataxia in animals recovering from acute infection. Lesions include a small amount of fibrin-rich fluid in the pleural, peritoneal, and pericardial cavities. Fluid may also be found in the joint capsules. Serofibrinous polysynovitis, polyarthritis, polytendovaginitis, and cellulitis have also been reported. Patchy edema may be seen in the lungs, and lymphadenitis is often observed. Petechial hemorrhages or edema occurs in the lymph nodes. Areas of focal necrosis are seen in the major muscle groups.

Host Response to Infection

Clinical signs of BEF are short-lived but severe with infected cattle exhibiting a biphasic or triphasic fever. Morbidity rates range from 1% to 100% depending upon the epidemiological factors, with mortality rates rarely reaching higher than 1%. Temperature peaks are approximately 12–18 hours apart. The first indication of disease may be fever accompanied by a dramatic reduction in milk production that may not return to normal until the next lactation. Clinical signs are more severe during the second fever with animals exhibiting increased heart rate, tachypnea, depression, anorexia, ruminal atony, serous, or mucoid discharge from nose and eyes, salivation, muscle twitching, shivering, joint pain, stiffness, and shifting lameness. In addition, there may be submandibular edema or patchy edema on the head. Animals may become recumbent for eight hours to days with temporary loss of reflexes. Some animals will not be able to rise. Within a day or two, animals begin to recover. Complications are rare but may include temporary paralysis, gait impairment, aspiration pneumonia, emphysema, and the subcutaneous accumulation of air along the back.

Laboratory Diagnosis

If BEF is suspected, notification of the responsible authorities should be the first step. All samples collected for laboratory examination need to be sent securely to prevent spread of the disease. BEF is confirmed by serological testing, and submitted samples should include approximately 20 ml of clotted blood and 5 ml of uncoagulated blood using an anticoagulant other than EDTA. Paired serum samples, to demonstrate a rising titer, are tested by virus neutralization tests or ELISA. Cross-reactivity to related rhabdoviruses may occur. Virus isolation from blood samples is difficult. BEF has been confirmed by inoculating susceptible cattle or weaned mice with uncoagulated whole blood.

Treatment and Control

Sodium hypochlorite and other disinfectants will destroy BEFV, but generally disinfection is unimportant as the virus is transmitted through mosquitoes or midges rather than by direct contact. Anti-inflammatory drugs and calcium borogluconate injections are recommended in some cases to treat fever, anorexia, muscle stiffness, ocular, and nasal discharge, ruminal stasis, and sternal recumbency. Immunity after infection has been reported, and vaccination has an important role in the management of BEF. There are killed and live vaccines currently being sold in Asia and Africa.

Diseases of Fish Caused by Rhabdoviruses

Several rhabdoviruses cause serious diseases and economic losses in wild and farmed fish. Three specific viruses that have a high economic impact on farmed and wild fish and are reportable to OIE will be mentioned here: SVCV, classified within the genus *Sprivivirus*, and IHNV and VHSV, both classified within the genus *Novirhabdovirus*.

Spring Viremia of Carp

SVCV causes a fatal disease in cyprinid fish. Common carp, including the variety called koi carp, are the main species infected by SVCV, although many species of fish in the minnow family are also susceptible to the disease as well as non-cyprinids, such as rainbow trout, pike, and penaeid shrimp. The disease has been reported in Europe, the Middle East, Asia, and in North and South America. First signs of the disease include a change in behavior, and fish and fish may begin to congregate in slow-moving water or lie on the bottom of ponds and streams. As the disease progresses, fish lie on their sides, become sluggish, and their rate of respiration decreases. Externally, fish may exhibit physical signs of darkening of the skin, swollen abdomen, exophthalmia, skin, or gill hemorrhages, and a protruding vent. Internally infected fish exhibit edema in body organs, hemorrhages in the swim bladder, and inflammation of the bladder. As indicated by the name, most outbreaks occur in the spring in cooler temperatures. When water temperatures rise to 10–17 °C, the immune system of carp can develop neutralizing antibody and suppress virus replication. Viral transmission of SVCV occurs horizontally through contact with virus-infected feces, urine, and gill mucous. Vertical transmission is a possibility as SVCV has been infrequently identified in ovarian fluids, but the lack of confirmed outbreaks in fry and fingerlings indicates that

it may not be an important route of infection. Diagnosis of SVCV can be confirmed through virus isolation, immunofluorescence antibody test (FAT), immunoperoxidase (IPT), or ELISA, though RT-PCR followed by sequencing is commonly employed. Prevention of SVCV includes using a source of water that is free from disease, especially in an endemic region; disinfection of eggs by iodophor treatment; regular physical and chemical disinfection of ponds and equipment; and proper disposal of infected fish. Effective experimental vaccines have been developed, but cost-effectiveness is currently unlikely to lead to commercial availability.

Infectious Hematopoietic Necrosis

IHNV infection results in a serious viral disease of salmonid fish. It is present in North America, Europe, and some Asian countries. Clinical infections are most common in young fish. IHNV can cause major financial losses for infected farms that raise young trout or salmon. In addition, epizootics have also been reported in wild salmon. Susceptible species to IHNV include members of the trout and salmon family. Experimental infections have also been reported in pike fry, sea bream, and turbot. Transmission occurs horizontally from clinically ill fish and asymptomatic carriers through infected feces, urine, sexual fluids, and external mucous either by direct contact or through the water. Vertical transmission has also been reported. The incubation period is between 5 and 45 days, and clinical signs include abdominal distension, exophthalmia, darkened skin, and pale gills. Infected fish are lethargic, exhibiting bouts of hyperexcitability and frenzy. Petechial hemorrhages are commonly seen at the base of the pectoral fins, mouth, skin behind the lateral line, muscles near the anus, and the yolk sac in sac fry. Surviving fish often have scoliosis. Postmortem lesions include lack of food in the digestive tract, pale kidney spleen, and focal necrosis on the liver. Petechiae are often seen in the internal organs. Hemorrhages may occur in the kidney peritoneum and swim bladder. Mortality reaching 50–90% in outbreaks have been reported. Clinical disease occurs when water temperatures are between 8 and 15 °C, although outbreaks have been reported in warmer water. Young fish, less than two months old, are most susceptible to disease. Fish that survive usually develop good immunity to IHNV, but they may also become viral carriers. Diagnosis is confirmed through virus isolation, FAT, and PCR. There are OIE-approved serological methods, such as serum neutralization (also approved by US and Canadian authorities) and an ELISA with a less than 24-hour turnaround time. If IHNV is suspected, appropriate authorities should be contacted. Outbreaks are controlled by culling, disinfection, and quarantines. Eggs should be disinfected with an iodophor solution, and virus-free water should be used to incubate eggs and raise fry. IHNV is inactivated by most common disinfectants including iodophors. A plasmid-based vaccine against IHNV has been developed and is applied once intramuscularly to fry at the hatchery.

Viral Hemorrhagic Septicemia Virus

VHSV affects salmonids and has increasingly been isolated from other marine and freshwater fish in the northern hemisphere. Septicemia causes a mortality up to 100%, particularly in young fish and naïve populations. The largest economic losses affect rainbow trout aquaculture, but also Japanese flounder and turbot operations. Distribution of VHSV infection with clinical importance in marine and freshwater fish in many countries contributes to its importance among fish diseases. Transmission is horizontal through contaminated water (urine, feces, and reproductive fluids from VHSV-diseased and -carrier fish). Vertical transmission has not been reported. The incubation period is between 7 and 14 days, occurring in water temperature of 12 °C, but prolonged incubation can occur at 1 °C. Clinical infection results in symptoms indistinguishable from other viral infections (as described above). Histologically, necrotic lesions are seen in the liver, spleen, and kidney, with hemorrhage typical. Fish that survive infection will have immunity. Diagnosis is based on virus isolation followed by serum neutralization per OIE, with confirmation by RT-PCR and sequencing, IFAT, or ELISA. Similar prevention methods as described for IHNV are used for VHSV outbreak prevention in farming operations. In addition, use of processed feed has been shown to reduce outbreaks. No commercial vaccines are currently available. Oral vaccination using an attenuated strain under investigation would offer the most viable approach.

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61

Coronaviridae and Tobaniviridae

Udeni B.R. Balasuriya, Yun Young Go, and Mariano Carossino

Introduction and Classification

The International Committee on Taxonomy of Viruses (ICTV) first established the order *Nidovirales* in 1996. All members of the order *Nidovirales* are enveloped viruses with linear, positive-sense, single-stranded RNA (ssRNA) genomes. They share a strikingly similar genome organization and replication strategy but differ considerably in genetic complexity and virion architecture. "Nido" is derived from the Latin word *nidus* for "nest," which refers to the 3′ co-terminal nested subgenomic viral mRNA produced during replication of these viruses. Initially, the order contained only two viral families, *Coronaviridae* and *Arteriviridae*. However, recent advances in next-generation sequencing (NGS) techniques and metagenomic analysis have identified additional viral genomes. Characterization of these novel viral genomes has profoundly changed the classification of these viruses. At present, eight suborders have been established under the order *Nidovirales*: *Abnidovirineae*, *Arnidovirineae*, *Cornidovirineae*, *Mesnidovirineae*, *Monidovirineae*, *Nanidovirineae*, *Ronidovirineae*, and *Tornidovirineae* (Figure 61.1). These eight suborders currently contain 14 viral families, 25 subfamilies, 39 genera, 65 subgenera, and a total of 109 viral species. However, the viral particle size, morphology, and genome size of viruses in these eight suborders vary greatly. Among all eight suborders of the order *Nidovirales*, *Cornidovirineae* harbors the most important veterinary and human viral pathogens (Figure 61.2). Avian infectious bronchitis virus (IBV, now designated Avian coronavirus) was the first coronavirus isolated from chicken embryos in 1937. This was followed by Mouse hepatitis virus (MHV) in the 1940s and other mammalian coronaviruses in the 1970s, including both animal coronaviruses (e.g., Porcine transmissible gastroenteritis [TGEV], Bovine coronavirus [BCoV], and Feline coronavirus [FCoV]), as well as human coronaviruses (HCoV; e.g., HCoV-OC43 and HuCoV-229E). Since the emergence of the human severe acute respiratory syndrome-related coronavirus (SARS-CoV) responsible for the severe acute respiratory syndrome (SARS) in the spring of 2003, coronaviruses became more recognized and generated significant interest among researchers. Coronaviruses are well-known agents of respiratory, enteric, and neurological disease in humans and domestic animals. They are the only nidovirus group reported to cause disease in humans.

The suborder *Cornidovirineae* contains one viral family, *Coronaviridae*, which is further divided into two subfamilies, *Orthocoronavirinae* and *Letovirinae*. The subfamily *Orthocoronavirinae* contains four genera: *Alphacoronavirus* (Alpha-CoV), *Betacoronavirus* (Beta-CoV), *Deltacoronavirus* (Delta-CoV), and *Gammacoronavirus* (Gamma-CoV) (Figure 61.3). This is the largest subfamily in the order *Nidovirales* and includes some highly pathogenic viruses, such as SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2. Alpha-CoVs and Beta-CoVs infect mammals, whereas Delta-CoVs and Gamma-CoVs mainly infect birds, but a few do infect mammals. In contrast, the subfamily *Letovirinae* contains one genus (*Alphaletovirus)*, one subgenus (*Milecovirus*), and one viral species (Microhyla letovirus 1 [MLeV]) that infects the ornamented pygmy frog (*Microhyla fissipes*).

Alphacoronaviruses

The genus Alpha-CoV contains 14 viral genera and 19 species and is the largest genus in the subfamily *Orthocoronavirinae*. Two human Alpha-CoVs, HCoV-229E and HCoV-NL63, belong to subgenera *Duvinacovirus* and *Setracovirus*, respectively. Genetic evidence has shown that HCoV-229E and HCoV-NL63 share common ancestors with bat CoVs, indicating the possibility of viral origin from bats.

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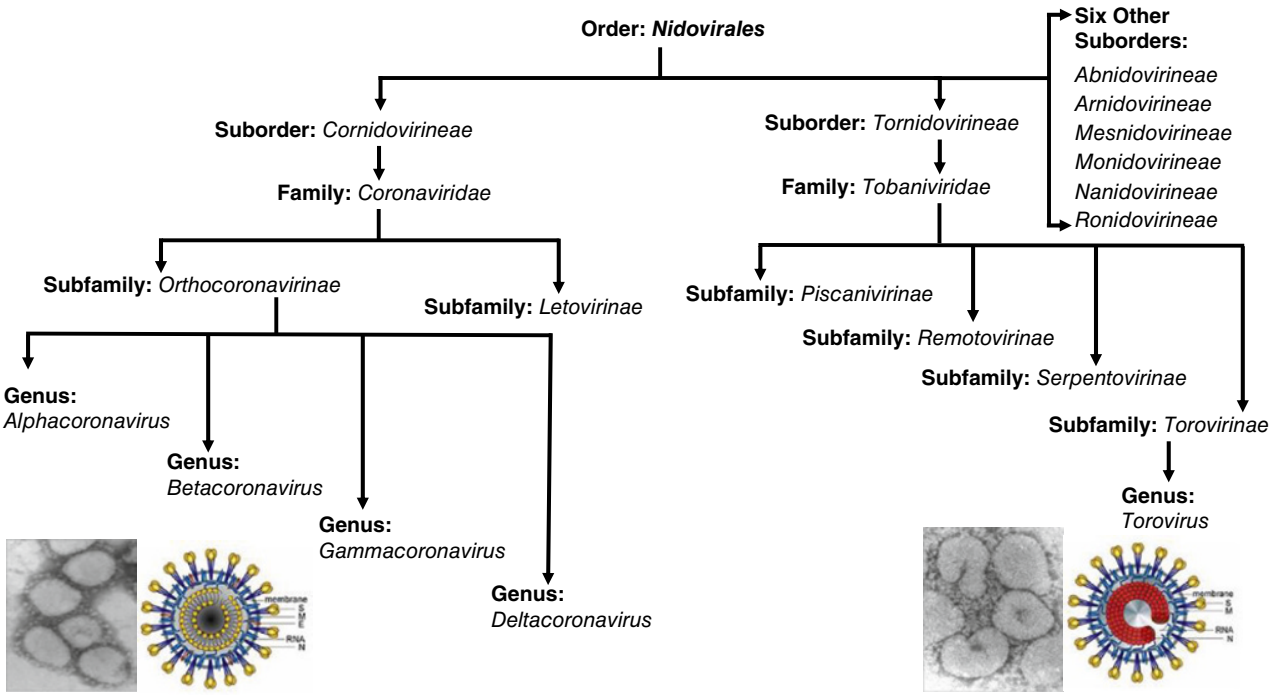


Figure 61.1 Taxonomic classification of the order *Nidovirales*. *Source:* Schematic diagram and electron micrograph pictures of virus particles were reproduced from Gorbalenya et al. (2006) (coronavirus and torovirus) with permission.

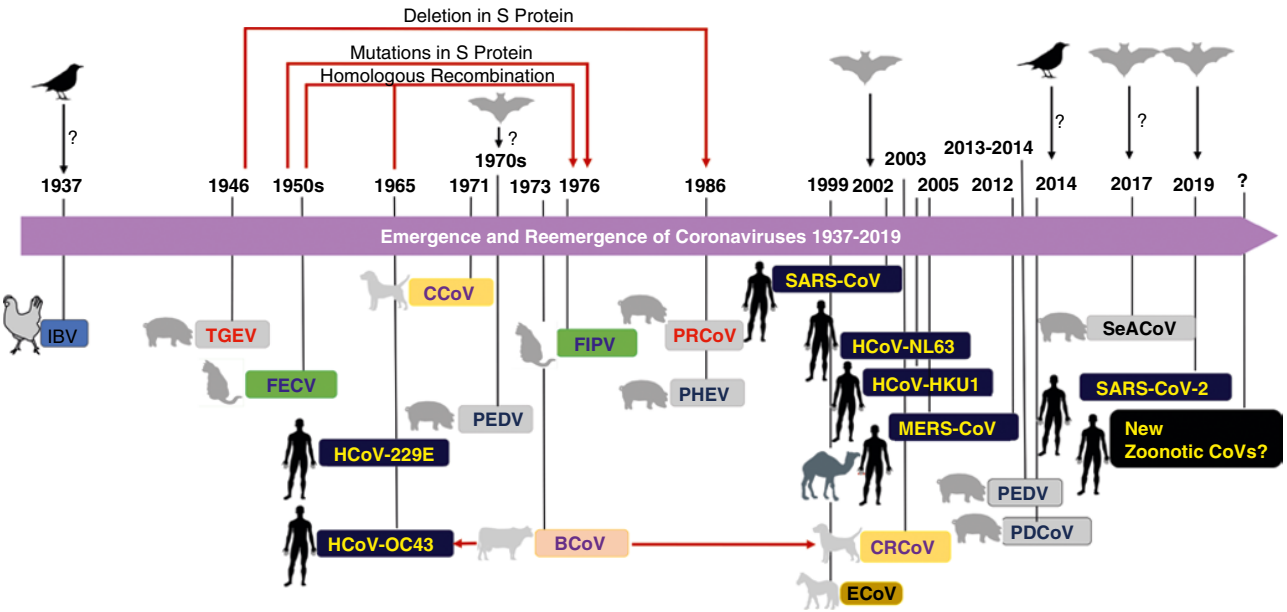


Figure 61.2 Timeline (1937–2019) depicting the emergence and reemergence of coronaviruses (CoVs) affecting farm animals, pets, and humans. Brown arrows indicate the emergence of new coronaviruses due to mutations or deletions leading to a change in tissue tropism or species jumping. CoVs in the same color (red, blue, and purple text) indicate a close genetic relationship due to either gene exchange or sharing common ancestors.

Moreover, coronaviruses (CoVs) similar to HCoV-229E were also detected in camelids, including alpacas and dromedary camels. The HCoV-229E-related CoVs isolated from dromedary camels can use human aminopeptidase N (APN) as a receptor and are also neutralized by antibodies to HCoV-229E in positive patient sera, suggesting a close antigenic relationship between HCoV-229E and 229E-related CoVs. Due to the long-term contact and habitat overlap between humans and camels, one of the conjectures about the transmission path of HCoV-229E is that the ancestor bat CoV was transmitted to camels first and then from camels to humans. In contrast, NL63-related CoVs

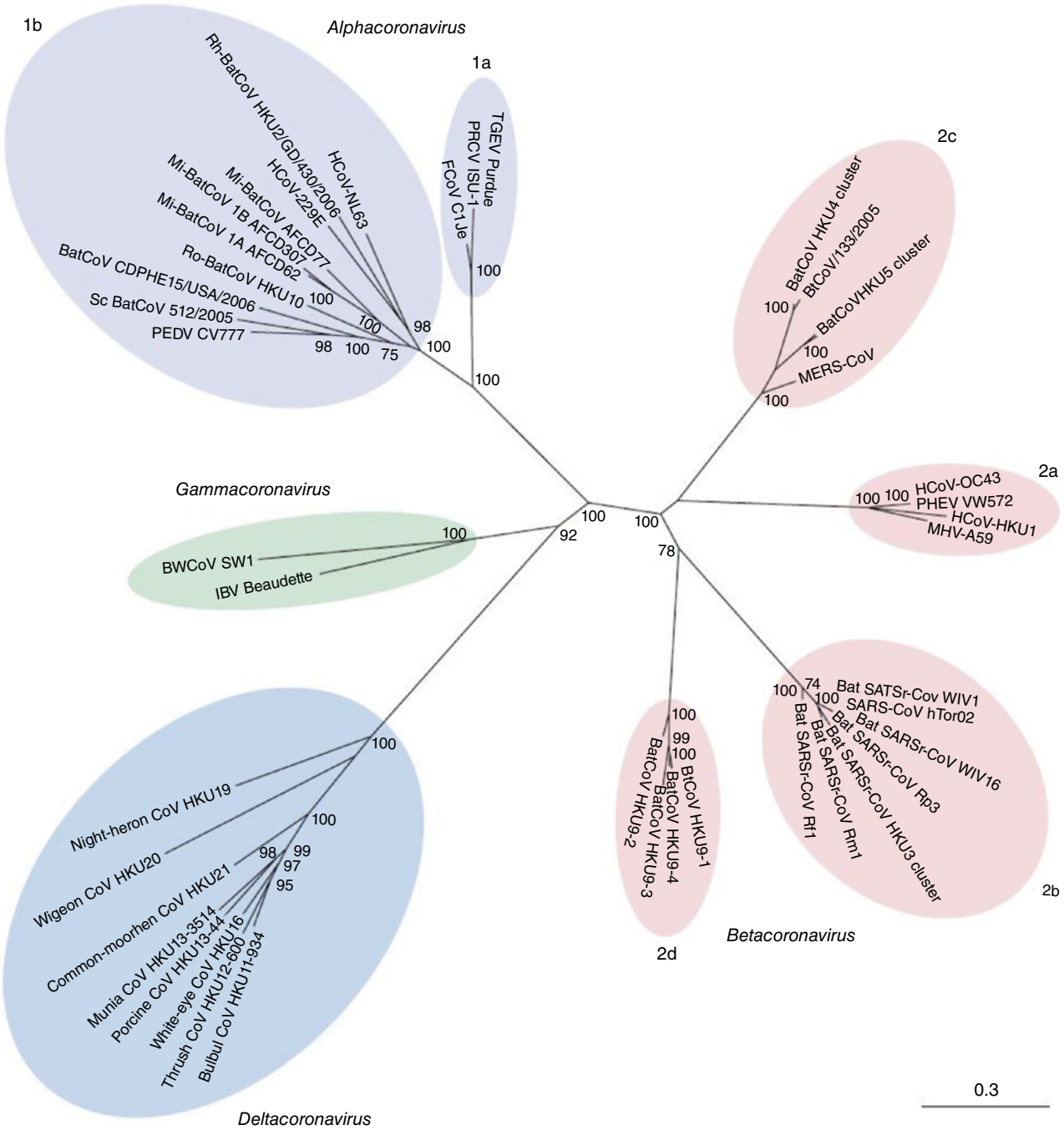


Figure 61.3 Phylogenetic relationships in the *Orthocoronavirinae* subfamily. The viruses in this subfamily are grouped into four genera (prototype or representative strains shown): Alpha-CoV (purple), Beta-CoV (pink), Gamma-CoV (green), and Delta-CoV (blue). Classic subgroup clusters are labeled 1a and 1b for the Alpha-CoVs and 2a–2d for the Beta-CoVs. The tree is based on published trees of *Orthocoronavirinae* and reconstructed with sequences of the complete RNA-dependent RNA polymerase coding region of the representative coronaviruses (maximum likelihood method under the GTR+I+Γ model of nucleotide substitution as implemented in PhyML, version 3.1). Only nodes with bootstrap support above 70% are shown. IBV, infectious bronchitis virus; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, mouse hepatitis virus; PEDV, porcine enteric diarrhea virus; SARS-CoV, severe acute respiratory syndrome-related coronavirus; SARSr-CoV, bat SARS- related coronavirus. *Source:* Phylogenetic tree was reproduced from Cui et al. (2019) with permission.

have not yet been detected in livestock or other animals, so the origin and intermediate host(s) of HCoV-NL63 are still unclear. Most of the Alpha-CoVs infecting animals are derived from bats (Tables 61.1 and 61.2). These include Porcine epidemic diarrhea virus (PEDV; first recognized in 1978 in Belgium), Swine enteric alphacoronavirus (SeACoV), also known as swine acute diarrhea syndrome coronavirus (SADS-CoV) or Porcine enteric alphacoronavirus (PEAV; first identified in Guangdong Province, China in 2016), TGEV (first detected in the United States in 1946),

Porcine respiratory coronavirus (PRCV), Canine coronavirus (CCoV), FCoV, Feline infectious peritonitis virus (FIPV), Mink coronavirus (MCoV), Ferret systemic coronavirus (FRSC), Ferret enteric coronavirus (FRECV), and Rat coronavirus (RCoV).

Betacoronaviruses

The genus Beta-CoV currently contains 5 subgenera and 14 species. This genus is well known because it includes the most pathogenic CoVs to human beings, such as SARS-CoV, MERS-CoV, and SARS-CoV-2, and many related bat CoVs (Tables 61.1 and 61.2). The other Beta-CoV species include *Rattus* coronavirus HKU24, Human coronavirus HKU1, Human coronavirus OC43 (HCoV-OC43), HKU23 in dromedary camels, BCoV in bovine and alpaca, Porcine hemagglutinating encephalomyelitis virus (PHEV), Equine coronavirus (ECoV), Giraffe coronavirus (GiCoV), Sable antelope coronavirus (SACoV), MHV, and Hedgehog coronavirus 1.

Table 61.1 Important animal, human and bat coronaviruses in the suborder *Cornidovirineae,* family *Coronaviridae*, and subfamily *Orthocoronavirinae.*

Genus[a]/species	Abbreviation	Natural host	Tissue affected/disease
Genus: *Alphacoronavirus*			
Transmissible gastroenteritis virus	TGEV	Pig	Enteric infection
Porcine respiratory coronavirus	PRCV	Pig	Respiratory infection
Porcine epidemic diarrhea virus	PEDV	Pig	Enteric infection
Swine enteric alphacoronavirus or Swine acute diarrhea syndrome or Porcine enteric alphacoronavirus	SeACoV or SADS-CoV or PEAV	Pig	Enteric infection
Canine coronavirus (Canine enteric coronavirus)	CCoV	Dog	Enteric and systemic infection
Feline infectious peritonitis virus	FIPV	Cat	Systemic /multi-organ infection
Feline coronavirus	FCoV	Cat	Enteric infection
Ferret enteric coronavirus	FRECV	Ferret	Enteric infection (epizootic catarrhal enteritis [ECE])
Ferret systemic coronavirus	FRSCV	Ferret	Systemic/multi-organ infection
Mink coronavirus	MCoV	Mink	Enteric infection (mink epizootic catarrhal gastroenteritis [ECG])
Rabbit coronavirus	RbCoV	Rabbit	Heart
Human coronavirus 229E	HCoV-229E	Human	Respiratory infection
Human coronavirus NL63	HCoV-NL63	Human	Respiratory infection
Scotophilus bat coronavirus 512	Sc-BatCoV-512	Lesser Asiatic yellow house bat	No clinical disease
Rhinolophus bat coronavirus HKU2	Rh-BatCoV-HKU2	Chinese horseshoe bat	No clinical disease
Miniopterus bat coronavirus HKU8	Mi-BatCoV-HKU8	Bent-winged bat	No clinical disease
Miniopterus bat coronavirus 1A	Mi-BatCoV-1A	Bent-winged bat	No clinical disease
Miniopterus bat coronavirus 1B	Mi-BatCoV-1B	Bent-winged bat	No clinical disease
Genus: *Betacoronavirus*			
Porcine hemagglutinating encephalomyelitis virus	PHEV	Pig	Enteric, respiratory and neurological infection
Bovine coronavirus	BCoV	Cattle	Enteric and respiratory infection
• Enteric bovine coronavirus	EBCoV		
• Respiratory bovine coronavirus	RBCoV		

(*Continued*)

Table 61.1 (Continued)

Genus[a]/species	Abbreviation	Natural host	Tissue affected/disease
Canine respiratory coronavirus	CRCoV	Dog	Respiratory infection
Equine coronavirus	ECoV	Horse	Enteric infection
Mouse hepatitis virus	MHV	Mouse	Hepatitis, enteric and neurological infection
Rat coronavirus	RCoV	Rat	
• Sialodacryoadenitis virus	SDAV		Sialodacryoadenitis – salivary and lacrimal glands
• Parker's rat coronavirus			Respiratory infection
	RCoV-P		
Human coronavirus OC43	HCoV-OC43	Human	Respiratory infection
Human coronavirus HKU1	HCoV-HKU1	Human	Respiratory infection
Middle East respiratory syndrome coronavirus	MERS-CoV	Human	Respiratory infection
		Dromedary camel	Respiratory infection
SARS-related coronavirus	SARS-CoV	Human	Respiratory infection
SARS coronavirus-2	SARS-CoV-2	Human	Respiratory infection
SARS-related *Rhinolophus* bat coronavirus	SARSr-Rh-Bat-CoV	Chinese horseshoe bat	No clinical disease
Tylonycteris bat coronavirus HKU4	Ty-BatCoV HKU4	Lesser bamboo bat	No clinical disease
Pipistrellus bat coronavirus HKU5	Pi-BatCoV HKU5	Japanese pipistrelle bat	No clinical disease
Rousettus bat coronavirus HKU9	Ro-BatCoV HKU9	Leschenault's rousette bat	No clinical disease
Genus: *Gammacoronavirus*			
Infectious bronchitis virus	IBV	Chicken	Respiratory, reproductive, and renal infection
Turkey coronavirus	TCoV	Turkey	Enteric infection
Beluga whale coronavirus SW1	BWCoV	Beluga whale	No clinical disease
Dolphin coronavirus	DCoV	Dolphin	No clinical disease
Canada goose coronavirus		Canada geese and Snow geese	Enteric infection
Duck coronavirus		Duck	No clinical disease
Guinea fowl coronavirus	GfCoV	Guinea fowl	Enteric infection
Genus: *Deltacoronavirus*			
Bulbul coronavirus HKU11	BuCoV HKU11	Chinese bulbul	No clinical disease
Thrush coronavirus HKU12	ThCoV HKU12	Gray-backed thrush	No clinical disease
Munia coronavirus HKU13	MunCoV HKU13	White-rumped munia	No clinical disease
Porcine delta coronavirus	PDCoV	Pig	Enteric infection

[a] There are several subgenera within a genus that are not identified in the table.

Table 61.2 Important human and animal coronaviruses by species affected.

Animal/viral species (Abbreviation)	Genus	Tissue affected/disease
Human		
Human coronavirus 229E (HCoV-229E)	*Alphacoronavirus*	Respiratory infection (common cold)
Human coronavirus NL63 HCoV-NL63	*Alphacoronavirus*	Respiratory infection (common cold)
Human coronavirus OC43 (HCoV-OC43)	*Betacoronavirus*	Respiratory infection (common cold)
Human coronavirus HKU1(HCoV-HKU1)	*Betacoronavirus*	Respiratory infection (common cold)
Middle East respiratory syndrome coronavirus (MERS-CoV)	*Betacoronavirus*	Respiratory infection (MERS)
Severe acute respiratory syndrome-related coronavirus (SARS-CoV)	*Betacoronavirus*	Respiratory infection (SARS)
SARS-related coronavirus-2 (SARS-CoV-2)	*Betacoronavirus*	Respiratory infection (Coronavirus Disease 2019; COVID-19)
Cat		
Feline coronavirus (FCoV)	*Alphacoronavirus*	Enteric infection
Feline infectious peritonitis virus (FIPV)	*Alphacoronavirus*	Systemic infection
Dog		
Canine coronavirus (CCoV; Canine enteric coronavirus)	*Alphacoronavirus*	Enteric and systemic infection
Canine respiratory coronavirus (CRCoV)	*Betacoronavirus*	Respiratory infection
Pig		
Transmissible gastroenteritis virus (TGEV)	*Alphacoronavirus*	Enteric infection
Porcine respiratory coronavirus (PRCoV)	*Alphacoronavirus*	Respiratory infection
Porcine epidemic diarrhea virus (PEDV)	*Alphacoronavirus*	Enteric infection
Swine enteric alphacoronavirus (SeACoV) or Swine acute diarrhea syndrome coronavirus (SADS-CoV) or Porcine enteric alphacoronavirus (PEAV)	*Alphacoronavirus*	Enteric infection
Porcine hemagglutinating encephalomyelitis virus (PHEV)	*Betacoronavirus*	Enteric, respiratory and neurological infection
Porcine delta coronavirus (PDCoV)	*Deltacoronavirus*	Enteric infection
Cattle and Horse		
Bovine coronavirus (BCoV) • Enteric bovine coronavirus (EBCoV) • Respiratory bovine coronavirus (RBCoV)	*Betacoronavirus*	Enteric and respiratory infection
Equine coronavirus (ECoV)	*Betacoronavirus*	Enteric infection
Laboratory Animals		
Ferret enteric coronavirus (FRECV)	*Alphacoronavirus*	Enteric infection (epizootic catarrhal enteritis [ECE])
Ferret systemic coronavirus (FRSCV)	*Alphacoronavirus*	Systemic infection
Mink coronavirus (MCoV)	*Alphacoronavirus*	Enteric infection (epizootic catarrhal gastroenteritis [ECG])
Mouse hepatitis virus (MHV)	*Betacoronavirus*	Hepatitis, enteric and neurological infection
Rabbit coronavirus (RbCoV)	*Alphacoronavirus*	Heart
Rat coronavirus (RCoV) • Sialodacryoadenitis virus (SDAV) • Parker's rat coronavirus (RCoV-P)	*Betacoronavirus*	 Sialodacryoadenitis – salivary and lacrimal glands and eyes Respiratory infection

(*Continued*)

Table 61.2 (Continued)

Animal/viral species (Abbreviation)	Genus	Tissue affected/disease
Marine Mammals		
Beluga whale coronavirus SW1 (BWCoV)	*Gammacoronavirus*	No clinical disease
Dolphin coronavirus (DCoV)	*Gammacoronavirus*	No clinical disease
Chicken, Turkey, and Wild Bird		
Infectious bronchitis virus (IBV)	*Gammacoronavirus*	Respiratory, reproductive and renal infection
Turkey coronavirus (TCoV)	*Gammacoronavirus*	Enteric infection
Canada goose coronavirus	*Gammacoronavirus*	Enteric infection
Duck coronavirus	*Gammacoronavirus*	No clinical disease
Guinea fowl coronavirus (GfCoV)	*Gammacoronavirus*	Enteric infection
Bulbul coronavirus HKU11 (BuCoV HKU11)	*Deltacoronavirus*	No clinical disease
Thrush coronavirus HKU12 (ThCoV HKU12)	*Deltacoronavirus*	No clinical disease
Munia coronavirus HKU13 (MunCoV HKU13)	*Deltacoronavirus*	No clinical disease

Gammacoronaviruses

The genus Gamma-CoV consists of three subgenera: *Igacovirus, Brangacovirus*, and *Cegacovirus*. Viruses of the *Igacovirus* subgenus primarily infect birds, such as chickens (*Gallus gallus*), turkeys, ducks, geese, pheasants, partridges, pigeons, and peafowl. The *Igacovirus* subgenus contains three species: Avian coronavirus, Avian coronavirus 9203, and Duck coronavirus 2714. IBV (Avian coronavirus) is the prototype virus of the *Igacovirus* subgenus, and there are various serotypes of IBV with varying virulence and pathogenesis. IBV is the causal agent of infectious bronchitis (IB), a disease prevalent worldwide and primarily affecting the upper respiratory tract, but several IBV serotypes also infect the kidney, digestive tract, and reproductive system.

Deltacoronaviruses

There are three subgenera (*Andecovirus, Buldecovirus*, and *Herdecovirus*) in the genus *Deltacoronavirus*, harboring CoVs infecting mammals (porcine, Asian leopard cat, and Chinese ferret badger) and birds (bulbul, thrush, munia, white eye, sparrow, magpie robin, night heron, wigeon, common moorhen, falcon, houbara, pigeon, and quail). Among mammalian Delta-CoVs, Porcine deltacoronavirus (PDCoV) has become one of the primary pathogens causing diarrhea in pigs since 2014. PDCoV was first discovered in a surveillance study in Hong Kong in 2012, and two strains with completed genomes were named HKU15-44 and HKU15-155. Subsequently, PDCoVs were reported in other countries, including the United States, South Korea, Japan, mainland China, Thailand, Laos, Vietnam, and other regions worldwide. Recently, three Haitian children with acute undifferentiated febrile illness were tested positive for PDCoV, the first report of a Delta-CoV infecting humans. Thus, the potential of cross-species transmission of Delta-CoVs to humans should not be neglected because of the close contact of people with pigs and other domestic animals.

It has been suggested that PDCoV and Asian leopard cat coronavirus (AlCCoV) evolved from a bird coronavirus, and the closest evolutionary species to PDCoV among bird coronaviruses are the sparrow delta coronavirus HKU17 (SpDCoV-HKU17). It has also been shown that SpDCoV-HKU17 in the United States shares approximately 83.9% whole-genome nucleotide identity and greater than 85% amino acid identity for S protein with PDCoV, which provides more evidence for the bird origin of PDCoV. However, no direct evidence shows that birds transmit PDCoV, and significant recombination events were only observed in porcine or bird Delta-CoV.

The human SARS outbreak in 2003, followed by the discovery of bat-SARS-related CoV, marked the beginning of the hunt for coronaviruses in humans, bats, birds, and other animals. Since then, several new coronaviruses have been identified in humans, bats, beluga whales, Asian leopard cats, and birds. As most of the new viruses have been identified in bats and wild birds, it has been hypothesized that human and animal coronaviruses had evolved from a diverse population of coronaviruses present in bats and wild birds (Figure 61.4). The increased diversity of coronaviruses in bats and birds could be due to several reasons:

i) Bats and birds involve highly diverse species. Bats represent 20% of the 4800 mammalian species, and there are about 10 000 bird species worldwide.

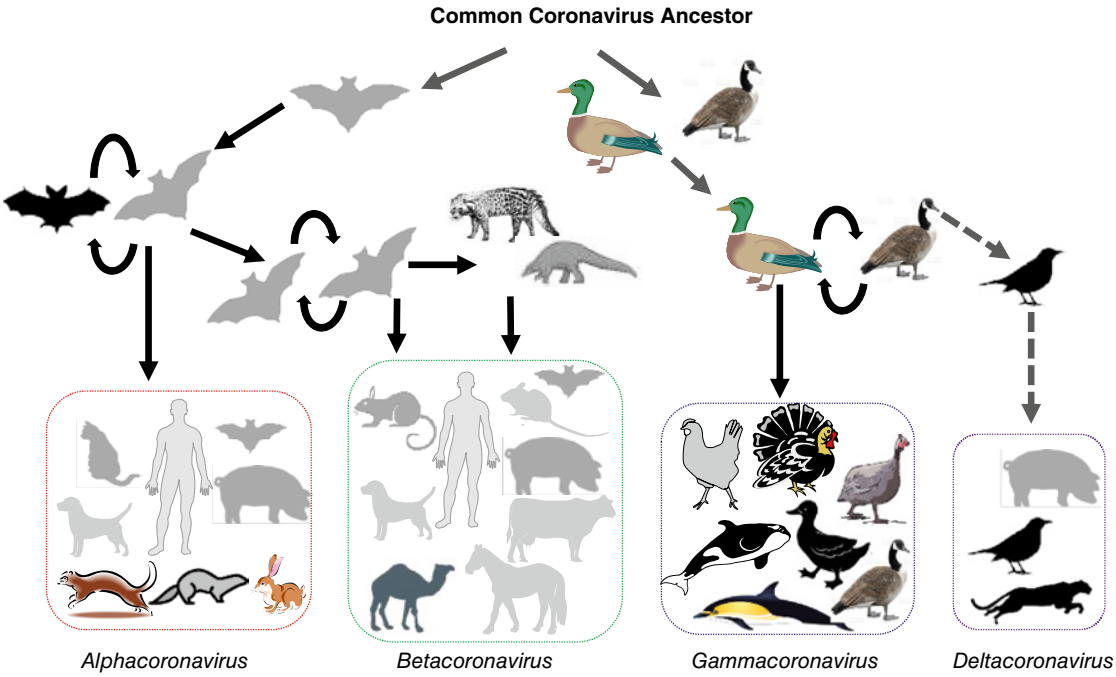


Figure 61.4 A proposed model of coronavirus evolution. Coronaviruses of bats and birds may have evolved from a common ancestor. Subsequently, coronaviruses from bats would have given rise to alphacoronaviruses and betacoronaviruses, whereas coronaviruses from birds would have given rise to gammacoronaviruses and probably to deltacoronaviruses (dashed arrows).

ii) Bats and birds can fly long distances. Bats have been found in habitats as high as 5000 m, and some birds can fly over 10 000 km during migration. This would allow bats and birds to exchange viruses with different species in close contact.
iii) Different environmental pressures (climate, food, shelter, and predators) would provide different selective pressures on establishing different coronaviruses in various species of bats and birds.
iv) Bat's immune system is specially adapted to tolerate viruses that allow them to stay healthy while hosting viruses that kill other animals, and
v) Finally, the habit of roosting (bats) and flocking (birds) in large numbers facilitates the exchange of viruses among individual bats and birds.

Recently, it has been proposed that the ancestor of the currently circulating coronaviruses must have infected a bat; then it jumped from the bat to a bird, or it infected a bird, which jumped from the bird to a bat and evolved dichotomously. Consequently, the bat coronavirus jumped into other species of bats, giving rise to Alpha-CoVs and Beta-CoVs. In turn, these bat coronaviruses jumped to other bat species and other mammals, including humans, with each interspecies jump evolving dichotomously. On the other hand, bird coronaviruses jumped to other species of birds and occasionally to some specific mammalian species, such as the whale and Asian leopard cat (e.g., Gamma-CoVs and probably Delta-CoVs, respectively). Furthermore, the genetic diversity of the coronaviruses results from the high error rate in the viral RNA-dependent RNA polymerase (RdRp), with mutation rates on the order of 1 per 1000–10 000 nucleotides replicated, as well as homologous and heterologous recombination mainly due to the large genome size, which imparts plasticity to the accommodation and modification of genes. The evolutionary history of coronaviruses and the identification of increasing numbers of more closely related coronaviruses from distantly related animal species clearly indicate their ability to jump between species. Therefore, coronaviruses have the potential to give rise to new veterinary and zoonotic pathogens, which can pose a significant threat to animal and public health.

In summary, coronaviruses infect a wide range of mammals and birds. They exhibit a marked tropism for the respiratory and gastrointestinal tract epithelial cells, and macrophages for some viruses. Coronaviruses cause a remarkably diverse spectrum of different diseases in different hosts (Table 61.2). They typically have a restricted host range, infecting only their natural host and closely related animal species. However, they can cross the species barrier (species jumping) and infect new hosts.

Other Suborders of the Order *Nidovirales*

In addition to the suborder *Cornidovirineae*, the order *Nidovirales* contains seven more suborders: *Ab-, Ar-, Mes-, Mo-, Na-, Ro-, and Tor-nidovirineae*. Members of these suborders and their hosts will not be discussed in this chapter. However, the members of the suborder *Arnidovirineae* and family *Arteriviridae* contains many important veterinary pathogens (Equine arteritis virus [EAV], Porcine reproductive and respiratory syndrome virus [PRRSV], Simian hemorrhagic fever virus [SHFV], and Lactate dehydrogenase elevating virus [LDV]) and therefore, they are discussed in a separate chapter. Furthermore, members of the suborder *Tornidovirineae* (family *Tobaniviridae*, subfamily *Torovirinae*) that infect horses, pigs, cattle, and humans will be briefly described here. The subfamily *Torovirinae* was once classified into the family *Coronaviridae*, and the prototype species of *Torovirus* is Equine torovirus (EToV), which was identified in the 1970s. In the 1980s, Porcine torovirus (PToV) and Bovine torovirus (BToV) were detected. Subsequently, torovirus-like (TVL) particles have been detected by electron microscopy (EM) in feces from dogs, cats, and turkeys. The toroviruses have a worldwide distribution, cause acute self-limiting gastroenteritis, and are a significant burden on the animal industry. There are three species, BToV, EToV, and PToV, in the subgenus *Renitovirus* of genus *Torovirus* in the subfamily *Torovirinae*. The recently identified toroviruses in snakes and turtles have not yet been classified. Human torovirus (HToV) has been identified as a potential pathogen inducing diarrhea in humans, specifically in young and/or immunocompromised individuals. Interestingly, close genetic and antigenic relationships between BToV and HToV have been demonstrated.

Virion Properties and Virus Replication

Coronaviruses

Coronaviruses have a unique morphological appearance, which is that of a crown. Thus, the name *coronavirus* was derived from the Latin word *corona* (Greek κορώνα), meaning "crown." They are spherical, enveloped virions with large club-shaped surface projections (peplomers) extending from the viral envelope (Figures 61.1 and 61.5). Virion size ranges from 100 to 160 nm in diameter. The coronavirus genome is a single-stranded (ss), positive-sense RNA molecule that ranges in size from 26.4 to 31.7 kb, and it is associated with the N phosphoprotein to form a long, flexible, helical nucleocapsid. However, in at least two coronaviruses (TGEV and MHV), the helical nucleocapsid is enclosed within an "internal core structure," 65 nm in diameter, that is spherical, or possibly icosahedral, in form. The virus core is surrounded by a lipoprotein envelope derived from the intracellular membranes acquired during virus budding from the cell. The envelope is studded with three to four viral proteins depending on the coronavirus. These include the long spikes (20 nm), which consist of the S (spike) glycoprotein and the short spikes consisting of the HE (hemagglutinin-esterase) glycoprotein, which are present in only some coronaviruses. The envelope also contains the glycoprotein M, a transmembrane protein, which is more deeply embedded in the envelope. As indicated above, the M and N proteins form an internal core structure in at least two coronaviruses. The small envelope protein E is present in much smaller amounts than the other viral envelope proteins. The E protein, together with the M protein, plays an essential role in coronavirus particle assembly. Coronaviruses have a buoyant density in sucrose of 1.15–1.20 g/cm^3. The buoyant density in CsCl is 1.23–1.24 g/cm^{3,} and the sedimentation coefficient (S20,w) is 300–500. Coronaviruses are sensitive to heat, lipid solvents, nonionic detergents, formaldehyde, oxidizing agents, and UV irradiation. Some coronaviruses are resistant to acid pH and/or desiccation.

The genome organization of the suborders *Cornidovirineae* (CoVs) and *Tornidovirineae* (ToVs) resembles the other members of the order *Nidovirales* (Figures 61.5 and 61.6). The coronavirus and torovirus genomic RNAs are 5′-capped, 3′-polyadenylated, and can function as an mRNA. Thus, the purified genomic RNAs of these viruses are infectious. The coronavirus genomes consist of a 5′-leader sequence (65–98 nucleotides [nts]) followed by an untranslated region (5′-UTR; 200–400 nts). At the 3′-end of the viral genome, there is another UTR of 200–500 nts (3′-UTR), followed by a poly-A tail of variable length. The 5′ and 3′-UTRs flank an array of multiple genes (open reading frames [ORFs]) whose number may vary among the members of the subfamily *Orthocoronavirinae*. The coronaviruses have 9–14 ORFs that encode viral nonstructural and structural proteins. The 5′-proximal two-thirds (~20–22 kb) of the genome is occupied by the two largest ORFs (ORF1a and ORF1b), which encode for the nonstructural proteins (nsps). ORF1a and ORF1b are connected by a −1 ribosomal frameshift site (RFS). The translation of genomic RNA starts at the start codon of ORF1a, resulting in the production of a polyprotein, pp1a. In some cases, specific RNA signals promote ribosomal frameshifting at a −1 RFS or "slippery" sequence between ORF1a and ORF1b, resulting in the C-terminal extension of pp1a with the ORF1b-encoded polypeptide, pp1ab. The pp1a and pp1ab are co- and posttranslationally processed by two virus-encoded proteases (papain-like protease [nsp3] and picornaviral 3C-like cysteine protease [nsp5]) to produce 16 nsps. nsp14 has RNA-dependent RNA

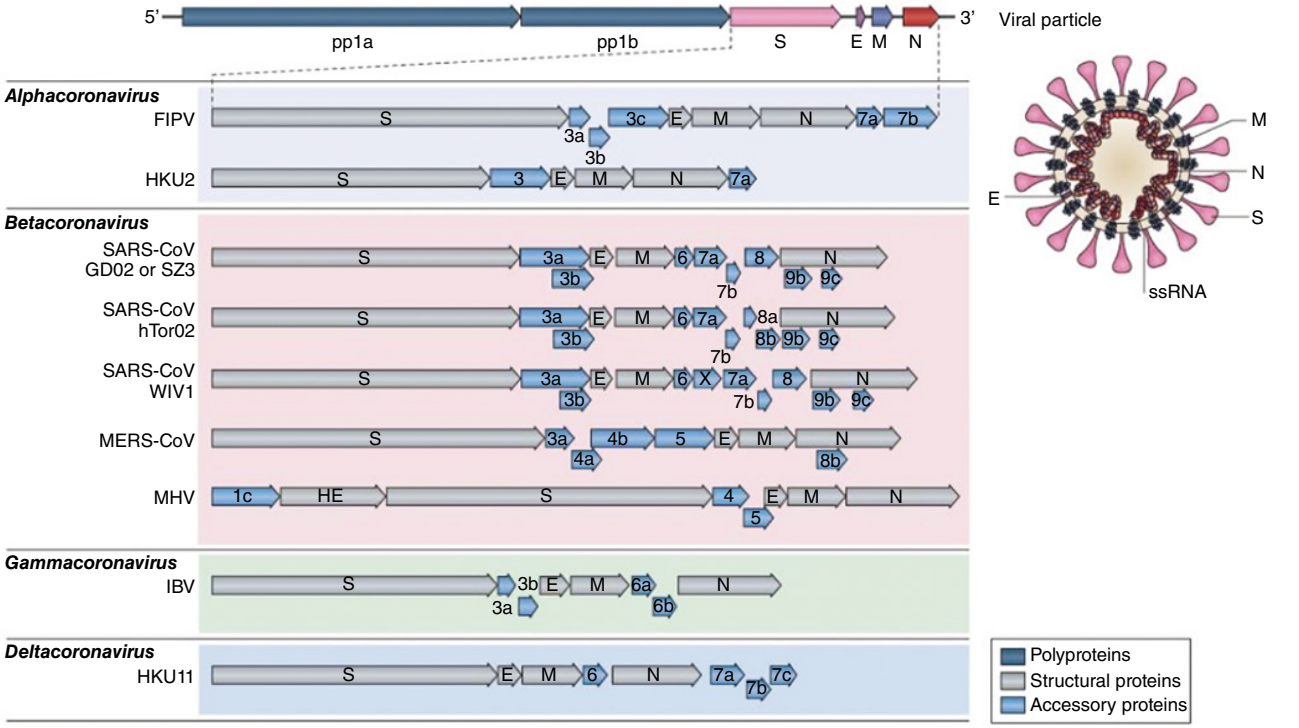


Figure 61.5 Genome organization of selected members of the subfamily *Orthocoronavirinae*. Coronaviruses contain a positive-sense, single-stranded RNA (ssRNA) genome 27–32 kb in size. The 5′-terminal two-thirds of the genome encodes a polyprotein, pp1ab, which is further cleaved into 16 non-structural proteins involved in genome transcription and replication. The 3′ terminus encodes structural proteins, including envelope glycoproteins spike (S), envelope (E), membrane (M), and nucleocapsid (N). In addition to the genes encoding structural proteins, there are accessory genes that are CoV species specific and dispensable for virus replication. Depiction of comparative analysis of prototypical and representative strains of four CoV genera: Alpha-CoV (Feline infectious peritonitis virus [FIPV] and Rhinolophus bat CoV HKU2); Beta-CoV (SARS-related CoV strains GD02 and SZ3 from humans infected during the early phase of the SARS epidemic and from civets, respectively; SARS-related CoV strain hTor02 from humans infected during the middle and late phases of the SARS epidemic; Bat SARS-related coronavirus [SARSr-CoV] strain WIV1; and Middle East respiratory syndrome coronavirus [MERS-CoV], and Mouse hepatitis virus [MHV]); Gamma-CoV (Infectious bronchitis virus [IBV]) and Delta-CoV (Bulbul coronavirus HKU11). *Source:* Schematic diagram was reproduced from Cui et al. (2019) with permission.

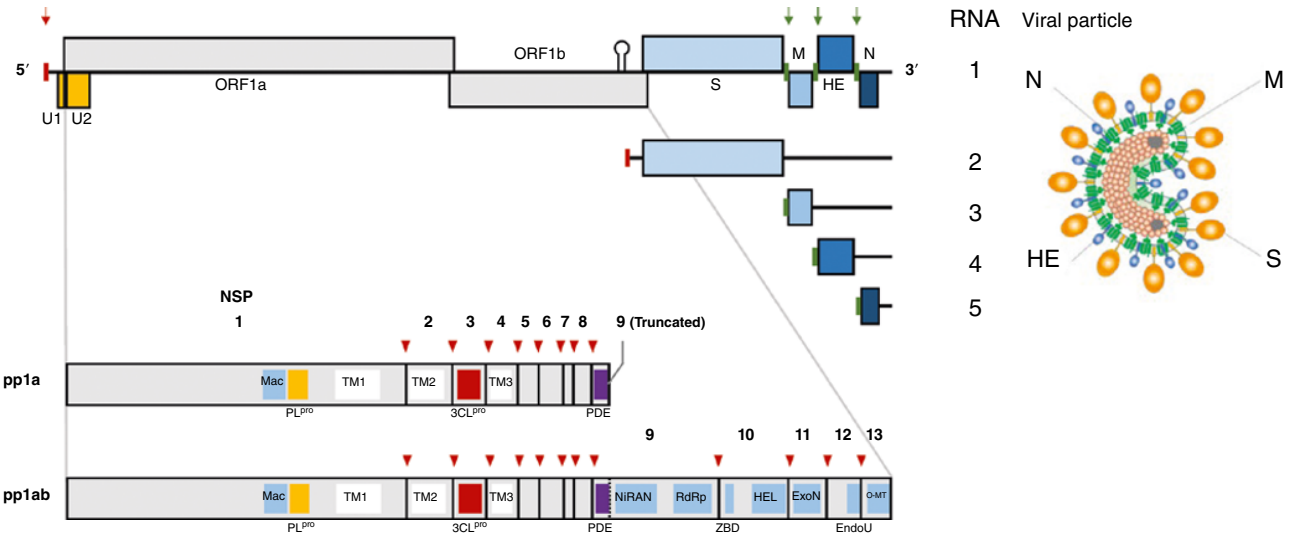


Figure 61.6 Genome organization of members of the subfamily *Torovirinae* (e.g., BToV genome is depicted). ToVs have a positive-sense, single-stranded RNA genome of about 28–30 kb with a 5′-cap structure and a 3′-poly (A) tail, consisting primarily of six conserved open reading frames (ORFs). The genome has 5′-UTR and 3′-UTR regions. The 5′-proximal two-thirds of the genome contains ORF1a and ORF1b, encoding replicase-transcriptase proteins similar to CoVs. These are synthesized as two large polyproteins; pp1a is translated from ORF1a and pp1ab from ORF1a/1b by −1 ribosomal frameshifting. These polyproteins are proteolytically processed into 13 nonstructural proteins (nsps). The remaining one-third of the genome encodes four structural (or accessory) proteins: S, M, HE, and N proteins in that order. *Source:* A schematic diagram of genome organization was reproduced from Ujike and Taguchi (2021) with permission.

polymerase activity. These products of the large replicase protein, possibly with other viral and cellular proteins, assemble into a replication/transcription complex (RTC) bound to modified intracellular membranes.

The structural proteins encoding ORFs are located in the 3′ one-third of the genome, for all coronaviruses, in the order 5′-S-E-M-N-3′. The ORFs encoding these proteins are interspersed with several ORFs encoding nsps (accessory proteins) and HE glycoprotein, which differ significantly among coronaviruses in number, nucleotide sequence, gene order, and method of expression (Figure 61.5). However, they are conserved within the same group of coronaviruses; thus, they are called group-specific proteins. When the HE protein is expressed, it is encoded 5′ to the S protein. The structural and accessory proteins are expressed from a 3′ co-terminal nested set of subgenomic mRNAs (sgmRNAs). Briefly, replication of the genomic RNA involves the synthesis of a full-length negative-stranded RNA that serves as a template for full-length genomic RNA. The structural and accessory proteins are expressed from multiple overlapping 3′ co-terminal sgmRNAs. Each sgmRNA has a common leader sequence at its 5′-end derived from the 5′-end of the genomic RNA. The sgmRNAs are synthesized from sg-negative-strand RNAs. The anti-leader sequences are added onto the 3′-ends of negative-stranded RNAs, which serve as templates for synthesizing sgmRNAs. Most of the time, one viral structural protein is translated from individual sgmRNAs. However, in some cases, there may be two ORFs carried on and translated from one sgmRNA.

The S glycoprotein forms the large peplomers on the virion surface, giving the virus its coronal or crown-like morphology when examined under the electron microscope. The S protein is a heavily glycosylated type I membrane glycoprotein that can be divided into three structural domains: a large N-terminal external domain further divided into two domains, S_1 and S_2; a transmembrane domain; and a short carboxy-terminal cytoplasmic domain. The S1 subunit forms the globular portion of the spike, and there is considerable sequence variation due to various deletions and substitutions within this region in different coronavirus strains. Thus, mutations in S1 sequences have been associated with altered antigenicity, pathogenicity, and cellular tropism of the virus. In contrast, the S2 subunit is more conserved and constitutes the stalk of the spikes. There is evidence that the mature S protein of some coronaviruses (e.g., SARS-CoV-2) naturally assembles into homo-trimers. In some coronaviruses (e.g., MHV and BCoV), the S protein gets cleaved by the cellular proteases during or after virus maturation, and the S1 and S2 subunits remain noncovalently associated in the viral spikes. The S protein of coronaviruses has several biological properties, which include binding to the specific cellular receptor(s), inducing neutralizing antibodies, eliciting a cell-mediated immune response, inducing fusion of the viral envelope with host cell membranes, inducing cell-to-cell fusion, and binding the Fc fragment of immunoglobulins (MHV and TGEV). The cellular receptors for many coronavirus S proteins have been identified. These include angiotensin-converting enzyme 2 (ACE2), the host cell receptor of SARS-CoV, SARS-CoV-2, and HCoV-NL63; and dipeptidyl peptidase 4 (DPP4), which is the receptor for MERS-CoV. Recently, full-length S protein or fragments of S protein (e.g., receptor-binding domain [RBD], the S1 subunit, N-terminal domain [NTD], and fusion peptide [FP]) have been used to develop a recombinant vaccine against SARS-CoV-2 and other coronaviruses.

The HE glycoprotein is found in all lineage 2a Beta-CoVs (Figure 61.3; e.g., BCoV, HCoV-OC43, MHV) and one member of the Gamma-CoVs (Turkey coronavirus [TCoV]) as a disulfide-linked dimer that forms short spikes on the virion surface. The absence of HE protein in some coronaviruses and the frequent tendency of its structure to be mutated or completely deleted during serial cell culture passage suggest that this protein is not essential for viral replication. The HE gene of coronaviruses is believed to be acquired from the Influenza C virus by heterologous recombination. The HE protein of coronaviruses has several biological properties, including hemagglutination, hemadsorption, and esterase activity that cleaves acetyl groups from N-acetyl-9-O-acetylneuraminic acid and may play a role in initial virus adsorption and entry or release from the infected cells.

The M protein of coronaviruses is glycosylated with a short amino-terminal domain exposed to the exterior surface, followed by a triple-membrane-spanning domain, an α-helical domain, and a large carboxy-terminal domain inside the viral envelope. The glycosylation of the amino-terminal of the M protein can either be O-linked (MHV) or N-linked (IBV, TGEV, and SARS-related CoV). In some coronaviruses, the carboxy-terminal of the M protein is exposed on the virion surface (TGEV). In MHV, monoclonal antibodies against M protein neutralize the virus. The M protein of coronaviruses interacts with both the N and S proteins and may play a key role in virus assembly. It may also play a major role in packaging viral RNA into nucleocapsids during virus assembly. The E protein of coronaviruses acts synergistically with the M protein during virus budding from the infected cells. In addition to this, E protein also functions as an ion channel. The N protein interacts with the viral genomic RNA to form the viral nucleocapsid. The N protein has three relatively conserved structural domains, and it interacts with M protein, leading to the incorporation of the nucleocapsid into the virus particle.

Toroviruses

Toroviruses are pleomorphic and measure 120–140 nm in diameter. Spherical, oval elongated, and kidney-shaped particles have been visualized under EM. Toroviruses are enveloped with a helically symmetric tubular nucleocapsid. The nucleocapsid forms a doughnut-shaped structure, and the envelope contains a large number of small spikes (15–20 nm) that resemble the peplomers of coronaviruses. Torovirus particles consist of at least four structural proteins: nucleocapsid protein (N), unglycosylated membrane protein (M), spike glycoprotein (S), and HE protein. ToVs have a positive-sense, single-stranded RNA genome of about 28–30 kb with a 5′-cap structure and a 3′-poly (A) tail, which consists primarily of six conserved ORFs (Figure 61.6). The genome has 5′-UTR (~820–850 kb) and 3′-UTR (~200 kb), respectively. The 5′-proximal two-thirds of the genome contains ORF1a and ORF1b, encoding replicase-transcriptase proteins similar to coronaviruses. These are synthesized as two large polyproteins: pp1a is translated from ORF1a and pp1ab from ORF1a/1b by −1 ribosomal frameshifting. These polyproteins are proteolytically processed into 13 nsps. The remaining one-third of the genome encodes four structural (or accessory) proteins: S, M, HE, and N proteins in that order. Toroviruses have a buoyant density of 1.14–1.18 g/cm^3 in sucrose and an estimated sedimentation coefficient (S20,w) of 400–500. Virus infectivity is stable between pH 2.5 and 9.7 but is rapidly inactivated by heat, organic solvents, irradiation, and most disinfectants.

Animal Diseases Caused by Members of the Genus *Alphacoronavirus*

Currently, four different Alpha-CoVs have been reported from swine, and these include TGEV, PRCV, PEDV, and SeACoV, also known as PEAV or SADS-CoV (Table 61.2). Alpha-CoVs of dogs and cats include CCoV, and FCoV (and its mutated form FIPV). Other Alpha-CoVs of animals include MCoV, FRSC, FRECV, rabbit coronavirus (RbCoV), and RCoV. Recently, new Alpha-CoVs have been reported in rats, voles, shrews, and camels.

Transmissible Gastroenteritis Virus and Porcine Respiratory Coronavirus

Disease

Transmissible gastroenteritis (TGE) is a highly contagious enteric disease of swine caused by TGEV. TGEV is antigenically related to coronaviruses of humans, dogs, and cats. Only one serotype of TGEV is recognized, but antibodies to TGEV can antigenically cross-react with PRCoV. However, some antigenic sites of TGEV are absent from PRCoV because of a deletion in the amino terminus of the S protein in PRCoV (see below). TGEV and PRCoV are inactivated by lipid solvents (ether and chloroform), sodium hypochlorite, quaternary ammonium compounds, iodines, heating at 56 °C for 45 minutes, and exposure to sunlight. Both viruses are stable when frozen but somewhat labile at room temperature. TGEV is stable for long periods when frozen at −80 °C, but when held at 37 °C for four days, there was a total loss of infectivity. In liquid manure, the infectious virus persists for more than 8 weeks at 5 °C, 2 weeks at 20 °C, and 24 hours at 35 °C. TGEV resists inactivation by trypsin and acidic pH (pH 3.0) and is relatively stable in pig bile. These properties allow the virus to survive in the stomach and small intestine.

TGEV is characterized by severe diarrhea, vomiting, dehydration, and high mortality in young piglets (less than two weeks of age). Mortality in older pigs (greater than five weeks) is usually low. Older growing and finishing pigs develop transient watery diarrhea, but vomiting is uncommon. TGEV infection of adult swine tends to be asymptomatic. Still, infected sows sometimes exhibit anorexia, diarrhea, fever, vomiting, and agalactia.

More than 35 years ago, PRCoV was detected in swine herds with minor respiratory symptoms. This virus is very similar to TGEV, but a major difference between the two porcine coronaviruses is a 672 base in-frame deletion located 59 bases downstream of the initiation codon of the S gene that encodes a truncated S protein in PRCoV with 1225 amino acids as opposed to 1447–1449 amino acids in TGEV Purdue and Miller strains. This difference involves the omission in the PRCoV S sequence of a continuous stretch of 224 residues starting at position five from the N terminus.

PRCoV is a natural deletion mutant of TGEV and has changed the tropism from porcine enteric epithelium to respiratory epithelium and alveolar macrophages. Because of this structural difference, TGEV but not PRCoV has a sialic acid-binding activity that allows the attachment to mucins and mucin-type glycoproteins. The sialic acid-binding activity may allow TGEV to overcome the mucus barrier in the gut and access the intestinal epithelium for initiation of infection. As PRCoV shares some epitopes for neutralizing antibodies with TGEV, it acts like a natural vaccine against TGEV, resulting in a drastic reduction of TGE outbreaks in Europe. Thus, PRCoV can infect pigs of all ages by aerosol or direct contact transmission. PRCoV infections are generally subclinical, but strains of the virus differ in the severity of the clinical signs they induce. Clinical signs include moderate to severe respiratory disease with interstitial pneumonia. In addition, PRCoV

infection can be concurrently associated with other respiratory virus infections such as PRRSV, which can alter the severity of the disease and associated clinical signs.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

TGE has only been described in swine; however, TGEV has been isolated from the feces of experimentally infected cats, dogs, foxes, and starlings (*Sturnus vulgaris*) for up to 20 days. Serological studies have also suggested natural infection of skunks, opossums, muskrats, and humans. Following experimental and natural infection, the virus has also been demonstrated in house flies (*Musca domestica Linnaeus*).

TGEV infection of swine occurs worldwide and is documented in North, Central, and South America, Europe, and Asia. Epizootics of TGE are often seasonal in the United States, occurring in the winter months. The primary mode of transmission appears to be ingestion of feed contaminated with infected feces (fecal–oral). Persistence of TGEV in nature is likely via the fecal carrier/shedding state in recovered swine; thus, the virus is maintained in endemically infected herds through ongoing fecal–oral infection of susceptible pigs. Infection of swine in endemically infected herds is often subclinical or mild, whereas the virus spreads rapidly in non-immune herds and can cause devastating disease outbreaks. In addition to the movement of infected swine, TGEV is potentially transmitted between herds by fomites and other animals.

PRCoV has been isolated in North America and Europe. Infected pigs shed viruses in their respiratory secretions, and PRCoV may persist in herds by continuous infection of newly weaned pigs. Although this is not an enteric pathogen, the virus has been demonstrated in feces by virus isolation and nested reverse transcription-polymerase chain reaction (nRT-PCR), which may suggest the possibility of fecal–oral transmission.

Pathogenesis and Pathology

TGEV survives in the gastrointestinal tract after ingestion because of its resistance to low pH and trypsin; thus, it passes through the stomach without inactivation. Six to twelve hours following intragastric inoculation, viral replication occurs in the villus epithelial cells of the small intestine with the highest titers of virus in the jejunum. TGEV infects and destroys the columnar epithelial cells lining the intestinal villi, resulting in atrophy of the villi. Villous blunting and increased crypt depth (as a consequence of replication of the progenitor cells in the crypts to repopulate the denuded villi) occurs 24–40 hours post infection and coincides with the occurrence of severe diarrhea. The loss of enterocytes lining the villi results in malabsorption and maldigestion, which in turn causes diarrhea and dehydration. Undigested lactose in the intestinal contents passes to the large bowel, where it exerts an osmotic effect that further exacerbates diarrhea in TGEV-infected swine. Affected piglets are dehydrated with fecal staining around the perineum. The typical lesions include thinning of the intestinal wall, villous atrophy, and gastrointestinal distension with yellow fluid containing curds of undigested milk.

PRCoV is transmitted via respiratory aerosols and droplets to susceptible in-contact animals. The virus replicates in the tonsils, mucosal epithelium of the nasal passages and the airways of the lungs, and the type I and the type II pneumocytes in the alveolar lining. This causes inflammation and necrosis of terminal airways of the lungs, leading to diffuse bronchointerstitial pneumonia. The severity of clinical signs and lesions may vary, and subclinical infection can occur in herds.

Host Response to Infection

Neutralizing antibodies develop within approximately seven days of TGEV infection of swine. The presence of secretory IgA plays a major role in protective immunity and viral clearance. Intramuscular immunization of pigs with TGEV results in the development of a humoral IgG response but not protective immunity. Conversely, pigs immunized orally with TGEV develop protective virus-specific IgA in their intestinal mucosal secretions. Infection of sows with TGEV results in the secretion of protective IgA in colostrum (so-called lactogenic immunity), which is protective in suckling pigs. Cell-mediated immunity also is likely important in the immune response to TGEV infection, as passive transfer of mononuclear leukocytes from immune donor pigs to susceptible histocompatible piglets results in reduced disease expression. In addition, high levels of type I interferon are produced by infected intestinal cells, which also may play a role in controlling viral replication. Pigs infected with PRCoV develop neutralizing antibodies to the virus. Antibodies against PRCoV provide partial protection against TGEV, and the incidence and severity of TGE may decline in swine herds with endemic PRCoV infection.

Laboratory Diagnosis

The clinical signs and atrophic enteritis caused by TGEV are frequently observed in other enteric infections (PEDV, PDCoV, rotavirus, and coccidia). Therefore, laboratory diagnosis is necessary for the definitive diagnosis of TGEV.

TGEV infection of young pigs is usually diagnosed through detection of viral antigen in mucosal scrapings or frozen sections of jejunum and ileum by

immunohistochemical (IHC) or immunofluorescent (IF) staining with virus-specific antibodies. Definitive diagnosis of TGEV can be made by viral isolation in cell cultures (pig kidney [PK], testis, or thyroid gland). EM or immuno-EM can also be used on fecal contents or intestines for diagnosis. Standard and real-time RT-PCR (RT-qPCR) assays for the detection and differentiation of TGEV and PRCoV are available.

TGEV has been propagated in various cell culture systems, including PK, testis, salivary gland, and thyroid gland; organ cultures of the esophagus, intestine, and nasal epithelium; canine kidney cell cultures; and embryonated chicken eggs (ECC) (amniotic cavity). PK and swine testicle (ST) cell lines have been the choice of cells for virus isolation from feces or gut contents of infected pigs. Development of cytopathic effect may require multiple passages in cell culture. PRCoV replicates in PK and ST cells, as well as a cat fetus cell line.

Serological diagnosis is appropriate when acute and convalescent sera are available. However, serological diagnosis is complicated because both PRCoV and TGEV induce neutralizing antibodies that cross-neutralize each other. Therefore, TGEV strains are not distinguished from nonenteropathogenic variant PRCoV by the virus neutralization test but can be differentiated by blocking enzyme-linked immunosorbent assay (ELISA).

Treatment and Control

Treatment of TGEV-infected pigs is usually unrewarding. Replacement of fluids and antibacterial drugs to reduce complications associated with enteropathogenic *Escherichia coli* may be beneficial. Inactivated and modified-live attenuated virus vaccines are available for vaccination of pigs to prevent TGE. These can be used for vaccination of newborn piglets or immunization of sows, or both. Vaccination of pregnant sows provides lactogenic immunity that is passively transferred to piglets via colostrum. Vaccines have been variably successful in preventing TGE. Oral immunization provides optimal stimulation of local immunity (secretory IgA) in the intestine. The practice of infecting sows with virulent TGEV at least three weeks prior to farrowing to induce an immune response by providing colostral immunity to piglets is complicated by the fact that this practice contaminates the environment with virulent TGEV that subsequently can spread the infection to susceptible pigs.

Porcine Epidemic Diarrhea Virus

Recently, a coronavirus has been isolated from swine with diarrhea, and it was designated PEDV. This virus is antigenically distinct from TGEV and PHEV and causes acute diarrhea, and/or vomiting, dehydration, and high mortality in neonatal pigs. There are two different genotypes of PEDV: the PEDV variant with multiple deletions and insertions in the S1 subunit of the spike (S) protein (S INDEL [G1b] genotype) and the non-S INDEL (G2b) genotype. Both genotypes were detected during the diarrheal disease outbreak in US swine in 2013–2014. The US G2b non-S INDEL strains were genetically closest to PEDV strains that emerged in China in 2010. In China, the G1b S INDEL PEDV strains were detected in 2011, probably resulting from recombination events between PEDV strains circulating in the field. Subsequently, similar PEDV strains have been identified circulating worldwide. The G2b non-S INDEL PEDV was highly virulent; in contrast, the G1b S INDEL PEDV caused milder disease.

PEDV infection of neonatal pigs causes fecal virus shedding (alongside frequent detection of PEDV RNA in the nasal cavity), acute viremia, and severe atrophic enteritis – mainly jejunum and ileum. Pathogenesis studies indicate that PEDV replicates in the small and large intestines, but lesions are confined to the small intestines. Affected pigs have small intestines distended with yellow fluid; the lesions are similar to those of TGE. There is moderate to severe villous atrophy with large numbers of PEDV-infected epithelial cells in the mid-jejunum to the ileum. Duration of diarrhea in infected nursing pigs varied from two to eight days. Prolonged fecal viral RNA shedding up to 15–26 days post infection (dpi) has been reported. Infected pigs are seroconverted at 7–14 dpi, with peak serum PEDV-specific IgG or IgA antibody titers at 21 dpi. Thus, PEDV-specific IgA effector and memory B cells in previously infected sows play a critical role in sow lactogenic immunity and passive protection of piglets. PEDV is primarily transmitted via the fecal–oral route, but airborne transmission via the fecal–nasal route may play a role in pig-to-pig and farm-to-farm spread.

PEDV particles can be demonstrated in the feces of infected pigs by direct EM, and the virus can be propagated in some African green monkey (Vero) cell lines but not in others. Viral growth depends on the presence of trypsin in the cell culture medium. Generally, field strains of PEDV need to be adapted to grow in cell culture, making it difficult to use as a routine diagnostic assay. Virus can be adapted to grow in primary porcine cell culture or Vero cells (African green monkey cells). A direct immunofluorescence assay (IFA) and immunohistochemistry (IHC) technique applied on a section of the small intestine are the most sensitive, rapid, and reliable methods of diagnosing PEDV in pigs. A serological diagnosis can be made by

demonstrating PEDV antibodies using IFA and ELISA. A multiplex RT-qPCR procedure developed for the simultaneous detection of PEDV and TGEV in preweaning pigs with diarrhea has been described.

Attenuated vaccines are available in some Asian countries to prevent PEDV infection in pigs. In the United States, inactivated vaccines are commercially available. However, control of the disease is also dependent on management and husbandry practices. Specifically, the enhanced biosecurity measures, such as decontamination and disinfection of transport trailers and other potential contaminated fomites, supply of secure feed or feed additives, and maintenance of strict hygiene measures on swine farms and farm workers, are critical to prevent direct or indirect transmission of PEDV.

Swine Enteric Alphacoronavirus

The SeACoV, which was first identified in Guangdong Province, China, in 2017, caused an acute diarrheal outbreak in suckling piglets. SeACoV is also known as the SADS-CoV or PEAV and is the fifth porcine CoV identified in piglets with diarrhea. SeACoV was the most recently recognized CoV of potential bat origin before the emergence of the new human severe acute respiratory syndrome CoV 2 (SARS-CoV-2), associated with the pandemic CoV disease 2019 (COVID-19). Sequence analysis has shown that the novel SeACoV sequence is similar to that of bat coronavirus HKU2. Thus, SeACoV may have originated from the interspecies transmission of bat-HKU2 from bat to swine. SeACoV is currently recognized as a regional epizootic virus, and it has not been reported in other provinces of China or in any other regions in the world.

SeACoV was first isolated from the feces of piglets with severe watery diarrhea and high mortality rates. The virus primarily replicates in the jejunum of infected piglets. Its clinical symptoms are similar to those of PEDV and TGEV, including severe diarrhea, vomiting, and dehydration. Like other porcine enteric viruses, SeACoV is transmitted through the fecal–oral route.

SeACoV can be diagnosed by virus isolation in Vero cells and confirmation by IFA using virus-specific antiserum. Diagnosis can also be made via detection of viral nucleic acid in clinical specimens using various molecular techniques (i.e. reverse transcription loop-mediated isothermal amplification [RT-LAMP] and RT-qPCR) or demonstration of virus-specific antibodies in pig serum by ELISA. Currently, there are no vaccines against SeACoV infection, and prevention and control of the disease depend on the implementation of enhanced biosecurity measures to prevent the direct and indirect spread of the virus.

Canine Enteric Coronavirus

Disease

CCoV infects domestic and wild canine species (Table 61.2). This virus was first isolated from dogs with acute enteritis. CCoV infection of dogs is highly contagious and generally causes inapparent or mild gastroenteritis. CCoV is an important enteropathogen of dogs and is widespread in dog populations, mainly in kennels and animal shelters. Clinical signs include anorexia, lethargy, vomiting, diarrhea, and dehydration. The virus is shed at high titers in the feces of infected dogs, leading to a typical fecal–oral route of transmission characterized by high morbidity and low mortality. Fatal infection usually occurs due to mixed infections with CCoV and canine parvovirus type-2, canine adenovirus type-1, or canine distemper virus (CDV). CCoV is antigenically related to other coronaviruses, including TGEV, feline enteric coronavirus (FCoV), and FIPV (mutated FCoV). Multiple antigenically and genetically distinct strains of CCoV have been recognized, including a novel respiratory coronavirus.

Based on genetic, antigenic, and biological properties, the canine enteric coronaviruses can be broadly classified into CCoV type I (CCoV-I) and CCoV type II (CCoV-II). The type I and type II CCoVs share up to 96% nucleotide identity. Subsequently, CCoV-II was divided into two subtypes, CCoV-IIa and CCoV-IIb, with the second of these arising as a result of a putative double recombination event between CCoV-II and TGEV (TGEV-like CCoV). In contrast to CCoV-I strains, some CCoV-IIa strains are considered highly virulent and cause fatal systemic disease in pups. A pantropic variant causing systemic disease in pups was identified in 2005 (CCoV CB/05). The virus was isolated from feces and various parenchymatous organs of affected puppies. Subsequently, the disease was reproduced after experimental infection of dogs, with young puppies showing more severe clinical signs. Pantropic CCoV-IIa strains (e.g., CB/05 and NA/09 strains) cause severe systemic disease in dogs, characterized by fever, lethargy, anorexia, depression, vomiting, hemorrhagic diarrhea, severe leukopenia, and neurological signs (seizures and ataxia) followed by death 48 hours after the onset of symptoms.

In addition, various recombinants between CCoV-I and CCoV-II strains and the TGEV spike protein gene have been reported. Although TGEV-like CCoVs were detected in the internal organs of naturally infected dogs, experimental infection failed to cause systemic involvement or virus dissemination through the blood. CCoVs are inactivated by lipid solvents and most disinfectants and are heat labile. The viruses are acid stable (pH 3.0) and retain infectivity under cool conditions.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

CCoV was first isolated in 1971 from an epidemic of diarrhea in dogs in Germany. The virus has since been recognized worldwide, including North America, Europe, Australia, and Asia. Infected dogs excrete high titers of virus in their feces for two weeks or longer, and fecal contamination of the environment is the primary source for its transmission via fecal–oral infection.

Pathogenesis and Pathology

Following an incubation period of one to four days, the virus causes infection of small intestinal epithelial cells that progressively passes through the gastrointestinal tract, leading to mild gastroenteritis. Diarrhea occurs 1–7 dpi, with the virus being shed in feces within one to two days following the appearance of clinical signs. Viral replication in the intestinal epithelium results in desquamation and shortening of the villi. Diarrhea associated with CCoV infection occurs as a consequence of intestinal maldigestion and malabsorption. The pups infected with the pantropic CCoV will develop severe lesions in the alimentary tract, tonsils, lungs, liver, spleen, and kidneys. The most prominent lesion described was hemorrhagic gastroenteritis. The other lesions include enlarged tonsils with multifocal hemorrhages, bronchopneumonia in the cranial and caudal lobes with effusions in the thoracic cavity, enlarged spleen with subcapsular hemorrhages, necrosis, and lipidosis with hemorrhages in the liver. In addition, infarction and hemorrhages have been reported in the cortex and medulla of kidneys. Although CCoV infection is widespread, mortality is typically very low. However, fatal disease in dogs commonly occurs as a consequence of mixed infections of CCoV with canine parvovirus type 2 (CPV-2) with canine adenovirus type 1 (CAdV-1) or with CDV.

Host Response to Infection

Mucosal immunity appears to be protective as dogs orally infected with CCoV become immune, whereas those immunized parenterally do not. It has been reported that immunity after natural exposure to enteric CCoV does not provide complete protection against the new pantropic CCoV infections.

Laboratory Diagnosis

Virus or viral antigens can be visualized by EM or fluorescent antibody (FA) staining of feces or necropsy tissues. Antiserum specific for CCoV is commonly used to aggregate virus prior to negative staining for EM. Virus can be isolated from feces or intestinal tissue on cell culture. Several primary (kidney and thymus) and continuous canine cell lines of thymus, embryo, synovium, and kidney (line A-72) are susceptible to infection. The virus also infects feline kidney and embryo fibroblast cell lines. RT-PCR (including RT-qPCR) assays have been developed to detect CCoVs in feces. CCoV-I can be distinguished from CCoV-II using genotype-specific conventional RT-PCR or RT-qPCR assays. Serum virus neutralization and ELISA tests for the detection of CCoV antibodies have been developed. An ELISA based on recombinant spike protein S for detecting and differentiating antibodies to TGEV-like CCoV from other CCoV-II strains has recently been described.

Treatment and Control

Treatment of CCoV-associated gastroenteritis is limited to relieving dehydration and electrolyte loss in severe cases. Inactivated and modified-live virus (MLV) vaccines are available for parental administration for protection against CCoV infection. However, their use is questionable due to the apparent importance of local immunity at the level of the intestinal mucosa.

Feline Enteric Coronavirus and Feline Infectious Peritonitis Virus

Disease

Feline infectious peritonitis (FIP) is a progressive, and highly fatal disease of domestic and some wild feline species. The signs are highly variable and reflect the tissues affected by the disease, but persistent fever, weight loss, lethargy, dyspnea, and abdominal distension are common clinical signs. The disease can occur in cats of all ages but it is especially common in young (<1 year of age) and very old cats. Two distinct forms of FIP are recognized: (i) an effusive (wet) and (ii) a noneffusive (dry) form. The effusive form is characterized by the accumulation of protein-rich fluid (effusion) in the peritoneal cavity. The noneffusive form is characterized by the formation of granulomas in internal organs, central nervous system (CNS), and eyes. Both forms of the disease are fatal. FIP has a highly complex pathogenesis that involves the mutation of relatively apathogenic FCoV into a mutated coronavirus (FIPV) that undergoes a change in cellular tropism and is able to replicate in macrophages to produce an immune-mediated disease in affected cats. FCoV seems confined to the intestinal tract and causes mild, often unapparent enteritis, especially in kittens. Comparative sequence analysis of FCoVs and FIPVs has shown that these viruses are genetically closely related but differ in their pathogenic potential (different pathotypes of the same virus). The FIPV efficiently infects macrophages and monocytes that escape from the intestine to cause lethal systemic disease with multi-organ involvement, in classical cases accompanied by

accumulation of abdominal effusion. One of the hypotheses is that the FIPV pathotype results from a mutation(s) in the FCoV genome.

The infection with FCoV initially occurs in intestinal epithelial cells, and it is known to cause persistent infections with chronic, often intermittent shedding of virus in feces. However, the FCoV mutates and acquires the ability to enter and replicate efficiently in the monocytes and macrophages of cats. Specific mutations in the FCoV genome change the cellular tropism and systemic spread of the newly selected FIPV and contribute to a change in virulence and FIP development. Interestingly, as the efficiency of FIPV replication in monocytes and macrophages increases, the ability to replicate in enterocytes decreases. Thus, FIPV is not shed or is shed at very low levels in the feces of cats, which may explain why FIP epidemics are uncommon compared to other feline viral infections (e.g., panleukopenia virus or feline calicivirus). Two significant mutations causing two alternative codons in the FIPV genome have been mapped to the S glycoprotein gene, specifically, in the FP of S. These nucleotide changes alter two amino acids within the region responsible for fusion between the viral envelope and the cell membrane. These two amino acid changes in the S protein lead to a change in cellular tropism and efficient replication of the virus in monocytes and macrophages, resulting in FIP development.

In addition, other mutations in the FIPV S protein gene correlate with increased virulence of FIPV. Specifically, changes within the furin recognition site on the S protein cleavage site (i.e. cleavage of S to S1 and S2) lead to modulation of the proteolytic processing of the protein critical for the development of FIP. Some mutations in the M gene of FIPV have also been correlated with the virulent phenotype of FIP. However, these mutations have not been found consistently in the M gene of all cases of FIP. Finally, the change in FIPV tropism is speculated to involve mutations in nsps such as 3c, 7a, and 7b. It has been shown that with deletions in the 3c gene, the virus is not shed in feces. Therefore, it is believed that an intact 3c gene was required for virus replication in the intestine but not in monocytes and macrophages. However, virulence determinants specifically associated with FIPV pathogenesis have not been completely identified. This may also explain why FIP outbreaks in multiple cats are rarely seen. Host factors such as genetic characteristics, age, and concurrent disease, especially immunosuppressive diseases, can predispose to FIP development. In addition, various factors that affect the cat's immune system status, including MHC diversity, cytokine production, and lymphocyte apoptosis, are critical factors for FIP development.

Environmental factors, including stress, seem to influence FIP development. For example, stress can increase glucocorticoid production, negatively impacting T lymphocytes (Th1), directly decreasing CMI and interfering with virus clearance. In addition, cats living or originating from multi-cat settings (e.g., shelters, breeding facilities, cat shows) are more likely to develop FIP. This is primarily because the likelihood of FCoV infection is higher where multiple cats live together. Other factors leading to FCoV exposure include a high density of cats, frequent introductions, reintroductions, the mingling of cats from different sources (e.g., shelters, breeding facilities, at cat shows), presence of chronic FCoV shedders, and inadequate disinfection.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

Based on serological differences, FCoV strains have been separated into two serotypes (more common FCoV type I and less common FCoV type II), both being able to cause two forms of FIP (dry and wet form). FCoV type I is difficult to grow in cell culture, whereas FCoV type II can be readily propagated. The FCoV type II is genetically more closely related to CCoV. It has been shown that FCoV type II emerged as a result of double recombination events between FCoV type I and CCoV type II. FCoV II may have further mutated to induce a severe form of FIP in cats, but the diseased cats do not spread the virus to other cats. FCoVs and FIPV are resistant to acid and trypsin but are readily inactivated by most disinfectants, including lipid solvents.

Infectivity for Other Species and Culture Systems

In addition to infecting domestic cats, FIPV has been associated with disease in wild *Felidae* such as lions, cheetahs, mountain lions, leopards, jaguar, lynx, caracal, sand cats, and Pallas's cats. Young pigs can be experimentally infected with FIPV, resulting in the development of lesions similar to those induced by TGEV. In addition, suckling mice are susceptible to infection, with viruses replicating in the brain. FIPV primarily replicates in macrophages, but the virus can be propagated *in vitro* in feline organ cultures, cell lines, and mononuclear phagocytes (FCoV type II).

Host–Virus Relationship

Distribution, Reservoir, and Transmission

FCoV and FIPV occur worldwide. FCoV spreads efficiently via the fecal–oral route, but in contrast, FIPV is not well transmitted. FCoV may persist subclinically for up to a year or longer, and these persistently infected cats serve as the virus reservoir, which allows horizontal spread between cats.

Pathogenesis and Pathology

The pathogenesis of FIP is complex, and much remains to be resolved. Initial FCoV infection is rarely characterized by apparent disease but results in persistent, low-level infection of macrophages in many tissues. The development of clinical FIP is associated with increased viral replication, usually secondary to some immunosuppressive event that suppresses cellular immunity. Increased virus replication results in the emergence of viral variants (FIPV) that replicate with increasing efficiency in macrophages, where they persist, safe from immune clearance. Antibody exacerbates the disease, which suggests that FIP is at least in part an "immune-mediated" disease. Virus-specific antibody actually facilitates the uptake of FIPV by phagocytes in which pathogenic strains of the virus replicate.

The "wet" form of FIP is an acute and rapidly progressive disease with a fatal outcome within weeks to two months. This form is associated with an ineffective cell-mediated immune response and a strong antibody response leading to the formation of immune complexes that deposit in blood vessel walls (type III hypersensitivity). The vascular damage leads to severe vascular leakage and formation of pleural, pericardial, and peritoneal effusions and fibrinous exudation. In contrast, the "dry" form of FIP is a chronic and slowly progressive disease with a fatal outcome in several months. As opposed to the "wet" form, it is associated with partial cell-mediated immunity in the absence of a humoral response by which T helper type 1 (Th1) lymphocytes attract macrophages inducing granulomatous perivascular inflammation (type IV hypersensitivity). Both "wet" and "dry" forms of FIP can occur concurrently and lead to the development of vasocentric granulomas (or pyogranulomas). It is proposed that deposition of complexes of FIPV and specific antibodies (immune complexes) in the walls of blood vessels is responsible for the characteristic perivascular location of these lesions. These perivascular granulomas can occur in the gastrointestinal tract, kidneys, liver, lungs, CNS, eyes, and lymph nodes of affected cats and are especially common on the serosa of the abdominal viscera in cats with the wet form of FIP.

Host Response to Infection

The basis of immunity to FIPV is poorly understood. Cats develop humoral and cellular immune responses soon after FCoV infection, and these responses hold the infection in check until some stress or concurrent infection causes immune suppression. Antibodies to FCoV cross-react with FIPV, and these cause disease expression rather than protection; this antibody facilitates viral uptake by phagocytic cells (antibody-dependent enhancement) where the virus effectively replicates, and viral antigens complexed with specific antibodies (and complement) contribute to immune-mediated vasculitis.

Laboratory Diagnosis

Antemortem diagnosis of FIP can be difficult. There is currently no single, fully accurate diagnostic test for the antemortem diagnosis of FIP. A combination of history, progression of the disease, complete blood count/biochemistry panels, fluid analysis (if effusions are present), and other complementary diagnostic tests may be needed. Noneffusive FIP is more difficult to diagnose and must be differentiated from other infectious, granulomatous, and neoplastic conditions. Serological diagnosis may be determined by viral neutralization, ELISA, or IFA techniques. A serum titer of greater than 1:1600 or 1:3200 (varies among labs) would support the diagnosis of FIP. However, serological testing can be confounded by previous exposure to FCoV and cross-reactivity or low levels of antibody titers in some cats with FIP. Therefore, serology is not recommended for the diagnosis of FIP. Hematological changes include non-regenerative anemia and neutrophilic leukocytosis with lymphopenia, eosinopenia, and monocytosis. Serum biochemistry changes, such as hypoalbuminemia and hyperglobulinemia with decreased albumin-to-globulin (A:G) ratio, bilirubinemia, and bilirubinuria, suggest FIPV infection in cats. Usually, there is an elevated total protein, and a decreased A:G, reflecting an increase in blood gamma globulins. An A:G greater than 0.6–0.8 has a high negative predictive value for FIP. Thus, finding a higher A:G helps rule out FIP when the prevalence in the population is low. However, none of these findings are pathognomonic on their own.

Several RT-qPCR assays for diagnosis of FIP have been developed, including assays targeting the S protein gene-associated mutations in the FIPV. Although these assays targeting the S protein mutations may be helpful, it is not clear that these mutations are present in every case of FIP, nor if these mutations may be present in asymptomatic virus-infected cats. A positive PCR assay for the spike mutation provides good evidence for FIP, but a negative test result could not rule out this diagnosis, especially when testing serum or plasma. Furthermore, it has also been demonstrated that identifying mutations within the spike protein gene does not significantly enhance FIP diagnosis compared with detection of FIPV alone by reverse transcription PCR. Thus, the peritoneal fluid remains the specimen of choice for diagnosing FIP by RT-qPCR, which is absent in the dry form (noneffusive) of the disease. With these caveats, RT-qPCR is valuable because it reveals the systemic spread and presence of the virus in various sites other than the intestine. However, false negative and false

positive test results are possible; therefore, it may not be possible to confirm FIP diagnosis with RT-qPCR alone. Nevertheless, when viral nucleic acid (i.e. RNA) is found in the blood, peritoneal effusion, CSF, or tissue sample by RT-qPCR, along with the other FIP indicators described above can confirm the diagnosis of FIP. Histopathology (with identification of pyogranulomatous inflammatory foci particularly with a vasocentric pattern) and IHC for the detection of FIPV in affected tissues (either biopsy specimens or postmortem tissues) remains the gold standard for diagnosis of FIP. Unfortunately, this involves the invasive collection of samples, which is often difficult in a sick and debilitated cat.

Treatment and Control

No treatment for FIP has been described that consistently reverses the disease process. There have been several advances in the treatment of FIP in cats. These new approaches could be divided into (i) modification of the immune response to the FIPV in infected cats and (ii) inhibition of FIPV replication using antiviral drugs. FIP is an immune-mediated disease, and corticosteroids have been used to suppress negative aspects of the immune response, including the humoral response. However, corticosteroid treatment may provide some palliative relief but does not affect the outcome. Therefore, corticosteroid treatment may be most useful when the lesions are focal and restricted to a single tissue, such as anterior uveitis. Other therapies include cytokines, interferon (feline and human recombinants), and Polyprenyl Immunostimulant to enhance the T-lymphocyte response to promote CMI in the FIPV-infected cats. Still, these immunotherapies have only shown variable success. Recently, antiviral drugs, specifically protease inhibitors targeting the viral proteases, have been used to reduce replication of the virus. Several antiviral compounds that inhibit virus protease activity both *in vitro* and *in vivo* have been identified. These antiviral drugs include GC376, GS5734, and GS441524, which inhibit disease development and remission in cats infected with FIPV. Currently, these drugs are not commercially available for the treatment of FIP in cats. A temperature-sensitive mutant FIPV is available for the intranasal vaccination of cats. The intranasal delivery prevents the induction of antibody responses while inducing a cell-mediated immune response. Its use is not recommended in seropositive cats. Its efficacy is questionable, and it has not been widely used. Control of FIP is best achieved by decontaminating infected premises, segregating cats by age groups, reducing stress factors, isolating serologically positive cats from those with no titer, and screening newly acquired cats for serum antibodies. Early weaning programs have also been proposed for breeding facilities in order to minimize early exposure of kittens to FCoV.

Ferret Coronavirus

Recently, a novel FRECV was identified in domesticated ferrets (*Mustela putorius furo*) associated with epizootic catarrhal enteritis (ECE). ECE, a relatively new disease of domestic ferrets, was first described in the spring of 1993 on the east coast of the United States. Since then, FRECV has spread across the United States and other countries. The disease is characterized by anorexia, lethargy, vomiting, and foul-smelling bright green diarrhea with high mucous content. The morbidity generally approaches 100%, but the overall mortality rate is low (<5%), with juvenile ferrets often developing only mild or subclinical disease. Clinical signs are more severe in older ferrets, and the mortality rate is usually higher. The microscopic lesions observed in affected ferrets were consistent with those described in the intestinal CoV infections in other species. The mucosa of the affected portion of the small intestine is usually highly hyperemic and thin. A diffuse lymphocytic enteritis with villous atrophy, fusion, blunting, and vacuolar degeneration and necrosis of the apical epithelium is often described. Another coronavirus has emerged in ferrets in the United States and Europe, causing systemic disease, FRSCV, characterized by FIP-like clinical signs and lesions. It occurs most frequently in young animals, and common clinical signs include anorexia, weight loss, diarrhea, and the presence of large palpable intra-abdominal masses. Gross lesions are characterized by widespread angiocentric granulomas (i.e. multisystemic granulomatous lesions) on serosal surfaces and within the parenchyma of the abdominal and thoracic organs as well as in the brain. Histopathologic examination shows a systemic pyogranulomatous inflammation involving the liver, kidney, spleen, pancreas, adrenal glands, mesenteric adipose tissue, lymph nodes, brain, and lungs. Comparative nucleotide sequence analysis has demonstrated that FRECV and FRSCV differ significantly in their S genes (79.5% identity). Phylogenetically, FRSCV is more closely related to FRECV than to other alphacoronaviruses.

Ferret coronaviruses can be diagnosed by demonstrating coronaviruses-like particles in clinical samples (e.g., feces and visceral organs) by EM or staining with cross-reactive anti-FCoV monoclonal antibodies (e.g., FIPV3-70). Viral antigen can also be demonstrated in formalin-fixed tissues by IHC. Microscopically, pyogranulomatous lesions can be observed in many visceral organs. The two ferret coronaviruses can be detected and distinguished by both conventional and RT-qPCR assays. Virus isolation in Madin-Darby canine kidney (MDCK), Crandell feline kidney (CrFK), Vero, and rabbit kidney (RK-13) cells has been unsuccessful.

Rabbit Coronavirus

RbCoV infection was first reported in 1961 by Scandinavian researchers who observed 50–75% mortality among laboratory rabbits. It was established that acute RbCoV infection targets the heart and results in virus-induced cardiomyopathy and congestive heart failure in rabbits. The affected rabbits develop pyrexia, pulmonary edema, pleural effusion, and cardiomyopathy (i.e. cardiorespiratory disease). RbCoV infection is used experimentally to produce a rabbit model of cardiomyopathy and has only been described in laboratory rabbits. Antibodies to the virus cross-react with human and other mammalian coronaviruses. RbCoV was demonstrated by EM examination of heart tissues of affected rabbits and by demonstrating complement-fixing antibodies to the human coronaviruses 229E and OC43 in surviving rabbits. Furthermore, immunofluorescence staining with anti-229E serum localized fluorescence in the interstitial tissue of the myocardium of affected rabbits. Antiserum to RbCoV cross-reacts with FCoV, CCoV, and TGEV by radioimmunoassay.

Animal Diseases Caused by Members of the Genus *Betacoronavirus*

Porcine Hemagglutinating Encephalomyelitis Virus

Disease

PHEV is the cause of the vomiting and wasting disease (VWD) of young swine characterized by encephalomyelitis, vomiting, and wasting. PHEV is antigenically related to BCoV. The virus hemagglutinates chicken, rat, mouse, hamster, and turkey erythrocytes. PHEV is sensitive to lipid solvents, including sodium deoxycholate. It is also heat labile and relatively stable when frozen. VWD occurs in pigs less than three weeks of age, although older swine may exhibit milder signs of the disease. VWD in young piglets is characterized by anorexia, lethargy, vomiting, constipation, and CNS disturbance (hyperesthesia, muscle tremors, paddling of the legs). Mortality is high, up to 100%; pigs also may develop chronic infections and eventually die from starvation or secondary infections. PHEV is the only known neurotropic coronavirus in pigs. The virus can spread from the primary replication sites through the peripheral nervous system to the CNS.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

PHEV was first isolated and associated with VWD in Canadian swine in 1958. Subsequently, the virus has been identified in swine in many areas of the world. Pigs are the only known host of PHEV, and subclinical or inapparent carrier states likely exist. PHEV is shed in nasal secretions, and horizontal aerosol and direct animal contact are mechanisms of transmission.

Pathogenesis and Pathology

The pathogenesis of PHEV infection has been characterized by experimental inoculation of colostrum-deprived day-old pigs. Following oronasal inoculation, primary viral replication occurs in the epithelial cells of the nasal mucosa, tonsils, lungs, and small intestine. The virus subsequently spreads along peripheral nerves to the CNS and infects the trigeminal ganglion and brainstem trigeminal sensory nucleus. Following peripheral viral spread, the virus infects well-defined nuclei of the medulla oblongata, progressing to the brainstem, spinal cord, and occasionally the cerebrum and cerebellum. PHEV spreading through the vagal nerve allows the virus to infect the vagal sensory ganglion and brainstem vagal sensory nucleus. The virus can also spread peripherally from the intestinal myenteric plexuses to the spinal cord's local sensory ganglia. Viral spreading through the vagal nerve infects the vagal sensory ganglion and brainstem vagal sensory nucleus. Vomiting is induced by viral replication in the vagal sensory ganglion (*ganglion distale n. vagi*) or by impulses of the vomiting center, generated by vagal ganglia-infected neurons. In addition, it has been suggested that virus-induced lesions in the stomach's myenteric plexus may contribute to gastric stasis and delayed stomach emptying. PHEV can also spread peripherally from the intestinal myenteric plexuses to the spinal cord's local sensory ganglia. PHEV replicates only in the cytoplasm of sensory neurons. Before disease expression, the viral antigen is present in the trigeminal, inferior vagal, superior cervical ganglia, solar and dorsal root ganglia of the lower thoracic region, and in the intestinal nerve plexuses. Infection in the brainstem is initiated in the trigeminal and vagal sensory nuclei and subsequently spreads to other nuclei and the rostral portion of the brain stem. Later stages of the infection may be characterized by viral replication in the cerebrum, cerebellum, and spinal cord; the virus is typically found in the nervous plexuses of the stomach at the later stages of infection.

There are few characteristic gross lesions in natural PHEV infections; mild catarrhal rhinitis is sometimes evident in encephalomyelitis cases, and gastroenteritis is sometimes observed in VWD. Lesions in the CNS are of a nonsuppurative encephalomyelitis characterized by perivascular cuffs of mononuclear cells, formation of glial nodes, neuronal degeneration, and meningitis. Respiratory tract lesions consist of focal or diffuse interstitial peribronchiolar pneumonia with cellular infiltrates composed of monocytes, lymphocytes, and neutrophils.

Host Response to Infection

Viral neutralization, hemagglutination inhibition (HI), and agar gel immunodiffusion (AGID) are used to quantify humoral immune responses. The clinical disease is self-limiting in pig populations due to the rapid development of maternal antibodies and transfer via colostrum.

Laboratory Diagnosis

Diagnosis of PHEV encephalomyelitis or VWD in piglets can be made by IHC staining of viral antigens in the tissues of affected pigs, virus isolation on primary PK, or pig thyroid (PT) cells, or demonstration of a rising antibody titer. PHEV grows in primary PK or PT cells with the formation of characteristic syncytia. IHC can detect PHEV antigen in the brain, spinal cord, and myenteric plexus of clinically affected animals. In addition, PHEV can be consistently isolated from the tonsils and respiratory tract (nasal and pharyngeal swabs, nasal mucosal, and lungs) but irregularly from the pons and medulla, hindbrains, and stomach wall. Oral fluids are a suitable specimen for routine PHEV diagnosis and surveillance. Viral isolates are is usually confirmed by direct immunofluorescence and hemadsorption. Also, viral RNA can be detected by standard RT-PCR and RT-qPCR. Antibodies to PHEV can be detected by VN or HI tests. Differential diagnoses must include Teschen-Talfan disease pseudorabies (Aujeszky's) disease, and porcine astrovirus type 3.

Treatment and Control

No effective treatment has been described for PHEV-induced encephalomyelitis or VWD. Clinical outbreaks are self-limiting. No vaccines are available, and good animal husbandry and biosecurity practices are necessary for preventing and controlling the disease.

Bovine Coronavirus

Disease

BCoV is a pneumoenteric virus that infects the upper and lower respiratory tracts and the intestine of cattle and wild ruminants. In cattle, BCoV can cause three different disease syndromes: neonatal diarrhea in newborn calves (one to three weeks, calf diarrhea [CD]), winter dysentery (WD) in adult cattle with hemorrhagic diarrhea, and respiratory infections in cattle of various ages. In addition, respiratory infections in cattle can occur as part of the bovine respiratory disease complex (BRDC) or shipping fever in feedlot cattle.

The CD is characterized by anorexia and a liquid, yellow diarrhea that persists for four to five days. WD is a sporadic acute disease in adult cattle characterized by explosive bloody diarrhea accompanied by decreased milk production, depression, and anorexia. The BCoV strains isolated from diarrheal or intestinal fluid are now identified as enteropathogenic bovine coronaviruses (EBCoV). Recently, other strains of BCoV have been identified as respiratory pathogens in cattle; these coronavirus strains have been isolated from the nasal secretions and lungs of cattle with severe shipping fever pneumonia and are designated as respiratory bovine coronaviruses (RBCoV). Respiratory disease caused by RBCoV typically occurs in calves aged six to nine months and is characterized by fever, nasal discharge, and respiratory distress. RBCoV alone or in combination with several other respiratory viruses (e.g., bovine respiratory syncytial virus, parainfluenza-3 virus, bovine herpesvirus) and viruses capable of mediating immunosuppression (e.g., bovine viral diarrhea virus) can precipitate BRDC. Furthermore, other predisposing factors allow commensal bacteria of the nasal cavity (e.g., *Mannheimia haemolytica, Pasteurella* sp., *Mycoplasma* sp.) to infect the lungs, leading to fatal fibrinous pneumonia associated with BRDC.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

BCoV is acid stable (pH 3.0) but is inactivated by lipid solvents, detergents, and high temperatures. Although there are significant phenotypic, antigenic, and genetic differences between the EBCoV and RBCoV strains, the precise relationship between enteric and respiratory strains of BCoV is uncertain. There is only one known serotype/genotype of BCoVs. As of now, no consistent antigenic or genetic markers have been identified to discriminate BCoV isolates from the three different clinical syndromes. BCoV is antigenically related to coronaviruses of other species. BCoV particles hemagglutinate erythrocytes from hamsters, mice, and rats.

Infectivity for Other Species and Culture Systems

BCoV has been propagated in suckling mice, and following such passage will infect suckling rats and hamsters by both intracerebral and subcutaneous routes. EBCoV has been propagated in Madin-Darby bovine kidney (MDBK), African green monkey kidney (Vero), bovine fetal thyroid (BFTy), and bovine fetal brain (BFB) cells. Trypsin treatment of the latter two fetal cell cultures enhances plaque formation and cell fusion. Some isolates are difficult to propagate *in vitro* and may require passage in the natural host. In contrast, only a human rectal tumor cell line (HRT-18) is permissive for the initial isolation of RBCoV.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

BCoV is distributed worldwide, and transmission of EBCoV is likely fecal–oral by ingestion of virus from contaminated feed, teats, and fomites. RBCoV is shed in respiratory tract secretions of infected animals and thus is spread horizontally by infected aerosols/respiratory droplets. However, RBCoV is also found in feces, leading to fecal–oral transmission. Bovine-like CoVs have also been identified in water buffalo calves and alpacas. Serological surveys using IFA have demonstrated the circulation of CoVs antigenically closely related to BCoV in captive and wild ruminants. Some of the CoVs from captive ruminants (sambar deer, white-tailed deer, elk, waterbuck, and giraffe) are biologically, genetically, and antigenically (cross-neutralizing) very closely related to BCoVs. Sequencing these closely related CoVs from captive ruminants has shown very close amino acid identity (93–99%) in some viral proteins to enteric and respiratory BCoV strains, further confirming their close genetic relationship. Thus, the possibility exists that wild and captive ruminants could transmit bovine-like CoVs to cattle or vice versa. Such interspecies transmission (species jumping) of BCoVs combined with their recombination ability would lead to the emergence of more genetically divergent CoVs. Sequence analysis has revealed that PHEV and HCoV-OC43 may have evolved from ancestral BCoV strains. Furthermore, the genetic and antigenic similarity between canine respiratory coronavirus (CRCoV) and BCoV has been demonstrated.

Pathogenesis and Pathology

Diarrhea develops within 24–30 hours following oral infection of calves with EBCoV. Four hours after the onset of diarrhea, the viral antigen is detectable in the epithelium of the small intestine and colonic crypts. Proteolytic enzymes facilitate the initiation of infection in the intestinal tract as trypsin treatment of coronaviruses in cell culture results in enhanced viral growth. The virus also infects the adjacent mesenteric lymph nodes. Destruction of the mature enterocytes that line the intestinal villi leads to atrophy and fusion of affected villi, subsequent intestinal maldigestion and malabsorption, rapid loss of fluids and electrolytes, and, in severe cases, dehydration, acidosis, shock, and death.

WD is a syndrome in adult cattle during which animals develop bloody diarrhea. It has high morbidity but low mortality (<2%). Grossly, the colon is filled with bloody contents, and histologically, the lesions are similar to those of CD but restricted to the colon. Although interstitial pneumonia is possible, the typical histological lesion in RBCoV-infected calves is bronchiolar necrosis. The virus typically replicates in the nasal and tracheal epithelium.

Host Response to Infection

Both EBCoV and RBCoV infections in calves result in a humoral immune response that can readily be quantitated by viral neutralization, HI, hemadsorption inhibition (HAI), and ELISA tests. As circulating antibodies do not protect calves from infection, local immune responses play an important role. In addition, neonatal ingestion of colostral IgA protects the intestinal lumen against EBCoV infection for a limited period.

Laboratory Diagnosis

Pneumoenteric BCoVs replicate in the upper respiratory and the intestinal tracts. Thus, the virus and viral nucleic acids (RNA) can be detected in nasal secretions and feces, with nasal shedding often preceding fecal shedding. Furthermore, the following specimens should be collected from animals with suspected respiratory (or enteric) BCoV disease for the detection of infectious virus, viral antigens, or viral RNA: tissues from the respiratory tract (e.g., nasal, pharyngeal, tracheal, and lung tissues) and the distal small intestine and colon. Diagnosis of EBCoV-induced neonatal diarrhea requires identifying the virus in fecal samples or intestinal sections. This can be achieved by viral isolation, EM, or FA, or IHC staining. Nasal swabs collected during the acute stage of upper respiratory tract disease are the specimens of choice for the diagnosis of RBCoV. BCoV can be isolated from the nasal secretions, lung homogenates, or feces using the HRT-18 cell line during the acute phase of the disease. Respiratory epithelial cells in the nasal swabs are spotted onto slides for examination by a direct FA test.

BCoV RNA in feces, nasal secretions, or tissues can be detected by conventional RT-PCR, nRT-PCR, and RT-qPCR assays (targeting most conserved genomic regions – ORF1ab or N genes). In serum, antibodies to BCoV can be detected using VN or HI tests that measure neutralizing or hemagglutinating antibodies, respectively. Because BCoV antibodies are widespread in cattle, it is important to test paired acute and convalescent serum samples for serological diagnosis of BCoV infections. Antibodies to BCoV in nasal secretions or feces can be quantitated in ELISA (overall or isotype-specific antibodies [IgM, IgA, IgG1, and IgG2]) or by using VN or HI tests.

Treatment and Control

Treatment is dictated by the severity and type of disease. Electrolyte solutions can be administered for dehydration in calves with diarrhea caused by EBCoV infections, and antibiotic therapy may be used to control secondary infections. The BCoV infections are best controlled by good management practices to minimize exposure to these viruses,

such as avoiding introducing new (infected) animals into an intensive calving operation. Although several licensed inactivated and modified-live vaccines against BCoV are currently available, there is insufficient data on the protective efficacy of these vaccines against CD/WD. It is challenging to control enteric disease by vaccination because very young calves are most affected before they have the opportunity to respond to vaccination. The alternative is to immunize the dam to increase antibody levels in colostrum. Similarly, no BCoV-vaccines have been developed to prevent BCoV-associated respiratory disease in young calves or BRDC of feedlot cattle. In summary, there are many challenges to developing effective vaccines against BRDC and BCoV diarrheal disease. These include the ubiquitous nature of BCoV infections, lack of comprehensive data on vaccine efficacy, short duration of post-vaccine mucosal immunity, immunologic immaturity of calves, and potential interference by maternal antibodies.

Canine Respiratory Coronavirus

In 2003, a new coronavirus was identified in the respiratory tract of dogs housed in a rehoming kennel in the United Kingdom. However, the oldest specimens (i.e. lung) that tested positive for CRCoV were collected in Canada in 1996. This virus was referred to as CRCoV. Nucleotide sequence analysis of CRCoV spike protein gene demonstrated 97.3% and 96.9% identity to the betacoronaviruses BCoV and HCoV-OC43, respectively. It has been shown recently that HCoV-OC43 has emerged after viral transmission from cattle to people. Interestingly, the genetic relationship of CRCoV to BCoV spike protein suggests that the virus was probably transmitted to dogs from cattle. The CRCoV shares only 21.2% amino acid identity to CCoV-I in the spike protein. CRCoV is responsible for mild respiratory disease in dogs. However, in most, cases, it contributes to the development of canine infectious respiratory disease (CIRD) with other canine respiratory pathogens (e.g., *Bordetella bronchiseptica, Streptococcus equi* subsp. *zooepidemicus. Mycoplasma cynos*, canine adenovirus type 2, canine parainfluenza virus, canine herpesvirus, reoviruses, and influenza viruses).

Serological studies have shown antibodies to CRCoV in dog serum samples surveyed in the United Kingdom, Canada, Ireland, Italy, United States, and Japan. CRCoV infection associated with CIRD in kennels can occur throughout the year, but it can be more common in the winter months of the year. Isolation of CRCoV in canine cell lines is difficult, but it can be isolated in a HRT-18 and its clone HRT-18G. However, not all strains of CRCoV can be isolated in HRT-18 or HRT-18G cells. Hemagglutination assay using chicken RBCs at 4 °C can be used to detect CRCoV-infected cell cultures. CRCoV nucleic acids can be detected in clinical samples by standard RT-PCR or RT-qPCR. CRCoV can also be detected by IHC on formalin-fixed tissues using an anti-BCoV cross-reactive antibody. Several ELISAs either using BCoV antigen or CCoV antigen have been developed. However, an ELISA assay using CRCoV antigen was more sensitive and specific than an assay based on BCoV. Antibodies to CRCoV can also be detected by using an IFA on CRCoV-infected HRT-18 cells. It is recommended to use RT-PCR methods or serology on paired serum samples to diagnose CRCoV infections because the sensitivity of virus isolation is low. Antibodies to CRCoV do not cross-react with enteric CCoVs. There is no specific treatment for infections caused by CRCoV, but dogs should be treated for other bacterial causes of CIRD. There are no vaccines against CRCoV, and vaccines against CCoV are unlikely to protect against CRCoV because of low antigenic similarity in the spike protein of these viruses.

The control of CRCoV requires thorough cleaning and disinfection of kennels after outbreaks of respiratory disease. The importance of fecal shedding and the potential transmission of CRCoV among dogs sharing common facilities, such as outdoor runs, have yet to be resolved. Other measures, such as washing one's hands after handling animals with respiratory disease, should also help reduce the virus spread. In addition, quarantine of newly arriving dogs in training kennels or shelters for at least two weeks will reduce the risk of CRCoV outbreaks.

Equine Coronavirus

Coronavirus-like agents have been identified by EM examination of fecal samples from foals and adult horses having enteric disease and fever. The first isolation and characterization of ECoV (NC99 strain) from feces of a diarrheic foal was described in 2000. Phylogenetic analysis has demonstrated that the ECoV NC99 strain is most closely related to BCoV, HCoV-OC43, and PHEV. In 2011, isolation of ECoV (the Tokachi09 strain) from adult horses with fever and enteric disease was described from Japan. Comparative nucleotide and amino acid analyses of nucleocapsid and spike protein genes showed that the Tokachi09 strain is very similar to the NC99 strain from North America (98.0% and 99.0% at the nucleotides, 97.3% and 99.0% at the amino acids, respectively). However, the Tokachi09 strain had a 185-nucleotide deletion four bases upstream from the stop codon of the spike gene, resulting in the absence of the ORF predicted to encode a 4.7 kDa nsps in strain NC99. Since 2010, ECoV has been involved in a rising number of enteric disease outbreaks characterized by fever, depression, anorexia, colic, and diarrhea in adult horses in the

United States, Japan, and Europe. Clinical cases are either sporadic or can occur as an outbreak. Outbreaks have predominantly been reported in riding, racing, and show horses and less frequently in breeding animals. Sporadic outbreaks of ECoV infection in adult horses could occur year-round, with increased reports during cold months of the year (October to April). The virus is transmitted by the fecal–oral route during outbreaks.

The main clinical signs of ECoV infection in adult horses include depression, anorexia, lethargy, fever (38.6–41 °C [median 39.9 °C]), diarrhea (the changes in fecal character vary from soft-formed to watery consistency), and colic. Diarrhea and colic are only observed in 25% and 18% of horses with other clinical signs, respectively. Systemic signs of anorexia and fever generally precede gastrointestinal signs. The horses with clinical signs consistently show leukopenia due to neutropenia and/or lymphopenia. A small percentage (3%) of infected horses could develop signs of encephalopathy, including circling, head pressing, ataxia, proprioceptive deficits, nystagmus, recumbency, and seizures. Although the clinical disease is apparent in 10–83% of ECoV-infected horses, some may remain asymptomatic after infection. The percentage of asymptomatic horses during an outbreak of ECoV has been highly variable (11% and 83%). The incubation period is two to three days, and the horses can be sick for a few days to one week. However, the ECoV morbidity rate is 10–80%, the mortality rate is low, and the horses recover fast. Generally, it is a self-limiting infection, and the ECoV outbreaks can last two to three weeks. There are few reports of gross and histopathological lesions described in ECoV-infected adult horses. However, one article described that ECoV-infected equids display severe diffuse necrotizing enteritis with marked villus attenuation, epithelial cell necrosis in the tips of the villi, neutrophilic and fibrin extravasation into the small intestinal lumen (pseudomembrane formation), as well as crypt necrosis, microthrombosis, and hemorrhage.

Enteritis and shedding of ECoV foals have been reported previously. However, ECoV shedding has also been detected in healthy foals, and this observation suggests that the virus commonly circulates among young horses with subclinical disease. In addition, sometimes ECoV infections in foals with a gastrointestinal disease can be associated with coinfections with other opportunistic secondary viral, bacterial, or protozoal infections as shown for enteric and respiratory coronavirus in other species.

The diagnosis of ECoV can be achieved by demonstrating coronavirus particles in feces under electron microscopic examination. However, it may be difficult to find a sufficient number of ECoV particles in diagnostic fecal specimens, and thus, it may be less rewarding. In addition, ECoV can be difficult to isolate and propagate in cell culture. Nevertheless, ECoV has been successfully isolated from feces by inoculating HRT-18 cells. Recently, conventional RT-PCR and RT-qPCR assays targeting the conserved nucleocapsid protein gene have been described. RT-qPCR can detect ECoV in the small intestine, gastrointestinal content, or feces, and coronavirus antigen can be demonstrated by IHC and/or direct FA testing in the small intestine. Antibodies to ECoV cross-react with the BCoV, and therefore, demonstration of rising neutralizing antibody titers (fourfold or more) to BCoV in paired serum samples indicates exposure to ECoV.

Mouse Hepatitis Virus

First identified in 1949, MHV is one of the most common viruses among wild and laboratory mice worldwide. MHV is highly contagious and causes explosive outbreaks of disease in mouse colonies throughout the world. It is also one of the most studied CoV in animals and is used as a model system to study multiple sclerosis and autoimmune hepatitis in humans. In addition, MHV-1 infection of A/J mice has been used as a mouse model for the pathogenesis of SARS-CoV in humans. Typically, MHV disease is not clinically evident, and most infected animals are asymptomatic. This represents a significant problem in animal facilities, where subclinical MHV infection could alter experiment outcome and reliability.

The severity of the clinical disease depends on several factors. These factors can be broadly classified into viral (strain, dose, and route of infection) and host factors (strain of mice, age, and immune status). There are many different strains of MHV, each with characteristic tissue tropism and associated clinical manifestations. For example, the A59 strain of MHV induces moderate to severe hepatitis, whereas the MHV-4 strain does not. Infection of mice with different virus strains can cause enteritis, hepatitis, nephritis, and demyelinating encephalomyelitis. Other strains of MHV cause respiratory and CNS disease in mouse colonies. A59 and JHM strains of MHV are the most commonly studied neurotropic strains of this virus. A59 also causes moderate to severe hepatitis and mild encephalitis and demyelination in the brain. A59 is a tissue culture adapted dual-tropic strain that infects the liver and the brain. CNS infection with neurotropic strains of MHV causes paralysis due to demyelination (used as a model of chronic demyelinating diseases of humans such as multiple sclerosis).

Strains of MHV are classified into two major groups: polytropic and enterotropic biotypes. Polytropic strains initially replicate in the nasal respiratory epithelium and disseminate to other organs via the blood, lymphatic system, and olfactory nerve. In contrast, enterotropic strains target intestinal mucosal epithelium and are characterized by

minimal or no dissemination to other tissues. Polytropic MHV strains associated with hepatitis may show necrotic foci on the liver surface, becoming hemorrhagic in immunodeficient mice, along with fibrin deposition and mild infiltration of mononuclear inflammatory cells. Mice can also develop hepatic nodular hyperplasia with fibrosis and parenchymal collapse. Histopathological evaluation of affected organs such as the liver, spleen, lymph nodes, thymus, bone marrow, and gut-associated lymphoid tissue will show multifocal to coalescing areas of acute necrosis as well as syncytia of parenchymal cells and endothelial cells. However, syncytia may not be so evident in immunocompetent mice. In addition, microthrombi in liver sinusoids might be present. MHV strain MHV-S can cause mild olfactory mucosal necrosis, neuronal necrosis of olfactory bulbs, and interstitial pneumonia. Mice infected with the MHV-1 strain develop interstitial pneumonia, hyaline membrane and fibrin deposition, infiltration with lymphocytes, macrophages, neutrophils, and death. MHV-JHM and MHV-A59 stains are predominantly neurotropic, and lesions are often seen in neonatal and immunocompromised mice. Experimentally infected mice may show vestibular signs and posterior paresis. The histopathological lesions may include necrotizing encephalitis, meningoencephalitis, optic neuritis, loss of retinal ganglion cells, retinal vasculitis and perivasculitis, neuronal cell loss and degeneration, apoptosis with infiltration of macrophages, neutrophils, and natural killer cells, as well as syncytia formation. In survivors, chronic demyelination is often seen, and for this reason, infected mice are used as an animal model for human multiple sclerosis. MHV-3 is neurotropic, depending on the strain of the mice infected. Lesions are characterized by meningitis, ependymitis, and leukoencephalitis, chronic thrombotic vasculitis of meningeal and parenchymal vessels in the brainstem.

The enteropathogenic strains of MHV cause severe diarrhea in suckling mice with nearly 100% mortality. The intestines are distended and filled with yellowish fluid. In neonatal mice, lesions due to enterotropic MHV strains are primarily located in the terminal small intestine, cecum, and colon. They are characterized by severe necrotizing enterocolitis with villous attenuation, syncytia of enterocytes and endothelial cells of mesenteric vessels, leukocytic infiltration, and occasionally hemorrhages. Eosinophilic intracytoplasmic inclusions may be present within enterocytes. Older mice develop jaundice, lose weight, and cease breeding. Characteristic histopathological lesions include blunted (club-shaped) intestinal villi with extensive syncytium formation. All mice develop acute hepatitis with focal hepatocellular necrosis and inflammatory cell infiltration. Lesions in adults are minimal, with enterocytic syncytia predominantly located in the cecum and ascending colon. Adult nude mice may develop chronic hyperplastic typhlocolitis and mesenteric lymphadenopathy. Rarely, some enterotropic MHV strains can disseminate and cause hepatitis and encephalitis. Mice that have recovered may develop residual lesions, such as reactive hyperplasia of spleen and lymph nodes, hematopoietic hyperplasia, perivascular cuffing in the lungs and brain, foci of inflammation in the liver, and intestinal mucosal hyperplasia.

MHV infection in mouse colonies is diagnosed with the detection of characteristic gross and histological lesions in the intestines and liver, although some strains of MHV are highly attenuated and cause minor pathology or disease. The diagnosis is confirmed by IHC and serology using an enzyme immunoassay. Viruses can be isolated using any of several mouse cell lines. In addition, there are standard and RT-qPCR assays to detect MHV RNA in clinical specimens.

The virus persists following epidemics in persistently infected mice that continually infect susceptible mice introduced into the colony. Control is achieved by breaking this cycle of transmission through strict quarantine and biosecurity measures.

Rat Coronaviruses

Sialodacryoadenitis virus (SDAV) is the etiologic agent of sialodacryoadenitis in laboratory rats. SDAV and Parker's rat coronavirus (PRC) are the two natural CoVs infections affecting rats, with SDAV being one of the most common infections in laboratory rats worldwide. They are antigenically related to MHV, BCoV, and HCoV-OC43. Although they are considered two different viruses, they produce overlapping lesions. These two prototype strains (i.e. SDAV and PRC) of RCoV have different tissue tropism and disease associations. Typically, PRC is associated with respiratory lesions, whereas SDAV primarily causes an inflammation of salivary and lacrimal glands but can also cause pulmonary disease in young rats. SDAV is characterized by high morbidity and negligible mortality.

SDAV is transmitted by aerosol, direct contact, and fomites. It is a severe, self-limiting inflammatory disease of the upper respiratory tract, salivary and lacrimal glands, and eyes of rats. All the salivary and lacrimal glands are generally enlarged by edema and inflammation. The SDAV clinical signs include cervical swelling due to edema and inflammation of the parotid and submandibular salivary glands, blepharospasm, sniffling, epiphora, and often chromodacryorrhea (i.e. dark red crusts around the eyes). During the acute infection, histopathological lesions of salivary and lacrimal glands are characterized by necrosis of the ductal epithelium, interstitial edema, infiltration with lymphocytes, plasma cells, macrophages, and neutrophils. In addition, lesions in the eyes,

such as keratitis sicca, hyphema, impaired intraocular damage, and megaglobus, have been reported. During the recovery phase (7 dpi), the salivary and lacrimal glands show nonkeratinizing squamous metaplasia of periductal and acinar epithelium, mild infiltration with mononuclear inflammatory cells, as well as periglandular, interlobular, and peri ductal fibrosis. SDAV has also been isolated from the lower respiratory tract and can cause mild interstitial pneumonia in young rats. In addition, it has been reported that SDAV can experimentally cause lesions to the CNS in animals during their first week of age. Microscopic lesions are represented by acute necrotizing encephalitis associated with minimal inflammatory response. The virus is highly contagious and causes high morbidity and low mortality in infected colonies. Lesions in the lacrimal duct may lead to corneal drying. Lesions typically resolve within two weeks. The virus is transmitted by aerosol, direct contact, and fomites.

PRC has only been isolated from the respiratory tract and causes fatal pneumonia in suckling rats. Following intranasal experimental infection, PRC replicates in the upper and lower respiratory tract, leading to interstitial pneumonia and alveolar edema, which resolves by day eight after infection. The lesions caused by PRC include necrotizing rhinitis, laryngitis, tracheitis, bronchitis, all with hyperplasia, flattening, and sloughing of respiratory epithelial cells. The histopathological lesions include interstitial pneumonia with leukocyte infiltration in the alveolar wall and type II pneumocyte hyperplasia.

Recently, a novel RCoV was discovered in China and named HKU24. However, although an enteric tropism has been suggested, there is no evidence that this virus causes disease in rats.

SARS Coronavirus and Middle East Respiratory Syndrome Coronavirus

During the winter of 2002 and 2003, SARS-CoV was first identified in China's Guangdong province as the causative agent of a contagious and fatal respiratory disease named SARS. Diffuse alveolar damage (DAD) leading to acute respiratory distress syndrome (ARDS) was the typical disease presentation in infected people. Subsequently, the virus spread to Hong Kong, Vietnam, Singapore, Canada, and elsewhere. In July 2003, at the end of this outbreak, 8422 human cases had been recorded, 916 (10.8%) of them fatal, in 29 countries across six continents. During the initial SARS outbreak in Hong Kong, it was first recognized that SARS-CoV could infect cats. Subsequently, experimental inoculation of cats and ferrets was performed. The infection did not result in clinical signs in cats but did lead to lethargy in a subset of ferrets and death in a single ferret. Across both species, the virus was observed to be shed from the pharynx. Although fecal shedding was not observed, viral RNA was detected by RT-PCR in the gastrointestinal tract. In addition, seroconversion was observed in naïve animals that were group-housed with experimentally inoculated animals (i.e. cats and ferrets). As for many other CoVs, bats are the proposed definitive reservoir hosts for SARS-CoV. Based on epidemiological data, civets and raccoon dogs were proposed as intermediate animal hosts in the live animal market, where SARS-CoV emerged as a human pathogen in China. This is another example of the significant ramifications of species jumping of CoVs.

Middle East respiratory syndrome (MERS) is a viral respiratory disease caused by a novel coronavirus (MERS-CoV) that was first identified in Saudi Arabia in 2012. The most significant outbreaks of MERS were seen in Saudi Arabia, the United Arab Emirates, and the Republic of Korea. Typical MERS symptoms in humans include fever, cough, shortness of breath, pneumonia, and gastrointestinal symptoms, including diarrhea. There are some laboratory-confirmed cases of MERS-CoV infection that are asymptomatic. The virus does not seem to pass easily from person to person unless there is close contact, such as when providing unprotected care to a patient. Since April 2012, 2589 cases of MERS-CoV, including 940 deaths (36%), have been reported. Current scientific evidence suggests that dromedary camels are a primary reservoir host for MERS-CoV and an animal source of MERS infection in humans. However, the exact role of dromedaries in the transmission of the virus and the exact route(s) of transmission are unknown. However, the consumption of raw or undercooked camel products, including milk and meat, carries a high risk of MERS-CoV infection.

Compared to SARS-CoV and SARS-CoV-2, MERS-CoV has not been shown to cause active infection in ferrets. In addition, there are no reports of SARS-CoV or MERS-like viruses transmitting to dogs. However, experimental infection and subsequent monitoring shedding and presence of the virus have identified that dromedary camels are the source of MERS-CoV-2 for human infections. The virus was also demonstrated in the tissues of rhesus macaques and common marmosets. In contrast, no virus was detected in the tissues of alpacas, llamas, and rabbits.

SARS Coronavirus-2

Etiologic Agent and Disease

The world is currently experiencing the devastating effects of the COVID-19 pandemic caused by the newly emerged SARS-CoV-2. The outbreak was first identified in Wuhan,

China, in December 2019 and recognized as a pandemic by the World Health Organization on March 11, 2020. The SARS-CoV-2 is highly contagious and primarily spreads from person to person via respiratory droplets generated during coughing, sneezing, and talking. The high transmissibility of the virus and the immunologically naïve population around the world has led to its rapid spread across the globe, causing 246 million cases and 5 million deaths in 220 countries as of October 2021. In addition, there are many reports of animals infected (naturally or experimentally) with SARS-CoV-2 around the world. It has been demonstrated that SARS-CoV-2 can infect companion animals (cats, dogs, and ferrets), animals in zoos and sanctuaries (lions, tigers, leopards, otters), nonhuman primates (gorillas and monkeys), farmed mink, and white-tailed deer in the United States. Most of these animals have become infected after contact with infected people, including owners, caretakers, or others in close contact. Infection in dogs and cats have caused increased concerns among animal owners about spreading the virus to humans (zoonosis). Although it is clear that SARS-CoV-2 could spread from infected people to animals (reverse zoonosis), there is no evidence at this time that animals play a significant role in spreading SARS-CoV-2 to people. As of November 2021, there have been 294 reported cases of SARS-CoV-2 infections in non-farmed animals (cats [51], dogs, [31], lions [35], tigers [30], minks [16], gorillas [13], snow leopards [10], otters [7], fishing cats [1], ferrets [1], cougar [1], coati [1] and binturong [1]) and 17 confirmed positive mink farms in the United States. The largest number of infected animals are reported in the states of Texas (101), New York (20), California (14), Colorado (13), Georgia (12), Florida (12), and Utah (12). Under experimental conditions, animals that appear to be susceptible to SARS-CoV-2 include cats, dogs, raccoon dogs, deer, deer mice, hamsters, minks, and ferrets, but not horses, cattle, sheep, pigs, or poultry. The original SARS-CoV-2 strain and its variants (Alpha [B.1.1.7 or UK variant], Beta [B.1.351 or South African variant], Gamma [P.1. or Brazilian variant], and Delta [B.1.617.2 or Indian variant]) have been reported to infect pets.

Cats and dogs with naturally acquired (as well as experimentally) SARS-CoV-2 infections are often asymptomatic. However, a combination of any of the following clinical signs has been reported in some naturally infected animals: fever, coughing, difficulty breathing (shortness of breath), lethargy, sneezing, nasal discharge, ocular discharge, vomiting, and diarrhea. The virus is shed for a limited period in respiratory secretions and feces of cats and dogs as determined by the presence of viral RNA. Both cats and dogs seroconvert following natural infection with the virus.

Experimental Infections

Recently, several studies described experimental infection of companion animals (i.e. cats, ferrets, and dogs), farm animals (i.e. pigs, sheep, and cattle), and wildlife (white-tailed deer, bats). After experimental inoculation, the studies have shown that cats and ferrets can be readily infected with SARS-CoV-2. Although clinical signs in ferrets and cats are variable, infected animals can transmit the virus to naïve animals of the same species under experimental conditions. Similarly, dogs can be experimentally infected with SARS-CoV-2, but they are less permissive than ferrets or cats.

Cats

It has been shown that experimentally infected domestic cats are highly susceptible to SARS-CoV-2 infection and can transmit the virus to naïve contact cats. However, all experimentally infected cats were clinically asymptomatic during infection but capable of transmitting SARS-CoV-2 to sentinels. Both experimentally infected and contact cats seroconverted to SARS-CoV-2. The virus replicated in the upper and lower respiratory tract, with evidence of viral antigen and viral RNA. Although fecal shedding was identified, viral replication in the gastrointestinal tract of infected cats remains controversial and has not been unequivocally demonstrated. Histological lesions are commonly characterized by rhinitis and adenitis of tracheobronchial glands, whereas the development of interstitial pneumonia seems sporadic/rare. Virus antigen was found in epithelial cells of the nasal turbinates, including the olfactory neuroepithelium, necrotic debris in the tonsil, and submucosal glands of the trachea and bronchi. There is one report describing viral antigen within enterocytes of the small intestine; however, this was not identified in the experimental animals evaluated by the authors. The virus has been isolated from the trachea, nasal turbinates, and esophagus of cats experimentally infected with SARS-CoV-2. However, infectious virus was not found in the lung or other organs of these cats. These studies have also demonstrated that cats previously experimentally infected with SARS-CoV-2 can be re-infected with SARS-CoV-2. However, the levels of virus shed by re-infected cats were insufficient for transmission to co-housed naïve sentinels. Thus, it was concluded that SARS-CoV-2 infection in cats induces immune responses that provide partial, non-sterilizing immune protection against reinfection.

Dogs

Similarly, dogs that have been experimentally infected are susceptible to SARS-CoV-2 but to a very mild degree (i.e. low susceptibility). In experimental infection studies, dogs

do not typically develop apparent clinical signs, nor do they readily transmit SARS-CoV-2 to naïve dogs. Viral shedding was not detected by plaque assay from any of the dogs at any point post infection. Similar to cats, dogs seroconvert following natural and experimental infection with the virus. In summary, there is no evidence that cats or dogs play a significant role in human infection.

Although as yet unconfirmed, there are several reports of animal-to-human (zoonotic) transmission in the Netherlands (mink) and Germany (cat). Based on the current literature, reverse zoonosis is possible if infected owners expose their domestic pets to the virus during acute infection. However, one has to be very cautious not to overinterpret the results from these studies because only a small number of experimental infection and transmission studies have been done under controlled conditions.

Other Species

Experimental infections of other animal species have been reported, including pigs, cattle, sheep, white-tailed deer, deer mice, raccoon dogs, chickens, ducks, and fruit and North American brown bats. The pigs infected with SARS-CoV-2 did not show any clinical signs or clear evidence of viral replication. Furthermore, no virus was isolated or antigen detected from specimens collected from the experimentally infected pigs, indicating that pigs do not act as amplifying hosts for SARS-CoV-2. However, pigs seroconverted following experimental infection.

Experimental inoculation of cattle demonstrated that they are susceptible to SARS-CoV-2 infection and mounted an antibody response against the virus (i.e. showed specific seroconversion by RBD-ELISA). This finding corresponds with a predicted medium susceptibility of cattle species based on computational modeling of their ACE2, the cellular receptor for SARS-CoV-2. However, only a few inoculated animals showed transient viral replication, and there was no transmission of SARS-CoV-2 to in-contact cattle. Thus, there is no indication that cattle play a role in the human pandemic, and no reports of naturally infected bovines exist.

Experimentally infected sheep show seroconversion without showing any clinical signs. The viral RNA was detected in nasal and oral swabs primarily at one-day post challenge (DPC) and later in the respiratory tract and lymphoid tissues at 4 and 8 DPC. Co-mingled naïve sheep did not seroconvert or show any evidence of clinical signs. Thus, like pigs and cattle, sheep may not play a role in transmitting the virus.

Recently, white-tailed deer fawns from the United States were shown to be susceptible to SARS-CoV-2. Subsequently, pregnant adult white-tailed deer were experimentally infected with SARS-CoV-2 to confirm their susceptibility to the virus. Findings from this study demonstrate that adult white-tailed deer are susceptible to SARS-CoV-2 infection and can transmit the virus through direct contact.

It has also been shown that deer mice are susceptible to SARS-CoV-2 infection. When deer mice were challenged with SARS-CoV-2, the virus replicated well in the upper respiratory tract, lungs, and intestines, with detectable viral RNA for up to 21 days in oral swabs and 14 days in lungs. The virus also entered the brain via the gustatory-olfactory-trigeminal pathway. However, the deer mice did not show any significant clinical signs of disease, and they did not succumb to infection.

In April and May 2020, outbreaks of acute respiratory disease due to SARS-CoV-2 were described in mink farms in the Netherlands. Subsequently, additional farm outbreaks here and in other countries were described, with mortalities ranging from 1.2% to up to 10% and seroconversion reaching over 93%. Affected farms had at least one worker with COVID-19. Infected mink developed severe acute interstitial pneumonia and DAD, histological features rarely encountered in other domesticated species following SARS-CoV-2 infection. These outbreaks also led to transmission from mink to humans, with the emergence of variant mink viruses, causing additional concerns (e.g., leading to complete culling of all mink in Denmark).

It has been shown that raccoon dogs are susceptible to SARS-CoV-2 infection, and they could transmit the virus to in-contact animals. Infected animals did not show any signs of illness. However, virus replication and tissue lesions occurred in the nasal conchae. The limited experimental studies have suggested that chicken and ducks are not susceptible to SARS-CoV-2 infection. Similarly, there is no evidence of horses getting infected with SARS-CoV-2. Experimental infection of fruit bats (*Rousettus aegyptiacus*) has shown transient infection of SARS-CoV-2. The virus was detected by RT-qPCR, IHC, and in situ hybridization in the nasal cavity associated with rhinitis. Viral RNA was also identified in the trachea, lung, and lung-associated lymphoid tissue. One of three contact bats became infected with SARS-CoV-2, indicating intraspecies transmission. In contrast, North American big brown bats (*Eptesicus fuscus*) experimentally infected with SARS-CoV-2 showed no evidence of infection, including no viral excretion, no detectable virus in tissues, and no signs of disease, pathology, or transmission. These data supported that North American brown bats are resistant to infection with the SARS-CoV-2.

Experimental Animal Models

It is imperative to develop experimental animal models to study SARS-CoV-2 pathogenesis and evaluate the efficacy of new drugs and vaccines. To this end, several animal

models have been developed, including humanized mice, Syrian hamsters, ferrets, and nonhuman primates (NHP). A significant number of scientific publications and review articles describe the outcome of SARS-CoV-2 infection in the above animal species.

Murine Models

The main limitation for SARS-CoV-2 infection of murine cells is the lack of appropriate cellular receptors to initiate viral infection. SARS-CoV-2 uses the cellular surface protein ACE2 to bind and enter cells, and murine ACE2 does not effectively bind the viral spike protein of the original wild-type SARS-CoV-2 strains. To solve this problem, three strategies have been developed.

i) Development of mouse-adapted SARS-CoV-2 virus by serial passage of the virus in mice or use of reverse genetics to modify the RBD of the virus to bind the mouse ACE2 protein.
ii) Genetic modification of the mice to express human ACE2 (hACE2). There are currently several transgenic models in which hACE2 is expressed by different promoters (keratin 18 [K18], CAG [consisting of the CMV immediate-early enhancer and the chicken beta-actin promoter], HFH4, FOXJ1, and the endogenous mouse ACE2 promoter). All of these mice are susceptible to infection by SARS-CoV-2, but the differences in hACE2 expression result in a pathogenic range from mild to lethal disease. In particular, with the exception of the model in which hACE2 is controlled by the mouse ACE2 promoter, all other models develop encephalitis after infection with SARS-CoV-2 (and original SARS-CoV). The SARS-CoV-2 infection of these mice results in highly lethal encephalitis. Some mice expressing hACE2 have been reported to succumb to severe pneumonia when the brain infection is not substantial. Alternatively, genetically engineered mice have been developed to mirror tissue-specific expression of hACE2, substituting the mouse ACE2 gene with the hACE2 gene (knock-in humanized mice). These hACE2 humanized mice support replication of SARS-CoV-2 in the respiratory tract with limited replication in other extrapulmonary organs, but they do not develop severe disease.
iii) Instead of permanent genetic modification of mice, scientists have generated mice susceptible to SARS-CoV-2 infection by sensitizing the respiratory tract of mice to SARS-CoV-2 replication through transduction with adenovirus (Ad5) or adeno-associated virus (AAV) expressing hACE2 under a CMV promoter. Following infection with SARS-CoV-2, Ad5-hACE2 mice develop a widespread infection of the lungs and histopathological changes consistent with viral pneumonia. Additional ongoing efforts to develop mouse models for SARS-CoV-2 infection studies involve mice humanized with hACE2 and human hematopoiesis (i.e. mice transplanted with human immune cells or human immune system [HIS]) and Collaborative Cross (CC) mouse studies.

In summary, various murine models for mild and severe COVID-19 have been developed. All will be useful for vaccine and antiviral evaluation, and some share features with the human disease. No murine model at present recapitulates all aspects of human COVID-19, especially unusual features such as the pulmonary vascular disease and hyperinflammatory syndromes observed in adults and children, respectively. However, continued refinement may eventually result in better animal models to study the pathogenesis of human COVID-19. Overall, these mouse models provide a stringent test for vaccine and therapeutic efficacy and may be helpful for pathogenesis studies in the future.

Syrian Hamster Model

In silico comparison of the human ACE2 sequence with that of the hamsters suggested that Syrian hamsters might be susceptible to SARS-CoV-2 infection. Subsequent experimental intranasal infection of Syrian hamsters showed mild to moderate disease with progressive body weight loss starting very early after infection (days 1–2 post inoculation). The hamsters consistently developed respiratory signs, including labored breathing, lethargy, ruffled fur, and hunched posture. After two weeks of infection, hamsters typically recover. Most importantly, SARS-CoV-2 infection in hamsters reflects some of the demographic differences of COVID-19 in humans. Thus, aged hamsters and male hamsters seem to develop more severe diseases than young and female hamsters. SARS-CoV-2 disease in hamsters is associated with high levels of virus replication and some of the histopathological changes similar to COVID-19 in humans (e.g., ground-glass opacities). Viral RNA is readily detected in the respiratory tract and other tissues, such as the small intestine, which could be useful for the evaluation of therapeutics and vaccines. Histologically, inflammatory infiltrate with abundant viral antigen expression and apoptosis was observed in the upper and lower respiratory tract with the development of necrotizing bronchointerstitial pneumonia starting at 2 dpi, being most severe at 4 dpi and resolving at 14 dpi. Among the non-respiratory tract tissues, only the intestine demonstrated viral antigen expression associated with severe epithelial cell necrosis, damaged and deformed intestinal villi, and increased lamina propria mononuclear cell infiltration. Virus transmission to cage mates has also

been observed, suggesting that hamsters could be helpful in transmission studies.

Ferret Model

It has been reported that following experimental mucosal exposure of ferrets to SARS-CoV-2, disease presentation could vary from asymptomatic or mild and may include lethargy, nasal discharge, wheezing, oropharyngeal mucus buildup, sneezing, and loose stool. SARS-CoV-2 virus shedding is observed in the nasal and oropharyngeal fluid. As with Syrian hamsters, virus replication is detected in the upper respiratory tract very early after infection (day 2) and is detectable during two weeks of infection. Virus replication in ferrets appears to be restricted to the respiratory and gastrointestinal tracts. Recent studies strongly indicate that SARS-CoV-2 infection in ferrets results in a predominantly upper respiratory tract infection. These findings make the ferret model especially suited to test the efficacy of mucosal vaccines and therapeutics to prevent upper airway infection and/or transmission. In addition, it has been shown that there was efficient virus transmission from experimentally infected ferrets to naïve cage mates. These studies indicated that airborne transmission of SARS-CoV-2 can occur and suggested that the ferret model may be helpful in further transmission studies.

Nonhuman Primate Model

NHPs (i.e. rhesus macaques, cynomolgus macaques, and African green monkeys) have been used as animal models for COVID-19. SARS-CoV-2 replicated to a very high level in the upper and lower respiratory tract leading to viral pneumonia with mild, variable clinical disease in infected NHPs. However, consistent severe clinical illness has not yet been observed in them. Following experimental infection with SARS-CoV-2, NHPs mount innate, humoral, and cellular immune responses and provide robust protection against rechallenge with the virulent virus. It has also been shown that older animals shed viruses from the nose and throat for longer than young adult animals. In rhesus macaques, higher viral loads were also detected in the lung tissue of aged animals. Furthermore, advanced age in rhesus macaques was also associated with increased radiological and histopathological changes.

Mink Model

Minks are naturally susceptible to SARS-CoV-2 infection, and there had been major COVID-19 outbreaks in mink farms in Europe and the United States. The minks get infected as a result of contact with SARS-CoV-2-infected farmworkers. In contrast to ferrets, minks displayed moderate to severe respiratory signs, including labored breathing, and some animals may die due to infection. SARS-CoV-2 was abundant in oropharyngeal and rectal swabs collected from dead animals from infected farms. Similar to ferrets, the viral loads in mink were higher in the oropharyngeal swabs than in the rectal swabs. Although minks may represent a suitable model for moderate to severe COVID-19, these animals are difficult to handle under laboratory conditions.

Laboratory Diagnosis

Numerous laboratories in the United States are currently testing animal specimens for SARS-CoV-2 using RT-qPCR and/or serological assays (SN and ELISA). Testing pets may be justified for certain animals in the following situations: Animals have clinical signs consistent with SARS-CoV-2, close contact with a person with suspected or confirmed COVID-19 or come from facilities associated with human disease outbreaks. Current RT-qPCR assays target ORF1b, spike protein gene, and the nucleocapsid gene of the virus. The preferred specimens to be submitted include nasal, oropharyngeal, and rectal swabs in molecular transport medium or viral transport medium and serum.

Treatment and Control

There is no specific antiviral treatment for pets diagnosed with COVID-19. The animals positive for COVID-19 should be separated (i.e. isolated) from people and other pets at home and provided with a separate litterbox. Avoid contact with the pet as much as possible, including petting, snuggling, being kissed or licked, and sharing food or bedding. Detailed guidelines on handling COVID-19 positive pets are found on the Centers for Disease Control and Prevention (CDC) and American Veterinary Medical Association (AVMA) websites. Zoetis has manufactured an experimental COVID-19 vaccine to protect more than 100 mammalian species in zoos, conservatories, sanctuaries, academic institutions, and government organizations. However, this vaccine is not available for routine vaccination of pets as it is currently authorized for experimental use only.

Animal Diseases Caused by Members of the Genus *Gammacoronavirus*

Avian IBV

Disease

Avian IBV (also known as Avian coronavirus) is one of the most significant causes of economic losses within the poultry industry, affecting the performance of both egg-laying

and meat-style (broiler) birds. Avian IBV causes respiratory disease in chicks 10 days to 4 weeks of age. However, all ages, sexes, and breeds are susceptible to infection, although mortality is low in birds greater than 6 weeks of age. The virus not only replicates in the upper and lower respiratory tract but also in the alimentary tract (e.g., esophagus, proventriculus, duodenum, jejunum, cecal tonsils, rectum, cloaca, and bursa of Fabricius) and other tissues such as the reproductive tract (e.g., oviduct, testes) and kidneys. Respiratory disease is characterized by respiratory distress, rales, coughing, nasal discharge, and depression. The clinical course lasts 6–18 days. Morbidity is 100%, and mortality may exceed 25%. Chicks with no maternal antibody may experience permanent oviduct damage and fail to lay eggs when they mature. Infection of the alimentary tract tissues does not manifest clinically. Nephritis is not uncommon among some of the IBV-infected broilers. IBV-associated renal disease is dependent upon the viral strain. Many viral strains with an affinity for the kidneys cause only mild or inapparent respiratory signs but can cause substantial mortality in susceptible birds. Infection of laying flocks results in a drop of egg production and hatchability. Pullets in good condition return to average production within a few weeks. Oviduct infection is believed to contribute to diminished egg production.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

IBV is antigenically distinct from other coronaviruses, and multiple distinct virus strains have been identified and grouped by serological techniques, polypeptide patterns in polyacrylamide gel electrophoresis (PAGE), oligonucleotide fingerprinting, and nucleotide sequencing. Strain-specific epitopes for viral neutralization and hemagglutination have been identified on the S1 protein (S protein is cleaved to generate two subunits, amino-terminal S1, and carboxy-terminal S2) with monoclonal antibodies. However, serotyping based on the reaction between an IBV strain and chicken-induced IBV serotype-specific antibodies has become less practical due to the emergence of an increasing number of antigenic variants with new neutralization phenotypes. In addition, a small percentage of amino acid changes in the S1 protein can result in a change in virus-neutralizing epitopes, leading to the emergence of new antigenic variants and serotypes. Thus, IBV variants may belong to over 100 serotypes. Genotyping of IBV strains has been achieved primarily by RT-PCR amplification and sequencing of the S1 subunit of the spike glycoprotein, which is the primary inducer of protective immunity and expresses most virus-neutralizing, including the serotype-specific, epitopes. In general, the IBV strains with a high level of homology in the S1 gene will provide a good level of cross-protection, which is not seen with strains with a low homology in this region.

Resistance to Physical and Chemical Agents

Most strains of IBV are inactivated within 15 minutes at 56 °C. The virus is relatively stable at cold temperatures. Stability at acidic pH is strain variable, with some strains surviving at pH 3.0 for three hours at 4 °C. Lipid solvents inactivate the virus.

Infectivity for Other Species and Culture Systems

The chicken is the only known natural host of IBV. However, quail and seagulls have been experimentally infected. Suckling mice can be infected by intracerebral inoculation. The virus can be cultivated in developing avian embryos, cell cultures, and organ cultures. Turkey embryos have also been successfully infected with IBV, but less efficiently. IBV can be grown in chick embryo cells (kidney, lung, and liver), embryonic turkey kidney cells, and monkey kidney (Vero) cells. Organ cultures have been used to propagate IBV, including tracheal and oviduct cultures.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

A large number of IBV variants exist around the world. Interestingly, some IBV variants are unique to a particular area, whereas others have a general distribution. The virus is likely maintained in persistently infected birds and/or continuous transmission cycles. The virus has been recovered for up to 49 days from infected chickens held in isolation and for even longer periods in those held under natural conditions. Viral transmission occurs by inhalation, with the respiratory tract being the primary site of infection. The virus is shed in respiratory and fecal materials, with subsequent spread by contaminated fomites and aerosols.

Pathogenesis and Pathology

The incubation period of IBV infection is 18–36 hours. Virus gains entry via the respiratory tract, and the respiratory form of IBV results in tracheitis and bronchitis. Mortality may be as high as 25% in young chicks. Strains exhibiting an affinity for the kidneys damage the renal tubules, resulting in renal failure. Chickens infected with nephrotropic viral strains develop renal tubular necrosis characterized by swollen and pale kidneys with the accumulation of uric acid crystals causing distension of the tubules and ureters. The infection produces a serous, catarrhal, or caseous exudate primarily in the trachea, nasal passages, and sinuses. In addition, the air sacs may contain caseous exudate, and small foci of bronchopneumonia may be apparent. Young

chicks may experience more severe infection, with lesions developing in the oviduct. Microscopic lesions of the respiratory tract include cellular infiltration, mucosal edema, vascular congestion, and hemorrhage.

Host Response to Infection

IBV elicits humoral and cellular immune responses. Humoral responses can be measured by ELISA, virus neutralization, and HI tests. Following infection with IBV, chickens first develop ELISA antibodies, followed by virus-neutralizing and HI antibodies. Titers of passively transferred maternal antibodies decrease to negligible levels within four weeks of hatching. Chickens recovered from natural infection are resistant to homologous viral challenges. The duration of immunity is variable and difficult to determine due to the diversity of IBV strains. Passive transfer of maternal antibody does not confer total protection to the chick but reduces disease severity and mortality. Local tracheal immunity appears to play a major role in resistance to IBV. The relative importance of humoral versus cellular immunity is unclear. However, it has been demonstrated that cytotoxic T-cell responses to IBV infection in chickens correlated with an initial decrease in infection and clinical signs. The observation that chickens may be protected without demonstrable antibodies suggests an important role for cell-mediated immunity.

Laboratory Diagnosis

Diagnosis of IB may be based upon direct visualization of viral antigen in tracheal smears using IHC or FA staining, detection of viral nucleic acid by RT-qPCR, viral isolation, or serology. In addition, IB must be differentiated from other acute respiratory diseases of birds, such as Newcastle disease, infectious laryngotracheitis, and infectious coryza.

Viral isolation is conducted by inoculating tracheal or respiratory exudates into the chorioallantoic sac of 10- to 11-day-old ECE. Serial passage in ECEs may be required before embryo dwarfing or mortality occurs. Recently, RT-qPCR assays for the detection of IBV nucleic acid have been described. Serological diagnosis of IBV infection requires paired serum samples and the use of IBV-specific viral neutralization, HI, AGID, or ELISA assays.

Treatment and Control

No specific treatment for IB or IBV infections is available. Proper husbandry practices that reduce environmental stress are logical. Control of IB may be approached through management procedures and vaccination. The spread of the virus may be reduced by strict isolation of an affected flock and restocking with day-old chicks reared in isolation. Attenuated and inactivated vaccines have been developed for the control of IB or IBV infection in poultry. Inactivated vaccines induce neutralizing antibodies, but their efficacy has been questioned. MLV vaccines attenuated by serial passage in ECE have reduced pathogenicity but also decreased immunogenicity. The most commonly used MLV vaccines contain derivatives of Massachusetts stain (Mass41, H120, and H52). Vaccination with MLV vaccines produces short-lived protection and declines after nine weeks. Consequently, commercial layers that are kept for a year or more should be vaccinated several times with MLV vaccines, perhaps with more than one serotype. Vaccines may be administered via aerosol or in drinking water. High passage vaccine viruses have reduced invasiveness and generally require aerosol administration.

The diversity of IBV strains and serotypes has made it challenging to develop efficacious vaccines. No single strain has been identified as capable of inducing more than limited protection to heterologous viruses. Multivalent vaccines are available, but prolonged reactions to vaccination and some interference between vaccine strains have been reported in certain instances. Vaccines have not been developed commercially with nephropathogenic strains of IBV. Inactivated and subunit vaccines have been developed against IBV, but they do not provide good protection against infection. Some inactivated autogenous vaccines specific for the variant virus in a particular geographic region have been used to control the disease with limited success.

Turkey Coronavirus

Disease

TCoV is the causative agent of coronavirus enteritis (CE) of turkeys. It is an acute and highly contagious disease of turkeys of all ages and is of major economic importance to the turkey industry. Synonyms of the disease include blue comb disease, mud fever, transmissible enteritis, and infectious enteritis. The disease primarily affects the alimentary tract and is characterized by depression, subnormal body temperature, anorexia, inappetence, loss of body weight, and wet droppings. Darkening of the head and skin and tucking of the skin over the crop are characteristics of infected growing turkeys. In addition, a rapid drop in egg production with the formation of chalky eggshells occurs in producing breeder hens. Morbidity is essentially 100%, and mortality varies with age and environmental conditions.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

TCoV is inactivated by lipid solvents and detergents but is resistant to acidic pH (pH of 3.0 at 20 °C for 30 minutes).

The virus is resistant to 50 °C for one hour. TCoV is apparently unrelated antigenically to other coronaviruses and is readily distinguished from IBV based on antigenic and biological differences. However, phylogenetic analysis based on the nucleocapsid gene has demonstrated that TCoV is more closely related to IBV than to other mammalian coronaviruses. TCoV agglutinates rabbit and guinea pig erythrocytes, but not those from cattle, horse, sheep, mouse, goose, monkey, rooster, or chickens.

Infectivity for Other Species and Culture Systems

TCoV infection is confined to turkeys, and virus replication occurs exclusively in the intestinal epithelium and the epithelium of the bursa of Fabricius. Using IHC, TCoV antigens can be demonstrated in these tissues. Although TCoV can infect chickens, experimental infections of pheasants, seagulls, and coturnix quail were refractory to infection. Laboratory propagation of the virus has been limited to turkey and chicken embryos, and attempts to grow TCoV in cell cultures have been unsuccessful.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

CE has been described in North America and Australia. The virus persists in turkeys for life following recovery from the disease. It is stable in frozen feces and survives throughout the winter months in infected droppings; thus, the transmission of TCoV is principally fecal–oral from the infected feces of carrier birds. Introduction of virus into a location may occur via carrier turkeys, feces-contaminated fomites, personnel and equipment, and possibly mechanical transmission by free-flying birds.

Pathogenesis and Pathology

The incubation period of CE varies from one to five days. Gross lesions are primarily confined to the intestinal tract, with petechial hemorrhages sometimes apparent on the serosal surface. Lesions are most distinct in the jejunum but may also occur in the duodenum, ileum, and cecum. Gas and fluid typically distend the small intestine and ceca. The breast muscles are typically dehydrated, and the carcass appears generally emaciated. Microscopic lesions include destruction of enterocytes lining the intestinal villi, leading to their shortening, loss of microvilli, and mononuclear cell infiltration of the lamina propria of the affected bowel.

Host Response to Infection

Turkeys respond to TCoV infection with a humoral and cellular immune response. Serum antibodies (IgM, IgA, and IgG) develop following infection, but only IgG is present by 21 days. Local IgA antibody appears in intestinal secretions and bile for at least six months. Passive transfer of maternal immunity has not been observed, and administration of antiserum to young poults does not afford protection.

Laboratory Diagnosis

Definitive diagnosis of CE requires identification of viral antigen in intestinal tissue sections by IHC staining, detection of viral nucleic acid by PCR techniques, viral isolation, or demonstration of serum antibody. The virus may be isolated by inoculation of embryonated turkey eggs or young poults, and its presence is confirmed by FA staining of infected tissues. Attempts to propagate TCoV in various avian and mammalian cell cultures have been unsuccessful. Serological diagnosis can be conducted on paired serum samples by viral neutralization or indirect FA tests.

Treatment and Control

No specific treatment effectively reduces the morbidity of CE, although various treatments may be used to prevent other intestinal infections. TCoV vaccines are not available, but prevention of infection is possible. The disease has been eliminated in some areas by depopulation and decontamination of affected premises. An alternative to viral elimination is exposing poults (five to six weeks of age) to recovered carrier birds under ideal environmental conditions to induce protective immunity. Such a program is recommended only on farms with persistent problems, when all other control methods have failed.

Animal Diseases Caused by Members of the Genus *Deltacoronavirus*

Porcine Deltacoronavirus

Disease

PDCoV is a newly emerged enteric pathogen of swine. It was first identified in China in 2012 and then in the United States in 2014. The virus has spread across the United States, Canada, and many other countries in Asia, including South Korea, and Vietnam, causing severe economic losses. The Porcine delta coronavirus disease (PDCoVD) is clinically characterized by watery diarrhea and vomiting in suckling pigs. PDCoV is the fourth enteric virus of pigs that causes diarrhea and high mortality in pigs, along with the other enteric coronaviruses of swine, including TGEV, PEDV, and newly emerged SADS-CoV. Thus, differential diagnosis of PDCoV, PEDV, TGEV, and SADS-CoV is critical in the regions where these porcine enteric viruses circulate in swine farms.

Etiologic Agent

The morphological and physicochemical properties of PDCoV are similar to those of other coronaviruses. Comparative nucleotide sequence analysis has demonstrated that all global PDCoV strains overall share high nucleotide identities. However, some strains have multiple mutations and deletions in their S, nonstructural genes, or 3′ untranslated region, which separate them into different phylogenetic groups (i.e. US and Chinese PDCoVs clades). It has been reported that there is no cross-reactivity of PDCoV with antibodies to PEDV and TGEV. However, antigenic cross-reactivity between a single epitope in N proteins of US PDCoV and PEDV strains has been reported. PDCoV is primarily transmitted by the fecal–oral route. Feces and/or vomitus and other contaminated fomites are major transmission modes of the virus.

Host–Virus Relationship

The clinical signs of PDCoVD in suckling and older pigs are similar but milder than those caused by PEDV and TGEV infections in suckling piglets. Compared with PEDV, the clinical signs caused by PDCoV infection are less severe, and the mortality rate of newborn piglets is 30–40%. However, co-infection with PEDV, TGEV, or porcine rotavirus is more common and leads to more serious clinical signs. Seronegative pigs of all ages are susceptible to PDCoV infection, and morbidity could reach up to 100% in piglets but can vary in sows. PDCoV infection can cause up to 40% mortality in suckling piglets. PDCoV causes acute, watery diarrhea, frequently accompanied by vomiting, leading to dehydration, bodyweight loss, lethargy, and death. Diarrhea could last for 5–10 days, with viral RNA shedding for up to 19–28 days in feces and up to 42 days in oral fluids. Gross lesions are limited to the gastrointestinal tract, mainly restricted to the proximal jejunum and colon. The lesions are characterized by thin and transparent intestinal walls with the accumulation of large amounts of yellow fluid. The stomach is frequently filled with curdled milk. The transparency and fragility of the affected regions of the intestine are milder compared to PEDV and TGEV infections. Histopathological lesions are similar to those caused by other porcine enteric coronaviruses, including acute, multifocal to diffuse, mild to severe atrophic enteritis in the proximal jejunum to the ileum. In addition, vacuolated enterocytes and massive exfoliation of enterocytes on the tips or the entire villi of jejunum and ileum are seen in acute infections. Atrophied villi are frequently fused and covered with an attenuated or regenerated epithelium. In addition, there is infiltration of macrophages, lymphocytes, and neutrophils in the lamina propria. No histological lesions are observed in the duodenum, which is consistent with the small number of PDCoV antigen-positive duodenal epithelial cells. The immune responses of pigs to PDCoV infection are largely undefined, but antibodies to the virus have been reported in serum. Young piglets can be protected by the transfer of maternal antibodies via colostrum and milk of immune dams.

Laboratory Diagnosis

Laboratory diagnostic techniques should be used to differentiate PDCoV from other porcine enteric viruses (TEGV, PEDV, SADS-CoV, and rotavirus). Definitive diagnosis of PDCoV infection includes detection of viral RNA or antigen in the feces or intestinal tissues from diarrheic pigs. Several RT-PCR and RT-qPCR assays have been developed to detect and differentiate PDCoV from other porcine enteric viruses. In addition, IFA or IHC using virus-specific MAbs or polyclonal antibodies are available to detect the virus in infected tissues. *In situ* hybridization also can be used to detect viral nucleic acid in infected tissues. Direct EM can be used to demonstrate PDCoV particles in feces, but immunoelectron microscopy using hyperimmune or convalescent sera is essential to differentiate PDCoV from TGEV and PEDV. The virus can be isolated from feces or intestinal tissues using LLC-PK or ST cells, but the success rate is low compared to the molecular techniques. Serological diagnosis can be conducted by IFA, VN, and ELISA assays.

Treatment and Control

There are no treatments or vaccines to control PDCoV infection in pigs. The prevention and control measures used to prevent other porcine enteric diseases are also applicable to PDCoV. These include implementing very high-level biosecurity measures to reduce PDCoV transmission via contaminated fomites.

Animal Diseases Caused by Members of the Genus *Torovirus*

ToVs have recently been classified into the new subfamily *Torovirinae* within the family *Tobaniviridae* in the suborder *Tornidovirineae*. ToVs are associated with enteric diseases in animals and humans, and they are recognized as pathogens of veterinary and medical importance. However, little attention has been paid to them because their infections are usually asymptomatic or not severe. Four species of toroviruses have recently been identified: BToV (also known as Breda virus [BRV]), PToV, EToV (also known as Berne virus [BEV]), and HToV (Table 61.3). ToVs are enveloped viruses

Table 61.3 Important animal and human toroviruses in the suborder *Tornidovirineae,* family *Tobaniviridae, and* subfamily *Torovirinae.*

Genus/species	Abbreviation	Natural host	Tissue affected/disease
Genus: *Torovirus*			
Equine torovirus	EToV	Horse	Enteric infection
Bovine torovirus	BToV	Cattle	Enteric infection
Porcine torovirus	PToV	Pig	Enteric infection
Human torovirus	HToV	Humans	Enteric infection

with a linear, non-segmented, positive-sense single-stranded RNA genome. Virions exhibit polymorphic shapes such as spherical, oval, rod- and kidney-shaped particles. Its nucleocapsid forms a helically symmetric tubular structure, a doughnut-like shape described by the Latin word "torus," which is the origin of the name *Torovirus*. EToV, the prototype of this family, was first isolated from a horse in Switzerland in 1972; BToV was isolated from calves in Iowa in 1979; and PToV was detected in piglets in the Netherlands in 1998. ToVs, particularly BToV and PToV, have been reported in many countries. Torovirus-like particles have been observed in fecal samples of horses, cattle, pigs, cats, dogs, and humans. HToV shows antigenic cross-reactivity to BToV or EToV, as demonstrated by immuno-EM, ELISA, HI test, and Western blot analysis. Due to its high prevalence, HToVs are thought to be the causative agent of human diarrhea. However, HToV has not yet been isolated in cell culture. In addition, serological evidence for torovirus infections has been demonstrated in horses, cattle, sheep, goats, pigs, rabbits, rats, and two species of wild mice. It has been speculated that toroviruses are associated with enteric disease in many animal species. Still, only the BToV has been ascribed to gastroenteritis in animals.

Equine Torovirus (EToV)

EToV (BEV) was originally isolated from the rectal swab of a diarrheic horse in cultured cells (equine dermis cells). Limited seroepidemiological studies indicate that the virus is present in the United States and Europe. However, experimental or natural infection by BEV does not induce obvious clinical signs, and it is likely of little pathogenic importance. Thus, much remains to be determined regarding the pathogenic significance of EToV infections of horses.

Bovine Torovirus (BToV)

BToV (BRV) was isolated from the feces of calves with severe diarrhea in Breda, Iowa, in 1979. BToV is antigenically divided into two serotypes, BRV1 and BRV2. Initially, BToV could only be propagated in gnotobiotic or colostrum-free calves. In 2007, a subcell line of human rectal tumor-18 cells (HRT18-Aichi) was demonstrated to be susceptible to BToV, allowing isolation and propagation of several other BToVs. Currently, BToV is the only ToV that has a reverse genetic system (i.e. an infectious cDNA clone of the virus). Seroepidemiological studies indicate that BToV infection of cattle seems to be common throughout the world. Diarrhea due to BToV infections has been reported both in young and old cattle. The clinical manifestations of BToV infections include watery diarrhea with dehydration, weakness, and depression. Predominantly affected are two- to three-week-old calves, and they tend to have only mild diarrhea by three to four months of age but continue to shed the virus. Diarrhea outbreaks associated with BToV in adult cattle have been described. Most of the affected cattle develop watery diarrhea, anorexia, and decreased milk production. BToV infections are usually limited to the intestine, but there is some evidence for respiratory tract involvement in adult cattle leading to pneumonia in older cattle. Recently, BToV nucleic acid has been detected by RT-PCR in nasal swabs collected from Japanese calves with respiratory symptoms, suggesting that this virus may be a predisposing factor and/or causative agent for bovine respiratory disease. BToV infects the epithelial cells of the small and large intestine extending from mid-jejunum to colon. Transmission of BToV probably occurs via the oral and respiratory routes. Following infection, cattle develop neutralizing antibodies to BToV, and these antibodies can cross-react with toroviruses from other animal species. Diagnosis of BToV infection is achieved by demonstration of virus particles with a doughnut-shaped nucleocapsid surrounded by an envelope containing a large number of small spikes that resemble the peplomers of coronaviruses. BToV from fecal samples of diarrheic cows in Japan has been isolated in HRT-18-Aichi cells. The diagnosis of BToV infection is mainly achieved by demonstration of antigen or demonstration of antibodies to the virus by immunologic methods. Viruses in fecal samples can be detected by indirect immunofluorescence using bovine anti-BToV antiserum. Antibodies to BToV can be demonstrated by neutralization assay or ELISA. Recently,

standard and RT-qPCR assays have been described to detect both BToV and PToV nucleic acid in fecal samples.

Porcine ToV (PToV)

PToV was originally detected and characterized in the Netherlands in 1998 from the feces of piglets that did not show any signs of enteritis or diarrhea. However, it since has been identified in pigs with diarrhea at the time of weaning. PToV has been identified from piglets in many countries globally in recent years. Although PToV is detected at a higher rate in piglets with diarrhea than in healthy piglets, its clinical relevance remains unclear. Coinfections with other enteric pathogens may exacerbate their signs. PToV has been detected in many countries, and a serological survey showed that most pigs over the age of 11 weeks were seropositive in Spain. PToV has not been propagated in cell culture. However, standard RT-PCR, RT-qPCR, RT-LAMP methods are available that target M and N genes to detect PToV in clinical specimens.

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62

Arteriviridae and Roniviridae

Udeni B. R. Balasuriya, Mariano Carossino, and Yun Young Go

Introduction and Classification

The virus families *Arteriviridae* and *Roniviridae* are included in the order *Nidovirales,* along with *Coronaviridae*. Recent advances in next-generation sequencing and viral metagenomics led to the discovery of many new viruses. Characterization of these novel genomes has profoundly changed the classification and nomenclature of the members of the order *Nidovirales* (see Chapter 61). The members of *Arteriviridae* and *Roniviridae* are enveloped viruses with linear, positive-sense, and multi-cistron RNA (ssRNA) genomes. The genomes contain a 5′-cap structure and a 3'-poly'-A tail and include untranslated regions (UTR) at their 5′- and 3′-termini. They share a strikingly similar genome organization and replication strategy to coronaviruses, but differ considerably in their genetic complexity and virion architecture (Figures 62.1 and 62.2 and Tables 62.1–62.3). The four arteriviruses of veterinary importance (Equine arteritis virus [EAV], Lactate dehydrogenase elevating virus [LDV] of mice, Porcine reproductive and respiratory syndrome virus [PRRSV genotypes I and II]) and Simian hemorrhagic fever virus [SHFV] were initially included in the single genus *Arterivirus*. However, in the new ICTV classification (2020), these viruses are assigned to three distinct subfamilies: *Equarterivirinae* (EAV), *Variarterivirinae* (LDV and PRRSV), and *Simarterivirinae* (SHFV). The subfamily *Equarterivirinae* includes the single genus *Alphaarterivirus* (species *Alphaarterivirus equid* [EAV]). The subfamily *Variarterivirinae* includes two genera: *Gammaarterivirus* (species *Gammaarterivirus lacdeh* [LDV]) and *Betaarterivirus* (subgenera *Eurpobartevirus-Betaarterivirus suid 1* [PRRSV-1] and *Ampobartevirus* (species *Betaarterivirus suid 2* [PRRSV-2]). The two genotypes of PRRSV (previously known as genotype I and genotype II) were promoted to species and are now identified as PRRSV-1 and PRRSV-2 (collectively known as PRRSV in the literature as well as in this chapter).

The arteriviruses primarily target macrophages in their respective hosts, and the disease outcome is highly variable and includes persistent asymptomatic infections, respiratory disease, reproductive failure (abortion), and lethal hemorrhagic fever. EAV, SHFV, LDV, PRRSV-1, and PRRSV-2 are capable of establishing asymptomatic, long-term infection or persistent infection in their respective natural hosts.

The family *Roniviridae* includes a single genus *Okavirus* with virus species that infect crustaceans (shrimp, prawns, and crabs). The name "Roniviridae" is derived from "rod-shaped nidovirus," referring to the virion morphology of viruses in the family. The name "Okavirus" is derived from the observation that these viruses can infect the shrimp lymphoid or "Oka" organ. Members of this genus include Yellow head virus (YHV) and Gill-associated virus (GAV), both of which infect and cause high mortalities in farmed black tiger prawns (*Penaeus monodon*) in Asia and Australia, respectively. Based on phylogenetic analysis of ORF1b, roniviruses are most closely related to other nidoviruses infecting arthropods, including members of the families *Mesoniviridae* (from mosquitoes) and *Euroniviridae* (from crustaceans). Therefore, the newly identified families *Euroniviridae* and *Roniviridae* are included in the new suborder *Ronidovirineae*.

Virion Properties and Virus Replication

Family *Arteriviridae*

Arterivirus virions are spherical, 45–74 nm in diameter (EAV 45–60 nm and PRRSV 50–74 nm), consisting of an isometric nucleocapsid of 25–39 nm in diameter, surrounded by a lipid envelope. However, recent cryoelectron tomography studies of PRRSV have shown that the

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nucleocapsid is a rather pleomorphic and "disorganized" core structure. The envelope includes 12–15 nm diameter ring-like surface projections. The large spikes that are typical of coronaviruses and roniviruses are absent from the arterivirus envelope. The arterivirus genome is a linear, positive-sense, ssRNA 12.7–15.7 kb molecule and includes 10–14 open reading frames (ORFs) (Table 62.2 and Figure 62.2).

The polycistronic arterivirus genomes contain 10–16 recognized ORFs, which encode nonstructural and structural proteins. The two most 5′-proximal ORFs (1a and 1b) occupy approximately three-fourths of the genome (Figure 62.2). The

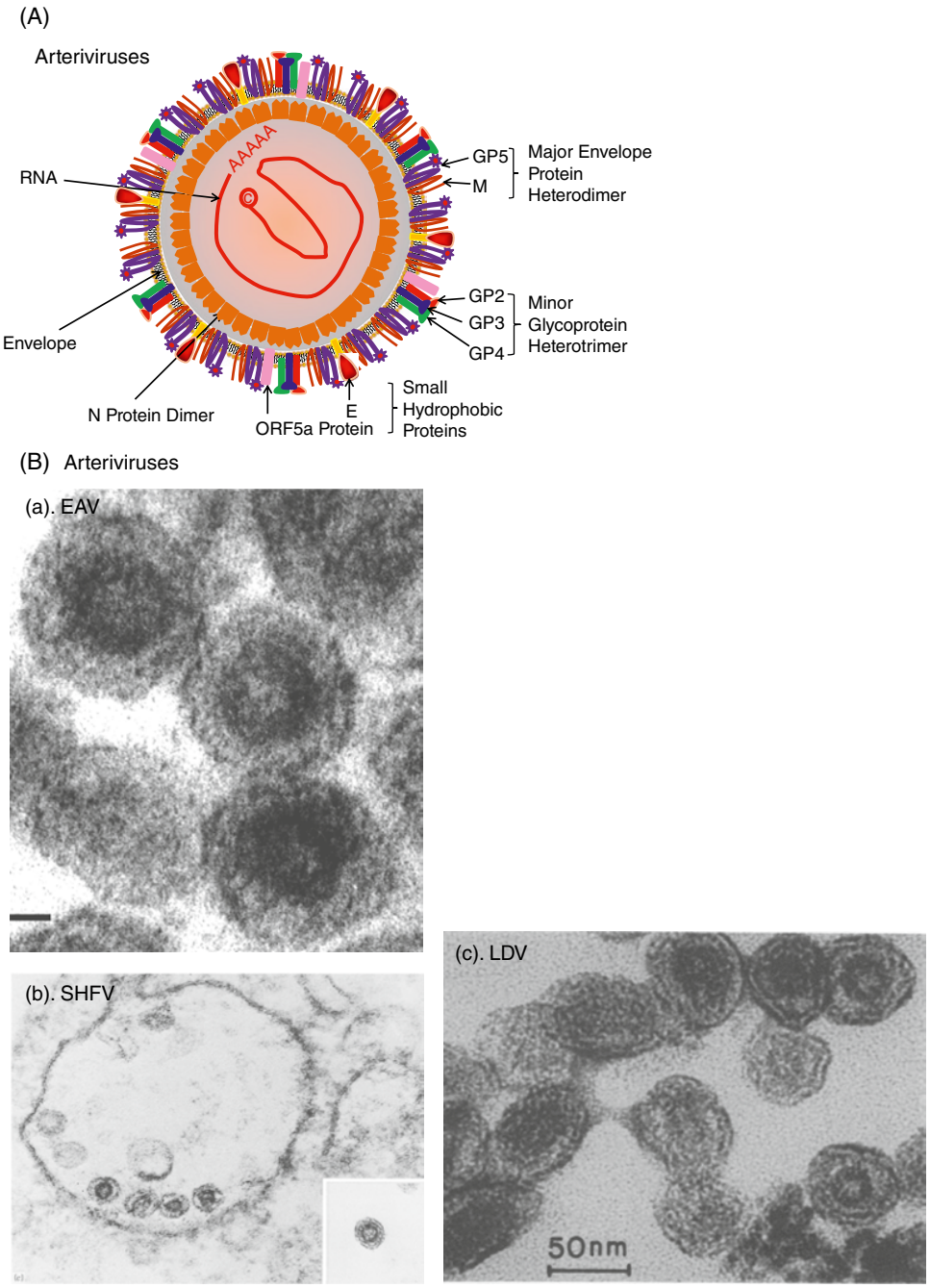
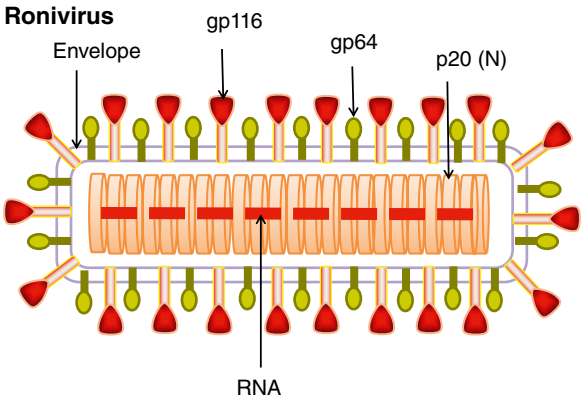


Figure 62.1 Structure and morphology of arteriviruses and roniviruses. (A) Schematic representation of arteriviruses (e.g., EAV); (B) electron micrographs of arteriviruses: (a) EAV, (b) SHFV, and (c) LDV; (C) schematic representation of ronivirus; and (D) electron micrograph of roniviruses (e.g., GAV. (Adapted from Snijder et al. 2005 (EAV and GAV), Gravell et al. 1980 (SHFV), and Brinton-Darnell and Plagemann 1975 (LDV).

(C)



Roniviruses

(D)



Figure 62.1 (Continued)

replicase ORFs 1a and 1b are translated to produce polyproteins pp1a and pp1ab, with the latter being a C-terminally extended version of the former. These two precursor proteins are extensively processed after translation into at least 13 nonstructural proteins (nsp1-12, including nsp7 α/β; Figure 62.3, Table 62.2) by three viral proteases (nsp1, nsp2, and nsp4). The region downstream of the replicase gene (3′ quarter of the genome) contains 8–12 relatively small ORFs, most of which have both 5′- and 3′-terminal sequences that overlap with neighboring genes. These genes encode mostly (or exclusively) structural proteins and are translated from sg mRNAs. Their organization is generally well conserved in the arterivirus genome. An exception is a region downstream of the replicase gene of SHFV, which contains three or four additional ORFs, which may have arisen from the duplication of ORFs 2a/b to 4. These duplicated genes are identified with a "prime" annotation (2b′, 2a′, 3′, and 4′ and they encode for E′, GP2′, GP3′, and GP4′, respectively).

The virions of EAV and PRRSV consist of a nucleocapsid protein (N; encoded by ORF7) and seven envelope proteins (E, GP2, GP3, GP4, ORF5a protein, GP5, and M; encoded by ORFs 2a, 2b, 3, 4, 5a, 5, and 6; Figure 62.2 and Table 62.2). The only exception is PRRSV, where ORF2a and 2b are swapped (encodes GP2 and E, respectively). The GP5 (glycosylated) and M (unglycosylated) are the major envelope proteins of EAV and PRRSV, and they form a disulfide-linked heterodimer in the mature virus particles. The GP5 protein contains the known neutralization determinants of EAV, PRRSV, and LDV, and it has been demonstrated that heterodimerization of GP5 and M proteins is critical for the authentic posttranslational modification (glycosylation) and conformational maturation of neutralization determinants in GP5 of both EAV and PRRSV. The M protein may act as an essential scaffold on which the GP5 protein folds from the epitopes that induce neutralizing antibodies in mice, horses, and pigs. In addition, the arterivirus envelope contains a heterotrimer of three minor membrane glycoproteins (GP2, GP3, and GP4) and two unglycosylated envelope proteins (E and ORF5a proteins).

Following the uncoating of the viral genome, arterivirus replication starts with the translation of the two large replicase polyprotein genes (ORF1a and ORF1b) located in the 5′ three-quarters of the genome through a ribosomal frameshifting. These two replicase polyproteins are processed into 13 or 14 cleavage products or nonstructural proteins (nsps) and a variety of processing intermediates by three viral proteases. Both the 5′-leader sequence (5′-UTR) and transcription regulatory sequences (TRSs) located upstream of each ORF play a critical role in genome replication and transcription of viral proteins. The TRSs are short-conserved sequence elements (5′-UCAAC-3′) that determine a base-pairing interaction between positive and nascent minus-strand RNA and are essential for leader–body joining. Arterivirus replication occurs in the cytoplasm of the infected cells. The viral structural protein-encoding genes are overlapping and located in the 3′ one-fourth of the genome (Figure 62.2). These structural protein genes are expressed from a 3′-co-terminal nested set of subgenomic mRNAs (sg mRNAs). All sg mRNAs have a common 5′- leader sequence derived from the 5′-UTR of the viral genome. For production of new genomic RNA, recognition of signals present close to the 3′ end of the antigenome is used. The N protein encapsidates the newly synthesized viral genomic RNA, and arteriviruses acquire their envelope during budding through the endoplasmic reticulum and/or the Golgi complex. After budding, progeny virus particles are transported in intracellular vesicles to the plasma membrane for release.

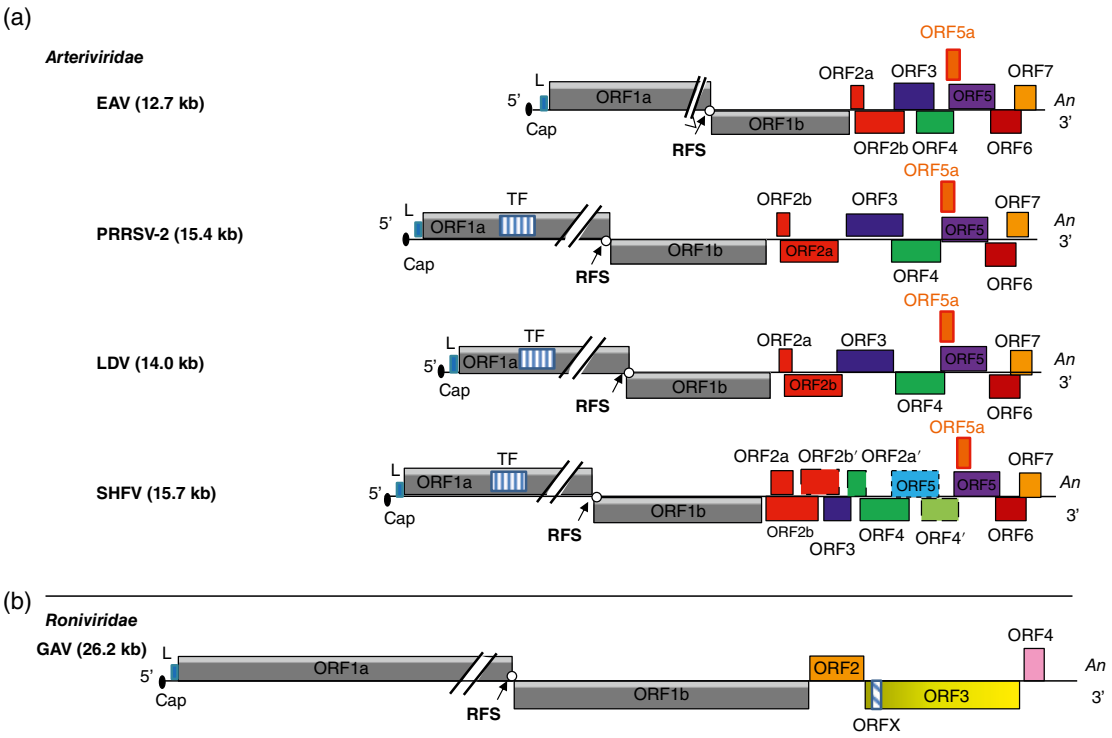


Figure 62.2 Schematic representation of genome organization of arteriviruses (a) and roniviruses (b). The genomic RNA of representative viruses of the families *Arteriviridae* (EAV, PRRSV-2, LDV, and SHFV) and *Roniviridae* (GAV) is shown to the same scale. The ORFs encoding replicase proteins and viral structural proteins are depicted. The duplicated SHFV structural protein ORFs are indicated by boxes with broken lines. The 5′-cap structure, 5′-leader sequence (L), ribosomal frameshift (RFS) of the ORF1a/1b, and the 3′ poly-A tail (A_n) are indicated. *Source:* Modified from Gorbalenya et al. (2006). The newly identified short transframe (TF) ORF on the nsp2-coding region of ORF1a is depicted with a hatched box. This TF ORF is conserved in the genomes of all currently known arteriviruses, except EAV and WPDV.

Table 62.1 Viruses of veterinary importance in the families *Arteriviridae* and *Roniviridae*.

Family/virus	Year of isolation	Natural host	Disease/tissue affected	Transmission
Family *Arteriviridae*				
Equine arteritis virus (EAV)[a]	1953	Horse, donkey, mule, and zebra	Equine viral arteritis (EVA) – respiratory disease (influenza-like disease), abortion, interstitial pneumonia in young foals	Aerosol, contact, fomites, venereal (breeding), embryo transfer, and congenital infection
Porcine reproductive and respiratory syndrome virus (PRRSV)[b]	1990–1992	Pig	Porcine reproductive and respiratory syndrome (PRRS) – respiratory distress, abortion, stillbirths, mummified fetuses	Aerosol, contact, fomites, venereal (breeding)
Lactate dehydrogenase elevating virus (LDV)[c]	1960	Mice	No clinical disease, but elevated lactate dehydrogenase elevating enzyme in blood	Biting and coprophagy
Simian hemorrhagic fever virus (SHFV)[d]	1964	Monkeys	Systemic hemorrhagic disease (SHF)	Aerosol, contact, fomites, biting, and iatrogenic
Family *Roniviridae*				
Yellow head virus (YHV)[e]	1990	Shrimp and prawns	Yellowing of the hepatopancreas	Horizontal (waterborne), cannibalism, and vertical
Gill-associated virus (GAV)[f]	1993	Shrimp and prawns	Gills and lymphoid organs	Horizontal (waterborne), cannibalism, and vertical

[a] EAV virulent Bucyrus strain (EAV-VBS) – DQ846750; cell culture-adapted Bucyrus strain – X53459 (=NC_001639).
[b] PRRSV-1 (Lelystad virus) – M96262; PRRSV-2 (VR-2332) – U87392.
[c] LDV Plagemann (LDV-P) – U15146 (=NC_001639); LDV-C (L13298).
[d] SHFV – AF180391 (=NC_003092).
[e] YHV(YHV1992) – FJ848673.
[f] GAV – NC_010306.

Table 62.2 Molecular properties of arteriviruses.

	Replicase proteins			Structural proteins		
Virus (genome size; bp)	**ORFs**	**Nsp name**	**Size (aa)**	**ORFs**	**Protein name**	**Size (aa)**
EAV	ORF1a	1–8/9	1727	2a	E	67
(12 704–12 731)	ORF1ab	10–12	3175	2b	GP2	227
				3	GP3	163
				4	GP4	152
				5a	ORF5a protein	59
				5b	GP5	255
				6	M	162
				7	N	110
PRRSV-1	ORF1a	1-alpha and 1-beta - 9/10	2504	2a	GP2	256
(15 047–15 465)	ORF1ab	10–12	3962	2b	E	73
				3	GP3	2254
				4	GP4	178
				5a	ORF5a protein	51[a]
				5b	GP5	200
				6	M	174
				7	N	123
PRRSV-2	ORF1a	1-alpha and 1-beta - 9/10	2397	2a	GP2	249
(15 111)	ORF1ab	10–12	3854	2b	E	70
				3	GP3	265
				4	GP4	183
				5a	ORF5a protein	43
				5b	GP5	201
				6	M	173
				7	N	128
LDV	ORF1a	1–9	2206	2a	E	70
(14 104)	ORF1ab	12	3616	2b	GP2	227
				3	GP3	191
				4	GP4	175
				5a	ORF5a protien	47
				5b	GP5	199
				6	M	171
				7	N	115
SHFV	ORF1a	1–9/10	2105	2a	E	80
(15 717)	ORF1ab	12/13	3594	2b	GP2	214
				3	GP3	179
				4	GP4	205
				2b′	E′	94
				2a′	GP2′	281
				3′	GP3′	204
				4′	GP4′	182
				5	GP5	278
				5a	GP5a protien	64
				6	M	162
				7	N	111

Molecular data are based on the following GenBank sequences: DQ846750 (EAV virulent Bucyrus strain), U87392 (PRRSV-NA [VR-2332]), M96262 (PRRSV-EU [Lelystad virus]), U15146 (LDV-P), and AF180391 (SHFV).

[a] Size varies between 46 and 51 amino acids among PRRSV-2 strains.

Table 62.3 Molecular properties of roniviruses.

	Replicase proteins		Structural proteins		
Virus (genome size; bp)	**ORFs**	**Size (aa)**	**ORFs**	**Protein name**	**Size (aa)**
YHV[a]	1a	4074	2	N	146
(26 652–26 673)	1ab	6692	3	gp116	1666[b,c]
				gp64	
				p20	
			4	Polypeptide	20
GAV[a]	1a	4060	2	N	144
(26 253)	1ab	6706	3	gp116	1640[a]
				gp64	
				p20	
			4	Polypeptide	83

[a] Molecular data are based on the following GenBank sequences: YHV (YHV1992)—FJ848673 and GAV (NC_010306).
[b] Polyprotein encoded by ORF3.
[c] There is an alternative reading frame in ORF3 identified as ORFX encoding a putative small double-membrane-spanning protein (px).

Family *Roniviridae*

Roniviruses are enveloped, bacilliform (40–60 nm × 150–200 nm) virions with rounded ends (Figure 62.1c,d). The nucleocapsid (20–30 mm in diameter) has helical symmetry and comprises coiled filaments of 5–7 nm diameter. The nucleocapsid is surrounded by the envelope, which has diffuse projections (approximately 8 nm thick and 11 nm in length) extending from the surface. The most economically important and well-studied roniviruses include YHV and GAV of black tiger shrimp. YHV can remain infectious for at least 72 hours in seawater (25–28 °C), and the virus can be effectively disinfected with 30 ppm calcium hypochlorite and sodium dodecyl sulfate. YHV is inactivated by heating for 15–30 minutes. Like other nidoviruses, the ronivirus genome is a positive-sense, single-stranded 26.2–26.6 kb RNA molecule that contains a 5′-cap structure and a 3′-polyadenylated tail, and includes five or six ORFs (5′-ORF1a–ORF1b–ORF2-ORFX–ORF3–(ORF4)– poly A-3′; Figure 62.2b). In GAV, ORF3 is followed by a sixth ORF (ORF4). It is important to note that the ronivirus genome organization differs significantly from vertebrate nidoviruses in several aspects: (i) the nucleoprotein gene (ORF2) is located upstream of the

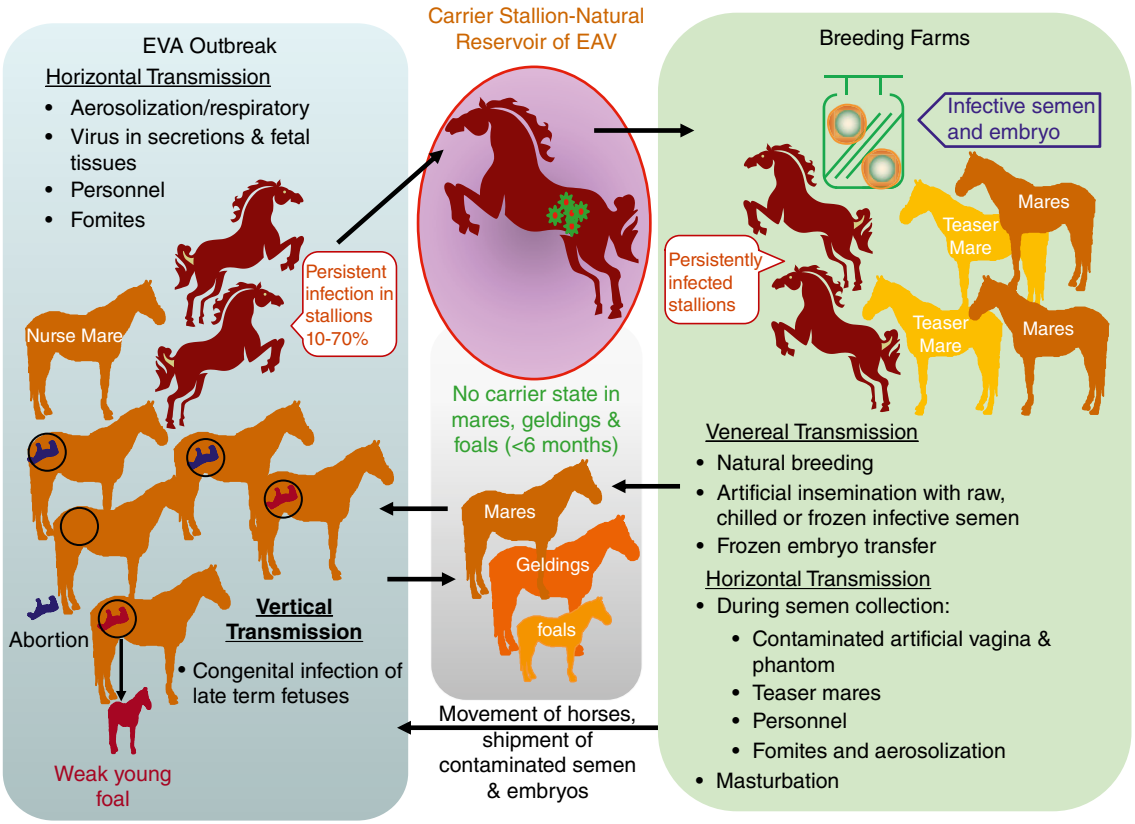


Figure 62.3 Transmission of Equine arteritis virus (EAV), depicting the central role of the carrier stallion in the maintenance and spread of the virus.

glycoprotein gene (ORF3), (ii) there is no discrete gene encoding a structural membrane (M) protein, and (iii) the virion envelope glycoproteins gp116 and gp64 are generated by proteolysis of a pp3 precursor polyprotein encoded by the ORF3 gene.

Similar to other members of the order *Nidovirales*, these viruses encode two large replicase polyproteins (pp) from their genomic RNA: ORF1a (pp1a) and ORF1ab (pp1ab) fusion products. The pp1a and pp1ab are processed into several nsps, including viral proteinases RdRp, helicase, and other conserved enzyme domains. Full-length genomic RNA (RNA1) and two sg mRNAs (sgmRNA2 and sgmRNA3) are produced during replication. Similar to arteriviruses, viral structural protein genes are expressed from the two nested sets of 3′-coterminal sg mRNAs (sg mRNA2 and sg mRNA3). Unlike the other nidoviruses, the N protein (p20; 20–22 kDa) of okaviruses is encoded by ORF2, which resides immediately downstream of the 5′-terminal ORF1a/ab replicase gene (Table 62.3). The ORF3 encodes a precursor polyprotein (pp3) that is cleaved to produce two envelope glycoproteins (gp116 [110–135 kDa] and gp64 [63–67 kDa]) and an approximately 25 kDa N-terminal triple-membrane-spanning protein (p25) of unknown function. There is no evidence that intermolecular disulfide bonds link gp116 and gp64 in the virus particle. ORFX is an alternative reading frame in ORF3 encoding a putative small double-membrane-spanning protein (px). In GAV, the ORF4-encoded polypeptide may be expressed in infected cells at an extremely low level, but its function is unknown. During viral replication, long and filamentous nucleocapsid precursors are formed in the cytoplasm of infected cells and acquire envelopes by budding at membranes of the endoplasmic reticulum or other cellular membranes. Newly budded mature enveloped virions often appear as multimers arranged in paracrystalline arrays (end-to-end) of virions within vesicles.

Arteriviruses

Equine Arteritis Virus

Disease

EAV is the causative agent of equine viral arteritis (EVA) in horses, a respiratory and reproductive disease that occurs worldwide. The clinical signs displayed by EAV-infected horses depend on various factors, including the genetics, age, and physical condition of the horse(s), challenge dose and route of infection, strain of virus, and environmental conditions. The vast majority of EAV infections are inapparent (or subclinical). However, acutely infected animals may develop a wide range of clinical signs, including pyrexia, depression, anorexia, dependent edema (scrotum, ventral trunk, and limbs), stiffness of gait, conjunctivitis, lacrimation, and swelling around the eyes (periorbital and supraorbital edema), respiratory distress, urticaria, and leukopenia. The incubation period of 3–14 days (usually six to eight days following venereal exposure) is followed by pyrexia of up to 41 °C (105.8 °F) that may persist for two to nine days. The virus causes abortion in pregnant mares, and abortion rates during natural outbreaks of EVA can vary from 10% to 60% of infected mares. EAV-induced abortions can occur at any time between 3 and 10 months of gestation. EAV infection can cause severe fulminating interstitial pneumonia in neonatal foals and progressive pneumoenteric syndrome in older foals. A high proportion of acutely infected stallions (10–70%) become persistently infected and shed the virus in semen for a long time. The maintenance of the carrier state in stallions is testosterone dependent. There is no evidence of any analogous persistent infection of mares, geldings, or foals. EAV exclusively persists in the reproductive tract of carrier stallions (mainly the ampullae of the vas deferens), which continue to shed virus in semen.

Etiologic Agent

Antigenic Properties

There is only one known serotype of EAV; however, field strains differ in their virulence and neutralization phenotype. Phylogenetic analysis based on ORF5 segregates EAV strains into North American and European lineages, with the European lineage further subdivided into two subgroups. Recently, using 170 EAV ORF5 reference sequences from horses and donkeys, it was demonstrated that donkey isolates from Chile and South Africa belong to a single monophyletic group (i.e. asinine cluster). Thus, this cluster represents a new *Alphaarterivirus* equid species present in South America and South Africa. Clearly, these asinine strains of EAV circulating in donkeys are distinct from well-characterized EAV strains prevalent in horses.

Resistance to Physical and Chemical Agents

Arteriviruses are readily inactivated by lipid solvents (ether and chloroform), common disinfectants, and detergents (e.g., 0.01% NP-40 or Triton X-100). Arteriviruses are also highly heat labile, and their half-life progressively decreases with increasing temperature. EAV survives 75 days at 4 °C, two to three days at 37 °C, and 20–30 minutes at 56 °C. Tissue culture fluid or organ samples containing EAV can be stored at −70 °C for years without significant loss of infectivity.

Infectivity for Other Species and Culture Systems

EAV can infect horses, donkeys, mules, and zebras, and it replicates primarily in macrophages, CD3+ T cells, and endothelial cells, but it also replicates in selected epithelial, mesothelium, and smooth muscle cells of the tunica media of smaller arteries, venules, and the myometrium. Unlike other arteriviruses, EAV replicates in various primary cultures, including equine pulmonary artery endothelial cells, horse kidney, rabbit kidney, and hamster kidney cells. It also replicates in established continuous cell lines from numerous species including baby hamster kidney (BHK-21), rabbit kidney-13 (RK-13), African green monkey kidney (VERO), rhesus monkey kidney (LLC-MK2), hamster lung (HmLu), SV-40-transformed equine ovary, equine dermal, canine hepatitis virus-transformed hamster tumor cells (HS and HT-7), and MARC-145 cells (rhesus macaque kidney cell line).

Host–Virus Relationship

Distribution, Reservoir, and Transmission

EAV is distributed throughout the world, although the seroprevalence of EAV infection varies between countries and horses of different breeds and ages in the same region. In the United States, about 70–90% of adult Standardbred horses are seropositive for EAV, compared to only 1–3% of the Thoroughbred population. Similarly, a high percentage of European Warmblood horses are seropositive for EAV. Seroprevalence to EAV increases with age, indicating that horses may be repeatedly exposed to the virus as they age. Persistently infected carrier stallions function as the natural reservoir of EAV and disseminate the virus to susceptible mares at breeding (Figure 62.3). The two principal modes of EAV transmission are horizontal transmission by aerosolization of infectious respiratory tract secretions from acutely infected horses and venereal transmission during natural or artificial insemination with infective semen from persistently infected stallions. Recently, it has been demonstrated that the virus can also be transmitted during embryo transfer from mares inseminated with infective semen. EAV can also be transmitted through indirect contact with fomites or personnel. Congenital infection results from transplacental transmission (vertical transmission) when pregnant mares are infected late in gestation.

Pathogenesis and Pathology

Most information on the pathogenesis of EAV infection is derived from experimental studies in horses inoculated intranasally with various strains of EAV. It is to be stressed that with the notable exception of fetal and neonatal infections, EAV infection of horses is very seldom fatal. The numerous publications describing lesions caused by the highly virulent horse-adapted virulent Bucyrus strain of EAV reflect a severe fatal infection that is not representative of the disease caused by field strains of the virus. Most of the field strains of EAV cause only mild to moderate clinical disease in infected horses; however, some strains of EAV can cause severe disease. Initial multiplication of the virus occurs in the alveolar macrophages in the lung after respiratory infection. The virus soon appears in the regional lymph nodes, especially the bronchial nodes. Within three days, the virus is present in virtually all organs and tissues (viremia), replicating in macrophages and endothelial cells. The clinical manifestations of EVA reflect endothelial cell injury and increased vascular permeability. *In vitro* and *in vivo* studies have demonstrated increased transcription of genes encoding pro-inflammatory mediators –interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α – following EAV infection, suggesting that these cytokine mediators are critical in determining the outcome of infection and severity of the disease.

Following natural EAV infection, the majority of the stallions can become persistently infected and continuously shed virus in their semen for either a short (ranging from several weeks to <1 year, defined as short-term shedders [STS] or short-term carriers) or a long time (> 1 year to lifelong; defined as long-term persistently infected [LTPI] shedders or carriers) without adverse effects on semen quality or reproductive capacity. The virus persists exclusively in the male reproductive tract (i.e., primarily in the ampullae) despite high levels of neutralizing and mucosal antibodies. Recently, dual immunohistochemical and immunofluorescence techniques were employed to unequivocally demonstrate that the ampulla is the main EAV tissue reservoir rather than immunologically privileged tissues (i.e., testes). The study revealed that EAV has a specific tropism for stromal cells (fibrocytes and possibly tissue macrophages) and $CD8^+$ T and $CD21^+$ B lymphocytes but not glandular epithelium. Persistent EAV infection is associated with moderate, multifocal lymphoplasmacytic ampullitis comprising clusters of B ($CD21^+$) lymphocytes, and significant infiltration of T (CD3+, $CD4^+$, $CD8^+$, and $CD25^+$) lymphocytes, tissue macrophages, and dendritic cells, (Iba-1^+ and $CD83^+$), with a low number of tissue macrophages expressing CD163 and CD204 scavenger receptors.

Furthermore, it has been demonstrated that the establishment of EAV LTPI correlates with the *in vitro* susceptibility of a subpopulation of $CD3^+$ T lymphocytes to EAV infection, suggesting that stallions with the $CD3^+$ T lymphocyte susceptibility phenotype are at higher risk of becoming LTPI carriers compared to those that lack this

phenotype. In addition, a genome-wide association study (GWAS) demonstrated that these phenotypes are associated with the *CXCL16* gene located in equine chromosome 11 (ECA11). Subsequently, studies have identified two allelic variants of *CXCL16* (namely $CXCL16^S$ and $CXCL16^r$) that differ by four non-synonymous nucleotide substitutions in exon 2. They show a strong association with the two $CD3^+$ T lymphocyte phenotypes (susceptible and resistant) and with either the establishment of LTPI ($CXCL16^S$) or the early viral clearance (STS) in stallions ($CXCL16^r$). These new findings implicated CXCL16 and its receptor CXCR6 (CXCL16-CXCR6 chemokine axis) in the pathogenesis of EAV LTPI in the stallion. Moreover, it has also been demonstrated that EAV LTPI is associated with the downregulation of a seminal exosome (SE)-associated miRNA (eca-mir-128) and an enhanced expression of CXCL16 in the reproductive tract, a putative target of eca-mir-128. Taken together, these findings suggest that SE eca-mir-128 is implicated in the regulation of the CXCL16/CXCR6 axis in the reproductive tract of LTPI infected stallions. Thus, the role of CXCL16 and its receptor CXCR6 (CXCL16-CXCR6 chemokine axis) in the pathogenesis of persistent EAV infection is under further investigation.

The characteristic histological feature of EVA is a severe necrotizing panvasculitis of small vessels and vascular leakage. Affected muscular arteries show foci of intimal, subintimal, and medial necrosis, edema, and infiltration of lymphocytes and neutrophils. EAV infection of pregnant mares can result in the abortion of fetuses, which are usually partially autolyzed at the time of expulsion. Aborted fetuses may exhibit interlobular pulmonary edema, pleural and pericardial effusion, and petechial and ecchymotic hemorrhages on the serosal and mucosal surfaces of the small intestine. Most often, EAV-infected fetuses do not typically exhibit either macroscopic or microscopic lesions but contain large viral loads (with titers that can be 1×10^9 plaque-forming units/g of tissue [PFU/g]) in most tissues, including lung, spleen, thymus, liver, kidney, and placenta (i.e. allantochorion and amnion). In addition, neonatal foals occasionally develop a very severe, acute bronchointerstitial pneumonia or pneumoenteric syndrome.

Host Response to Infection

Animals that recover from EAV infection or those vaccinated with either inactivated or attenuated strains of EAV develop neutralizing antibodies and are resistant to subsequent challenges with EAV. Neutralizing antibodies are detected within one to two weeks following exposure to the virus, reach maximum titers from two to four months, and persist for three years or more. The serological response to EAV is primarily mediated by virus-specific IgM and IgG1. Except for persistently infected stallions, EAV is eliminated from the tissues of infected horses by 28 days after the exposure. Foals born to immune mares are protected against clinical EVA by passive transfer of neutralizing antibodies in their colostrum. Neutralizing antibodies appear a few hours after colostrum feeding, peak at one week of age, and gradually decline to extinction from two to seven months. The innate immune response to EAV infection is not fully characterized, but studies have shown that the virus inhibits type I interferon (IFN) production in infected cells. Recent studies have demonstrated that nsp1, nsp2, and nsp11 can inhibit type I IFN activity. Of these three nsps, nsp1 has the most potent inhibitory effect by inhibiting IFN synthesis. The failure to induce type I IFN in EAV-infected cells may allow the virus to subvert the equine innate immune response. Recently, it has been demonstrated that EAV elicits a mucosal antibody response in the reproductive tract during persistent infection with homing of plasma cells into accessory sex glands. EAV-specific immunoglobulin isotypes in seminal plasma included IgA, IgG1, IgG3/5, and IgG4/7. Interestingly, seminal plasma IgG1 and IgG4/7 possessed virus-neutralizing activity, whereas seminal plasma IgA and IgG3/5 did not. However, virus-neutralizing IgG1 and IgG4/7 in seminal plasma are not effective in preventing viral infectivity. There is very little work done on the equine cellular immune response to EAV infection. The specific viral protein targeted by the cytotoxic T lymphocyte response of EAV-infected horses remains to be thoroughly characterized.

Laboratory Diagnosis

It is to be stressed that EVA is subclinical or mild in horses and that other, more important, viral respiratory and reproductive infections clinically resemble EVA. Specifically, differential diagnosis of EVA should include the following viral respiratory tract infections: Equine herpesviruses 1 and 4, Equine influenza virus, Equine rhinitis A and B viruses, Equine adenovirus, and Getah virus. It may also include equine infectious anemia, African horse sickness, purpura hemorrhagica, urticaria, and toxicosis due to hoary alyssum (*Berteroa incana*).

The laboratory diagnosis of EVA is currently based on the following laboratory tests: virus isolation (VI); viral nucleic acid detection using standard reverse transcription-polymerase chain reaction (RT-PCR), real-time (RT-qPCR) or RT-insulated isothermal PCR (RT-iiPCR) or i*n situ* hybridization (ISH; conventional or RNAscope®); viral antigen detection in tissues by immunohistochemistry (IHC); and/or serological demonstration of rising neutralizing antibody titers (fourfold or greater) in paired serum samples taken at a

21- to 28-day interval using the virus neutralization test (VNT). The VNT is the principal serological assay used to detect evidence of EAV infection by most laboratories around the world, and it continues to be the current World Organization for Animal Health (OIE) prescribed standard test for EVA (gold standard). In addition, several laboratories have developed and evaluated enzyme-linked immunosorbent assays (ELISAs) to detect antibodies to EAV using a whole virus, synthetic peptides, or recombinant viral proteins (e.g., GP5, M, and N) as antigens. However, various studies have shown that the source of antigen and the sera evaluated can markedly influence the results obtained with EAV protein-specific ELISAs and competitive ELISA. Therefore, none of these ELISAs or recently described microsphere immunoassay (Luminex) has yet been shown to be of equivalent sensitivity and specificity to the VNT. Recently, a commercial competitive enzyme-linked immunosorbent assay (cELISA) was developed and validated to detect EAV-specific antibodies and could be considered an alternative.

Suitable clinical samples to attempt virus isolation or detection of viral nucleic acids include nasopharyngeal swabs or nasal washings, conjunctival swabs, whole blood (in EDTA or citrate), and tissues of aborted equine fetuses (lung, spleen, lymph nodes, and placenta). A semen sample may be submitted for virus isolation and viral nucleic acid detection in seropositive stallions with unknown vaccination history. Tissue samples from foals and aborted fetuses (lung, spleen, lymph nodes, and placenta) could also be submitted for histopathological and IHC/ISH evaluation in 10% neutral buffered formalin.

Carrier stallions are first identified by serological tests as they are always seropositive, and persistent infection is confirmed by VI from semen in cell culture, by test breeding using seronegative mares (and monitoring these for seroconversion to EAV after breeding), or by RT-PCR or real-time RT-PCR to identify viral nucleic acid in semen. Identification of the carrier stallion is of critical epidemiological importance in the prevention and control of EAV infection. VI is currently the OIE-approved gold standard for detecting EAV in semen and is the prescribed test for international trade. However, it has been demonstrated that at least one of the rRT-PCR assays described in the literature has equal to or higher sensitivity than VI for the detection of EAV nucleic acid in semen samples.

Treatment and Control

Treatment of EVA is general supportive care until the acute phase passes. Adequate rest should be provided, particularly to breeding stallions and to horses in training. The use of systemic antibiotics is unrewarding and usually contraindicated, except in pneumonitic foals at risk of secondary bacterial infection. The main goal for the treatment of EVA is to recover from disease and avoid virus spread. Currently, there are no means available for eliminating the carrier state in stallions persistently infected with EAV other than surgical castration. However, preliminary data supports that GnRH (gonadotropin-releasing hormone) vaccines or antagonists can temporarily limit the shedding of the virus in the semen of carrier stallions. Furthermore, peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) targeting the genomic 5′-terminus of EAV could cure persistently infected HeLa cells under *in vitro* conditions. The PPMO-cured HeLa cells were free of infectious virus, viral antigens, and EAV nucleic acid as measured by plaque assay, indirect immunofluorescence assay, and rRTPCR, respectively. Although these findings demonstrate that PPMOs can be used to eliminate persistent EAV infection in cell culture, the efficacy of PPMO against EAV *in vivo* remains to be addressed.

There are currently two commercial vaccines that are widely used for protective immunization of horses against EAV infection: (i) ARVAC® (Zoetis, Kalamazoo, Michigan, United States), a modified-live virus (MLV) vaccine available in the United States, and (ii) Artervac® (Zoetis, Kalamazoo, Michigan, United States), a killed virus vaccine available in the European Union. The MLV vaccine is administered intramuscularly to horses, but it is not recommended for use in pregnant mares, especially during the last two months of gestation or in foals less than six weeks of age. The MLV vaccine is safe and effective for breeding mares and stallions in early gestational pregnant mares, geldings, fillies, and colts. It is recommended that foals be vaccinated at six months of age. Colts should be vaccinated prior to the onset of puberty, as this prevents them from becoming persistently infected carriers. Thus, protective immunization of prepubertal colts is central to control the spread of EAV infection. Prepubertal colts could be genetically tested for the presence of the CXCL16 genotype ($CXCL16^{S/S}$ or $CXCL16^{S/r}$) using an allelic discrimination real-time PCR. Colts that carry the susceptible genotype ($CXCL16^{S}$) should be vaccinated after six months of age. If no genetic testing is to be performed, all colts should be vaccinated between 6 and 12 months. All seronegative mares being bred to a persistently infected stallion should be vaccinated 21 days before natural breeding or artificial insemination (AI). Those previously vaccinated should be given a booster immunization. Horses that travel for competitions or are co-mingled with outside horses should be vaccinated. Experimental EAV vaccines have also been developed

recently using recombinant DNA technology, but none have been released to the market.

Outbreaks of EVA can be prevented by identifying persistently infected stallions and the institution of biosecurity and management practices to prevent the introduction of EAV-infected horses. Carrier stallions should be kept physically isolated and bred only to seropositive mares from previous natural exposure or vaccination. In addition, mares should be kept isolated from other seronegative horses after being bred by carrier stallions.

Porcine Reproductive and Respiratory Syndrome Virus

Disease

Clinical signs of porcine reproductive and respiratory syndrome (PRRS) are extremely variable and influenced by strains of the virus, the immune status of the herd, and management practices. Low-virulence strains of PRRSV may result in widespread infection of swine with minimal occurrence of disease, whereas highly virulent strains can cause severe clinical disease in susceptible herds. All ages of pigs are susceptible to infection with PRRSV in immunologically naive herds. Acute infection follows exposure to PRRSV and virus replication in lung and lymphoid tissues. Viremia is usually detected 6–48 hours post infection, and viral load peaks by 4–14 days post infection (DPI). After peak viremia, virus titers in serum drop rapidly, and most pigs are no longer viremic by 21–28 DPI. The infection of swine with PRRSV-1 and PRRSV-2 results in reproductive failure in sows, respiratory illness in growing pigs, and is usually asymptomatic in boars. PRRSV infection can be divided into three stages: acute infection, persistence, and extinction. Acute PRRSV infection of susceptible pigs is characterized by anorexia, fever (39–41 °C [102.2–105.8] °F), dyspnea, and lethargy. Affected pigs are lymphopenic and exhibit transient cutaneous hyperemia or cyanosis of extremities most visible on the ears, snouts, mammary glands, and vulvas. Pigs infected with the highly virulent strain of PRRSV that emerged in China in 2006 develop a prolonged high fever (41–42 °C [105.8–107.6 °F]), severe respiratory signs, red discoloration of the body, and blue ears associated with increased mortality (20–100%), and morbidity (50–100%). Transplacental transmission of PRRSV occurs most efficiently in the third trimester of pregnancy (usually after 100 days of gestation), and the abortion rate in affected sows can range from 10% to 50%. Sow mortality is considerably lower. Affected litters contain a variable mixture of normal pigs, weak small pigs, stillborn pigs, and partially or completely mummified fetuses (so-called SMEDI; stillbirth, mummification, embryonic death, and infertility). Infected sows can also exhibit nervous signs such as ataxia and circling. PRRSV-infected boars may continue to shed viruses in their semen for an extended time (i.e. persistently infected boars). The extinction phase begins when the virus is cleared from lymphoid tissues and viral shedding ends, which may take as long as 251 days post exposure to the virus.

Etiologic Agent

Antigenic Properties

The European – PRRSV-1, prototype LV – and North American – PRRSV-2, prototype VR-2332 virus – isolates of PRRSV represent genetically and antigenically distinct species of the same virus. Both viruses are associated with outbreaks of similar reproductive and respiratory disease in pigs; however, there is only 55–70% nucleotide identity in the various genes of viruses of the two species. Phylogenetic analysis based on ORF5 sequences from GenBank has identified three subtypes of PRRSV-1 circulating in the world. Subtype I includes PRRSV strains from Western European countries, China, South Korea, Thailand, Czech Republic, Poland, Russia, and the United States. Subtype II includes PRRSV strains from Eastern European countries (Lithuania, Russia, and some strains from Belarus), and subtype III includes strains from only Belarus. Subtype I is further subdivided into 12 clades (A–L). Clade A includes the LV that was first isolated in the Netherlands in 1990 and other PRRSV strains from many Western European countries, and some strains from the United States and Thailand. PRRSV-1 has been introduced into five non-European countries in North America (the United States and Canada) and Asia (Thailand, China, and South Korea). PRRSV-2 circulates primarily in North America and Asia. Phylogenetic analysis of PRRSV-2 strains has identified nine lineages. Seven of the nine lineages contained PRRSV strains isolated mainly from North America, whereas two lineages (3 and 4) contained strains isolated mainly from Asia. Numerous Asian isolates of PRRSV were found within the seven North American lineages (1, 2, and 5–9), indicating the introduction of North American strains to Asian countries. PRRSV was first isolated in China in 1995, and the virus (identified as genotype 2) has evolved quickly. Subsequently, highly pathogenic-PRRSV (HP-PRRSV) variants emerged in 2006, causing outbreaks of a fatal disease. The whole-genome-based phylogenetic analysis of Chinese PRRSV strains and representative North American and European strains suggests that the newly emerged highly virulent virus has evolved from a Chinese

strain circulating in 1996 (PRRSV CH-1a). The full-length genome sequence analysis of the virus revealed two distinct deletions in the nonstructural protein 2 (nsp2) in comparison to all previously reported North American genotype PRRSVs. In the following years, this HP-PRRSV variant spread to all Chinese swine-producing areas and surrounding Asian countries. PRRSV-1 and PRRSV-2 have been shown to recombine under *in vitro* and natural conditions. Deletions, insertions, and recombination events have been the main driving forces of PRRSV evolution in nature. However, the increase in recombinant strains and high rates of recombination (e.g., recombination between field strains and MLV vaccine strains) in recent years has played a significant role in the evolution of PRRSV around the world.

New PRRSV-1 and PRRSV-2 variants are constantly emerging due to the high mutation rate of the virus and genetic recombination between field strains and MLV vaccines. These new variants precipitate new outbreaks of PRRS in endemic countries. The emergence of new variants has also made it extremely difficult to develop new vaccines to provide complete protective immunity against heterogeneous virus strains or variants circulating in various countries. Similarly, the emergence of new PRRSV-1 and PRRSV-2 variants has complicated the development of highly specific and sensitive laboratory diagnostic tests for PRRS.

Resistance to Physical and Chemical Agents

PRRSV infectivity is lost within one week at 4 °C but is stable for a long time (months to years) when frozen (−20 or −70 °C). PRRSV is rapidly inactivated by low and high pH (<6 and >7.5).

Infectivity for Other Species and Culture Systems

PRRSV primarily infects pigs, but recently, chickens and ducks exposed to PRRSV in drinking water shed the virus in their feces, suggesting that they are susceptible to infection. American PRRSV-2 isolates replicate in porcine alveolar macrophages (PAM), PSP-36 cells, African green monkey cell line (MA-104), and derivatives thereof (CL2621 or MARC-145). Most, if not all, European PRRSV-1 isolates replicate best or exclusively in PAMs. However, European PRRSV isolates have been adapted to grow in the CL2621 cell line. Vaccine strains of PRRSV replicate much more efficiently (100–1000 times) in derivatives of monkey kidney cell lines than in PAMs. Recently, a PAM cell line derived from the lung lavages of porcine fetuses (ZMAC; ATCC PTA-8764) has been developed, and this new cell line is highly susceptible to PRRSV infection. Furthermore, genetically modified cell lines expressing CD163 (i.e. one of the cellular receptors of the virus) are permissive to PRRSV infection.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

PRRSV appears to be endemic in virtually all swine-producing countries globally, and PRRS has now emerged as the most prevalent swine disease worldwide. The original source of the virus and the circumstances under which it was introduced into the domestic swine population are unknown. PRRSV has been demonstrated in samples collected from wild boars in Europe and North America by RT-PCR. Transmission of PRRSV usually occurs by close contact between infected and uninfected animals. Swine are susceptible to PRRSV by several routes of exposure, including oral, intranasal, vaginal, intramuscular, and intraperitoneal. PRRSV is shed in respiratory tract secretions, saliva, semen, mammary secretions, urine, and feces of infected animals. Susceptible pigs are naturally infected by inhaling infectious aerosols or ingestion of PRRSV-contaminated food (horizontal transmission). Congenital infection results from transplacental transmission (vertical transmission) of the virus. Transmission of PRRSV to females has been demonstrated during breeding with semen from persistently infected carrier boars. PRRSV can also be transmitted through indirect contact with fomites or personnel, and some pigs harbor the virus in their tonsils long after the virus is cleared from other tissues. There is evidence that flies and mosquitoes might serve as mechanical vectors of PRRSV. PRRSV could establish a chronic, persistent infection in lymph nodes (e.g., inguinal, mandibular and sternal), tonsils, and the male reproductive tract. The virus has been isolated from pigs' tonsils and lymph nodes for up to 157 DPI. Low-level replication of the virus occurs in the lymphoid tissues, and thus, the shedding of PRRSV in oropharyngeal secretions is considered long-lasting. PRRSV has been detected in semen samples of experimentally infected boars for variable periods from 4 to 92 DPI. The most extended period where RT-nested PCR has detected PRRSV nucleic acid in semen is up to 92 DPI. However, using VI and swine bioassay, it has only been possible to detect the virus in semen samples lasting from 4 to 42 DPI. The persistence stage begins when viremia ends and clinical signs subside. Similar to EAV, PRRSV shedding in semen occurs in the absence of viremia and in the presence of neutralizing antibodies in serum. The presence of PRRSV in the semen of infected boars could

be either due to virus replication and shedding from the reproductive tract (epididymis and bulbourethral glands) or direct dissemination via infected monocytes and macrophages from tissues other than those of the reproductive tract. Shedding of the virus in semen is of particular concern because of the widespread use of artificial insemination in swine breeding.

Pathogenesis and Pathology

PRRSV replicates in macrophages and dendritic cells, especially those in the lungs and lymphoid tissues, and to a lesser extent, other tissues. Viremia occurs soon after infection and can last for one to two weeks in mature animals and eight weeks in young pigs. In general, the duration of viremia is no longer than 28 DPI, but viral RNA has been detected in serum by RT-PCR up to 251 DPI. Gross lesions are usually observed in only a few organ systems (e.g., respiratory and lymphoid). Microscopic lesions of PRRS include diffuse interstitial pneumonia, perivascular cuffing in multiple organs, vasculitis, and encephalitis. Lymphoid tissues exhibit lymphoid hyperplasia and lymphocyte apoptosis. Clinical outbreaks of PRRSV can be complicated by bacterial pneumonia, septicemia, or enteritis.

The clinical outcome of PRRSV infection is highly variable, ranging from mild, subclinical infections to acute deaths of adult animals. The differences in clinical signs in infected pigs are attributed to numerous factors, including host genetics, management practices, and virulence phenotype of the infecting PRRSV strain. Several mechanisms cause the clinical signs and pathological lesions due to PRRSV. These include the apoptosis of PRRSV-infected macrophages and surrounding cells (indirect apoptosis), induction of pro-inflammatory and immunomodulatory cytokine production (IL-1, IL-6, IL-10, IL-12, TNF-α, and IFN-γ), polyclonal B cell activation and decrease in bacterial phagocytosis, and killing by macrophages leading to increase in bacterial respiratory disease and septicemia. Recently, it has been demonstrated that PRRSV infection modifies the porcine innate immune response and alters disease outcomes in pigs coinfected with other respiratory viral pathogens such as Porcine respiratory coronavirus. Variation in genetic resistance/susceptibility to PRRS has been reported among different breeds of pigs. The breeds that show more resistance to PRRS have increased cell-mediated immune response with the increased number of IFN-γ -secreting cells. Similarly, reduced or delayed PRRSV replication in some pig breeds was associated with a high level of TNF-α and IL-8 mRNA expression. In addition, specific swine major histocompatibility complex – swine leukocyte antigen class I, II, and III (SLA I, II, and III) – alleles influence the susceptibility/resistance to PRRS, and studies have shown that SLA class I and II alleles regulate the level of circulating virus and antiviral immune responses in pigs. Using the CRISPR/Cas9 technique, researchers have recently knocked out the CD163 gene or the region that interacts with the viral envelop proteins (i.e. SRCR5 domain) to generate pigs genetically resistant to PRRSV infection. Subsequent studies by the same and other investigators confirmed that the PRRSV-resistant knockout pigs have healthy performance characteristics similar to wild-type pigs.

Host Response to Infection

The humoral immune response to PRRSV varies significantly between pigs. Some reports suggest that protective immunity is strain specific, with some degree of heterologous protection against other strains of PRRSV. It has been shown that passive transfer of PRRSV antibodies fully protected pregnant sows against challenges with virulent PRRSV. Piglets born to immune sows acquire anti-PRRSV antibodies by ingestion of colostrum, and these maternal antibodies persist in piglets up to six to eight weeks of age. Pigs infected with PRRSV produce various virus-specific antibodies; virus-specific IgM appears five to seven days after infection and IgG by 7–14 days. ELISA antibody titers peak by five to six weeks after infection and persist thereafter. However, PRRSV-neutralizing antibodies appear slowly, usually between four and five weeks post infection, and do not peak until approximately 10 weeks after infection. The appearance of neutralizing antibodies is associated with the clearance of PRRSV from the lungs of infected pigs. Despite the significant role that neutralizing antibodies seem to play in protection, their effectiveness might be limited against heterologous PRRSV strains.

The T cell ($CD4^+$ and $CD8^+$)-mediated immune response is delayed in pigs infected with PRRSV, and IFN-γ -producing T cells appear between 4 and 12 weeks after infection. IL-12, one of the critical modulators of T_{H1}-type cellular response, is produced at a lower level following PRRSV infection and along with the increased levels of IL-10 may be capable of shifting the immune response toward a less effective T_{H2} response. How conserved the T cell epitopes are or whether they could provide cross-protection against different PRRSV strains is yet to be determined.

In recent years, the innate immune response to PRRSV infection has been extensively studied. These studies have shown that the virus attenuates the innate immune response by blocking IFN-α production in infected cells (e.g., macrophages) to evade the antiviral cytokine response. As a result of the diminished innate immune response, adaptive immune responses are compromised, leading to weak cell-mediated responses, the delayed appearance of

the neutralizing antibodies leading to prolonged viremia and persistent infection of the pigs. However, virus strain differences and cell-type differences regarding response to PRRSV-mediated type I IFN suppression have been reported. Interestingly, PRRSV has developed strategies to subvert the innate, humoral, and cell-mediated immunity responses of pigs, making the development of a vaccine(s) against PRRS extremely difficult (Table 62.4).

Table 62.4 Immune evasion mechanism of PRRSV.

Viral strategies	Mechanism of immune evasion	Viral proteins involved	Consequences
Evasion of innate immune response			
Downregulation of IFN-α production pathway and interference with IFN-α-signaling pathway	Block dsRNA-induced IRF-3 and IFN promoter activation	Nsp1	Decreased innate immune response
		Nsp1	Minimal IFN-α response in infected lung macrophages
	Inhibition of STAT1, STAT2, and ISG3 in IFN-signaling pathways	Nsp2	IFN suppression
		Nsp2	Delayed humoral and cell-mediated immune responses
	Inhibition of ISG15-dependent RIG-I and JAK1 pathways	Nsp11	IFN antagonist
	Interfering with NF-κB-signaling pathway		
	Inhibition of IRF3 phosphorylation and nuclear translocation		
Reduction in NK cell activity	Reduction in NK cell-mediated cytotoxic function	?	Failure to kill virus-infected cells and decreased IFN-γ production leading to decreased innate immune response
Interference with antigen presentation by dendritic cells and macrophages	Apoptosis of infected dendritic and macrophage cells, downregulation of CD11b/c, CD14, CD80/86, SLA class I, and SLA class II	?	Downregulation of antigen presentation and induction of IL-10 and downregulation of inflammatory cytokine production and T cells
Induction of IL-10 and suppression of IL-12	Direct targeting of IL-10 promoter by the Nucleocapsid protein	Nucleocapsid	Suppression of T cell responses leading to decreased IFN-γ production
Evasion of adaptive immune response			
Genetic and antigenic variation	Viral quasi-species due to high error rate of viral RdRp and lack of proofreading capability	GP3[a], GP4[a], and GP5[b]	Lack of cross-protection due to variation in neutralization epitopes (NEs)
		Nsp2, GP3, and GP5	
		Nsp2	Persistence of the virus
	Intragenic recombination	Nsp2, GP2, GP3, and GP5	Lack of conserved B cell epitopes
	Variation in other B cell epitopes		Lack of conserved T cell epitopes
	Variation of T cell epitopes		
Glycan shielding of neutralizing epitopes	Masking of neutralizing epitopes	GP5	Delayed neutralizing antibody response, decreased sensitivity of the virus to neutralization, and persistence of the virus
"Decoy" epitope	Masking of neutralizing epitopes	GP5	Delayed neutralizing antibody response and persistence of the virus
Interference with T cell response	Induction of regulatory T cells (Treg; CD4+ CD25+ Foxp3+)	?	Delayed T cell response

?, not known.
[a] Variation in NEs of PRRSV-1 strains only.
[b] Variation in NEs of PRRSV-1 and PRRSV-2 strains.

Laboratory Diagnosis

Diagnosis of PRRSV may be complicated as many infections are inapparent. However, PRRS should be considered when clinical signs of respiratory disease are associated with reproductive failure in a herd. PRRSV antibodies are detected using various serological assays, including ELISA, microsphere immunoassay (Luminex), immunofluorescence assay, immunoperoxidase monolayer, and serum virus neutralization assay. Serological examination of acute and convalescent pig sera may provide evidence of seroconversion. VI from clinical specimens such as bronchoalveolar lavage fluid, lung, lymph node, buffy coat, and serum can be done in PAM, ZMAC, MA104, and its derivatives and PSP-36 cell lines. However, different strains or isolates of PRRSV vary in their ability to replicate in different cell types. Various PCR assays (e.g., standard RT-PCR, RT-nested PCR, and real-time RT-qPCR) have been developed to detect viral nucleic acid in the blood, semen, tissue homogenates, pulmonary lavage fluid, oropharyngeal scrapings, oral fluid, and other clinical specimens. These assays are highly specific and sensitive and give a more rapid diagnostic turnaround time than VI in cell culture. Whereas VI amplifies infectious virus, PCR detects viral RNA in clinical specimens. There are currently several commercial real-time RT-qPCR assays for diagnosing PRRSV and discriminating between PRRSV-1 and PRRSV-2 strains.

The PRRSV strains circulating in swine farms were genetically characterized by ORF5 Sanger sequencing in diagnostic laboratories. However, recent advances in next-generation whole-genome sequencing have allowed direct RNA sequencing to reliably detect the PRRSV strain present in clinical specimens with high accuracy. Importantly, whole-genome sequencing has a more profound discriminatory power and provided more information when compared to classical ORF5 Sanger sequencing. In addition, NGS has allowed differentiation of multiple coinfecting strains present in clinical specimens. Overall, direct viral RNA sequencing followed by bioinformatic analysis has proved to be a promising approach for identifying the viral strain(s) involved in clinical infections, allowing for more precise prevention and control strategies during PRRSV outbreaks. Furthermore, NGS sequencing results could identify any recombination event between field strains of PRRSV and current MLV vaccines.

Treatment and Control

Both modified-live attenuated and killed vaccines are available to prevent PRRSV infection in pigs. They can be used to immunize sows or weanling piglets; however, there is considerable variation in the relative safety and efficacy of vaccines. MLV vaccines induce long-lasting protection compared to killed vaccines but do not completely prevent reinfection with wild-type virus and subsequent virus transmission. There is sufficient evidence to show that vaccine-derived viruses are being spread in farms and that homologous recombination between circulating field viruses with the vaccine virus is generating new genetic variants of PRRSV. Furthermore, there have been reports of under-attenuation and reversion of MLV vaccine strains to a more virulent type that can be spread from vaccinated to unvaccinated swine. The MLV vaccines reduce disease occurrence and severity and the duration of viremia and virus shedding. The killed vaccines are used for vaccinating sows and gilts to reduce reproductive losses caused by PRRSV. Current PRRSV vaccines do not allow discrimination between vaccinated and naturally infected animals. Therefore, several subunit vaccines and genetically modified-live marker vaccines have been developed to differentiate vaccinated animals from naturally infected pigs. One approach has been to use reverse genetic systems to engineer chimeric infectious cDNA clones of virulent and attenuated strains of the virus to establish attenuated MLV (marker) vaccines of PRRSV. However, the use of these subunit and recombinant vaccines in the field has been debated between vaccine developers, swine practitioners, and animal health authorities.

Although many commercial MLV and killed vaccines are available against PRRSV, there have been new PRRS outbreaks and a steady increase in PRRS incidence in the United States and around the world. These MLV vaccines provide protective immunity for PRRS virus strains that are highly homologous but limited protection against genetically divergent strains, which are constantly undergoing a high rate of mutation. Thus, generation of PRRS vaccines with a high level of genetic homology to a given field virus is difficult in a short time: fully licensed vaccines, based on either the MLV or killed format of currently circulating PRRSV strains, require several years of development followed by a few more years of regulatory testing to be approved for sale by the USDA. Thus, by the time a vaccine is approved and released for commercial use, its genome and composition have become antiquated due to the genetic diversification of the myriad of field virus variants whose genomes have substantially evolved over time by mutation and recombination events. In addition, the use of these commercial MLV vaccines presents some risks, including the shedding of vaccine virus that infects non-vaccinated pigs, persistent viral infection, and the potential of reversion of the attenuated vaccine to its original wild-type virulent phenotype. Because of these limitations, a rational approach to solving the problem of the high

antigenic diversity exhibited by PRRSV in the field makes the development of effective vaccines against PRRSV complicated. Thus, to overcome these problems, the swine industry aimed at developing effective autogenous (killed virus) vaccines for PRRSV. Autogenous biologicals are custom inactivated vaccines consisting of herd-specific antigens derived from a pathogen (i.e. PRRSV) isolated from the same herd that will be used as an immunogen. Autogenous vaccines for PRRSV are prepared using a virus strain(s) isolated from the animal(s) of an infected herd and used in the herd of origin. Thus, the viral antigens contained in the vaccine will perfectly match the viral antigens expressed by the virus that is causing the disease. As a result, small commercial companies could quickly develop these vaccines to be used in PRRS outbreaks. Most importantly, the USDA Center for Veterinary Biologics (CVB) approves the use of autogenous products when commercially licensed products are not available or when commercially licensed products have not provided adequate protection, as is the case with PRRSV vaccines. However, using an autogenous vaccine against PRRSV has advantages and disadvantages, and these need to be considered before implementing such vaccination programs in affected herds.

There are no specific treatments for PRRS in pigs. However, a PPMO targeting a highly conserved sequence in the 5′-terminal region of the PRRSV genome that inhibits virus replication in culture has been used as a candidate for experimental treatment of young pigs. Prevention and control programs have been developed to eradicate the virus from infected herds. The objective of PRRSV prevention programs is to stop the introduction of either the virus into negative herds or new strains into PRRSV-infected herds. Implementation of strict biosecurity and herd closure measures whereby the herd is closed to new introductions for a period during which the resident virus dies out has effectively controlled PRRSV infection from sow herds.

Lactate Dehydrogenase Elevating Virus

The murine arterivirus LDV was first isolated from tumor-bearing laboratory mice with elevated plasma lactate dehydrogenase (LDH) enzyme activity but was later found to be endogenous in house mouse populations (*Mus musculus domesticus*) in several countries, although the worldwide incidence is not known. LDV causes lifelong asymptomatic, persistent infections in mice that can only be recognized by elevated levels of plasma LDH. Attempts to infect *Peromyscus* mice, rats, guinea pigs, and rabbits with LDV have been unsuccessful. LDV replicates in permissive macrophages in the spleen, lymph nodes, thymus, and liver of infected mice. The subpopulation of macrophages that are permissive to LDV infection is also responsible for the normal clearance of the LDH enzyme from circulation. The continuous destruction of these macrophages by LDV leads to elevated levels of LDH in blood, hence the name of the virus. The persistent infection that characterizes LDV infection of mice is maintained by replicating LDV in new permissive macrophages that are continuously regenerated from apparently nonpermissive precursor cells. Other than the elevated LDH level and subtle changes in host immunity, persistent LDV infection of mice is generally asymptomatic, and infection of laboratory mice is now very uncommon.

Anti-LDV antibodies begin to appear four to five DPI and peak at three to four weeks. Antibodies that neutralize LDV appear in mice only after four weeks post infection, and these antibodies are directed against the GP5 protein of the virus. The replication of LDV in macrophages allows it to avoid host defense mechanisms, although the precise mechanism of immune evasion and persistent infection is still unclear. It has been shown that most virus stocks contain both neuropathogenic (LDV-C and LDV-v) and non-neuropathogenic (LDV-P and LDV-vx) variants. LDV-P and LDV-vx variants establish persistent infection in mice by resisting neutralization. Neuropathogenic variants are strongly suppressed in immunocompetent mice because of their sensitivity to neutralization. At the same time, the non-neuropathogenic, neutralizing antibody-resistant variants predominate in persistently infected mice. The infection of mice of certain strains (C58, AKR, PL/J, and C3H/FgBoy) by neurovirulent strains of LDV can lead to fatal age-dependent poliomyelitis (ADPM). ADPM develops only in aged mice that become spontaneously immunosuppressed at 6–12 months of age or in experimentally immunosuppressed younger mice.

LDV transmission between mice is relatively inefficient, despite the lifelong viremia in infected mice and the secretion of the virus in urine, feces, and saliva. Feces contain high titers of the virus, and thus coprophagic behavior of mice probably plays an important role in the transmission of the virus. LDV transmission from mother to offspring through the placenta or via breast milk is highly efficient if the mother is immunologically naive. However, transmission via these routes from persistently infected mothers is rare because anti-LDV antibodies block transplacental transmission of the virus and its release into milk. In general, horizontal transmission of LDV between mice is restricted by mucosal barriers, although horizontal transmission of LDV occurs among laboratory mice fighting and biting one another. Sexual transmission of LDV among mice has not been demonstrated.

LDV can only be quantified by an end-point dilution assay in mice based on the increase in plasma LDH activity that accompanies LDV infection. The presence of LDV in mice or other materials is readily detected by injecting plasma, tissue homogenates, or other materials (e.g., transplantable mouse tumors) into groups of two to three mice and assaying their plasma LDH activity four to five days later. LDV also can be detected by IFA and *in situ* hybridization with cDNA probes. Transplantable mouse tumors can be readily freed of LDV by a three-week *in vitro* propagation or passage in a host animal other than mice. LDV is not stable at −20 °C and rapidly loses its infectivity.

Simian Hemorrhagic Fever Virus

SHFV was first isolated from rhesus macaques suffering from hemorrhagic fever in primate research centers in the Soviet Union and the United States. African monkeys of three genera – African green monkey (*Cercopithecus aethiops*), patas (*Erythrocebus patas*), and baboon (*Papio anubis*) – are persistently infected in the wild population with low-level viremia, and they do not exhibit any clinical signs of disease. Accidental transmission of SHFV from African monkeys to any of three species of Asian macaque monkeys (*Macaca mulatta*, *Macaca arctoides*, and *Macaca fascicularis*) results in a generally fatal hemorrhagic fever. In macaques, clinical signs of simian hemorrhagic fever (SHF) include anorexia, fever, cyanosis, skin petechia, and hemorrhages, nosebleeds, facial edema, bloody diarrhea, dehydration, adipsia, and proteinuria. Death generally occurs 5–25 days after the onset of clinical signs, and mortality approaches 100% in macaques. The high lethality observed in macaque monkeys may be due to the extreme sensitivity of their macrophages to cytocidal infection by SHFV. Infection of captive patas monkeys with SHFV from persistently infected patas monkeys results in persistent infection without any clinical signs. However, infection of captive patas monkeys with SHFV from diseased macaque monkeys results in transient mild disease, indicating the selection of more virulent variants during epizootics in macaque monkeys. The humoral immune response against SHFV varies with the species of monkeys and the infecting virus strain. More virulent strains of SHFV induce neutralizing antibodies in patas monkeys seven days after experimental infection. However, only low levels of anti-SHFV antibodies are found in many persistently infected patas monkeys. Neutralizing antibodies against one strain of SHFV do not completely neutralize other strains, indicating variation in the neutralization determinants of individual strains of SHFV.

Between 1964 and 1996, there had been at least 15 more SHF outbreaks in research facilities, most of which had connections to Indian suppliers of nonhuman primates. Some of the monkeys in these outbreaks showed central nervous system signs, which were not seen in the earlier outbreaks. This observation suggested that different strains of SHFV may have caused the various outbreaks (e.g., simian hemorrhagic encephalitis virus [SHEV]). Until recently, only three distinct arteriviruses are known etiologic agents of past SHF epizootics in Asian macaques colonies, but only one, SHFV strain, has been isolated in cell culture. These include SHFV, SHEV, and Pebjah virus (PBJV). However, recent advances in next-generation sequencing have allowed scientists to identify at least nine additional distant relatives of these three viruses in apparently healthy African nonhuman primates.

The prevalence and incidence of SHFV infection among African monkeys in endemic areas of Africa are unknown; however, persistent subclinical infections in wild patas monkeys appear to be high. The method of transmission of SHFV among African monkeys in the wild is unclear. Infection most likely occurs through wounds and biting, but sexual transmission has not been ruled out. SHFV is not transmitted transplacentally from persistently infected mothers to their offspring. Several epizootics of SHF in captive macaque colonies have originated from the accidental mechanical transmission of SHFV from asymptomatic, persistently infected African monkeys. Once illness becomes apparent in the macaque colony, the SHFV spreads rapidly throughout the colony, most likely by direct contact and aerosols. There are no vaccines to prevent SHFV infection in monkeys.

Persistently infected monkeys can be identified by the presence of SHFV that replicates in primary cultures of peritoneal macrophages from rhesus and patas monkeys. The most sensitive method for detecting persistent infection in monkeys is experimental inoculation of macaque monkeys, and there are currently no molecular diagnostic assays for detecting SHFV. Indirect immunofluorescent assay, ELISA, and neutralization assays are available for serological diagnosis of SHFV infection. However, these assays are unreliable because of the low level of anti-SHFV antibodies found in many persistently infected monkeys. Accidental transmission of SHFV from persistently infected African monkeys to macaque monkeys in primate centers can be minimized by strict adherence to proper sanitary conditions and animal care practices.

Wobbly Possum Disease Virus

A novel, fatal neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*) was first identified in 1995 in a research facility and subsequently in free-living

possums in New Zealand and termed wobbly possum disease (WPD). Later, it was shown that it is caused by the Wobbly possum disease virus (WPDV). Based on the genomic sequence analysis, WPDV was closely related to the family *Arteriviridae*. Under experimental conditions, the disease was reproduced following inoculation of healthy possums with blood, urine, tissue homogenates, and homogenized mites derived from WPD-affected possums. These were administered through multiple routes. Transmission of WPDV was also observed in possums in close contact with infected possums. Recently, antibodies to WPDV have been demonstrated in the Australian common brushtail, mountain brushtail (*Trichosurus cunninghami*), and common ringtail (*Pseudocheirus peregrinus*) possums using ELISA.

WPD in New Zealand brushtail possums is clinically characterized by early behavioral changes followed by progressive cachexia and neurological signs such as intentional tremors, ataxia, difficulties climbing, and occasionally presumed blindness. A similar clinical syndrome has been described in common brushtail possums in Tasmania, and another syndrome, characterized predominantly by blindness, has been observed in mainland Australia.

The hallmark histological lesion in WPDV-affected possums in New Zealand is the presence of variable size infiltrates of mononuclear inflammatory cells in multiple tissues, including the liver, spleen, kidneys, choroids, and brain. However, in mainland Australia, where blindness is predominantly observed, the pathology described in possums presumably affected by WPD is a non-suppurative inflammation primarily limited to the brain, choroid, and optic tract.

Roniviruses

Yellow-Head Virus and Gill-Associated Virus

YHV was first detected in 1990 in black tiger shrimp (*Penaeus monodon*) farmed in Central Thailand. Since then, YHV has spread into Southeast Asia and the Indo-Pacific region. In 1993, a virus morphologically identical to YHV was detected in wild and farmed *P. monodon* in Australia and was given the name Lymphoid organ virus (LOV) based on the lesions identified in the lymphoid organs. Subsequently, in 1995 and 1996, an apparent pathogenic form of this virus was detected in high levels in the gills of moribund, farmed *P. monodon* displaying YHD-like histopathological lesions and named GAV. It is now evident that LOV and GAV correspond to the same virus and cause the mild and/or chronic form of infection in *P. monodon*; GAV has become the accepted name for both of these viruses. Recently, phylogenetic analysis based on a 661 nucleotide region of ORF1b for 21 viruses identified eight genotypes in the YHV complex. These genotypes include 1 (subtypes 1a and 1b; YHV), 2 (GAV), 3 (YHV-3), 4 (YHV-4), 5 (YHV5), 6 (YHV-6), 7 (YHV-7), and 8 (YHV-8). These genotypes vary in geographic distribution. Viruses assigned to genotypes 1, 2, 3, and 5 have been detected in shrimp from various Southeast Asia and China locations. There is evidence of naturally occurring genetic recombination involving genotypes 1, 2, 3, and 5. Genotype 4 has been detected only in India, genotype 6 only in East Africa, genotype 7 only in Australia, and genotype 8 only in China. The geographic range of roniviruses mirrors that of their primary penaeid hosts, extending from eastern Africa across the Indo-Pacific to South Asia, Australia, and some islands in the South Pacific, and northward throughout Southeast Asia to East Asia. YHV-8 has been reported to infect Chinese white shrimp (*Penaeus chinensis*). YHV has also been detected in cultured blue shrimp (*Penaeus stylirostris*) from Mexico.

YHV is a major pathogen of farmed *P. monodon* shrimp and is listed by the OIE as a notifiable disease in shrimp. YHV and GAV are transmitted horizontally via several routes, including exposure to the free waterborne virus during cohabitation and cannibalism of infected carcasses. There is no direct evidence of transmission of YHV vertically, but there is substantial evidence of vertical transmission of GAV in the wild and farmed *P. monodon* on the East Coast of Australia. Under experimental conditions, YHV appears to be more virulent than GAV and causes 100% mortality within three to four days of infection in *P. monodon*. In contrast, with GAV, the mortality occurs 7–14 DPI. Similarly, farm outbreaks are presented as a chronic disease involving the progressive appearance of relatively low numbers of moribund shrimp. Shrimp with YHD show a pale or bleached appearance of the body with yellowish discoloration of the cephalothorax due to yellowing of the hepatopancreas (HP). The HP of infected shrimp is swollen and soft compared to that of normal shrimp. The tissue distribution and histopathology seen in YHD infections are very similar and include necrosis of gill lamellae, HP, lymphoid organ, and heart. Histologically, GAV differs from YHV in that the lesions are limited to the gills and the lymphoid organ. Moreover, intensely basophilic inclusions found in the lymphoid organ of YHV-infected shrimp are not as evident in GAV-infected shrimps.

The diagnosis of GAV and YHV is achieved by routine hematoxylin and eosin histopathological examination and transmission electron microscopy (TEM). The diagnosis can be further confirmed by *in situ* hybridization with virus-specific probe and immunohistochemical

staining with polyclonal rabbit antiserum or monoclonal antibodies (Mabs) to either of these viruses. A nitrocellulose enzyme assay using rabbit polyclonal antibodies and a Western immunoblotting assay using Mabs have been described to detect YHV in gill tissues and hemolymph samples of *P. monodon*. Recently, several RT-PCR, RT-nested PCR, real-time RT-PCR, insulated isothermal RT-PCR (iiRT-PCR), and loop-mediated isothermal amplification (LAMP) assays have been developed to detect GAV and YHV in samples. The Western immunoblotting assay is highly specific and is recommended by the OIE as a confirmatory diagnostic test for YHV infection in combination with *in situ* nucleic acid hybridization, TEM, and RT-PCR. The control of YHV and GAV in *P. monodon* is mainly achieved by adapting various management strategies, including virus exclusion or prevention through the use of specific pathogen-free and/or specific pathogen-resistant shrimp breeding stocks.

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63

Reoviridae

Barbara S. Drolet, Bethany L. McGregor, Lee W. Cohnstaedt, William C. Wilson, and D. Scott McVey

Viruses in the Reoviridae family infect a wide range of species, including mammals, fish and shellfish, insects, plants, protists, and fungi. All of the viruses in this family have nonenveloped, double capsid, icosahedral structure (Figure 63.1) and a segmented genome of double-stranded RNA (dsRNA). The number of individual genome segments varies between genera. The family is grouped into 15 genera: *Orthoreovirus*, *Orbivirus*, *Rotavirus*, *Aquareovirus*, *Coltivirus*, *Cardoreovirus*, *Mimoreovirus,* *Mycoreovirus*, *Seadornavirus*, *Oryzavirus*, *Cypovirus*, *Dinovernavirus*, *Idnoreovirus*, *Phytoreovirus*, and *Fijivirus*. The mycoreoviruses infect fungi. The latter six genera are confined to insects, plants, or both. The *Coltivirus* genus includes Colorado tick fever virus, which is a tick-transmitted human pathogen. Orthoreoviruses and rotaviruses infect a wide variety of vertebrate species. The host range of orbiviruses includes both vertebrates and invertebrates, with bluetongue virus serving as the prototype. The aquareovirus host range includes fish and shellfish, with golden shiner virus serving as the prototype. Table 63.1 includes a list of genera and species of veterinary importance.

Orthoreoviruses

Mammalian Orthoreoviruses

Disease

Mammalian orthoreoviruses (genus *Orthoreovirus*) have been isolated from the respiratory and/or gastrointestinal tract of many animal species, including nonhuman primates, rodents, horses, cattle, sheep, swine, cats, and dogs. Orthoreoviruses are usually isolated from healthy animals and are designated "respiratory enteric orphan" viruses because they are typically not associated with any disease. However, orthoreoviruses are sometimes isolated from animals with mild respiratory and/or enteric disease, and orthoreovirus infection of infant (neonatal) mice can cause severe systemic disease. Experimental infection of kittens with orthoreovirus serotype 3 has caused conjunctivitis, photophobia, gingivitis, serous lacrimation, and nasal discharge. All three serotypes of orthoreovirus have been isolated from sheep, and experimental infections with serotype 1 have been reported to cause enteritis and pneumonia.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

There are three serotypes (1, 2, and 3) and many strains of mammalian orthoreoviruses. They all possess a genome of 10 distinct segments of dsRNA. The genome segments are of different sizes (grouped as large, medium, and small). Each encodes a single protein except the S1 gene, which includes two distinct open reading frames. The complete orthoreovirus particle has no envelope and exhibits icosahedral morphology with a diameter of approximately 85 nm. The orthoreovirus particle consists of eight structural proteins arranged into inner and outer protein capsids (coats). The inner protein core contains the viral RNA-dependent RNA polymerase (RdRP; transcriptase), as well as other enzymes that mediate mRNA synthesis and capping, helicase activity, and other functions that are necessary for virus replication. The predominant outer coat protein, sigma 1, is the primary determinant of virus serotype and hemagglutination and is also the cell attachment protein. Enzymatic digestion of the outer capsid protein sigma 3 from intact orthoreovirus particles generates infectious subviral particles, and removal of the outer capsid proteins – sigma 1, sigma 3, and mu 1 – generates core particles. All three particles are important in the lifecycle of orthoreovirus replication. Genetic diversity of strains of orthoreovirus occurs through accumulation of

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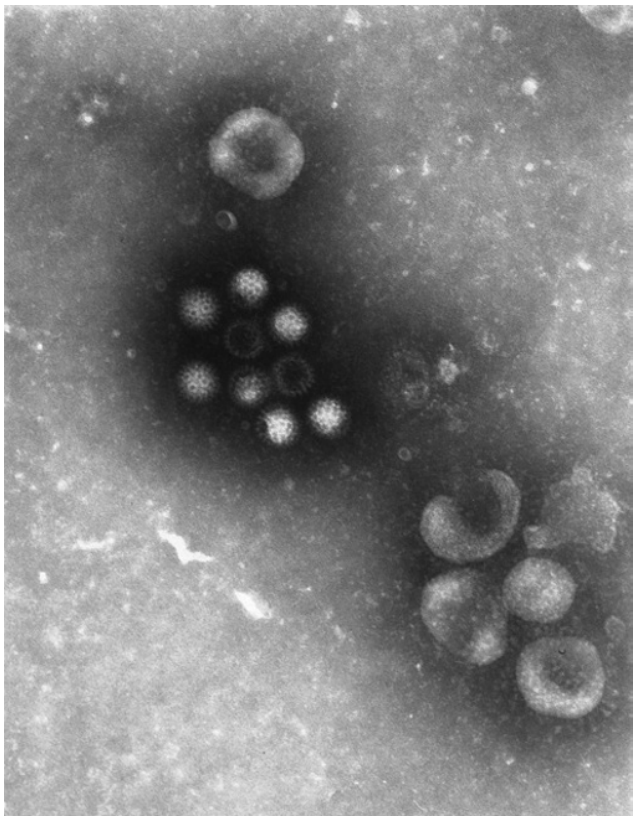


Figure 63.1 Negatively stained preparations of porcine rotavirus, with coronaviruses on the lower right side of the figure. *Source:* Courtesy of Dr. Richard Hesse, Kansas State University.

Table 63.1 Genera within the Reoviridae that include viruses relevant to veterinary medicine.

Genus	Serogroup	Minimum number of serotypes
Orthoreovirus	Mammalian	3
	Avian	11
Orbivirus	Bluetongue	29 (possibly 34)
	Epizootic hemorrhagic disease	8 (possibly 9)
	African horse sickness	9
	Equine encephalosis	7
	Palyam	11
Rotavirus	Five major groups	Uncertain
Aquareovirus	Not designated	Uncertain

mutations within individual viral genes due to the poor fidelity of the RdRP (genetic drift), and by the exchange of entire gene segments (reassortment) between viruses during coinfections with more than one orthoreovirus strain or serotype. Strains of reovirus that vary in virulence have been identified by sequence analysis of individual viral genes and proteins. Orthoreoviruses are stable at low temperatures (4 °C to room temperature) and are resistant to high temperatures (55 °C) for short periods of time. Orthoreoviruses are also resistant to detergents, many disinfectants, and are stable over a wide pH range (pH 2 to 9). They are inactivated by exposure to 95% ethanol and 10% household bleach.

Distribution, Reservoir, and Transmission

Mammalian orthoreoviruses have a wide geographic distribution and are commonly present in river water, untreated sewage, and stagnant water, likely reflecting fecal contamination by infected animals and/or humans. The mode of transmission is by direct contact or exposure to materials contaminated by virus-infected feces (oro-fecal) and/or respiratory discharge. Orthoreoviruses infect most mammals, and they replicate in a variety of cell cultures.

Pathogenesis and Pathology

In murine studies, orthoreoviruses infected either intestinal or respiratory epithelial cells after oro-fecal enteric or aerosol respiratory infection, respectively. Initial virus replication occurs in regional lymphoid tissues after infection of either the gastrointestinal (Peyer's patches) or respiratory (bronchus-associated lymphoid tissues) tracts. The virus sometimes gains entry to the systemic circulation in infected neonatal mice, leading to pancreatitis, myocarditis, myositis, encephalomyelitis, or hepatitis, with the specific disease process and pathogenesis reflecting the properties of the individual infecting strain of orthoreovirus as well as the age and resistance of the infected mouse. Orthoreoviruses can cause encephalitis and hepatitis in nonhuman primates.

Host Response to Infection

Both respiratory and enteric infection of mice with mammalian orthoreoviruses results in induction of humoral and cellular immune responses, natural killer (NK) cells, as well as interferons (IFN) and other cytokines, all of which are potentially important in clearing infection.

Laboratory Diagnosis

Orthoreovirus infections can be diagnosed by virus isolation or detection, and by serology. Virus can be isolated from tissues and from rectal, nasal, and throat swabs by cell culture techniques, although blind passage may be required before cytopathic effect (CPE) becomes visible. Virus isolates can be serotyped by either hemagglutination inhibition (HI) or virus neutralization (VN) testing with serotype-specific antisera. Orthoreoviruses can be identified in tissues or cell culture by immunofluorescent antibody staining (IFA) or immunohistochemistry (IHC).

Serological testing is done using paired sera and VN or enzyme-linked immunosorbent assay (ELISA).

Treatment and Control

As *Orthoreovirus* infection in mammals is usually mild, treatment is not required. No vaccines or control measures have been described and are unlikely to be developed in the future unless they are determined to be of greater importance as animal pathogens.

Avian Orthoreoviruses

Disease

Avian orthoreoviruses (genus *Orthoreovirus*) are of economic significance to the poultry industry. Systemic orthoreovirus infections of poultry can cause a variety of clinical syndromes, including gastroenteritis, hepatitis, myocarditis and pericarditis, pneumonia, and ill-thrift. Acute orthoreovirus infections also lead to increased mortality and carcass condemnations in affected flocks, as well as poor growth and food conversion efficiency (stunting syndrome). Arthritis and tenosynovitis are common in birds that survive acute infection. Orthoreoviruses are a major cause of avian arthritis, principally in broiler chickens and less often in layer birds and turkeys.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

The avian orthoreoviruses largely resemble their mammalian counterparts but differ in that they produce cell fusion (syncytia) in avian cell culture, lack hemagglutinating activity, and are typically unable to grow in mammalian cell lines. Avian and mammalian orthoreoviruses exhibit varying degrees of antigenic relatedness. At least 11 serotypes of avian orthoreovirus have been described, and strains within these serotypes vary significantly in their virulence. All share common antigens as determined by agar gel immunodiffusion (AGID) and complement fixation (CF) assays.

Infectivity for Other Species and Culture Systems

Avian orthoreoviruses replicate in embryonated chicken eggs (ECE), primary avian cell cultures, and, once adapted, certain established mammalian cell lines.

Distribution, Reservoir, and Transmission

Avian orthoreoviruses are prevalent worldwide in chickens, turkeys, and other avian species. They persist in nature through environmental contamination and by continued transmission of the virus from persistently infected birds. Transmission occurs both horizontally and vertically. Horizontal transmission is predominantly by the oro-fecal route and occurs through both direct and indirect contact. Vertical transmission from hen to offspring has been demonstrated following oral, tracheal, and nasal inoculation of breeder chickens.

Pathogenesis and Pathology

Infection with avian orthoreoviruses is usually inapparent. Occurrence of disease is dependent on the age of the bird at infection (young birds are predisposed), the virulence of the infecting virus strain, and the route of exposure. Orthoreovirus-induced arthritis is initially characterized by acute inflammation within affected joints that progresses to pannus formation with erosion of articular cartilage. Thus, orthoreovirus-induced arthritis in chickens somewhat mimics human rheumatoid arthritis. Gross lesions in affected chickens often also include extensive swelling of the digital flexor and metatarsal extensor tendons that can lead to chronic hardening and fusion of the tendon sheaths.

Host Response to Infection

Antibody responses to avian orthoreoviruses have been demonstrated by AGID, CF, and VN tests. The mechanisms responsible for protective immunity are poorly defined, and variable degrees of cross-strain protection have been reported.

Laboratory Diagnosis

Orthoreovirus-induced avian arthritis must be differentiated from arthritis and synovitis caused by other viruses and bacteria. A definitive diagnosis requires demonstration of orthoreovirus infection by direct IFA staining of tissues (tendon sheaths), virus isolation, or serology.

Treatment and Control

There is no treatment for avian viral arthritis. Due to the environmental stability and both vertical and horizontal transmission, eradication from commercial flocks is difficult. Infection is best controlled by proper management procedures and vaccination with available attenuated or killed vaccines.

Orbiviruses

Orbiviruses are important pathogens of livestock. Fourteen serogroups have been described, and five are of real or potential veterinary significance: (i) Bluetongue virus (BTV), (ii) Epizootic hemorrhagic disease virus (EHDV), (iii) African horse sickness virus (AHSV), (iv) Palyam virus, and (v) Equine encephalosis virus (EEV). Like mammalian orthoreoviruses, orbiviruses have a nonenveloped,

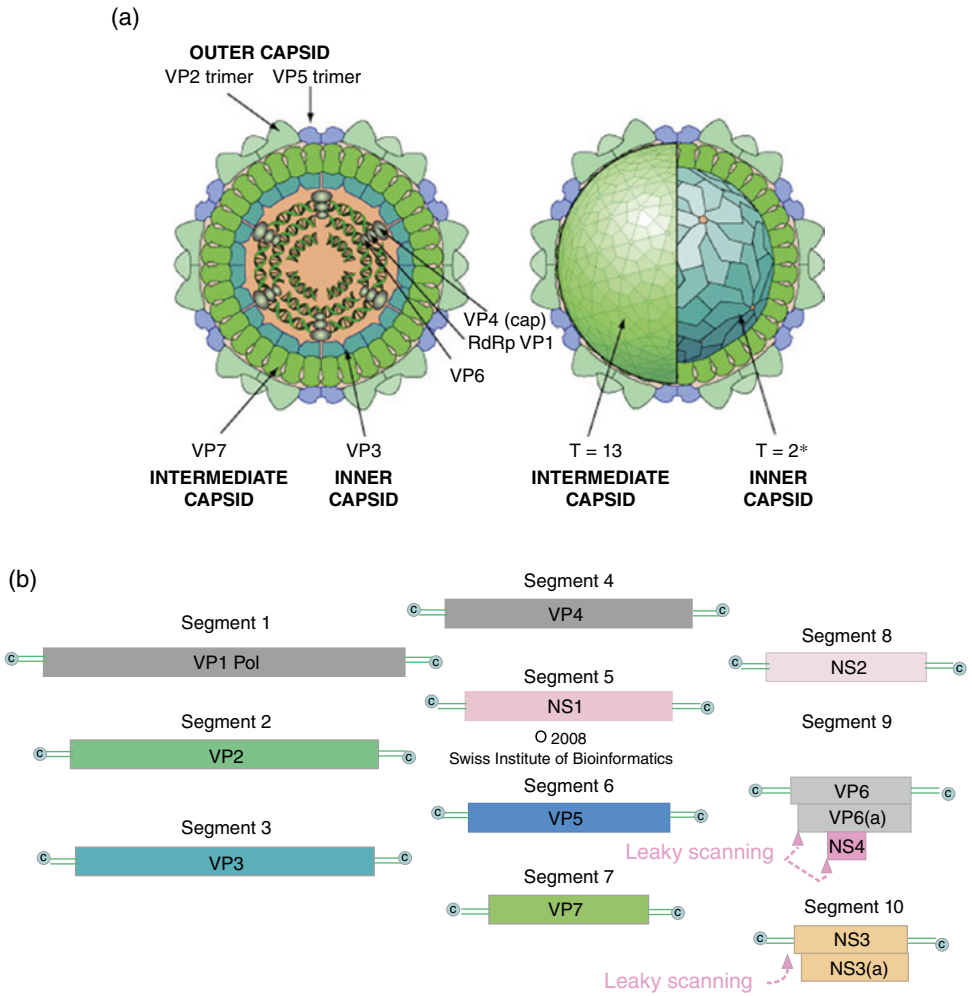


Figure 63.2 Orbivirus (a) capsid structure, and (b) double-stranded RNA segmented genome. *Source:* Copyright @ Swiss Institute of Bioinformatics 2008 with permission.

double capsid icosahedral structure, possess a segmented dsRNA genome (10 segments) (Figure 63.2) encoding 12 proteins (Table 63.2), and replicate in the cytoplasm of infected cells. Orbiviruses are distinguished from orthoreoviruses and rotaviruses in that they replicate in both insects and mammals, and they are not enteric pathogens of vertebrates. Orbiviruses replicate within the digestive tract of the hematophagous (blood feeding) insects that transmit these viruses between susceptible mammalian hosts.

Bluetongue Virus

Disease

Bluetongue (BT) is an arthropod-transmitted virus disease of domestic and wild ruminant species caused by BT virus (BTV). BT occurs most commonly in sheep (soremuzzle, catarrhal fever) and certain species of wildlife, particularly white-tailed deer. BT in sheep and deer is characterized by congestion, hemorrhage, and ulceration of the mucous membranes of the mouth, nose, and upper gastrointestinal tract (Figure 63.3). Other characteristic lesions include hyperemia of the coronary band and necrosis of both cardiac and skeletal muscles. BTV infection of sheep and deer is sometimes fatal, with terminal occurrence of disseminated intravascular coagulation. Sheep that survive severe bouts of BT are frequently emaciated, weak, and lame, and have a protracted convalescence during which they are susceptible to secondary infections. Breaks may occur in the wool fiber of convalescent sheep. Cattle are commonly infected with BTV in endemic areas, but clinical disease is uncommon for most serotypes. Vaccine strains of BTV, and those propagated in cell culture, can cross the placenta of pregnant sheep and cattle to infect the developing fetus, leading to fetal death, abortion, stillbirths, or teratogenic defects.

The major economic impact of BT is that it is a reportable disease by almost all regulatory authorities, along with

Table 63.2 Molecular constituents of orbiviruses.

Gene	Protein	Role
1	VP1	RNA polymerase; minor component of viral core particle
2	VP2	Receptor binding; serotype determination; component of outer capsid
3	VP3	Interacts with genomic RNA; structural component of viral core particle
4	VP4	RNA capping enzymes; minor component viral core particle
5	VP5	Structural interactions with VP2; component of outer capsid
6	NS1	Virus tubules; not a virion component
7	VP7	Group antigen; structural component of viral core particle
8	NS2	Binds RNA; virus inclusion bodies; nonstructural
9	VP6	Helicase; binds RNA; minor component of viral core particle
10	NS3/3A	Virus egress from infected cells; nonstructural
	NS4	Virus egress from infected cells; nonstructural



Figure 63.3 Sheep with bluetongue disease demonstrating (a) depression, salivation, and dyspnea; (b) death with typical cyanosis of the tongue; (c) hyperemia of the coronary band; and (d) ulceration and hemorrhage from the nasal cavity mucosa. *Source:* Courtesy of the USDA, ARS, Arthropod-Borne Animal Diseases Research Unit, Manhattan, KS, and Dr. Timothy Graham.

foot-and-mouth disease and at least 14 other diseases that are considered to have major adverse economic and societal ramifications. As a consequence, the international movement of ruminants and their germplasm from BTV-endemic countries is regulated and controlled. BTV recently was recognized as a potential pathogen of carnivores. Inadvertent infection of pregnant dogs with a BTV-contaminated vaccine caused abortion and death. Serological evidence of BTV infection has also been demonstrated in African carnivores.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

The 80–85 nm diameter, nonenveloped, icosahedral, double capsid BTV particle is composed of seven structural proteins, two of which form the outer coat and five the inner core. The outer capsid protein, VP2, contains the serotype-specific epitopes recognized by neutralizing and HI antibodies (Figure 63.2). The other outer coat protein, VP5, contributes to the conformation of the neutralizing epitopes on VP2. Four nonstructural (NS) proteins also occur in BTV-infected cells, with NS-1 forming cytoplasmic microtubular structures that are characteristic of orbivirus-infected cells. The core protein VP7 contains epitopes that are common to all serotypes and strains of BTV, which is the basis of group-specific serological assays such as AGID and competitive ELISA (cELISA).

There is considerable heterogeneity within the BTV serogroup, with over 30 distinct serotypes, each with potentially distinct biological properties. As with the orthoreoviruses, this genetic diversity has arisen as a consequence of both genetic drift and the reassortment of gene segments (genetic shift) during mixed infections of either insect or ruminant hosts with more than one BTV serotype or strain. Interestingly, recent sequence and phylogenetic analyses have shown that prolonged co-evolution of BTV with the different species of vector insects that occur in various regions of the world has resulted in strains of BTV that are unique to each region, so-called virus "topotypes."

Infectivity for Other Species and Culture Systems

BTV commonly infects domestic (sheep, cattle, and goats) and wild (deer, antelope, big horn sheep, etc.) ruminants. The viruses can be adapted to grow in suckling mice, ECEs, and a variety of mammalian and insect cell cultures. Replication of BTV is facilitated by incubation temperatures of 37 °C in mammalian cultures, 33.5 °C in ECEs, and 27–30 °C in insect cultures.

Distribution, Reservoir, and Transmission

Bluetongue virus has been isolated from ruminants in all continents except Antarctica. Infection occurs throughout tropical, subtropical, and temperate regions of the world, coincident with the distribution of susceptible ruminants and competent vector insects (*Culicoides* spp.). With the exception of some recently isolated atypical serotypes in goats, the virus is not contagious between ruminants; rather, infection occurs only following the bites of BTV-infected *Culicoides* insects. Subclinical and/or asymptomatic BTV infection of ruminants occurs throughout endemic regions of the world. Outbreaks of BT occur only sporadically, most often at the northern and southern extremities of the virus's range following incursions of BTV into immunologically naive populations of ruminants.

BT is transmitted by *Culicoides* biting midges. *Culicoides* become persistently infected with BTV after feeding on a viremic ruminant (Figure 63.4). These hematophagous insects are true biological vectors of BTV. They obtain the virus from blood while feeding on an infected animal and then transmit it to other ruminants after an extrinsic incubation period of approximately five days. During this period, the virus infects and disseminates from the midgut of the insect to its salivary glands. Replication of BTV within the insect vector is dependent on the ambient temperature; thus, increased replication of BTV in vector insects occurs at higher temperatures, but the increased temperature can shorten the lifespan of these insects. Transmission of BTV can occur year-round in climates that permit *Culicoides* activity in all seasons, with the virus persisting in a perpetual vector–ruminant cycle of infection.



Figure 63.4 Midge acquiring a blood meal. *Source:* Photograph courtesy of the USDA ARS Arthropod-Borne Animal Diseases Research Unit, Manhattan, KS.

In contrast, transmission of BTV is highly seasonal in regions of the world at the northern and southern extremities of the virus's range (approximately latitudes of 35°S and 45°N). In these areas, BTV transmission typically occurs only in the late summer and fall when vector populations peak and when ambient temperatures are highest (likely reflecting the influence of temperature-dependent virogenesis). The traditional global range of BTV has recently expanded into Mediterranean Europe after the northern spread of competent vectors, perhaps because of global warming.

With over 1400 identified *Culicoides* species worldwide, not all are competent vectors of BTV. Vector species vary by geographical location and dictate which viral serotypes circulate in any geographic region depending on their susceptibility to infection and ability to transmit each serotype. The most important confirmed vectors of BTV include *Culicoides sonorensis* (North America), *Culicoides insignis* (North America, South America), *Culicoides obsoletus* (Europe), *Culicoides imicola* (Africa, Asia, Europe), *Culicoides brevitarsis* (Australia, Asia), *Culicoides wadai* (Australia, Asia), and *Culicoides fulvus* (Australia, Asia). Studies to confirm vector competence are challenging in this insect group, and it is highly likely that additional competent *Culicoides* vectors exist around the world.

Pathogenesis and Pathology

Sheep and some species of deer are very susceptible to most BTV serotypes, whereas cattle infections are typically subclinical. The pathogenesis of BTV infection appears to be similar in all ruminant species. The virus multiplies initially in the lymph node(s) draining the site of infection, and viremia occurs as early as three days later with a subsequent febrile response. Upon systemic distribution, virus replicates in mononuclear phagocytic cells and the endothelium of small blood vessels, resulting in vascular injury, thrombosis, and infarction of the affected tissues; these include the upper gastrointestinal and respiratory tracts, the coronary bands, the heart, and skeletal muscle. Disseminated intravascular coagulation likely contributes to the vascular injury, hemorrhage, and tissue infarction that are characteristic of severe cases of BT in sheep and deer.

Viremia in BTV-infected ruminants is highly cell associated. Virus is initially associated with all blood cell types, and the titers of virus in each cell fraction reflect the proportion of each cell type in blood; thus, BTV is initially most associated with platelets and erythrocytes, and less so with leukocytes. However, late in the course of infection, virus appears to be exclusively associated with erythrocytes. It is this association that facilitates both prolonged infection and viremia of ruminants as well as infection of the hematophagous *Culicoides* insect vectors that feed upon them. BTV infection of ruminants can persist for up to approximately 60 days; however, with the exception of one unconfirmed report, persistent BTV infection of ruminants does not occur.

The gross lesions of BT include facial edema and hyperemia, with or without hemorrhage (petechial and ecchymotic) of the oral and nasal mucosa, skin, and coronary band. Ulcerations and erosions also may occur in and around the mouth, especially on the hard palate. Hemorrhages at the base of the pulmonary artery are very characteristic of severe cases of BT. Petechial hemorrhages may also occur in the myocardium, pericardium, skeletal musculature, and the tissues of the upper gastrointestinal tract.

Host Response to Infection

BTV-infected ruminants develop both humoral and cellular immune responses. Virus-neutralizing (serotype-specific) and non-neutralizing (group-specific) antibodies develop 7–14 days after infection. However, virus can coexist in blood with high titers of neutralizing antibody for several weeks because of the intimate association of BTV with the cell membrane of infected erythrocytes. Limited degrees of cross-serotype viral neutralization may occur following infection of an animal with a single BTV serotype, and subsequent exposures to additional serotypes can generate production of a set of broadly cross-reactive neutralizing antibody. However, immunization of sheep and cattle provides only homologous, serotype-specific protection.

Laboratory Diagnosis

Initial diagnosis of BT in sheep and deer is based on the characteristic clinical signs of affected animals in known BTV-endemic areas. BT typically occurs in the late summer and fall. Confirmation of the diagnosis requires virologic testing, usually by either polymerase chain reaction (PCR) assay or by virus isolation using inoculation of susceptible ECEs, suckling mice, or cell cultures. The cell cultures most often used include Vero and BHK and most recently *Culicoides* cell cultures. Multiple blind passages are often required before CPE is observed in mammalian cells and, although highly susceptible and therefore useful for viral amplification from low-titer diagnostic samples, no CPE is observed in *Culicoides* cells. Upon adaptation to cell culture, virus can be identified by IFA staining or VN assays. BTV is commonly detected in the blood of healthy ruminants in endemic areas, especially if sensitive nested PCR assays are used as these can detect BTV nucleic acid for up to 200 or more days after infection. Thus, the mere demonstration of BTV or BTV nucleic acid in the blood of ruminants certainly is not proof of disease

causality. Serological diagnosis can be performed using tests for group-specific (AGID, CF, ELISA, IFA) or type-specific (VN, HI) antibodies. Paired serum samples are required to demonstrate seroconversion or an increase in titer. A single serological test is often meaningless because a high proportion of ruminants are seropositive in BTV-endemic areas, and the vast majority of these animals never experience overt clinical disease following BTV infection.

Treatment and Control

Treatment of clinically ill animals is guided by disease severity, and treatment of severe cases involves replacement fluid therapy to treat dehydration and acidosis, minimization of environmental stress, and treatment of secondary infections. Transmission risk of BTV to unaffected ruminants can be reduced by moving animals indoors where insect vectors are not present, but that is often not a feasible option and can be hazardous to animals in regions with high environmental temperatures. Prophylactic vaccination of sheep with attenuated strains of BTV has been practiced for many years in endemic areas of South Africa and North America. The only vaccine approved for use in the United States is an attenuated modified-live vaccine for serotype 10. The South African vaccine incorporates five different serotypes in three doses for a total of 15 serotypes, starting with the most attenuated and ending with the least attenuated. Potential disadvantages of live attenuated vaccines include reversion to virulence and their ability to be transmitted in nature. Furthermore, live attenuated vaccines potentially can reassort their genome segments with field strains of BTV to create novel variant viruses. Finally, live attenuated vaccine strains of BTV repeatedly have been shown to be able to cross the placenta to cause fetal death or injury, whereas field strains of the virus typically cannot. The exception to this was the recent BTV serotype 8 outbreak in Europe where vertical transmission in cattle was observed. Recombinant baculovirus-expressed virus-like particles recently have been used as a BTV vaccine, avoiding the problems inherent to live attenuated vaccines. Control measures also include destruction of insect vector habitats and the use of insecticides/repellents.

Epizootic Hemorrhagic Disease and Ibaraki Viruses

Disease

Epizootic hemorrhagic disease (EHD) is an arthropod-transmitted orbiviral disease primarily of wild ruminants caused by EHD virus (EHDV). EHDV is an especially important cause of disease and mortality in white-tailed deer in North America and, to a lesser extent, pronghorn antelope and bighorn sheep. Although EHDV infection of domestic ruminant livestock is common in endemic areas, it is not regarded as a significant pathogen. A notable exception is a 2012 outbreak of EHDV serotype 2 (EHDV-2) in cattle in Nebraska and South Dakota, and Ibaraki disease of cattle in Japan and Korea. The causative agent, Ibaraki virus, is closely related to EHDV-2. EHDV closely resembles BTV in terms of its epidemiology, including dissemination by *Culicoides* insect vectors, pathogenesis of infection in ruminants, replication strategy, molecular structure, and methods of diagnosis.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

The nonenveloped, icosahedral, double capsid viral particle (80–85 nm diameter) and proteins of EHDV and Ibaraki viruses are similar to BTV, encoded by a 10-segmented dsRNA genome (Figure 63.2). As with BTV, there is considerable heterogeneity within the EHDV serogroup due to genetic drift and genetic shift, with eight distinct serotypes, each with potentially distinct biological properties. There currently are seven recognized serotypes of EHDV (1, 2, 4, 5, 6, 7, and 8) – eight if Ibaraki virus is classified as an EHDV serotype 7, as an has recently been proposed.

Infectivity for Other Species and Culture Systems

The *Orbivirus* EHDV commonly infects wild and captive cervids, especially white-tailed deer, but can cause mild or subclinical disease in cattle. Unlike BTV, EHDV is not a pathogen of sheep. Viruses can be adapted to grow in mammalian and insect cell cultures. Cultures of pulmonary endothelial cells are exquisitely sensitive to infection.

Distribution, Reservoir, and Transmission

Like BTV, EHDV infection occurs throughout tropical and temperate regions of the world, and EHDV infection of ruminants has been described in Africa, Asia, and the Americas. With the exception of Ibaraki virus, vaccines are not widely available for EHDV. The virus is not contagious between animals and is only transmitted via the bite of an infected *Culicoides* vector. Thus, disease prevalence is coincident with the geographic distribution of competent *Culicoides* vectors. Subclinical and/or asymptomatic infection occurs throughout endemic regions, providing viral reservoirs for feeding midges, which become transmission competent after a five-day extrinsic incubation period. As with BTV, transmission can occur year-round in climates with *Culicoides* activity in all seasons but is restricted to late summer and fall vector seasons in extreme northern and southern latitudes of the virus's range (35°S and 45°N). Many of the same *Culicoides* species that have been implicated as vectors of BTV are also considered likely vectors of EHDV. However, documentation of vector competence is

known only for a few species. *C. sonorensis* is a confirmed EHDV vector in North America, and field isolations have been made from *C. brevitarsis* in Australia and midges in the *C. schultzei* group in Africa.

Pathogenesis and Pathology

EHD clinical disease shares many features with BT. EHD in white-tailed deer closely resembles fulminant BT with hyperemia, hemorrhage and ulceration of the upper gastrointestinal tract, necrosis of cardiac and skeletal muscle, and terminal disseminated intravascular coagulation with widespread bleeding (Figure 63.5). Additional features of Ibaraki disease in cattle include marked dysphagia as a consequence of necrosis of muscles of the larynx, pharynx, esophagus, and tongue.

Host Response to Infection

As with BTV, both humoral and cellular immune responses develop in response to infection with serotype-specific and group-specific antibodies 7–14 days post infection. The association of EHDV with erythrocytes results in persisting viremia in the presence of circulating antibody and facilitates uptake of virus by blood-feeding *Culicoides* vectors.

Laboratory Diagnosis

Initial diagnosis of EHD in deer is based on the characteristic clinical signs of affected animals in endemic areas. Antigen detection diagnostics include virus isolation in susceptible cell cultures (Vero, BHK, *Culicoides*) with IFA or VN confirmation, or by serogroup- or serotype-specific PCR assays. As EHDV nucleic acid can persist in the blood of ruminants, positive PCR results are not proof of disease causality. As for BTV, serological diagnosis can be performed using tests for group-specific (AGID, CF, ELISA, IFA) or type-specific (VN, HI) antibodies. In endemic regions, a single serological test is often meaningless because a high proportion of deer are seropositive. Detection of antibodies in cattle is common in endemic areas, but the vast majority of these animals never experience overt clinical disease.

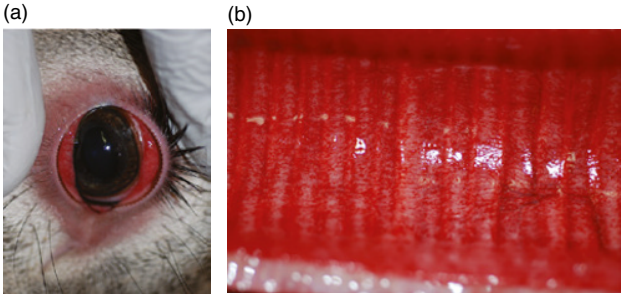


Figure 63.5 Enzootic hemorrhagic disease. (a) Photograph of a white-tailed deer that died nine days after experimental infection with EHDV showing edema and congestion of the conjunctiva. (b) Photograph of a white-tailed deer that died seven days after experimental infection with EHDV showing severe congestion and hemorrhage of the tracheal mucosa. *Source:* Courtesy of Dr. Mark Ruder of the University of Georgia and the USDA ARS Arthropod-Borne Animal Diseases Research Unit, Manhattan, KS. Reprinted with permission, Ruder (2012).

Treatment and Control

There is no specific treatment for EHD. Treatment of severe cases involves replacement fluid therapy to treat dehydration and acidosis, minimization of environmental stress, and treatment of secondary infections. Recent advances have been made for EHDV vaccines based on baculovirus-expressed VP2 subunits and DNA-based reverse genetics, but these are not yet commercially available. Both of these vaccine platforms avoid problems inherent to live attenuated vaccines, as discussed above for BT. Vector control measures include destruction of insect habitats and the use of insecticides/repellents.

African Horse Sickness Virus

Disease

African horse sickness (AHS) is a highly infectious, *Culicoides*-transmitted orbiviral disease of equids, including horses, mules, and donkeys. The causative agent, AHS virus (AHSV), is zoonotic, although fatal infection of humans is rare. Fatal AHSV infection of dogs also has been described. AHS of horses varies greatly in severity, depending on the infecting strain of virus and the susceptibility of the infected horse. There are several distinct forms of AHS, including (i) a peripheral form characterized by edema of the head; (ii) a central form characterized by pulmonary edema, high fever, severe depression, coughing, discharge of fluid from the nostrils, and rapid death of many affected horses; (iii) an intermediate form characterized by fever, edema of the head and subcutis (supraorbital edema is highly characteristic), and significant mortality of affected horses; and (iv) a horse sickness fever form characterized by a low-grade fever and congested mucous membranes.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

Similar to BTV and EHDV, the orbivirus AHSV is a nonenveloped, icosahedral, double capsid particle (55–70 nm diameter) with seven structural proteins and four nonstructural proteins encoded by a 10-segmented dsRNA genome (Figure 63.2). Nine serotypes of AHSV have been identified by cross-neutralization studies in mice. All types share common group-specific antigens.

Infectivity for Other Species and Culture Systems

AHSV infections have been documented in horses, donkeys, elephants, camels, mules, zebras, goats, dogs, and large African carnivores such as lions. Clinical disease has been described in horses and dogs. The virus can be propagated in suckling mice and adapted to grow in ECEs and cell cultures (Vero and BHK).

Distribution, Reservoir, and Transmission

AHS occurs throughout southern Africa, with periodic epizootics in northern Africa, the Middle East, Asia (the Indian subcontinent), and, on occasion in the past, Mediterranean Europe (the Iberian Peninsula). Like BT and EHD, AHS is transmitted by *Culicoides* insects with outbreaks occurring most commonly in the late summer and fall in endemic areas. The distribution of AHSV is dependent on the presence of competent insect vectors and ambient temperatures that facilitate temperature-dependent virogenesis within these vectors. Currently, *C. imicola* and *C. bolitinos* are the only confirmed vectors of AHSV, though other competent vectors throughout Africa and worldwide likely exist. It is believed that *C. imicola* may also have played a part in the 2020 AHS outbreak that occurred in Thailand, although it is unclear if other species also played a role. Zebras have been implicated as potential mammalian reservoirs of AHSV in southern Africa, because infection is asymptomatic, and viremia is more prolonged in zebras than in horses. Dogs may become infected with AHSV by eating infected horse meat, and serological surveys have shown that antibodies to AHSV are common among large wild carnivores in southern Africa. Renewed interest in AHSV as a threat to US equids has resulted from the recent emergence of the virus in Malaysia, a region from which many race and sport horse importations occur.

Pathogenesis and Pathology

The incubation period of AHS is generally less than seven days following the bite of an AHSV-infected *Culicoides* insect. The incubation period is shortest in susceptible horses infected with highly virulent strains of AHSV, and mortality can reach 95%. AHSV replicates in mononuclear cells of the lymph nodes, spleen, thymus, and pharyngeal mucosa, and in the vascular endothelium. The lesions of AHS result from vascular injury to small blood vessels, although it is uncertain whether the vascular injury that characterizes AHS is a result of only direct virus-mediated endothelial injury, or if vasoactive mediators released from AHSV-infected mononuclear phagocytic cells also contribute. The severe central form of AHS is characterized by pulmonary edema, hydrothorax, and hydropericardium, with epicardial and endocardial hemorrhages. Subcutaneous edema can be extensive in horses that suffer a more protracted form of the disease.

Host Response to Infection

All serotypes of AHSV share common serogroup antigens and induce development of antibodies that may be recognized by CF, AGID, and indirect IFA. Viral-neutralizing antibodies also develop following infection. These are predominantly serotype specific, but some cross-neutralization activity has been observed.

Laboratory Diagnosis

Presumptive diagnosis is made based on clinical signs, lesions seen during necropsy, and the presence of competent insect vectors. Field diagnosis of AHS, especially in nonendemic areas, should be supported by viral isolation or serology to distinguish it from other infections that can produce similar clinical signs. Laboratory confirmation of viral antigen can be done by virus isolation, PCR, and IFA of infected cultures or tissues. The presence of virus also can be rapidly identified in tissue specimens using an AHSV-specific capture ELISA. Intracerebral inoculation of suckling mice with blood or tissue suspension is a very sensitive method of isolating AHSV, although time-consuming serial passage may be required for viral adaptation. Cell cultures are not as efficient in isolating virus as mouse or horse inoculations. Serological diagnosis requires paired serum samples for demonstrating seroconversion or an increase in antibody titer. Assays routinely used for such purposes include competitive ELISA and CF, which are group-specific tests and detect antibodies to AHSV regardless of the infecting virus serotype, and VN assay, which is very sensitive but serotype specific.

Treatment and Control

No specific treatment for AHS is available. Hyperimmune horse serum confers transient protection. Minimizing exposure to *Culicoides* biting midges can decrease the risk of transmission but is not always logistically feasible and can cause animal welfare concerns in high-temperature environments. Both polyvalent and monovalent attenuated vaccines are available for AHS, as well as a monovalent inactivated vaccine. Annual vaccination of horses with the polyvalent attenuated virus vaccines that include the nine recognized serotypes of AHSV is widely practiced in southern Africa, and to control incursions of AHSV into Europe. There are several potential problems inherent to use of multivalent modified-live, or attenuated AHSV vaccines, including lack of protection against all serotypes of the virus, reversion to virulence of vaccine strains of virus, acquisition and dissemination of vaccine viruses by vector

insects, and reassortment of gene segments between different virus strains/serotypes during mixed infections. Control measures also include destruction of insect vector habitats and the use of insecticides/repellents.

Equine Encephalosis Virus

Equine encephalosis virus EEV was first described in South Africa in the 1800s but has since been detected in Ghana, Ethiopia, Gambia, and Israel. The encephalosis name is misleading as the disease is not typically neurological. It has been isolated from horses with hepatic lipidosis and vague neurological signs, but its importance as a primary pathogen is uncertain. The virus has also been isolated from aborted fetuses. Typically, infections result in mild clinical disease, but severe cases can include a short period of fluctuating fever and inappetence. Nasal congestion can result in elevated heart and respiratory rates. A red-brown discoloration of the mucous membranes can be seen. Severe clinical disease is rare but can include facial swelling, respiratory distress, and petechial hemorrhages of the conjunctivae. Sero-prevalence studies have shown that EEV infection is very widespread and common among horses in southern Africa. As with the other members of the genus *Orbivirus*, the EEV nonenveloped particle contains a 10-segmented dsRNA genome encoding seven structural and four nonstructural proteins (Figure 63.2). There are seven recognized serotypes of EEV that share common group antigens and closely resemble AHSV. The epidemiology of EEV infection is also like that of AHSV, with transmission by *Culicoides* insects, primarily *C. imicola*, but no direct transmission between animals. The majority of animals recover without sequelae. Anti-inflammatories or appetite stimulants may be administered, and antibiotics prescribed to treat or prevent secondary bacterial infections. With no vaccines available, control measures typically rely on insect habitat mitigation and reducing midge feeding when feasible with housing, repellents, and fans.

Palyam Virus

Palyam viruses, as with the other members of the genus *Orbivirus*, have a nonenveloped, icosahedral, double capsid structure with a 10-segmented dsRNA genome that encodes seven structural and four nonstructural proteins (Figure 63.2). To date there are 11 known serotypes. Palyam viruses are disseminated by *Culicoides* insect vectors, although the breadth of vector species is not well known. Infection with Palyam virus causes abortion and teratogenesis among cattle in Africa, Asia, and Australia. Fetuses that survive infection prior to mid-gestation may develop brain malformations, including hydranencephaly and/or false porencephaly. The pathogenesis and teratogenic effects of one serotype, Chuzan (Kasaba) virus which infects cattle and yaks, has been well described. First reported in Japan as the causative agent of disease that resulted in abortion, stillbirth, and congenital malformation in cattle, it is now widespread in many countries in Asia and has most recently been confirmed in yaks in China. No vaccines are available. Control measures rely on reducing midge populations with habitat mitigation and insecticides.

Culicoides Biting Midge Orbivirus Vectors

Ecology and Biology

The aquatic-terrestrial *Culicoides* biting midge life cycle progresses from egg, to larva, to pupa, to adult; although the egg, larval, and pupal stages are overall poorly understood. Larvae are typically semi-aquatic and are often found at the soil–water interface of diverse water bodies which can include streams, ponds, springs, marshes, roadside ditches, puddles, and animal hoofprints. Some species are found in more novel larval habitats, such as *C. obsoletus* (Europe), which is found extensively in manure piles on farms; *C. paraensis* (South America) which is commonly found in piles of discarded cacao hulls and banana stalks; and several species that use naturally occurring holes in trees. Larval development ranges in length from less than a week to several weeks, depending on factors such as species, ambient temperatures, and habitat conditions. Some species are also believed to overwinter as diapausing larvae for several months. The pupal stage is typically much shorter, often lasting just days, although it can extend to several weeks depending on conditions.

Most adult biting midges are crepuscular to nocturnal in behavior, flying to find mates, feed, and oviposit primarily in evening and overnight hours. Only females feed on blood in order to develop eggs. Although males may be attracted to hosts for purposes of finding females to mate with, they do not blood-feed. Both males and females also feed on nectar sources for additional energy. Although midges do not typically disperse long distances for host-seeking or oviposition (at most 2–3 km), there is evidence that they can be carried passively on wind currents for thousands of kilometers. This has been proposed as a mechanism of orbiviral disease introduction into novel areas. Most adults likely have a short lifespan of two to three weeks, though some midges can survive for months as adults, taking numerous blood meals.

Biting Midge Vector Management

In the absence of effective vaccines to prevent orbiviral diseases, vector control using an integrated pest management (IPM) approach is the most sustainable method of reducing pathogen transmission. An IPM approach uses a

combination of environmental, cultural, biological, genetic, and chemical tools to manage pest populations below an injurious or economic threshold.

Unfortunately, environmental management or larval habitat removal has been ineffective at reducing *C. sonorensis* populations in California and the *C. avaritia* complex in Europe. Furthermore, there are no chemical treatments currently registered for larval habitats. Cultural control or stabling of animals daily during peak midge biting times (usually the crepuscular hours) if feasible may reduce biting midge contact, but it has not consistently reduced transmission during viral outbreaks transmitted by *C. imicola*. Currently there are no biological (e.g., natural predators or parasites) or genetic (e.g., sterile insect technique) tools available, but the basic research has been initiated to create Wolbachia-infected midges and RNAi tools. This leaves chemical control or pesticide application as the last and most common method of reducing contact between vector insect species and host animals. On-animal treatments (e.g., ear tags and pour-ons) and feed-through treatments (e.g., novaluron and ivermectin) do not act fast enough to protect animals from being bitten, although they may kill the biting midge after it has fed, reducing further transmission and impacting population densities. Aerial pesticide application or space spraying, when applied correctly and timed when the biting midges are flying, has been shown to be effective at temporarily suppressing biting midge numbers and at stopping viral transmission during outbreaks.

Rotaviruses

Disease

Rotaviruses cause nausea, vomiting, enteritis, watery diarrhea, and a low-grade fever in many mammalian species (including humans) and in birds. Rotavirus infections are an important cause of diarrhea in young farm animals, including calves, lambs, foals, and piglets. The virus infects mature adsorptive cells at the tips of the intestinal villi with resulting malabsorption, maldigestion, and diarrhea. The clinical severity of the infection depends on factors such as the age and susceptibility of the affected animal, the virulence of the infecting strain of rotavirus, and the presence of other enteropathogenic organisms. Dehydration is the most common cause of death related to rotavirus infection.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

Rotaviruses represent a distinct genus within the family Reoviridae. The viruses have a nonenveloped, icosahedral, double capsid structure containing an 11-segmented dsRNA genome encoding six structural proteins, and six nonstructural proteins that are produced in rotavirus-infected cells but that are not incorporated into virions (Figure 63.6, Table 63.3).

The rotaviruses are currently organized into nine major groups (A–J) (Table 63.4). There are two distinct serotypes within Type A based on two proteins on the surface of the virus: the glycoprotein VP7 and the protease-sensitive

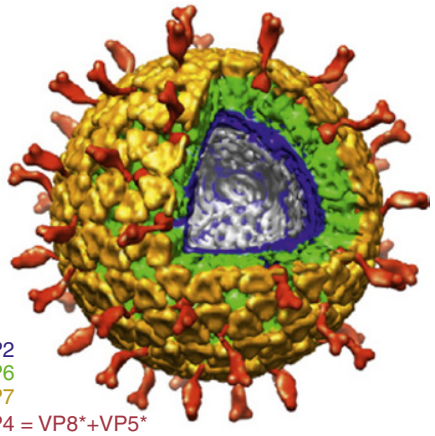


Figure 63.6 Diagram demonstrating the structure of a rotavirus, demonstrating outer capsid and the inner core particle.

Table 63.3 Molecular constituents of rotaviruses.

Gene	Protein	Role
1	VP1	Vertices of the core; RNA-dependent RNA polymerase
2	VP2	Forms inner shell of core; binds viral RNA genome
3	VP3	Vertices of the inner core; guanylyl transferase capping enzyme
4	VP4	Surface spike protein; cell attachment; virulence
5	NSP1	Binds RNA; blocks interferon response
6	VP6	Inner capsid protein; highly antigenic; used to identify species
7	NSP3	Binds mRNA; shuts down host cell protein synthesis
8	NSP2	Binds RNA; required for viral replication and packaging
9	VP7	Surface glycoprotein; determines G-type of strain; neutralization antigen
10	NSP4	Enterotoxin; induces diarrhea
11	NSP5	Binds RNA; accumulates in the viroplasm
	NSP6	Binds RNA; encoded by additional open reading frame in gene 11

Table 63.4 Types and host species of rotaviruses.

Types	Primary host species
A	Humans
A – Serotype G, based on VP7	
A – Serotype P, based on VP4	
D, F, G	Birds
H	Pigs
I	Cats
J	Bats

protein VP4. Because these two genes are passed on separately to progeny virus, different combinations can be found and vary between and within countries and years, similar to what is seen with influenza viruses. Rotaviruses within each group share common antigens, can reassort their genome segments during mixed infections, have considerable sequence homology of conserved viral genes, and tend to infect the same species of animals. Neutralizing antibodies are induced by the outer capsid proteins VP7 and VP4, whereas VP6 expresses determinants common to each rotavirus group and subgroup.

Infectivity for Other Species and Culture Systems

Although rotaviruses obtained from one species can sometimes infect other species, strains are largely species specific in their tropism. Rotaviruses are difficult to propagate in cell culture. A major advance in their propagation was the discovery that low concentrations of trypsin are required to initiate virus infection and replication in cell culture. Trypsin cleaves the viral outer coat protein, VP3, to facilitate infection of cell cultures, and once adapted, rotaviruses grow well in cell culture. The most common cell lines used are kidney epithelial cells, especially the rhesus monkey kidney cell line MA104.

Distribution, Reservoir, and Transmission

Rotaviruses are distributed worldwide and occur in many different animal species. High titers of virus are excreted in the feces of infected animals, and they are very stable in the environment. Transmission to other animals occurs by ingestion of virus, following either direct or indirect fecal-oral transmission.

Pathogenesis and Pathology

The pathogenesis of *Rotavirus* infection is similar regardless of the animal species affected. After oral infection, the virus infects the mature adsorptive epithelial cells that line the apical (lumenal) aspects of the intestinal villi. The infection progresses from the upper to the lower portions of the small intestine, and in some species, to the colon. Destruction of these mature villus enterocytes leads to villus atrophy, and the mature absorptive cells that line the villi are replaced by more immature cells from the intestinal crypts. This leads to maldigestion, small intestinal malabsorption, and diarrhea. Interestingly, the NSP4 protein alone can induce intestinal hypersecretion from crypt cells, suggesting that both malabsorption and hypersecretion of fluid and electrolytes contribute to the diarrhea of rotaviral enteritis. The disease is worst in young animals and can rapidly lead to fatal acidosis, dehydration, and hypovolemic shock. Animals that die of rotavirus enteritis are dehydrated and have very liquid intestinal contents. Diarrhea is fluid and yellow/white (the so-called white scours), which frequently stains the perineum of affected animals. Histological lesions include villus atrophy with loss of mature adsorptive cells covering the villi, and hyperplasia of the immature cells within the intestinal crypts.

Host Response to Infection

Animals infected with a rotavirus develop local and systemic humoral immune responses that can be identified by various serological techniques. Viral-neutralizing antibody is serotype-specific and directed at VP4 and VP7. The Rotavirus ELISA detects antibodies to group and subgroup determinants. Local immunity within the bowel is very important in preventing severe rotaviral enteritis in young animals; thus, ingestion of colostrum with high titers of rotavirus-neutralizing antibody provides temporary immunity against disease in neonates. Following infection, rapid innate immune responses involving IFN types I and III and Th1, Th2 cytokines inhibit viral replication and recruit macrophages and NK cells to the infected cells.

Laboratory Diagnosis

Diagnosis of rotavirus-induced diarrhea requires identification of the virus in feces or in tissues obtained at necropsy. Electron microscopy (EM), immuno-EM, and IFA staining of feces and/or intestinal tissue sections all facilitate direct visualization of virus or viral antigens, but virus in feces is most easily detected by antigen-capture ELISA. The ELISA is very sensitive and, depending on the capture antibody used, can distinguish different serotypes. Direct examination of the dsRNA genome of rotaviruses in the feces of animals can be done by polyacrylamide gel electrophoresis, which also identifies the specific group that is present. Antigen detection with PCR assays is also available.

Virus isolation is usually performed on MA104 cells in the presence of low concentrations of trypsin. Avian

rotaviruses are isolated on primary chicken embryo liver and kidney cells. Serological diagnosis can be done by ELISA or VN assays; however, the utility of data obtained usually is uncertain because of the widespread distribution of infection, which means that a high proportion of animals are seropositive regardless of disease status.

Treatment and Control

Treatment of clinically ill animals is guided by disease severity, and treatment of severe cases involves replacement fluid therapy to treat dehydration and acidosis, minimization of environmental stress, and treatment of secondary infections. Control is often difficult because the stability of the virus in feces leads to long-term environmental contamination. Although often challenging to implement, stringent sanitation practices can minimize exposure. Vaccine strategies are best directed at the dams of suckling neonates, to ensure that high titers of antibody are present in the colostrum and milk of these animals.

Aquareoviruses

Aquareoviruses are morphologically and physiochemically similar to orthoreoviruses, with nonenveloped, icosahedral, double capsid structure (75 nm diameter), but have 11 segments of dsRNA encoding 12 proteins. Seven of the 12 proteins are structural, with VP7 representing the major capsid protein. Seven genotypes (A–G) have been proposed for *Aquareovirus*. Viruses in this genus in general have low or no pathogenicity for fish and shellfish, but some types can cause hemorrhagic disease, hepatitis, pancreatitis, and necrosis in the parenchymal organs of infected fish with high mortality in fish hatcheries. Grass carp hemorrhage virus is the most pathogenic of the aquareoviruses. Various fish or shellfish cell lines incubated at 16 °C can be used to propagate aquareoviruses and can induce syncytia formation. Viruses attach to host cell receptors and enter by endocytosis. Replication occurs in the cytoplasm. Viruses exit the host cell by monopartite, non-tubule guided viral movement. Fish, shellfish, and crustaceans are natural hosts.

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Birnaviridae

Melissa Kennedy and Donald L. Reynolds

The family Birnaviridae includes seven genera: *Avibirnavirus* (infects poultry), *Aquabirnavirus* and *Blosnavirus* (infect fish), *Ronavirus* (infects the rotifer *Brachionus plicatilis* used for rearing fish larvae), *Telnavirus* (infects a marine bivalve mollusk), and *Entomobirnavirus* and *Dronavirus* (infect insects).

Picobirnaviruses were originally thought to belong to the family Birnaviridae because they possess characteristics similar to birnaviruses. However, the picobirnavirus virions are much smaller in diameter (35 nm) compared to birnavirus virions (60–65 nm). They have now been placed into their own family (Picobirnaviridae) and have one genus (*Picobirnavirus*). Picobirnaviruses are known to infect humans, other vertebrate animals, and invertebrates. Although they have been associated with clinical cases of gastroenteritis in vertebrates, their role in disease is unclear.

Infectious bursal disease (IBD) virus (genus *Avibirnavirus*) is the best-studied virus in the family Birnaviridae and causes significant disease in chickens. Infectious pancreatic necrosis virus (IBNV) (genus *Aquabirnavirus*) is a significant pathogen of salmonid fish. These two viruses of veterinary importance are discussed.

Infectious Bursal Disease

Disease

IBD, also known as Gumboro disease, after the town in Delaware (North America) where it was first identified, is an economically important viral disease of young chickens. The Infectious bursal disease virus (IBDV) replicates in immature B lymphocytes in the bursa of Fabricius (BF) and causes B lymphocyte destruction. The BF is responsible for the development and maturation of B cells and the generation of a diverse antibody repertoire in young chickens. Therefore, B cell destruction leads to reduced immunologic responsiveness. The virus can cause relatively high mortality in chickens two to six weeks of age, and profound immunosuppression in birds infected earlier in life. The clinical disease in birds over three weeks of age is characterized by soiled vent feathers (birds often peck at their own vents), diarrhea, depression, anorexia, trembling, severe prostration, dehydration, and eventual death. In the United States, economic losses are typically due not so much to increased mortality, but rather increased morbidity attributed to immunosuppression that leads to vaccination failures, secondary bacterial infections, decreased weight gains, and carcass condemnations. Highly virulent strains of IBDV that cause high mortality in affected flocks have become increasingly important in Europe and other regions of the world. Although these strains, referred to as very virulent IBD (vvIBD) are antigenically similar to the classical strains, they can infect chicks in the face of maternal immunity previously protective against the classical strains. Infection of birds less than three weeks of age results in economically devastating inapparent infection. Such birds are extensively immunocompromised and exhibit increased susceptibility to a variety of other infectious diseases. Furthermore, affected birds respond poorly to vaccination.

Etiologic Agent

Physical, Chemical, and Antigenic Properties

IBDV particles are non-enveloped with icosahedral symmetry (60 nm in diameter; Figure 64.1). The genome includes two segments of double-stranded RNA (dsRNA), designated A and B, that encode five proteins. Segment A has two open reading frames and encodes a nonstructural protein (VP5) as well as a polyprotein that is cleaved into

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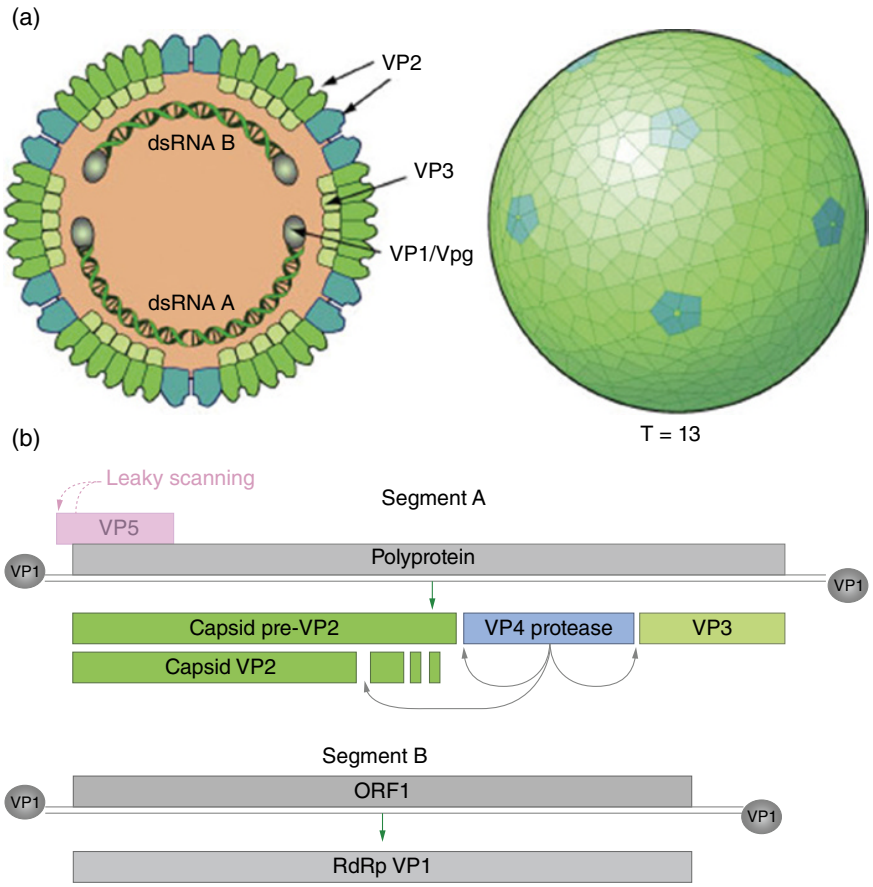


Figure 64.1 (a) Birnaviruses are non-enveloped, have a single-shelled (T = 13 icosahedral symmetry) capsid about 70 nm in diameter, and are composed of 260 trimers of VP2 that form spikes projecting radially from the capsid. The peptides are derived from pre-VP2 C-terminal cleavages that remain associated within the virion. VP3 forms a ribonucleoprotein complex with the genomic RNA. Minor amounts of VP1 are also incorporated in the virion. (b) The genome is segmented linear dsRNA: 2 segments (A,B) encode for 5–6 proteins. VP1 is found in a free form and covalently attached at the 5′ genomic RNA end (VPg). Segments sizes are about 2.3–3 kb. The total genome size is about 6 kb. *Source:* From: https://viralzone.expasy.org. ViralZone, SIB Swiss Institute of Bioinformatics.

two structural proteins (VP2, VP3) and the viral protease (VP4). Gene segment B encodes the viral RNA-dependent RNA polymerase (VP1). There are two serotypes of IBDV and marked antigenic and genetic variation within each type. Serotype 2 strains, which primarily infect turkeys, neither cause disease nor protect against serotype 1. Strains of IBDV serotype 1 cause disease in chickens throughout the world and are classified into three pathotypes: classical virulent, antigenic variant, and very virulent. RNA viruses, such as IBDV, are known to have high genetic mutation rates. In addition, mutations in the VP2 hypervariable region of the virus, genetic reassortment events, and homologous recombination within segments also occur, and all these events lead to increased variation in the virus. Extensive usage of live vaccines in the field is thought to favor the emergence of new strains of IBDV that evaded vaccine-induced immunity. A recent global molecular epidemiological study on IBDV isolated from four continents revealed that 60–70% of circulating IBDV isolates were very virulent (vv) strains.

Resistance to Physical and Chemical Agents

IBVD is extremely stable and resists inactivation following acid treatment (stable at pH 3), lipid solvents, various disinfectants, and heat (survives 60 °C for 30 minutes). The virus can remain viable for at least six months in dry litter and more than one year in unused dry chicken houses.

Infectivity for Other Species and Culture Systems

IBVD principally affects chickens, although turkeys, ducks, and some other species of domestic and wild birds also can be infected with it. The virus can be propagated in embryonating chicken eggs, with subsequent embryo mortality, as well as in a variety of avian cell cultures.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

IBD occurs worldwide in intensive poultry-raising areas. The virus persists in nature because of its stability. The virus has reportedly persisted in poultry houses, following depopulation, for over 100 days. There is no evidence of a true carrier state in birds.

Transmission of IBDV occurs by ingestion of virus from feces or feces-contaminated fomites, feed, and water. There are no reports of vertical transmission of IBDV.

Pathogenesis and Pathology

Initial replication of IBDV occurs in the intestinal tract (gut-associated lymphoid cells) within hours of ingestion of the virus, from where it disseminates to numerous tissues, including the BF. Other lymphoid organs such as the thymus, spleen, and tonsils also may be affected, and disseminated lymphoid atrophy is especially characteristic of birds infected with very virulent strains of IBDV.

The striking age-dependent nature of the response of chickens to IBDV likely reflects the maturation of the BF at the time of infection – stem cells and peripheral B cells do not support replication of the virus. That is, birds are most susceptible to IBD from two to six weeks of age, whereas bursectomized birds of the same age do not develop disease. The BF is the site of high viral replication that leads to a secondary viremia and spread of the virus to additional tissues, causing mortality. Birds older than six weeks also do not develop severe disease, nor do those infected prior to two weeks of age. The impaired immunologic responsiveness that occurs in chicks infected early in life has been attributed to bursal injury, which results in failure to seed B lymphocytes to peripheral lymphoid organs and diminished humoral immunity. Cellular immunity also is reduced in IBDV-infected young chicks.

Gross lesions may include dehydration, darkened pectoral muscles, and hemorrhages in the pectoral and leg muscles. The appearance of the BF is dependent on the state of disease. The BF initially becomes enlarged due to edema and hyperemia, but this is rapidly followed by progressive atrophy approximately eight days after infection. Bursal necrosis and hemorrhage are characteristic of advanced disease.

Histologically, the epithelial surfaces of the BF have multiple erosions, and there is extensive necrosis of lymphocytes within lymphoid follicles so that these become depleted of lymphocytes. Edema and infiltration of heterophils occurs initially in the affected BF, followed by formation of cystic cavities bound by columnar epithelial cells and lymphoid depletion. Pathology in other lymphoid organs is less severe and recovery more rapid, except in birds infected with very virulent strains of IBDV (see Figures 64.2 and 64.3).

Host Response to Infection

Following infection, birds develop a humoral immune response that can be measured by viral neutralization, agar gel immunodiffusion, and enzyme-linked immunosorbent

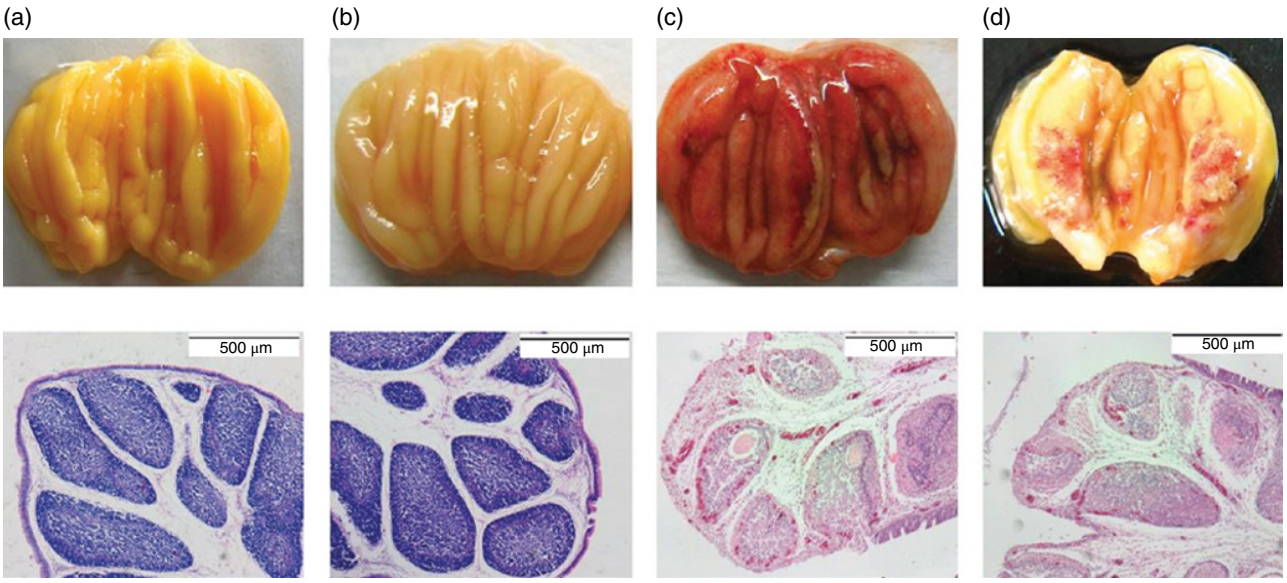


Figure 64.2 Gross morphology and histopathology of bursa of Fabricius follicle. Bursa follicle from (a) normal birds or (b) vaccinated birds, (c) control, or (d) PBS after the virulent IBDV challenge. *Source:* From Xu et al. (2011). BioOne, with permission.

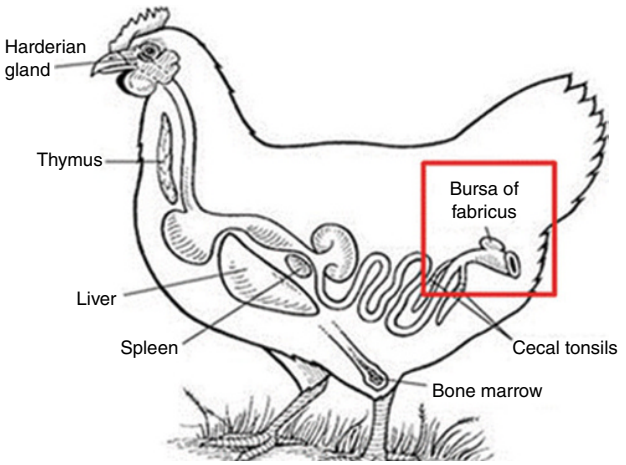


Figure 64.3 Location of the bursa of Fabricius in a chicken.

assay (ELISA) tests. In birds that recover, the BF may become repopulated with B lymphocytes. Although T lymphocytes are required for protection against IBD and viral clearance, they can cause bursal damage and slow recovery through cytokines and cytotoxic responses. Adult birds transfer maternal antibody to developing embryos, and if present in sufficient titer, the antibody protects the hatched chick for variable periods of time.

Laboratory Diagnosis

Field diagnosis can usually be made based upon the characteristic clinical signs and high morbidity and rapid recovery of most affected birds. Atrophy of the BF is characteristic of inapparent IBDV infection in young chicks. Definitive diagnosis of IBD can be carried out by direct fluorescent antibody (FA) staining of sectioned tissues or virus isolation from the bursa and spleen. Isolation can be made by inoculation of embryonated chicken eggs or cell cultures. Serology is also useful for diagnostic purposes. Types and subtypes of IBDVs can only be distinguished through neutralization assays. Increasingly, molecular diagnostic assays using reverse-transcription polymerase chain reaction are being utilized for IBD diagnosis, as well as strain characterization. Strain characterization is important for differentiation between field and vaccine strains and is accomplished by using molecular methods to target differences between strains in the VP2 region.

Treatment and Control

No treatment has been described for affected birds. Control of the disease may be facilitated by proper sanitation practices. Vaccination is the principal method of controlling IBDV, and its success depends on the choice of vaccine strain, vaccination schedule, and the circulating field virus strains. Vaccines include live attenuated virus, inactivated virus, and recombinant viral vectored vaccines. Immunization of breeder flocks is performed to facilitate passive transfer of immunity to chicks to provide protection during the first four to five weeks of life to protect against subclinical infection. Vaccination of chicks is also practiced, but to be effective, levels of maternal antibody must be considered at the time of vaccination. Vaccinating chicks with high maternal antibody levels may not develop anticipated immunity due to maternal antibodies interfering with the vaccine virus. Recombinant and immune-complex vaccines can be administered even in the presence of maternally derived antibodies, *in ovo* at 18 days of incubation or to 1-day-old chicks. The vaccine strains are classified as "mild," "intermediate," or "intermediate plus/hot" based on their increasing ability to overcome residual maternally derived antibodies and to replicate and cause lymphocytic depletion in the BF. Vaccination against the vvIBD strains encounters many challenges as they can break through protective antibodies, and therefore require more efficient vaccination approaches.

Birnavirus Infections of Fish

Infectious pancreatic necrosis virus (IPNV) is the cause of a highly contagious and fatal disease of salmonid fish (trout and salmon) that is increasingly important to the aquaculture industry worldwide. The disease is especially severe in fish less than six months old, but also occurs in older fish following stresses such as that associated with relocation from fresh to salt water. Affected fingerlings appear dark in color and swim with a rotating action (commonly known as whirling and/or corkscrew swimming). Anorexia is a typical clinical sign in post-molt salmon and non-salmonid species. Petechial hemorrhages may be present in the abdominal viscera of affected fish, along with necrosis of the pancreas. IPNV causes immunosuppression, and bacterial infections are a frequent sequela. The virus also affects commercially raised yellowtails with high mortality and is classically referred to as viral ascites due to accumulation of fluid in the abdomen (ascites) and associated abdominal distension. Eels can be infected, with resulting clinical disease. Fish that survive infection with IPNV may become persistently infected lifelong asymptomatic carriers and infect other fish, especially under hatchery conditions. The virus is very stable in the environment. It can be identified in the tissues of affected or carrier fish by virus isolation in fish cell cultures or

reverse-transcriptase-polymerase chain reaction (RT-PCR) techniques. Up to 10 serotypes of IPNV have been described, with the neutralizing epitopes being mapped to VP2. Efforts to develop effective vaccines are complicated by the multiple serotypes of IPNV and the difficulty of immunizing very young fish, which are especially susceptible to the virus. Control is based on husbandry efforts that maximize hygiene and sanitation and minimize crowding, stress, and introduction of infected replacement stock and/or eggs. Vigorous identification and culling of carrier fish is advocated as a method of controlling the disease.

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Retroviridae

Jean-Pierre Frossard

Viruses in the Retroviridae family are enveloped, with a single-stranded RNA genome, using an RNA-dependent DNA polymerase (reverse transcriptase [RT]). The family name is derived from the Latin *retro*, meaning "backward," a reference to the RT activity. A number of these viruses are associated with disease in humans and a wide range of animals. The disease manifestations include lymphomas, leukemias, sarcomas, and other tumors, immunodeficiencies, neurological disease, arthritis, and a variety of acute diseases resulting from tissue damage. Retroviruses exist as exogenous infectious viruses, transmitted horizontally or vertically, and also as endogenous proviruses that are transmitted through the germline of infected animals.

Classification

According to the Baltimore classification, based on mRNA synthesis processes, members of the *Retroviridae* belong to the sixth Baltimore group, as they have a positive-sense single-stranded RNA genome with a DNA intermediate in their replication cycle.

The Retroviridae family was historically divided into three subfamilies: Oncovirinae, Lentivirinae, and Spumavirinae. It is now classified into just two subfamilies: Orthoretrovirinae and Spumaretrovirinae. These comprise 11 genera and at least 68 species (Table 65.1).

The genera within Orthoretrovirinae are *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, and *Lentivirus*. The genera within Spumaretrovirinae are *Bovispumavirus*, *Equispumavirus*, *Felispumavirus*, *Prosimiispumavirus,* and *Simiispumavirus*. Viruses of veterinary importance are found within the Orthoretrovirinae subfamily. Infections with spumaviruses are not characterized by any clinical disease. The subfamily name is derived from the Latin *spuma*, meaning "foam," as the viruses often cause a characteristic "foamy" appearance in cell culture where multinucleated vacuolated giant cells are formed.

General Features of Retroviruses

Morphology

Virions are enveloped and spherical in shape with a diameter between 80 and 200 nm. The virion surface features glycoprotein projections, about 8 nm in length. The nucleocapsid makes up the internal core of the virions. This varies in shape between the genera, from spherical to polyhedral or cone shaped, depending on the genus. The organization of the proteins in the capsid follows the principle of fullerene arrangement, with a hexagonal capsid lattice containing 12 pentamers forming a closed shell structure. The resulting shape is eccentric for the betaretroviruses, concentric for alpharetroviruses, gammaretroviruses, deltaretroviruses, and spumaviruses, and rod or truncated cone shaped for the lentiviruses. This ultrastructural morphology forms the basis for a historical, but still used, typing system of retroviruses. An immature state with an intracellular double membrane lacking budding forms is type A. Extracellular virions with an eccentric spherical core are type B, those with a concentric spherical core are type C, and those with a cylindrical or conical core are type D. Figure 65.1 shows electron microscopy images of representative retroviruses.

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Table 65.1 Classification of retroviruses, with subfamilies, genera, and species.

Subfamily: Orthoretrovirinae			
Genus:	*Alpharetrovirus*	Species:	Avian carcinoma Mill Hill virus 2 Avian leukosis virus Avian myeloblastosis virus Avian myelocytomatosis virus 29 Avian sarcoma virus CT10 Fujinami sarcoma virus Rous sarcoma virus UR2 sarcoma virus Y73 sarcoma virus
Genus:	*Betaretrovirus*	Species:	Jaagsiekte sheep retrovirus Langur virus Mason-Pfizer monkey virus Mouse mammary tumor virus Squirrel monkey retrovirus Gunnison's prairie dog retrovirus (proposed)
Genus:	*Gammaretrovirus*	Species:	Chick syncytial virus Feline leukemia virus Finkel-Biskis-Jinkins murine sarcoma virus Gardner-Arnstein feline sarcoma virus Gibbon ape leukemia virus Guinea pig type-C oncovirus Hardy-Zuckerman feline sarcoma virus Harvey murine sarcoma virus Hervey pteropid gammaretrovirus (proposed) Kirsten murine sarcoma virus Koala retrovirus Moloney murine sarcoma virus Murine leukemia virus Porcine type-C oncovirus Reticuloendotheliosis virus *Rhinolophus ferrumequinum* retrovirus (proposed) Snyder-Theilen feline sarcoma virus Trager duck spleen necrosis virus Viper retrovirus Wooly monkey sarcoma virus
Genus:	*Deltaretrovirus*	Species:	Bovine leukemia virus Primate T-lymphotropic virus 1 Primate T-lymphotropic virus 2 Primate T-lymphotropic virus 3
Genus:	*Epsilonretrovirus*	Species:	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1 Walleye epidermal hyperplasia virus 2
Genus:	*Lentivirus*	Species:	Bovine immunodeficiency virus Caprine arthritis encephalitis virus Equine infectious anemia virus Feline immunodeficiency virus Human immunodeficiency virus 1 Human immunodeficiency virus 2 Jembrana disease virus Puma lentivirus Simian immunodeficiency virus Visna-maedi virus

(*Continued*)

Table 65.1 (Continued)

Subfamily: Spumaretrovirinae			
Genus:	*Bovispumavirus*	Species:	Bovine foamy virus
Genus:	*Equispumavirus*	Species:	Equine foamy virus
Genus:	*Felispumavirus*	Species:	Feline foamy virus
Genus:	*Prosimiispumavirus*	Species:	Brown greater galago prosimian foamy virus
Genus:	*Simiispumavirus*	Species:	Bornean orangutan simian foamy virus Central chimpanzee simian foamy virus Cynomolgus macaque simian foamy virus Eastern chimpanzee simian foamy virus Grivet simian foamy virus Guenon simian foamy virus Japanese macaque simian foamy virus Rhesus macaque simian foamy virus Spider monkey simian foamy virus Squirrel monkey simian foamy virus Taiwanese macaque simian foamy virus Western chimpanzee simian foamy virus Western lowland gorilla simian foamy virus White-tufted-ear marmoset simian foamy virus Yellow-breasted capuchin simian foamy virus

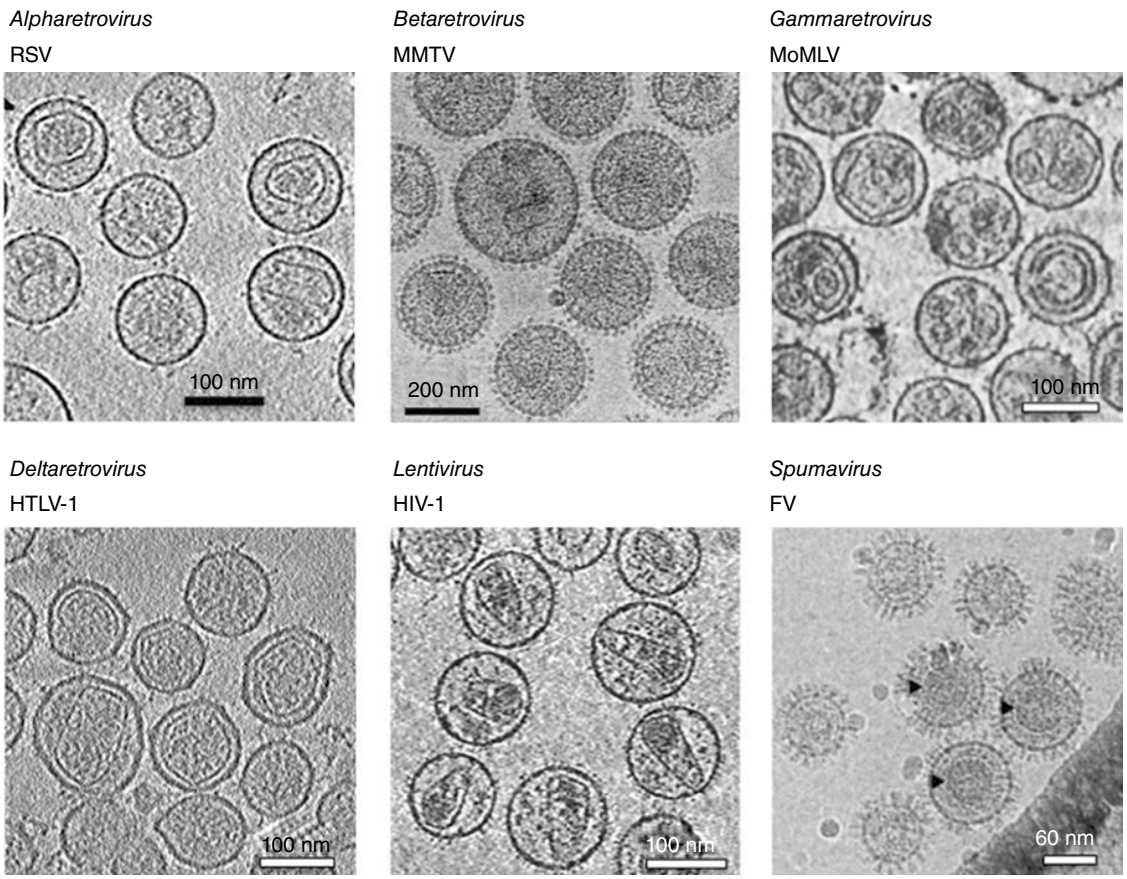


Figure 65.1 Morphology of mature retrovirus virions. The cryo-EM images and cryo-ET cross sections of the viruses display the heterogeneous morphology and size of the viral core in six genera of the Retroviridae. RSV: Rous sarcoma virus; MMTV: mouse mammary tumor virus; MoMLV: Moloney murine leukemia virus; HTLV-1: human T-cell leukemia virus type 1; HIV-1: human immunodeficiency virus type 1; FV: Foamy virus. The black arrowheads show regular Gag assemblies in the wild type virus. *Source:* Reproduced with permission from Zhang et al. (2015), AIMS Press.

Physicochemical and Physical Properties

The buoyant density of virions is between 1.16 and 1.18 g/ml in sucrose.

Being enveloped, virions are relatively resistant to UV light, but are sensitive to heat, detergents, and formaldehyde.

The virions are made up of RNA (2%), proteins (60%), lipids (35%), and carbohydrates (3%).

The genome consists of a dimer of linear single-stranded positive-sense RNA, between 7 and 13 kb in size. Each RNA molecule is polyadenylated at the 3′ end and is capped at the 5′ end. The dimers are joined near their 5′ ends. Additional host RNA or DNA is sometimes packaged alongside the viral RNA genome. As reverse transcription of viral RNA is a late step in the viral life cycle of the Spumaretrovirinae, reverse-transcribed double-stranded DNA is sometimes packaged as well as or instead of the RNA in these virions.

For most retroviruses, there are two envelope proteins, the surface protein (about 100 kd, involved in binding the virus to the cell receptor) and transmembrane protein (about 50 kd, involved in attaching the surface protein to the envelope). Some spumaretroviruses feature an additional leader peptide. There are several non-envelope structural proteins, including the matrix (about 15 kd, joining the core and envelope), capsid (about 25 kd, forming the major structural element of the virus core), and nucleocapsid (about 5–10 kd, interacting directly with the viral RNA) proteins. Nonstructural proteins include the protease (which cleaves the Gag and Pol polyproteins during virus assembly and maturation), RT (RNA-dependent DNA polymerase with RNase H activity, requiring a divalent cation to function), and integrase enzyme (which covalently links the viral DNA into the host's genome during provirus integration). In some viruses, there is also a dUTPase enzyme (needed for virus replication in nondividing cells), or an additional protein of undetermined function. Deltaretroviruses, epsilonretroviruses, lentiviruses, and spumaretroviruses also feature further nonstructural proteins such as transcriptional transactivators and proteins involved in RNA nuclear export, as well as proteins enhancing viral replication in specific cell types, and some interfering with host immune responses.

The envelope surface protein and often the transmembrane protein are glycosylated. Cellular glycolipids and some glycoproteins are also incorporated into the mature virions. Similarly, lipids (usually phospholipids) from the plasma membrane of the host cell are also incorporated in the envelope of mature virions, where they are arranged in a bilayered structure similar to the cellular outer membrane from which the viral envelope is derived. The typical arrangement of retroviral proteins in the virion is shown in Figure 65.2.

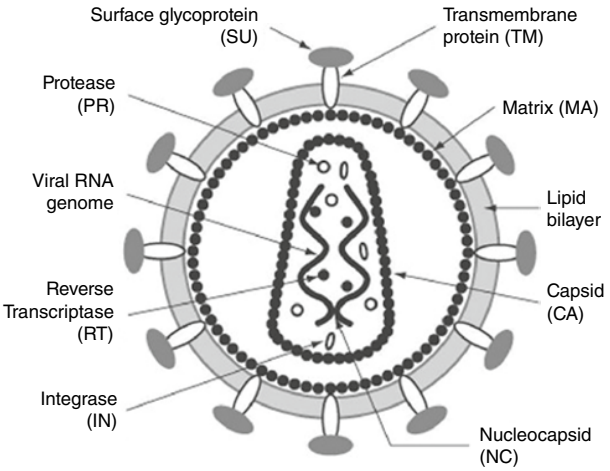


Figure 65.2 Schematic representation of a typical retrovirus virion, illustrating the disposition of structural and enzymatic proteins. Within the virion core, the dimeric RNA genome is most likely completely coated with the nucleocapsid product of the gag gene. The two-letter abbreviation for retroviral proteins is indicated in parentheses. *Source:* Reproduced with permission from The-Crankshaft Publishing; http://what-when-how.com/molecular-biology/retroviruses-part-1-molecular-biology.

Genome Organization and Replication

The organization of the retrovirus genome includes a minimum of four genes, always in the order 5′-*gag-pro-pol-env*-3′. Additional genes for nonstructural proteins, such as *tax* and *rex*, are present in some retrovirus genomes, in which case the viruses are called complex. See Figure 65.3 for a representation of the genome organization for each genus. Cell-derived sequences are sometimes inserted into the retrovirus genome, often forming a fused gene with a viral structural gene which is then translated into a single chimeric protein. Alternatively, some of these cell-derived sequences replace deleted viral sequences, usually then producing replication-defective viruses.

The life cycle of retroviruses is usually described with an early phase comprising the steps of infection from initial binding to the host cell and internalization, followed by integration of the viral cDNA into the cell genome, and then a late phase including the expression of viral genes and ending with the release and maturation of progeny virions. Figure 65.4 displays a general model of retroviral replication.

Replication of the retrovirus RNA genome occurs through a DNA intermediate. This is generated by reverse transcription of the viral RNA into cDNA using the 3′ end of the tRNA as primer to synthesize the negative-sense cDNA. That small initial product is transferred and then primes further cDNA synthesis from the 3′ end of the genome, utilizing the long terminal repeats (LTRs) at each

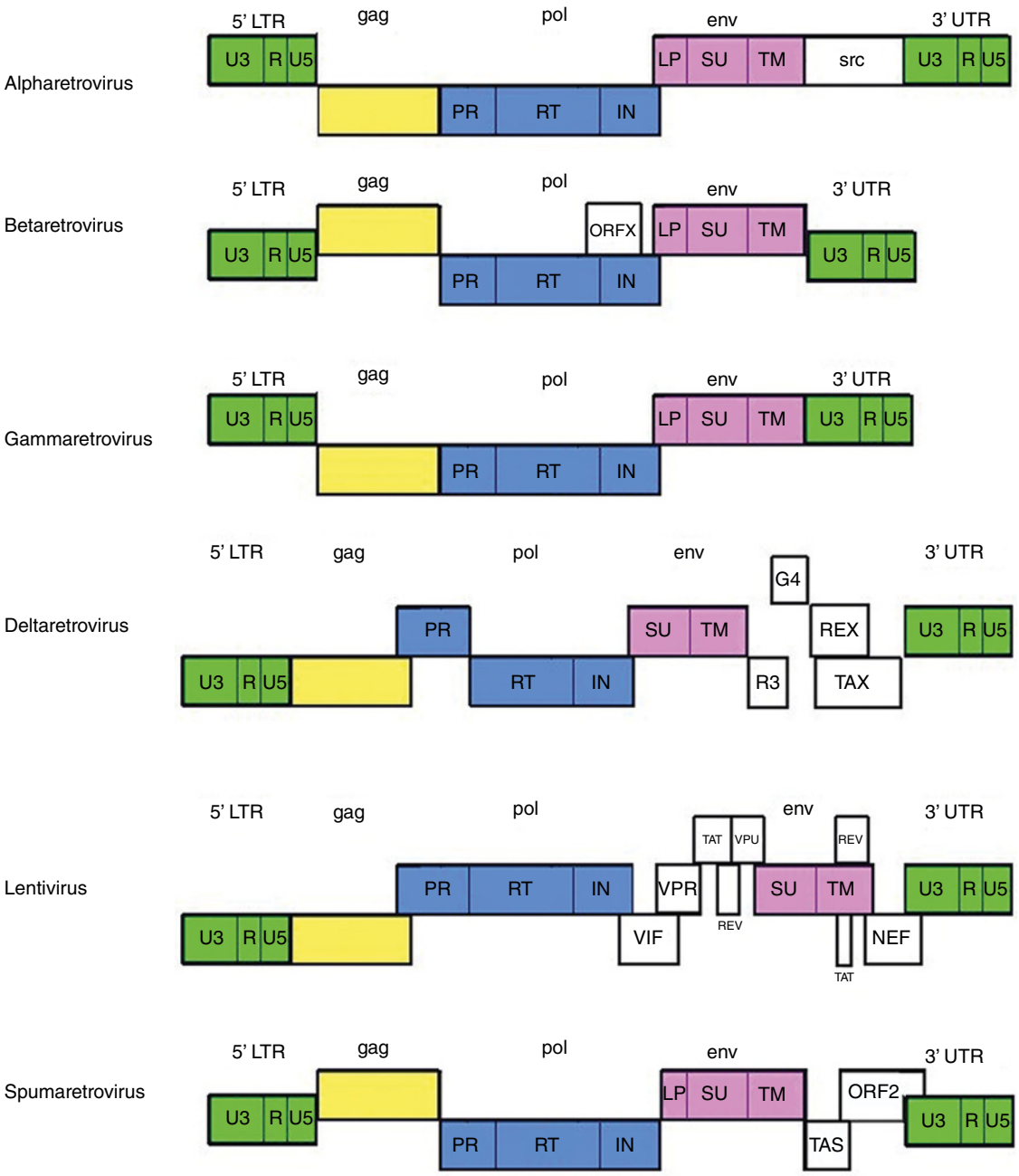


Figure 65.3 Genome organization of representative retroviruses from six genera. The schematic includes the following genetic regulatory areas: Internal promoter (IP), Long terminal repeat (LTR), Unique 3′ region (U3), LTR repeat region (R), and LTR unique 5′ region (U5). It also includes genes encoding the following products: Gene of unknown function (ORFX), Integrase (IN), Leader peptide (LP), Open reading frame 2 (ORF2), Protease (PR), Regulator of expression of virion proteins (REV, REX), Reverse transcriptase-RNAse H (RT), Surface glycoprotein (SU), Transcriptional transactivators (TAS, TAT, TAX), Transmembrane protein (TM), Tyrosine kinase (SRC), Viral infectivity factor (VIF), Viral protein R (VPR), and Viral protein U (VPU).

end of the viral RNA. The required digestion of the viral RNA is carried out by the RNase H component of the viral RT. A product of this hydrolysis then primes the positive-sense cDNA synthesis using the negative-sense DNA copies. The resulting double-stranded DNA derived from the viral genome includes LTRs made up of unique sequences from the 3′ and 5′ ends of the viral RNA, flanking a repeated sequence found near both ends of the RNA. In spumaretroviruses, this process takes place at the time of virion assembly, whereas for the remainder of the retroviruses it occurs following virion entry and uncoating in the host cell.

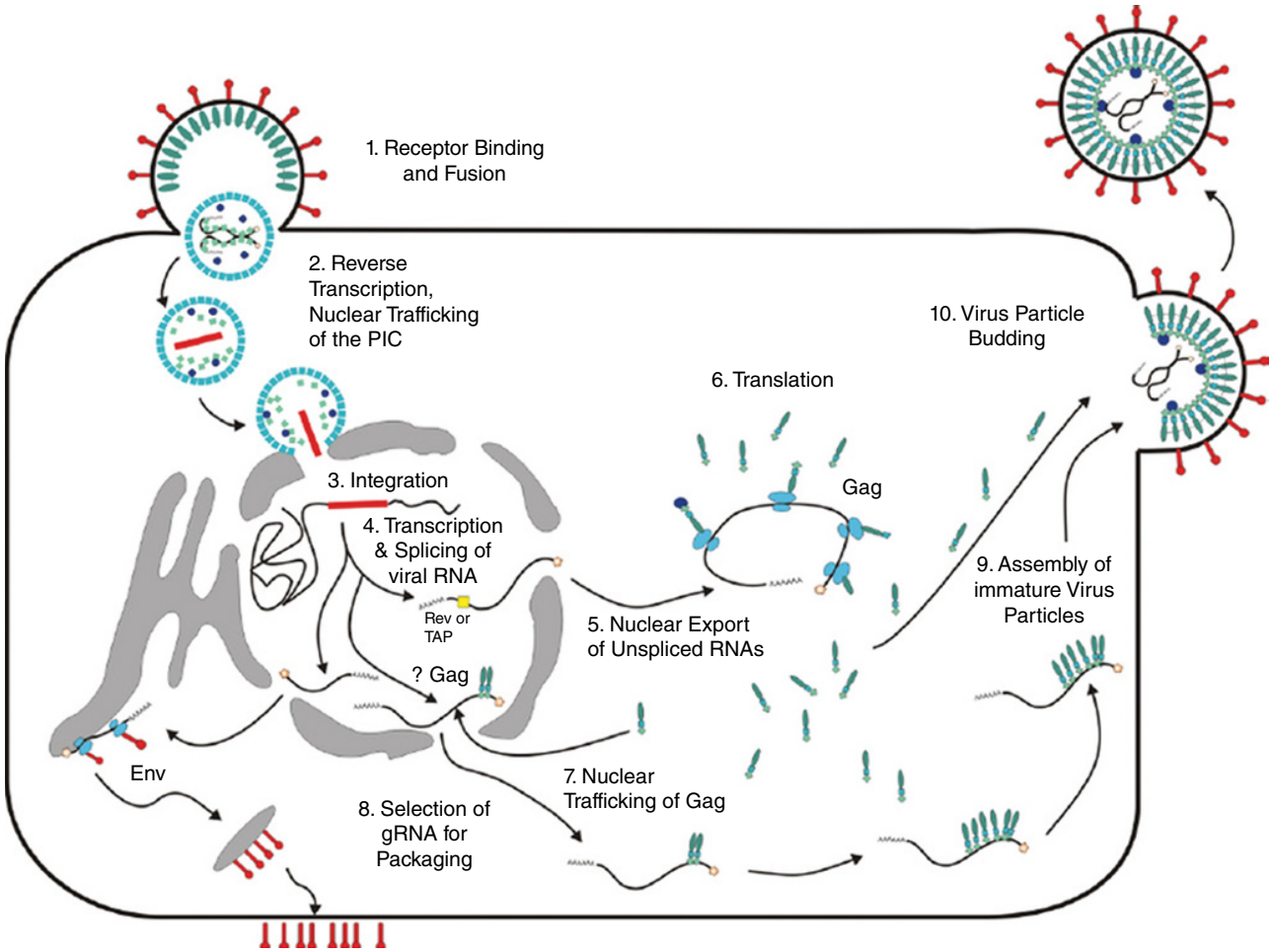


Figure 65.4 Model of retrovirus replication. Retroviral infection starts with binding of the viral Env protein to a cell surface receptor and fusion of the viral envelope with the cell membrane (step 1). The viral RNA genome is reverse-transcribed into DNA (step 2) to form the provirus, which is integrated into the host cell genome (step 3). Viral RNA is transcribed by the host polymerase, leading to translation of the viral proteins (step 4). A portion of the viral RNA is exported from the nucleus (step 5) to serve as a template for Gag and Gag-Pol translation (step 6). The Gag proteins of some retroviruses traffic through the nucleus during assembly (step 7). Genomic RNA is transported to the cytoplasm for packaging (step 8). The Gag-gRNA complex is transported to the plasma membrane (step 9), where additional Gag molecules bind the viral RNP to complete assembly of the immature virus particle, which buds from the plasma membrane (step 10). *Source:* Reproduced with permission from Stake et al. (2013).

The retroviral RT enzymes are devoid of $3' \rightarrow 5'$ exonucleolytic proofreading activity, and hence a mutation rate ranging from 2×10^{-5} to 6×10^{-6} per nucleotide per replication cycle is observed. The tendency for template switching exhibited by the RT leads to a relatively high rate of recombination in retroviruses. These factors result in increased genetic diversity within retroviruses.

The double-stranded DNA resulting from reverse transcription is integrated into the host cell genome by a mechanism involving the viral integrase, where it is stably maintained as a provirus. The ends of the viral DNA are joined to cellular DNA, with two bases from the ends of the viral DNA being removed, and a short duplication of cellular DNA sequences being generated at the integration site. Integration seems to occur in a non-sequence-specific manner, but the integration site may be guided by the structural characteristics of the neighboring chromatin. As a result, some retroviruses do exhibit a distinct preference for integration sites. The provirus may remain latent for long periods of time and will be replicated alongside the rest of the cellular DNA. Host cell enzymes replicate the integrated viral DNA, after which the cellular RNA polymerase II transcribes the proviral DNA into mRNA molecules and RNA genomes that are packaged into virions. The process is controlled by transcriptional signals in the viral LTRs, and for some retrovirus species, it is regulated by virus-encoded transactivators. The release and maturation of progeny virions relies on budding from either the

plasma membrane or intracellular membranes, followed by pinching off or fission. Finally, fully infectious particles with a lipid bilayer coat acquired from the host cell are released.

Oncogenesis by Retroviruses

Oncogenic retroviruses are classified into two main categories, depending on their mechanisms of tumorigenesis. A number of acute-transforming retroviruses, containing transduced host proto-oncogenes, are responsible for rapid tumor formation and subsequent death. However, the majority of retroviruses only lead to tumor formation after a period of latency, through a process of insertional mutagenesis of the host genome, leading to activation of a cellular proto-oncogene. The same genes are affected in each case, being either mutated or differently expressed. The affected genes are typically linked to cell cycle control or mitogenic signaling.

Viruses associated with leukemia and with sarcoma in birds were discovered in 1908 and 1910. Avian leukosis virus (ALV) was shown to induce lymphomas by insertional mutagenesis in B-cells. Work with the avian Rous sarcoma virus (RSV) led to the discovery of the viral oncogene *src*, encoding a tyrosine kinase. Further research with other retroviruses has led to the discovery of additional oncogenes. The analyses of viral oncogenes ultimately led to the discovery of cellular equivalents involved in human and veterinary cancers, with, or without virus associations.

In the highly oncogenic retroviruses, the virus genome incorporates all or part of an oncogene, often replacing viral gene sequences, resulting in a replication-incompetent virus. The viral oncogene is directly responsible for the transformation of a cell. So far, more than 20 different retroviral oncogenes have been identified, all of which have a matching gene in the host cell's normal genome. The cellular gene is termed a c-oncogene or proto-oncogene, while the viral version is called a v-oncogene. In the healthy cell environment, these genes are usually involved in growth regulation pathways, and include protein kinases, growth factors or their receptors, GTP binding proteins, and transcriptional activation factors. Expression of the viral oncogenes is regulated by the retroviral LTR rather than by the normal cellular mechanisms, thereby resulting in very much higher levels of expression than normal. Depending on the characteristics of the viral oncogene, which may be mutated, truncated, or fused to another viral sequence, its expression will have differing effects in the host cell. This could include abnormal growth of the cell, triggering the process of neoplasia.

The replication-incompetent retroviruses require a helper virus that is replication competent to supply the deleted viral gene products. The helper virus is most commonly a closely related retrovirus that does contain the normal *gag-pro-pol-env* genes. As the defective, highly oncogenic virus genome is then packaged within a virion made up of the structural proteins of the helper virus, termed a pseudotype virus, the host range of the resulting virus is determined by the helper virus's characteristics. In practice, infection with a defective, highly oncogenic sarcoma virus will usually cause multiclonal tumors to appear in the host within days or weeks, being rapidly fatal, and providing little opportunity for transmission to other hosts.

In contrast, the non-acutely transforming, weakly oncogenic retroviruses cause neoplasia less quickly and efficiently than the highly oncogenic viruses. These do not contain any oncogene sequences, and are replication competent, therefore do not require a helper virus for productive infection. Tumor formation in the host follows proviral insertion close to a cellular oncogene, resulting in clonal proliferation of the affected cell. This is termed insertional or cis-activational oncogenesis. In ALV infection, for example, the provirus often integrates next to or within the cellular *c-myc* gene. This can result in abnormal transcription of the oncogene, either by read-through from the retroviral promoter, or by enhancer activity by the retroviral LTR. As provirus integration occurs in locations throughout the host genome, integration in close proximity to a cellular oncogene is a relatively rare event. Oncogenesis is therefore much less frequent and much less efficient than with the retroviruses containing v-oncogenes. The resulting tumors are clonal in nature because, although many cells are infected with virus, only a single cell may have undergone insertional oncogenesis, progressing to a tumor.

A novel mechanism for the inactivation of host tumor-suppressor genes has been described in ALV-induced tumors. A common integration cluster in ALV-infected B-cells, associated with metastatic, long-latency lymphomas, has been identified as a precursor of cellular microRNA miR-155.

The members of the *Deltaretrovirus* genus contain the *tax* regulatory gene, which encodes a transactivator that upregulates retroviral transcription by binding to specific DNA sequences in the LTR of the virus. The Tax protein can sometimes also bind to transcriptional activator sequences for cellular genes, and can then disrupt the normal regulatory pathways of the cell. The integration site of the provirus is irrelevant in this case, as it is the expressed viral gene product which exerts its effect on the host. The progression to tumor formation is multifactorial, with host gene mutations also being required, which explains the long latency period in deltaretroviral infections such as bovine leukosis.

Yet another method of retrovirus-mediated oncogenesis has been described, with direct involvement of the retroviral envelope protein in oncogenic transformation having been demonstrated in some cases. The Env protein of the

Jaagsiekte sheep retrovirus (JSRV) has been shown to interact with cellular adaptors, altering host signaling cascades. A similar process has also been proposed for a human endogenous retrovirus. Another putative mechanism of Env-mediated oncogenesis involves the cellular entry receptor for JSRV, Hyaluronidase 2.

The study of oncogenic retroviruses and the processes leading to tumor formation under either natural or experimental conditions has led to significant gains in the understanding of not only the viruses, but also of neoplasia, and cell biology in general.

Avian Sarcoma/Leukosis Virus – *Alpharetrovirus* Genus

Disease

The avian sarcoma/leukosis viruses (ASLV) include ALV, Avian sarcoma virus (ASV) and RSV. ALV is the type species of the *Alpharetrovirus* genus of the family Retroviridae. Avian retroviruses were first described in 1908, when it was shown that a form of leukemia and lymphomas in chickens could be transmitted through the transfer of cell free-filtrates. A second virus linked to sarcomas in chickens was then described by Rous in 1911.

Infection with ALV may lead to lymphoid leukosis, also known as big liver disease. Erythroid and myeloid leukosis may also occur. Tumors are formed in various organs, including the bursa, spleen, liver, kidneys, and reproductive organs. The disease presentation, including the number and type of tumors, is dependent on the strain of virus, the route of infection, and the breed and age of chicken. The principal clinical signs observed are diarrhea, dehydration, decreased appetite, weight loss, weakness, depression, and enlarged bursa, kidneys, or liver. Subclinical infection, which is most frequent, still leads to delayed sexual maturity, and decreased egg production and quality.

Etiologic Agent

Classification

ASLVs are in the *Alpharetrovirus* genus of the Retroviridae family. They exhibit C-type morphology. The viruses infecting chickens are classified into seven distinct subgroups (A, B, C, D, E, J, and K) on the basis of surface glycoprotein antigens, interference patterns, and their host range in chicken cells of different phenotypes. Group E comprises endogenous retroviruses (ERVs) found in the genome of chickens. Groups F, G, H, and I comprise the ERVs found in ring-necked and golden pheasants, Hungarian partridge, and Gambel's quail, respectively. The sarcoma viruses contain one or two oncogenes including *myc*, *myb*, *fps*, *yes*, *jun*, *ets,* and *erbB* in their genome, originating from host cellular genes that have been incorporated into the virus by gene capture. RSV contains the *v-src* oncogene, a mutated version of the *c-src* tyrosine kinase gene found in chicken cells. With the exception of some strains of RSV, the sarcoma viruses are replication deficient, requiring a helper virus to replicate.

Physical, Chemical, and Antigenic Properties

ASLVs are enveloped viruses with a dense core, and small spikes on the lipid envelope. Although all ASLVs share a common group-specific antigen (p27) present in the core, their surface glycoproteins feature five hyper variable regions: vr1, vr2, hr1, hr2, and vr3. Accordingly, ASLVs from different subgroups tend not to cross-neutralize except for partial cross-neutralization between subgroups B and D.

Resistance to Physical and Chemical Agents

ASLVs are readily inactivated by most commercial disinfectants, except those based on phenol and bisphenols, or single ammonium compounds. The viruses are rapidly inactivated in the environment at ambient temperature.

Infectivity for Other Species and Culture Systems

In addition to domestic chickens, there is evidence of ASLV infection in other species of wild and domestic fowl, including partridges; ring-necked, golden, and green pheasants; as well as Chinese quail.

Culture of the viruses *in vitro* is primarily carried out using chicken embryo fibroblast cells. Hamster, rat, or mouse embryo fibroblasts can be used with some of the sarcoma virus strains. *In vitro*, some RSV strains have been shown to infect and transform mammalian cells, including human cells. They have also been shown experimentally to induce sarcomas and other tumors in very young mammals, including primates. There is no evidence that exogenous ASLVs present a risk to humans, despite long-term exposure of humans to the viruses from live poultry, poultry meat, and eggs, and historically some live vaccines.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

ALV is found in commercial chickens worldwide. Subgroup A and J viruses are most frequent, whereas subgroups B, C, and D occur more rarely. Endogenous ALVs are ubiquitous. Some breeding companies have implemented eradication programs with success.

Chickens are the natural hosts for ASLVs, but partridges, pheasants, and quail may also be infected.

ASLVs can be transmitted both horizontally and vertically. Vertical transmission occurs when virus present in the magnum of the hen's oviduct passes into the egg albumen and ultimately to the embryo. Even though semen may contain ASLVs, infected males are not known to transmit the infection to their progeny, but they can infect hens. Due to the absence of neutralizing antibody production, congenitally infected chicks remain viremic for life. Horizontal transmission is observed following direct contact between birds, or indirectly by exposure to contaminated material. As virus is shed in feces and saliva, the fecal–oral route of transmission is important, although oculonasal, respiratory, and skin routes have also been implicated. The viremia in horizontally infected birds is usually transient, due to the production of antibodies. Birds infected at a younger age, in the absence of maternal antibodies, are more likely to develop tolerance, with persistent viremia, and tumors. The presence of ERV increases the risk of horizontal infection with exogenous ASLVs. Endogenous ALVs are transmitted genetically, but some may be released in an infectious form and can therefore be horizontally transmitted.

Pathogenesis and Pathology

The pathogenesis of ASLV infection of chickens depends largely on the strain of virus and its oncogenicity. Viruses containing a v-oncogene are more likely to cause neoplasms in infected birds, whereas other strains may cause disease by insertional oncogenesis.

Lymphoid leukosis is a clonal malignancy of the bursal-dependent lymphoid system of birds. The transformation events take place 4–8 weeks following infection, in the bursa, but are usually not detectable until the birds reach the age of 14 weeks. The resulting tumors are composed almost entirely of B-cells expressing IgM on their surfaces.

Erythroblastosis, or erythroid leukosis, results in the enlargement of the spleen, liver, and sometimes kidneys due to the proliferation of erythroblasts, also affecting the bone marrow. Affected chickens can develop anemia and thrombocytopenia. Hemorrhages can develop in muscles or in the abdomen following rupture of the liver.

ALVs from group J can cause two forms of myeloid leukosis: myelocytomatosis and myeloblastosis. These are seen mainly in adult broiler breeders, but sometimes in younger chickens. Bone marrow is the target. Myelocytomas, comprising differentiated myelocytes, are present on the bone surfaces, including the sternum, vertebrae, ribs, mandible, and nares. Other tissues such as the trachea, spleen, liver, or ovaries can also be affected, being enlarged due to tumorous infiltration. Myeloblastosis manifests as a myeloid cell leukemia with an enlargement of the liver and spleen. The liver features intravascular infiltration of immature myeloblasts and promyelocytes, followed by extravascular accumulations of these cells. The spleen features accumulations of myeloid tumor cells. A marked myeloid cell leukemia is observed.

ALVs from group J are associated with a variety of other tumors, alone, or in combination with leukosis. These can include fibrosarcoma, chondroma, hemangioma, histiocytic sarcoma, mesothelioma, myxoma, nephroblastoma, and osteoma.

Although tumors only develop in a small percentage of infected birds, subclinical disease featuring lowered egg production and quality in the absence of tumor formation is more common, and more economically important.

Host Response to Infection

Following experimental infection with an ALV-J strain, it has been shown that two antiviral interferon-stimulated genes were expressed at high levels in infected peripheral blood lymphocytes. By 7 days post infection, a CD8+ T-cell response was detectable, while antibody responses were seen from 21 days post infection in some chicks, but at variable levels. As viremia levels decreased from 14 days post infection, and were generally eliminated by 21 days post infection, it is clear that antibodies were not solely responsible for viral clearance. It is more likely that thymus-derived CD8+ T-cells were responsible.

ASLV-infected birds can be classified into four categories: no viremia, no antibody (V-A-), no viremia, with antibody (V-A+), viremia, with antibody (V+A+), or viremia, no antibody (V+A-). Genetically resistant birds and those in a flock free of infection are in the V-A- category. The majority of susceptible and exposed birds are in the V-A+ category. A small proportion, less than 10%, of susceptible and exposed birds are in the V+A- category, and are likely to transmit the infection to chicks. Birds in the V+A+ category are usually those in the process of clearing an acute infection, and are likely to become V-A+. The V+A- birds are usually infected vertically, and immunologically tolerant to the virus, but they can also arise if chicks are infected at a very young age. They are a frequent source of infection for chicks through their eggs.

Laboratory Diagnosis

Although clinical history and presentation, gross pathology, and histopathology can be suggestive of ALV involvement in lymphomas and tumors, laboratory testing is usually required for confirmation. In the laboratory,

serology assays, polymerase chain reaction (PCR), virus isolation, and immunohistochemistry are most frequently used for diagnosis.

Virus isolation is considered as the gold standard test, as all strains of ASLVs can be identified in this way. The most useful samples for this are tumor tissue, serum or buffy coat, cloacal or vaginal swabs, egg albumin, or embryo material. Chicken embryo fibroblast cells free of endogenous ASLVs are used to isolate the virus. Material from the cultures is then examined to detect the presence of ASLV group-specific (p27) antigen. This can be done using the complement fixation test, direct, or indirect fluorescent antibody tests, radioimmunoassay, or ELISA tests. The subgroup classification of ALV isolates can be determined in a number of ways. The viral interference assay can be used to establish the ability of a virus to interfere with focus formation in fibroblast cultures by RSV of known subgroups. Virus neutralization assays, which use different chicken antisera with known subgroup neutralizing activities, can be used to determine a virus's susceptibility to neutralization by each one. Host range assays can be used to categorize isolates according to their ability to be cultured in cells of varying ALV subgroup susceptibilities. Specific PCR assays have also been developed to distinguish the ALV subgroups.

The p27 ASLV antigen can be detected in vaginal or cloacal swabs from chickens or in albumen using ELISA tests. PCR testing can detect integrated virus sequences, whereas RT-PCR can be used to detect viral RNA from infectious virus.

Virus neutralization and antibody ELISA tests are used to detect antibodies against ALV. These are commonly used in surveillance or eradication programs rather than for routine diagnosis.

Treatment and Control

There is no treatment or vaccine available. Control of the infection and disease relies on eradication programs. These are usually carried out in primary breeding stocks, where breeders are screened for virus shedding by testing albumen of eggs. Negative eggs are hatched and the chicks kept in isolation, then being used as the breeders for a leukosis virus-free flock. Flocks entirely free of virus can be generated in this way within three years or less, but some breeders are satisfied just to reduce levels of virus in their flocks.

Genetic factors related to increased resistance to infection of some chickens are being investigated, with the aim of breeding less susceptible birds. The cellular receptors for each virus type have been the primary targets for this approach. The production of transgenic chickens expressing ALV proviral genes including the envelope has also been investigated as an approach to disease control, as these have been shown to block infection by inducing superinfection resistance.

Jaagsiekte Sheep Retrovirus – *Betaretrovirus* Genus

Disease

Ovine pulmonary adenocarcinoma (OPA) is also called "jaagsiekte," after the Afrikaans words for "chase" (jaag) and "sickness" (siekte). This is due to the respiratory signs in diseased sheep resembling an animal out of breath from being chased. OPA was first described in England in 1888, and then in South Africa in 1891. It has also been known as ovine pulmonary carcinoma, or sheep pulmonary adenomatosis. The disease is caused by the JSRV, also called OPA virus. The infection only affects sheep, and sometimes goats.

OPA is always fatal, and initially resembles a nonspecific progressive pneumonia. It is characterized by weight loss and breathing difficulties, sometimes progressing to the production of clear or frothy fluid from the lungs, discharged through the nostrils. Coughing and fever are not usually observed. The disease has a long incubation period, with clinical signs usually observed in adult sheep over two years of age. The sheep may survive for many weeks after the signs of disease appear or may die suddenly within a day or two. The lesions caused by OPA predispose affected animals to secondary bacterial pneumonia, especially pasteurellosis. In some countries, JSRV infection has been implicated in almost 70% of all tumors found in sheep.

OPA, and JSRV infection have proved to be useful models for investigations of human lung carcinogenesis.

Etiologic Agent

Classification

JSRV is a member of the Retroviridae family, Orthoretrovirinae subfamily, and *Betaretrovirus* genus. In addition to the exogenous and transmissible form of JSRV, there is also an endogenous form (enJSRV), found in 20 or more copies in the genome of all sheep and goats.

Physical, Chemical, and Antigenic Properties

The structure of JSRV is inferred to be similar to that of other betaretroviruses, with a capsid surrounded by an envelope containing glycoprotein spikes.

Infectivity for Other Species and Culture Systems

Although explant cultures of tumor tissues from young lambs have been used to replicate JSRV for a short period, no susceptible cell culture system has yet been developed. *In vivo*, sheep are the primary host for the virus, but goats can also be infected.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Although OPA has not been found in Australia or New Zealand and has been eradicated from Iceland, it is otherwise found worldwide. Accurate data for national prevalence and distribution are largely unavailable, with OPA likely under-reported, but infection rates are thought to vary between 1% and 20% in many countries.

The reservoir for JSRV is sheep, many of which are asymptomatic.

JSRV is transmitted mainly through respiratory aerosols from infected animals. Milk and colostrum have also been shown to be a source of infectious virus, confirming that suckling is another natural route of transmission for JSRV.

Pathogenesis and Pathology

JSRV infects lung epithelial cells as well as some lymphocytes and myeloid cells expressing hyaluronidase 2 as a receptor. Expression of the transmembrane portion of the viral envelope protein has been shown to activate signaling cascades that promote cellular proliferation and subsequent transformation of the cells. The resulting tumor cells proliferate along the alveolar walls before eventually spreading to the local lymph nodes. The tumors exist as small nodules or as large solid areas involving the ventral parts of one or more lobes of the lung (see Figure 65.5). The uncontrolled proliferation of columnar-shaped type II pneumonocytes in the bronchioles (Clara cells) leads to the

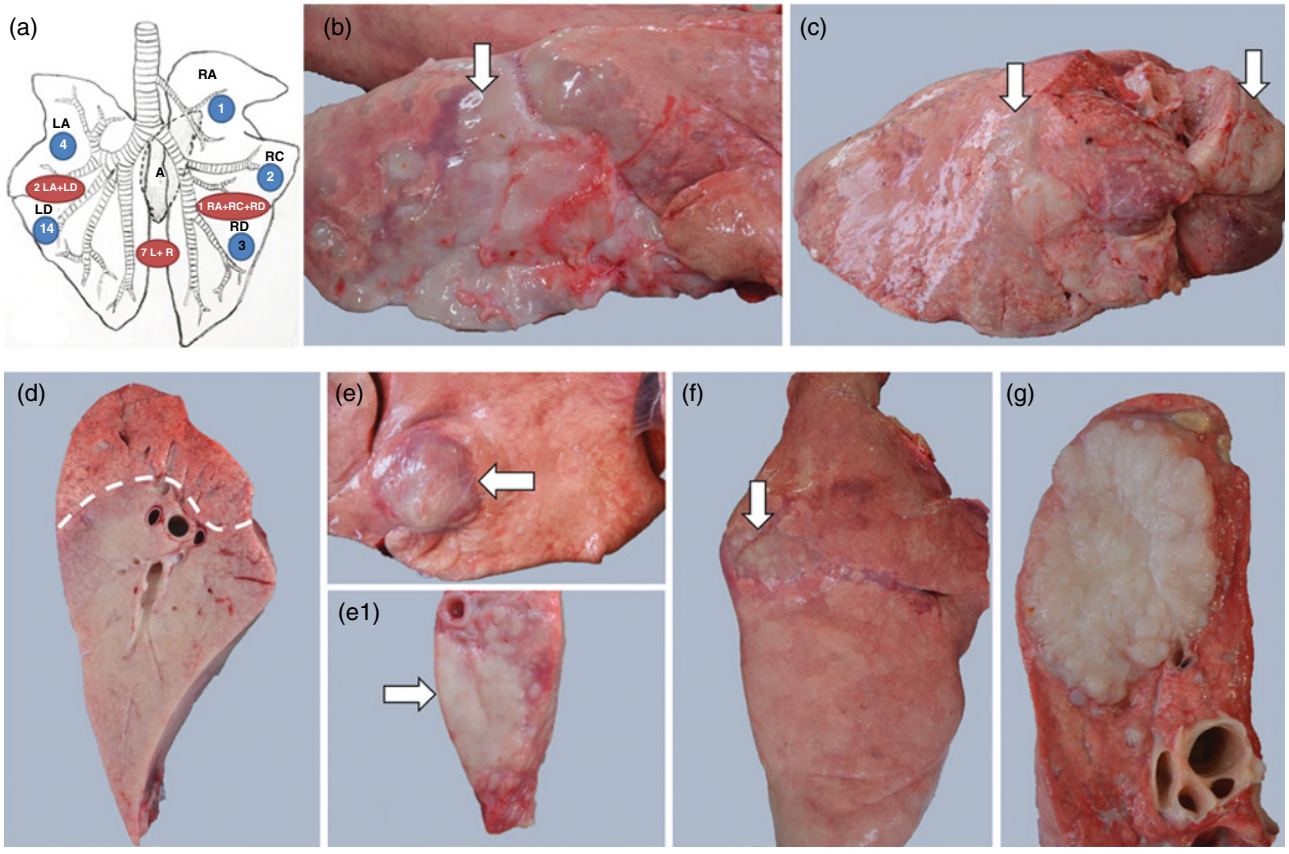


Figure 65.5 Gross features of spontaneous OPA in sheep. (a) Distribution of pulmonary lesions. (b) Classical form of OPA, involving the diaphragmatic lobe (arrow). (c) Classical form of OPA, affecting multiple pulmonary lobes (arrows). (d) Classical form of OPA on cut surface, showing the junction (interrupted line) between the tumor and normal tissue. (e and e1) Atypical form of OPA, showing a subpleural white-gray nodule (arrow). On the cut surface, the tumor is pearly white and dried (arrow). (f) Gross aspects of pulmonary myxoma-like nodule (arrow). (g) The myxomatous mass shows a multilobular, white-gray, gelatinous feature on the cut surface. *Source:* Reproduced with permission from Toma et al. (2020), BioMed Central Ltd.

observed histology changes. As the tumor expands, it begins to interfere with normal respiration and fluid accumulates in the lung, eventually leading to overt clinical signs of disease. The tumor mass may eventually fill 60% of the lung volume. In the terminal stages of disease, large volumes of fluid may flow freely from the nostrils when the head is lowered. The "wheelbarrow test" consists of raising the hindquarters of a sheep while the head is lowered to evaluate the fluid exudate. A proportion of animals with advanced disease will not exhibit this characteristic production of fluid, but these still serve as effective sources of infection for other animals, as they do shed virus.

Host Response to Infection

Notably, there is no detectable immunologic response at any stage following JSRV infection. This is most likely explained by the establishment of immune tolerance due to the presence of endogenous JSRV in the animals. Expression of proteins from the endogenous virus in the fetal thymus at the time of T-cell development and the downregulation of major histocompatibility class-I expression in tumor cells have both been suggested as mechanisms for this immunologic tolerance.

Laboratory Diagnosis

JSRV infection fails to elicit a specific immune response in sheep, possibly due to the expression of very closely related proteins from the endogenous virus in fetuses. Serology testing for diagnosis is therefore not an option. Although PCR testing to detect viral nucleic acid is possible, the small number of infected cells circulating in the blood leads to a low sensitivity of PCR for diagnosis. Although bronchoalveolar lavage fluid is a better test sample for PCR, it is not practical for diagnostic testing. Diagnosis therefore relies on clinical and pathological findings, with the possibility of postmortem laboratory confirmation by histopathological examination of affected lung tissue.

Treatment and Control

There is no effective treatment for OPA disease or JSRV infection. Antibiotic treatment can be used for sheep with secondary bacterial infection. Effective control of the infection is hampered by the lack of practical diagnostic tests. Standard biosecurity protocols usually rely on screening of animals for infection prior to bringing them on farm, but this is more difficult for OPA. Instead of using laboratory tests for such screening, it is instead carried out on the basis of weight loss and respiratory signs in the animals, thereby unavoidably excluding some uninfected animals. Alternatives continue to be explored, including ultrasound screening, to allow more accurate and earlier identification of OPA lesions in infected animals. Maintaining closed flocks reduces the chance of infection. Regular inspection of adult sheep is recommended for affected flocks, with any sheep exhibiting weight loss and respiratory disease being isolated and veterinary advice sought. It has been observed that keeping sheep in single-age groups is effective in reducing the incidence of clinical disease. As the main route of transmission of the virus is through aerosols, with close contact during housing or trough feeding increasing the rate of transmission, indoor housing of sheep must be considered a risk factor. In the United States, this disease is reportable in many states.

Mason-Pfizer Monkey Virus – *Betaretrovirus* Genus

Disease

The Mason-Pfizer monkey virus (MPMV) was discovered in 1970 in breast tumor tissue from a rhesus macaque (*Macaca mulatta*). It is categorized as a simian retrovirus serotype 3, and is representative of the other viruses in the genus. Simian retrovirus serotype 1 (SRV-1) was first identified in the early 1980s at the National Primate Research Center in California in a rhesus macaque. Simian retrovirus serotype 2 (SRV-2) was first discovered in endemic infections of pig-tailed monkey (*Macaca nemestrina*), cynomolgus macaques, and a Japanese macaque (*M. fuscata*) at the research center in Washington, as well as in rhesus and Celebes black macaques (*M. nigra*) at the Oregon research center. Serotype 4 (SRV/D-Tsukuba) was described in Japan in 2010 from cynomolgus macaques and then at the University of California. A further subtype, serotype 5, was identified at the Beijing Primate Center. SRV-6 was identified in Indian langurs (*Semnopithecus entellus*) and SRV-7 in Indian rhesus monkeys.

The viruses cause an often fatal immunosuppressive disease in Asian macaques. Infected animals develop a wasting disease followed by opportunistic infections. Clinical manifestations include fever, weight loss, diarrhea, pneumonia, enteritis, and rashes, as well as anemia, lymphopenia, granulocytopenia, and thrombocytopenia.

Etiologic Agent

Classification

MPMVs are members of the Retroviridae family, Orthoretrovirinae subfamily, and *Betaretrovirus* genus. They include the Human type D retrovirus, Simian

endogenous retrovirus, Simian endogenous retrovirus Vero ATCC CCL-81, Simian retrovirus 1, Simian retrovirus 2, Simian retrovirus srv-2 (isolate 2R-18B1), Simian SRV-like type D retrovirus, Simian type D retrovirus -Tsukuba, and unidentified Simian type D retrovirus.

Physical, Chemical, and Antigenic Properties

The MPMV is enveloped, about 125 nm in diameter, with an icosahedral capsid. The viral envelope is made up of a lipid bilayer derived from host cell and virus-encoded proteins. Two types of virus particles are recognized. A-type particles are found in the cytoplasm, and are small, ring-shaped structures that are non-infectious. D-type particles are found extracellularly, having matured and become infective.

Resistance to Physical and Chemical Agents

In common with other enveloped viruses, MPMVs are inactivated by lipid solvents and detergents. The viruses are also sensitive to high temperatures.

Infectivity for Other Species and Culture Systems

At least eight different species of macaques are susceptible to infection with MPMVs. Although *in vitro* studies have shown a theoretical possibility of zoonotic transmission of SRV-4 infection from Japanese macaque cells to human cells, this has not been demonstrated *in vivo*. Screening of humans for MPMVs has shown that these infections are very rare or nonexistent in the population. Serological surveys have shown only limited reactivity against MPMVs in human sera.

MPMV strain isolates replicate effectively in primary cells, including both T- and B-lymphocytes and macrophages. A variety of human and nonhuman primate cell lines of T- and B-lymphocytes, macrophages, and fibroblasts support the culture of MPMVs.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

MPMV and other SRVs are prevalent among wild Asian and colony-born macaque monkeys, including cynomolgus and rhesus macaques. Endogenous SRVs are found in both New and Old World monkeys, including African baboons as well as Asian langur and South American squirrel monkeys. African monkeys are not naturally infected with exogenous virus, however. Up to 90% of some wild and captive nonhuman primate populations are thought to be infected, but the prevalence varies widely based on the location and the species studied.

The viruses are secreted in the blood, urine, saliva, and lacrimal secretions of infected animals, including healthy carriers. Biting, scratching, grooming, and fighting, with resultant inoculation of saliva or blood are the most frequent methods of transmission. Although blood from viremic animals carries the highest risk of infection, blood from non-viremic animals with integrated provirus in cells can also transmit infection. Contaminated instruments or equipment can also spread this virus among animals. Vertical transmission has also been shown to take place, with transplacental transmission in females that are viremic during pregnancy.

Pathogenesis and Pathology

Various outcomes are possible following MPMV infection, including viremia with clinical disease, a transient viremia and quick recovery, an intermittent viremia followed by latency, or a persistent viremia with virus shedding leading to an asymptomatic carrier state. The viruses exhibit a broad cellular tropism including T- and B-lymphocytes as well as macrophages in the lymph nodes, spleen, and thymus, and the epithelial cells of the gastrointestinal tract, salivary glands, and choroid plexus of the brain.

Lymphoid hyperplasia is usually seen in acute infections, whereas a marked lymphoid depletion in the lymph nodes is observed in the terminal phases of fatal disease. Anemia and/or neutropenia are frequently observed, possibly due to apoptosis of infected hematopoietic stem cells. Retroperitoneal fibrosis (fibromatosis) and B-cell lymphomas are seen in cases of coinfection with MPMVs and a herpesvirus. The immunosuppression in infected animals is linked to a high incidence of subcutaneous fibrosarcomas, and coinfections with other pathogens. Lesions are often seen in the gastrointestinal tract, including goblet cell hyperplasia, villous blunting, and enteritis.

Host Response to Infection

The host immune response to MPMV infection includes the development of neutralizing antibodies. Animals with a strong antibody response often show a reduced viremia, while many of the viremic animals are seronegative.

Laboratory Diagnosis

Testing for MPMV or other SRV infections can be carried out by virus isolation, antibody screening (ELISA or Western blotting), or PCR. As not all infected animals produce detectable levels of antibodies or at least exhibit a considerable delay between infection and seroconversion, parallel testing for both antibodies and virus is

recommended. Serological results are not always simple to interpret, and significant levels of false positive and false negative results have been reported. As antibody levels can vary inversely with viral titers, asymptomatic animals with low or undetectable antibody levels sometimes exhibit high levels of viremia. PCR testing is now commonly used for screening or diagnosis. Some PCR assays differentiate the various subtypes of virus.

Treatment and Control

There are concerns over the inadvertent use of MPMV or other SRV-infected animals in biomedical research, and the potential for this to affect study results. As a result, and due to continuing infections in recently imported macaques, there is a drive to establish and maintain specific retrovirus-free breeding colonies. Of course, control of MPMV and SRV infections also improves the welfare of the animals, and potentially improves safety for researchers and animal handlers by reducing the risk of transmitting infection to humans. The prevention of further spread of viruses in group-housed monkeys by identifying affected animals has been aided by the use of sensitive and specific PCR assays in systematic screening protocols.

Research studies have shown that some nucleoside/nucleotide RT inhibitors such as zidovudine, zalcitabine, and 2′,3′-deoxyadenosine and some HIV-1 integrase strand transfer inhibitors including elvitegravir and raltegravir are active against MPMV and other SRV infections, at least *in vitro*.

A variety of experimental vaccines have been developed against MPMV and other SRVs. A formalin-inactivated vaccine against SRV-1 produced in human cells has been shown to protect macaques against lethal challenge. Another experimental vaccine against SRV-2 based on recombinant vaccinia virus expressing SRV-2 envelope glycoprotein was shown to protect against lethal challenge, in correlation with the generation of neutralizing antibodies. Additional experiments with recombinant vaccinia virus expressing SRV-1 or MPMV gp70 and gp20 proteins also showed protection against homologous challenge, but not against the more divergent SRV-2 virus. These vaccines remain experimental, however.

Feline Leukemia/Sarcoma Virus – *Gammaretrovirus* Genus

Disease

Feline viral leukemia is caused by the Feline leukemia virus (FeLV). This was the first feline retrovirus to have been discovered, being identified in a cluster of cats with lymphosarcoma in the early 1960s. This gammaretrovirus is one of the most important pathogens of cats worldwide. FeLV should be considered a differential for any cat with nonspecific clinical signs.

Infection with FeLV most often results in regressive or abortive infection, but sometimes in progressive infection, and very rarely in focal infection. Infection may cause a variety of clinical signs associated with several fatal syndromes. The most common outcomes of progressive infection are immune suppression, bone marrow suppression, and lymphoma. Although the prognosis following progressive infection is usually poor, some animals remain healthy for years prior to developing clinical disease. The age at which cats become infected influences the disease outcome, as do the virus strain and the level of early immune response. Experimentally, FeLV-related disease was shown to develop in almost all cats infected as newborns, in 85% of cats infected between the ages of 2 weeks to 2 months, and in just 15% of cats infected between the ages of 4 and 12 months. The risk of disease from exposure of adult cats is therefore much lower than for kittens.

Immunosuppression is a feature in all cats with progressive FeLV infection. Clinical signs may not be evident, but may include thymic atrophy, lymphopenia, neutropenia, impaired neutrophil function, and the loss of T-lymphocytes. Secondary infections are thus more likely in FeLV-infected cats.

Nonregenerative anemia is another common feature of FeLV progressive infection, resulting from chronic inflammatory responses, bone marrow suppression, myeloproliferative disease, thrombocytopenia, or neutropenia.

The development of various types of tumors is associated with FeLV infection, including leukemia and lymphoma, as well as other non-hematopoietic malignancies.

Other more unusual clinical presentations of disease include chronic enteritis due to the degeneration of intestinal epithelial cells and crypt necrosis, inflammatory and degenerative liver disease, fading kitten syndrome, and reproductive disorders such as fetal resorption, abortion and neonatal death, and neurological syndromes presenting as anisocoria, mydriasis, Horner's syndrome, urinary incontinence, abnormal vocalization, hyperesthesia, paresis, or paralysis.

Etiologic Agent

Classification

FeLV is a member of the Retroviridae family, Orthoretrovirinae subfamily, and *Gammaretrovirus* genus. Six subgroups have been proposed, based on viral interference and antibody neutralization tests as well as genetic similarities: A, B, C, D, E, and T.

In addition to the exogenous and transmissible form of FeLV, there is also an endogenous form, which is a replication-defective provirus found in domestic cats. The endogenous and exogenous forms are 86% similar in their nucleotide sequence, with the majority of the differences being found in the LTR and env regions. Expression of provirus DNA from the endogenous form is restricted to the subgenomic transcripts, thereby preventing the assembly of infectious virions.

Further, the Feline sarcoma virus (FeSV) is a result of recombination between FeLV and proto-oncogenes from the cat genome. This hybrid virus lacks the FeLV *pol* gene and part of the *env* gene, thus being replication defective, but highly oncogenic. FeSV is not thought to be transmissible.

Physical, Chemical, and Antigenic Properties

FeLV is spherical, 80–100 nm in diameter, consisting of an envelope, nucleocapsid, and nucleoid. The virions have small distinctive glycoprotein spikes that evenly cover the surface of the virion.

Resistance to Physical and Chemical Agents

In common with other enveloped viruses, FeLV is sensitive to inactivation by lipid solvents and detergents. The virus is inactivated within 1–2 hours in the environment, and is very rapidly inactivated by heating to 56 °C.

Infectivity for Other Species and Culture Systems

In addition to domestic cats, some other members of *Felidae* can also be infected with FeLV, including guignas, the lynx, jaguars, and the Florida panther. Zoonotic transmission to humans has never been observed.

FeLV-A has only been cultured in feline cells. FeLV-B and FeLV-C, however, have been cultured in a range of cells including human, canine, and porcine as well as feline.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

FeLV infections occur worldwide, with prevalence rates in the United States between 2% and 3%. The prevalence, particularly in Europe, has decreased considerably in recent years with the increased use of vaccines, as well as testing and segregation procedures.

Viremic cats are the source of infection, with shedding in saliva, tears, nasal secretions, feces, urine, and milk. Transmission occurs mainly through grooming activities or bites. Risk factors are young age, high population density, and poor hygiene.

Pathogenesis and Pathology

Upon contact with oral, ocular, or nasal membranes, FeLV initially replicates in oropharyngeal lymphoid tissue. It is then transmitted by infected blood mononuclear cells to cells in the lymph nodes, spleen, epithelial cells of the intestine and bladder, salivary glands, and bone marrow. Viral replication in the bone marrow takes place in neutrophils and platelet precursors, resulting in a secondary viremia and systemic infection. FeLV is then also found in secretions from these tissues as well as in peripheral blood leukocytes and platelets. Acute disease is seen two to six weeks following infection, with fever, malaise, lymphadenopathy, and lymphopenias. In the absence of an effective immune response, animals then become persistently viremic, leading to the progressive phase of disease. This prolonged viremia leads to infection of the salivary glands and intestinal linings, with large amounts of virus then being secreted in saliva, feces, urine, and milk. In these cases, the typical clinical signs of FeLV infection may not manifest until many months or even years of viremia.

There are four recognized presentations of FeLV-induced disease: feline lymphosarcoma, feline myeloproliferative diseases and anemia, immunopathologic disease, and feline fibrosarcoma. Lymphosarcoma usually originates in T-lymphocytes, and may be multicentric, thymic, alimentary, or unclassified. It usually presents as firm, homogenous pale tissue. Myeloproliferative diseases and anemia result from the viral transformation of cells in the bone marrow, including erythroid progenitors, granulocytic myeloid progenitors, granulocytic myeloid and erythroid progenitors, and fibroblasts. Accordingly, erythremic myelosis, granulocytic leukemia, erythroleukemia, or myelofibrosis may develop, leading to the presence of large numbers of neoplastic cells in the bone marrow, additional cytopenias, a nonregenerative anemia, and immunosuppression. Immunopathologic disease may result from the accumulation of FeLV antigen/antibody complexes in glomerular capillaries, inducing glomerulonephritis. Lymphocyte depletion can result from antibody-dependent cytotoxicity targeting FeLV antigens on lymphocyte surfaces. The emergence of FeSV as a result of FeLV infection can lead to the development of fibrosarcoma, with frequent metastases.

Host Response to Infection

The progression of FeLV infection in cats is largely dependent on the immune response to that infection, which in turn depends on the age of the animal, the exposure dose, and the virus strain. The antibody responses primarily target the major envelope glycoproteins of the virus. Abortive

infection occurs following infection with a low dose of FeLV or following a rapid, effective, and specific immune response. Regressive infection occurs in the context of an effective immune response later in the infection, around the time of FeLV infiltration into bone marrow. Animals with regressive infection are viremic for a period of time (weeks to months), after which it is controlled and virus is no longer detectable in blood, although it may remain as a provirus in some tissues, and therefore lead to positive PCR results. Focal infections are characterized by virus replication in specific tissues, such as the eyes or bladder, with only low levels of viral antigen being produced, leading to variable results on diagnostic testing. Progressive infection is the result of an insufficient immune response. Cats with progressive infection are continuously viremic and develop FeLV-associated diseases. As a result of infection, the numbers of circulating neutrophils and lymphocytes may be reduced, and the remaining cells may be dysfunctional. Complement levels in infected cats are often lower than normal, leading to further immunodeficiency. In the presence of low antibody levels and high viral antigen levels, the formation of immune complexes is encouraged, also leading to the development of immune-mediated disease.

Laboratory Diagnosis

The most commonly used diagnostic tests for FeLV infection are the ELISA, the immunofluorescent assay (IFA), and PCR. Virus isolation is possible as well, but rarely used. All of these tests are designed to detect viral antigen or nucleic acid rather than antibodies. The ELISA tests use blood or serum samples, detecting FeLV proteins, usually p27. Saliva or eye secretions are sometimes used for testing, but are not considered reliable samples. The ELISA will not detect regressive infections. A positive ELISA result does not differentiate between acute or persistent infection. IFA testing is carried out on blood samples and detects structural proteins of the virus in lymphocytes. It will not give a positive result for transient, primary infections, but only for persistent infections. However, both false positive and false negative results are possible with IFA. PCR testing is usually carried out on blood, but bone marrow, and other tissues can also be used. Because of its sensitivity, PCR testing can detect FeLV regressive infections, and is often used to resolve conflicting ELISA or IFA results. Any kitten that tests positive by ELISA should be retested by ELISA after a period of three to six months to confirm a progressive infection, or by PCR to detect a regressive infection. A negative ELISA and negative PCR result from blood following a previously positive ELISA result suggest abortive infection (or in rare cases, focal infection). An algorithm for diagnostic testing or suspect retroviral infection in cats is shown in Figure 65.6.

Additional diagnostic approaches are used, such as cytology and histopathology analyses on samples from tumors, bone marrow, lymph nodes, or body cavity fluids, to identify malignant lymphocytes. Blood cell counts can reveal changes such as leukopenia, or decreased packed cell volume and total protein levels resulting from anemia.

Treatment and Control

At present there is no effective cure for FeLV infection. Infected cats may live for years without major disease complications with routine prophylactic care, good husbandry, minimal stress, and avoidance of secondary infections. The efficacy of antiviral drugs is limited, and they are often associated with severe side effects, so are not usually used. When needed, treatment is targeted to specific problems such as secondary bacterial infections or severe anemia, and is supportive in nature.

Control of the disease is linked to the prevention of infection resulting from close contact with FeLV-infected cats, or shared items such as litter trays or food/water containers. Screening cats for FeLV infection prior to bringing them into a shared home or space is important in this context. Separation of infected and non-infected cats is the most reliable way to eliminate the potential for FeLV transmission.

Effective vaccines of various types are available against FeLV, but do not offer complete protection against infection. Among the potential side effects of vaccination is the development of vaccine-associated sarcomas at the injection site, leading to efforts to develop effective vaccines without adjuvants. This is one of the core vaccines in the United States for kittens and cats under a year old, with annual boosters recommended for high-risk animals such as those that have access to the outdoors and those in multi-cat households, potentially living with an infected cat.

In catteries, control of FeLV infection is best accomplished through regular test and removal procedures, combined with vaccination.

Bovine Leukemia Virus – *Deltaretrovirus* Genus

Disease

The first report of bovine leukosis was made in 1871, describing the presence of yellow nodules in the enlarged spleen of cattle. The majority of cattle infected with Bovine leukemia virus (BLV) remain asymptomatic. In 10–20% of infected cattle, a persistent lymphocytosis will develop. In just 5% of

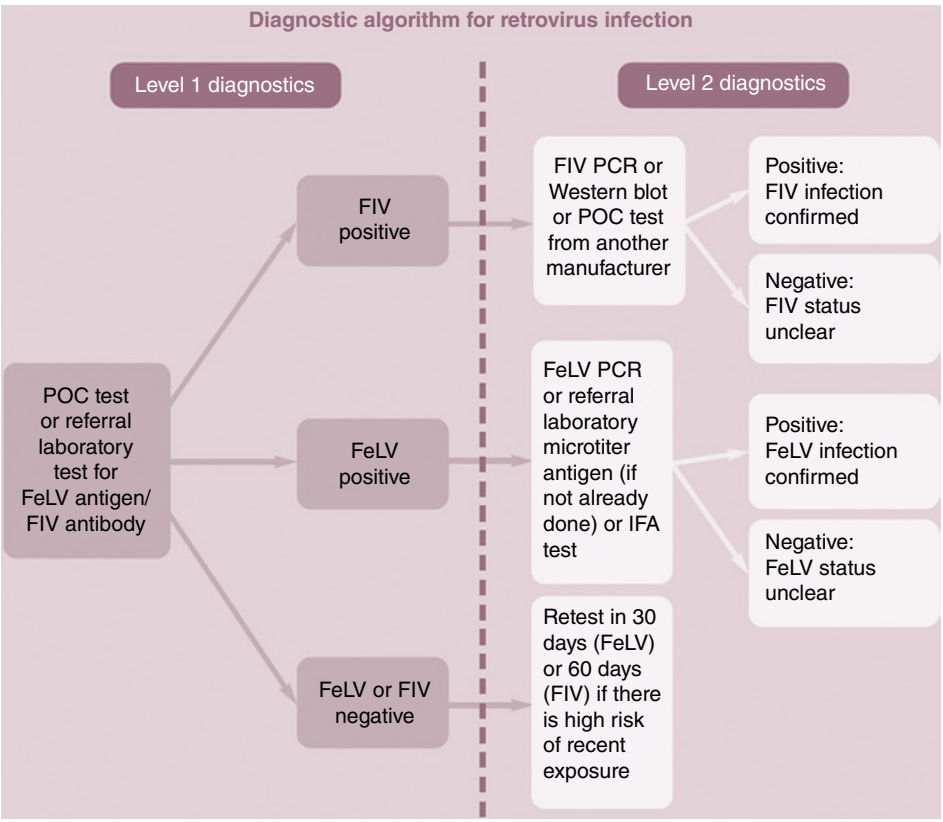


Figure 65.6 Diagnostic algorithm for feline retrovirus infection. Level 1 diagnostics might be sufficient in circumstances where the test results are consistent with the clinical signs. Level 2 diagnostics are appropriate to clarify the infection status in other cases. This diagnostic algorithm will correctly identify the true infection status for most cats. Regressive FeLV infections, recent exposure, atypical responses, and changes in the immune response over time complicate the interpretation and reliability of tests performed at a single timepoint. *Source:* Reproduced with permission from Little et al. (2020).

infected animals, usually 4–8-year-old cattle, lymphosarcoma will develop, termed enzootic bovine leukosis (EBL). The tumors may be found in the uterus, abomasum, heart, spinal canal, lymphoid tissue behind the eye, or blood, resulting in quite diverse clinical signs including fever, loss of appetite, weight loss, lymphadenopathy, lymphoid leukemia, respiratory distress, tachycardia, bloat, jugular vein distention, edema, decreased milk production, infertility, rear limb weakness, or paralysis, and protruding eyeballs. Although both beef and dairy cattle are susceptible to infection with BLV, EBL is much more common in dairy animals, due to the specific management practices in dairy farms.

Etiologic Agent

Classification

BLV is an exogenous virus in the *Deltaretrovirus* genus of the Retroviridae family, closely related to the Human and Simian T-cell leukemia viruses (HTLV I to III and STLV). BLV may integrate into the genomic DNA of B-lymphocytes as a provirus, or may exist in unintegrated forms.

Physical, Chemical, and Antigenic Properties

The BLV genome is just over 8700 nucleotides in length, and in common with other retroviruses, includes the *gag*, *pro*, *pol*, and *env* structural genes. There are additional genes encoding four regulatory proteins: the transactivator protein Tax, the Rex protein, and two accessory proteins R3 and G4.

Resistance to Physical and Chemical Agents

BLV is readily inactivated by most commercial disinfectants, including bleach, alcohol, and those containing detergents. Colostrum can be heat treated (low temperature pasteurization, 63 °C for 30 minutes) to inactivate BLV. Standard pasteurization of milk is also effective to inactivate the virus.

Infectivity for Other Species and Culture Systems

Natural infection with BLV only occurs in cattle (*Bos taurus* and *Bos indicus*), water buffaloes, zebu, yak, and capybaras. Experimental infections have been carried out in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats, and buffaloes. Most of

these are inapparent, resulting only in an antibody response. Infected sheep, however, develop tumors more often than cattle. Some rabbits have been shown to develop a fatal AIDS-like disease following BLV infection.

BLV can be cultured *in vitro* in cells from a wide variety of species, including cattle, human, simian, dog, goat, and horse.

The zoonotic potential of BLV is controversial, with varying results from several studies. The presence of BLV or BLV-infected cells in the milk of most naturally infected cows provides an opportunity for exposure to the virus for humans consuming non-pasteurized milk or milk products. Many studies have therefore targeted farmers and butchers, based on their potentially higher rates of exposure. Efforts to detect anti-BLV antibodies in humans were initially negative, but recent more sensitive tests have shown potentially significant rates of seropositivity, although cross-reactivity with anti- HTLV-1 and 2 antibodies cannot be ruled out. It has been suggested that antibodies could be generated in response to the consumption of beef or dairy products, even when any associated BLV has been denatured by heat. Some studies have identified an association between the presence of BLV in breast tissue and breast cancer, whereas other studies have failed to substantiate this link. The potential for BLV involvement in human disease thus remains unresolved.

BLV can be cultured on sheep and cattle cells of the monocyte/macrophage lineage in various stages of differentiation. Initial isolation is usually by co-cultivation of peripheral leukocytes from BLV-infected animals with a cell line such as sheep kidney cells. The formation of syncytia and cytoplasmic vacuolization is usually observed in infected cultures.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Although BLV has been present worldwide, many countries have introduced eradication programs, with Denmark, the United Kingdom, Australia, and New Zealand being particularly successful. The prevalence of BLV infection therefore now varies from country to country, with generally high levels in Eastern Europe and North and South America, and lower levels in Africa, the Middle East, Australasia, and Asia. Prevalence increases with age, and tends to be higher in dairy herds, increasing in line with the size of the herd. Rates of infection can be as high as 88% of the dairy herds, and 43% of dairy cows being infected, and 38% of beef herds and 10% of beef cattle. A 2018 study in the United States showed that 94.2% of dairy herds had at least one BLV antibody positive cow detected, whereas the average within-herd prevalence was 46.5%.

Transmission of BLV is primarily through the transfer of cells from infected animals in material such as blood, colostrum, and milk. Horizontal transmission of BLV is linked to procedures such as vaccination, dehorning, tattooing, and castration. Transmission by biting insects has also been documented, for example, by horn flies. Vertical transmission has been demonstrated perinatally and postnatally. Perinatal infection can occur both *in utero* and during birth. Contaminated placental and cord blood has been implicated, as well as colostrum and milk (particularly bulk milk). Conversely, colostrum containing antibodies against BLV can also protect calves from infection. Some sources of commercial colostrum replacer have been shown to contain antibodies against BLV as well as BLV genetic material, and its consumption has been associated with seropositivity of calves, potentially confounding screening and diagnostic testing programs.

BLV-infected cattle with virus-induced persistent lymphocytosis are major reservoirs of the virus and pose the greatest risk for transmission.

Pathogenesis and Pathology

The principal targets of BLV infection are B-lymphocytes, but monocytes, some T-lymphocytes, and granulocytes can also be infected. The initial viremia is relatively short-lived, as the cellular and humoral immune responses rapidly reduce the viremia and proviral load. Further spread of the infection is largely through clonal expansion of infected host cells, with very limited viral protein or RNA expression. These infections are most often asymptomatic.

A proportion of infected animals go on to develop a persistent lymphocytosis, due to a permanent and relatively stable non-malignant proliferation of untransformed B-lymphocytes, usually in the CD5+ IgM+ subset. During persistent lymphocytosis, animals may suffer from immunologic dysfunctions, sometimes leading to opportunistic infections such as mastitis.

In a small percentage of infected animals, following a period of latency of at least a year and up to eight years, a malignant B-cell lymphosarcoma will develop. The resulting lesions may either appear as firm pale tumor masses or as a diffuse tissue infiltrate. A range of organs and tissues may be affected, including visceral and peripheral lymph nodes, the abomasum, heart, intestines, liver, spleen, uterus, kidneys, or spinal cord. Histologically, the tumor masses comprise densely packed, monomorphic lymphocytic cells. Clinical signs will vary greatly, depending on the organs affected.

Host Response to Infection

Following infection with BLV, cattle, and sheep respond by producing antibodies, primarily targeting the structural

envelope gp51 and capsid p24 proteins. This response persists throughout the animal's life, with evidence of the ongoing stimulation of the immune system by BLV antigens. In some animals, BLV infection can lead to immune suppression through disruption of proper immune cell signaling molecules and cytokine production, disturbance of immune cell proliferation and apoptosis and typical lymphocyte ratios, and killing of activated, infected cells. A reduction of IgM-producing cells in the spleen and lymph nodes has been noted, with consequently lower levels of circulating IgM and a decrease in CD4+ T-lymphocyte numbers in peripheral blood. Overall, BLV-infected animals tend to show a diminished response to other infections.

Laboratory Diagnosis

Diagnosis of BLV infection may rely on the detection of BLV antigens or antibodies against the virus. In terms of disease, persistent lymphocytosis is identified through hematology tests, whereas lymphosarcoma is identified through histological examination of tissue biopsies.

Serology is most often carried out to diagnose BLV infection, using either an agar gel immunodiffusion (AGID) test (where available, and as required by some countries for import/export testing) or an ELISA test. Antibodies usually become detectable from 3 to 12 weeks post infection and remain so for the life of the animal, with antibodies against gp51 appearing first, followed by those against p24. Serological testing is not reliable in calves under six months of age that have ingested colostrum or milk, as maternal antibodies will be detectable.

PCR testing is used to detect proviral DNA of BLV in peripheral blood lymphocytes. This is a useful in detecting recent infections prior to seroconversion, and infections where maternal antibodies may confound serological testing.

Treatment and Control

There is no treatment for BLV infection, and once infected, animals remain so for life. One compound, valproate (a lysine deacetylase inhibitor), has been shown to lower the provirus load, and to induce regression of leukemia/lymphoma in some cases, but there are reported issues with chemoresistance.

In the absence of an approved vaccine against BLV infection, management practices are required to control the infection. They include the identification of BLV-infected cattle followed by either slaughter or segregation of positive reactors. It is then possible to manage infected and non-infected animals separately to prevent potential exposure of non-infected animals to the virus. Establishing processes to prevent the transfer of blood from infected animals to naive animals is essential in any control program. These include cleaning and disinfection of equipment and facilities. The control of biting insects is recommended to help minimize the spread of BLV between animals. Research is underway to evaluate the potential of controlling BLV infection by genetic selection of resistant cattle.

Walleye Dermal Sarcoma Virus – *Epsilonretrovirus* Genus

Disease

Walleye dermal sarcoma (WDS) was first described in 1969 in walleye (*Sander vitreus*) from Oneida lake in the state of New York, and has since been reported in other parts of North America. The disease is caused by Walleye dermal sarcoma virus (WDSV).

WDS is characterized by warty growths, gray-white, or pinkish in color, on the skin and fins of fish. The disease is not normally fatal to fish. Unusually, the disease appears to be seasonal, with lesions rarely observed in the summer, but with a high incidence in the late fall through to the spring.

Etiologic Agent

Classification

WDSV belongs to the genus *Epsilonretrovirus* in the family Retroviridae. It is one of three currently recognized epsilonretroviruses.

Physical, Chemical, and Antigenic Properties

WDSV virions are spherical and enveloped, with a diameter of 80–100 nm. In addition to the traditional retrovirus *gag*, *pro*, *pol,* and *env* genes, three additional open reading frames, called *orf a*, *orf b,* and *orf c*, are present, encoding viral accessory proteins. These have been found to regulate host and viral gene expression, promoting cell proliferation, blocking apoptosis, and later inducing apoptosis by altering the mitochondrial membrane potential.

Infectivity for Other Species and Culture Systems

Dermal sarcoma can be experimentally transmitted to several perch species, sauger (*Stizostedion canadense*), and yellow perch (*Perca flavescens*). There is no association of WDSV with disease in humans.

In vitro culture systems are very limited. Production of WDSV has been restricted to explant cultures of naturally infected walleye cells from tumors and the infection of walleye fibroblast cells with virus from tumors.

Examples include the W12 walleye fibroblast cells and the WC1 line, neither of which are productively infected. More recently, a virus-positive culture of spring tumor explant cells (STECs) has been established that does produce WDSV.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

WDS is found predominantly in the upper Midwest and Great Lakes region, and throughout Minnesota. It has been reported that up to 27% of walleye are affected with WDSV annually in Oneida lake. WDSV infection has only been found naturally in walleye. There is no other known reservoir.

The virus spreads through contact with water containing infectious virus or by direct contact between fish, for example, during the spring spawning run when fish congregate in high numbers.

Pathogenesis and Pathology

WDS presents as cutaneous mesenchymal neoplasms 0.2–1.0 cm in diameter, on the surface of the scales throughout the fish's epidermal surfaces. The tumors often coalesce as they expand. They are made up of non-encapsulated nodular masses fibroblasts. Fall tumors are highly vascularized due to a network of capillaries, whereas regressing spring tumors are usually pale with ulcerated surfaces. Perivascular accumulation of lymphocytes and aggregates of inflammatory cells within the dermis surrounding the mass is sometimes seen. Invasive tumors are very rare. Figure 65.7 shows an example of the gross pathology of the cutaneous tumors.

Immediately following infection with WDSV, virus expression is limited, associated with tumor progression, but this is followed by a switch to extensive virus expression associated with tumor regression. The fully developed tumor, with high levels of cell-associated virus, is instrumental in virus transmission as the tumor mass is shed into the surrounding water at the time of greatest fish-to-fish contact in the spawning season. Secondary bacterial and fungal infections sometimes develop at the sites of dislodged growths.



Figure 65.7 Gross pathology of tumors from experimental transmission of walleye dermal sarcoma with cell-free filtrate from regressing tumors. An individual tumor and a large mass of coalescing tumors are visible. *Source:* Reproduced with permission from Rovnak et al. (2010).

Host Response to Infection

It appears that fish develop tumors only in one season, remaining tumor-free in following years. This suggests the presence of an immune response, and its action in tumor regression. Early in the infection, WDSV accessory proteins downregulate the viral promoter and the host NF-κB-dependent promoters, helping to limit the innate immune response, and ensure only low levels of virus expression during tumor development. This restricts both humoral and cell-mediated immune responses otherwise associated with virus production. Later in the infection, another WDSV accessory protein is expressed alongside the production of increased levels of infectious virus and the initiation of tumor regression. The host immune response then serves to reject the tumor mass, and subsequently protect against the formation of new tumors.

Laboratory Diagnosis

Histopathology is usually sufficient for diagnosis. Clinically, the regression of the tumors in springtime is also specific for WDS. Definitive diagnosis relies on identification of the virus by RT-PCR, or by electron microscopy.

Treatment and Control

There is no practical method of treatment or control of this disease in the natural environment.

Small Ruminant Lentiviruses (Visna/Maedi/Progressive Pneumonia Viruses and Caprine Arthritis Encephalitis Virus) – *Lentivirus* Genus

Disease

Although it is now recognized that small ruminant lentiviruses (SRLVs) each infect multiple species, they were first thought to be species specific. Maedi was first described as a respiratory disease of sheep in South Africa in 1915. Ovine progressive pneumonia was identified in 1923 in Montana. Visna (wasting) was described in Icelandic sheep in 1933. A viral leukoencephalomyelitis observed in the United States in 1974, and a chronic arthritis in goats were both attributed to the caprine arthritis encephalitis virus (CAEV). Other historical names for the associated

diseases include Montana progressive pneumonia, Marsh's progressive pneumonia, chronic progressive pneumonia, La bouhite, Graff-Reinet disease, and Zwoegersiekte. SRLVs cause progressive multisystemic chronic diseases encompassing wasting, respiratory distress associated with pneumonia, encephalitis, arthritis, and mastitis, thereby affecting both the welfare and the production value of infected animals. Sheep are mainly affected by respiratory and neurological disease, both eventually being fatal. Adult goats are affected by chronic arthritis, whereas kids will develop encephalomyelitis. Mastitis affects all species.

Etiologic Agent

Classification

SRLVs belong to the genus *Lentivirus* in the family Retroviridae. Maedi-visna virus (MVV) was the first lentivirus to be isolated and characterized, in 1957. SRLV strains are classified into four genotypes: A–C and E. Genotypes A and B refer to MVV-like and CAEV-like viruses, respectively. Genotype A is the most heterogeneous group and has been subdivided into at least 20 subtypes. Genotype B comprises three subtypes. SRLVs restricted to specific geographic locations have been assigned to genotype C (two subtypes from Norway), genotype D (from Switzerland and Spain, but now re-assigned to genotype A), and genotype E (two subtypes from Italy). Recombination can occur between different SRLVs, creating new variants.

Physical, Chemical, and Antigenic Properties

SRLV virions are typically between 90 and 120 nm in diameter, with an envelope and an icosahedral capsid core. The envelope comprises a membrane bilayer of host cell-derived phospholipids within which viral glycoproteins are inserted. In addition to the standard retrovirus genes, accessory, or regulatory, genes include *vif*, *vpr-like*, which was initially likened to *tat* in other lentiviruses, and *rev*, which is involved in the regulation of viral expression.

Resistance to Physical and Chemical Agents

SRLVs are similar to other lentiviruses, being susceptible to standard commercial disinfectants. The virus is inactivated in colostrum heated to 56 °C for one hour, and does not survive for more than a few days in the environment, particularly under hot, dry conditions.

Infectivity for Other Species and Culture Systems

SRLVs are associated mainly with sheep and goats. Classical MVV strains occur mainly in sheep, but some other genotype A viruses infect both species. Classical CAEV strains (genotype B), as well as genotype E viruses, are mainly found in goats. Genotype C viruses affect both sheep and goats. This host specificity is not absolute however, with some strains having been shown to transmit from one species to the other. The genetic background of the animals is important, with some breeds of sheep being particularly susceptible to infection, including the Icelandic sheep. Wild species of small ruminants including wild ibex (*Capra ibex*), Rocky Mountain goats (*Oreamnos americanus*) and Mouflon (*Ovis gmelini*) are also susceptible to infection with SRLVs.

Wild relatives of small ruminants can be infected by SRLVs from domesticated animals. One virus, thought to be CAEV, caused disease in several captive Rocky Mountain goats (*O. americanus*) that had been fed goat milk as kids, and in another Rocky Mountain goat housed with this group. Mouflon (*O. gmelini*) can be infected experimentally with SRLVs, and genotype B viruses have been detected in wild ibex (*C. ibex*) in contact with goats. Serological evidence of SRLV infections has also been reported in some species including mouflon, ibex, and chamois (*Rupicapra rupicapra*). Preliminary evidence suggests that some viruses in wild ruminants may be distinct from CAEV and MVV.

In vitro, SRLVs infect cells derived from many vertebrate species, but they only replicate reliably in sheep or goat cells. Monocyte-derived cells are the primary targets for infection, with replication mainly in mature macrophages. Numerous other cells types can be infected, but with much lower viral replication rates: fibro-epithelial synovial membrane cells, choroid plexus cells, skin fibroblasts, spleen cells, corneal cells, testicle cells, goat endothelium, mammary gland epithelial cells, granulose cells, oviduct cells, microglial cells, tubular epithelial cells, hepatocytes, cardiac myocytes, third eyelid cells, and cells from lung explants.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Based on serological data, SRLV infections are present worldwide. Some countries are free of MVV, including Iceland, New Zealand, and Australia, but they are not free of CAEV. Individual prevalence rates have been reported between 2.5% and 71%, with even higher rates at the flock level.

Infected animals remain so for life. SRLVs can be transmitted to lambs, kids, or other susceptible species in milk or colostrum. The viruses have also been found in respiratory secretions and feces, with infections having occurred through intranasal or intra-conjunctival contamination, as well as through water contaminated with feces. Transmission is vastly increased where animals are kept in close proximity, such as indoor housing, highlighting the importance of transmission through respiratory secretions. Iatrogenic spread is also known through the reuse of needles or other items contaminated with blood and tissues. SRLVs are also found in semen and the female reproductive tract, but sexual transmission seems to be rare.

Pathogenesis and Pathology

The majority of SRLV infections are asymptomatic. In animals with clinical signs, the disease can take a variety of forms depending on the virus strain and host factors such as the immune response and genetic profile regarding resistance or susceptibility to the virus.

Maedi is the most common disease presentation in adult sheep following MVV infection. It is characterized by wasting and progressive dyspnea. The disease is eventually fatal, due to anoxia or secondary bacterial pneumonia.

Visna also occurs in adult sheep. Initially observed in Iceland in the 1950s, it is less common than maedi. The disease progression usually begins with subtle neurological signs such as hindlimb weakness, trembling of the lips or a head tilt, accompanied by loss of condition. The signs then progress to ataxia, incoordination, muscle tremors, paresis, and paraplegia, particularly in the hind legs. MVV-infected sheep sometimes develop progressive arthritis followed by severe lameness.

Encephalomyelitis may be observed in goats infected with CAEV, typically in kids two to six months of age. This presents as lameness, ataxia, hindlimb placement deficits, hypertonia, and hyperreflexia. The condition worsens, sometimes leading to paralysis. Some affected animals become depressed or exhibit other neurological signs such as head tilting or circling. In older goats, disease presents as lameness and knuckling, eventually leading to paralysis over weeks to months. MVV infection of goats may also result in neurological signs.

Adult goats infected with CAEV more commonly develop chronic polyarthritis, with synovitis and bursitis. This begins with distension of the carpal joint capsule and accompanying lameness, progressing to walking with front legs flexed, and eventually recumbence. The appearance of a CAEV-infected goat is shown in Figure 65.8. In rare cases, CAEV infection of goats has been associated with chronic interstitial pneumonia and progressive dyspnea.

SRLVs can also cause chronic indurative mastitis with a swollen, firm mammary gland and decreased milk production in infected animals. In wild animals, SRLV infection has been associated with chronic pneumonia, wasting, neurological signs, and joint lesions.

Lesions due to infection with SRLVs result from both the immune response to infection and the viral replication itself. The tissues most often infected include the lungs, mammary glands, nervous system, and joints. Lymphoid tissue hyperplasia may be observed in kidneys, liver, and heart. Respiratory signs are usually caused by meningoencephalitis, astrocytosis, microgliosis, and focal secondary demyelination in the brain and the spinal cord. Lesions in the joints result from infiltration of the synovial membrane by mononuclear cells, followed by villous hypertrophy, angiogenesis, and fibrosis, mineralization, and necrosis of synovium and joint capsule.

Host Response to Infection

SRLVs have a tropism for monocytes, macrophages, and dendritic cells, but can also infect microglia, endothelial cells, fibroblasts, and epithelial cells of other tissues. Cells



Figure 65.8 Appearance of a goat infected with Caprine arthritis encephalitis virus (advanced stage) in which the animal is hitting its head against the pen wall (left picture), is displaying depressive behavior (middle picture), and has joint inflammation (right picture). *Source:* Reproduced with permission from Schultz et al. 2020, Elsevier.

of the pulmonary or intestinal mucosa are targeted initially, with the infections spreading through dendritic cells to the lymph nodes and then beyond via infected macrophages. Viral antigens are recognized by T-cells, stimulating the production of interferons, thereby attracting other inflammatory cells. This progressive inflammation leads to many of the clinical signs observed. Decreased T-cell proliferative responses have been shown to correlate with the development of arthritis in goats infected with CAEV. An antibody response is detectable from three weeks post infection. Sheep and goats respond differently, however, based on antibody and provirus detection in milk. In sheep, the increasing presence of antibodies in the udder correlates with a decrease in provirus load, whereas this is not observed in goats.

Laboratory Diagnosis

SRLV-induced diseases can be diagnosed by both serological and virological methods.

Serological testing is challenging due to the extensive antigenic variation in the SRLVs, the existence of maternal antibodies, the sometimes lengthy lag period between infection and antibody production, and the fluctuation of antibody levels over time. Serological analyses are carried out principally using ELISA or sometimes AGID where available, with radioimmunoprecipitation (RIPA) and Western blot (WB) often used as confirmatory tests.

Molecular detection of SRLV proviral DNA in fluids and tissues (milk, blood, lungs, mammary glands, or synovial membranes) is carried out using PCR. This offers the advantage of detecting infection prior to seroconversion, but the genetic diversity of SRLVs complicates the development of PCR assays. It is therefore suggested that molecular and serological testing both be carried out.

Virus isolation may be attempted by co-culturing peripheral blood or milk leukocytes from live animals, or fresh tissues collected at necropsy, with susceptible sheep or goat cells, but this is not routinely carried out for diagnosis.

Treatment and Control

Due to the impact of disease caused by SRLVs, the World Organization for Animal Health (OIE) has included it in the list of notifiable terrestrial and aquatic animal diseases.

There is no effective vaccine or treatment for SRLV infections. Management processes are therefore used to minimize the effects of clinical signs and the economic losses. Supportive therapy may be offered for individual animals, but will not prevent the progression of disease. This can include making animals with arthritis more comfortable by regular foot trimming and administration of non-steroidal anti-inflammatory drugs. Of course, antibiotics can be used for secondary bacterial infections in cases of mastitis or pneumonitis.

As there is evidence of a genetic basis for susceptibility to infection by SRLV, at least in sheep, selective breeding is used as a control strategy. As SRLV introduction into a flock is mostly through contact with infected animals, screening of animals prior to adding them to the herd is an important control method. It is recommended to avoid mixing sheep and goats, or feeding milk or colostrum from one species to another. The elimination of infected animals, replacing them with tested, uninfected animals is another method of control. A nationwide eradication program in Iceland has been successful.

Equine Infectious Anemia Virus – *Lentivirus* Genus

Disease

Equine infectious anemia (EIA) is a potentially fatal disease of equids (horses, mules, and donkeys). It was first described in France in 1843, and was later named "swamp fever" in 1903 by Torrance. There are three recognized forms of the disease: acute, chronic, and inapparent.

In the acute form, animals may present severe signs such as fever with sweating, anorexia, a serous discharge from the nose, thrombocytopenia, and severe anemia, but often only an elevated temperature is noted. The signs appear rapidly, and the animals may die within two to three weeks, or may recover after a week.

If an animal survives the first acute bout of disease, it may develop a recurring clinical disease 2–4 weeks later. The signs may include fever rising to 105 °F or, rarely, as high as 108 °F, dropping back to normal until the onset of another episode, as well as petechial hemorrhages often on the mucous membranes, depression with dejected and generally listless presentation, weight loss, edema under the skin in the legs and under the chest and other underbody surfaces, and anemia, sometimes leading to cardiovascular anomalies such as an irregular heartbeat. The cycle of fever and clinical signs can recur many times, with each episode usually lasting less than a week, and an interval of several weeks between cycles. The frequency and severity usually decrease after 6–8 episodes.

By far the majority of horses are inapparent carriers of the virus. They show no overt clinical abnormalities as a result of infection and survive as reservoirs of the infection for extended periods. They typically have lower concentrations of virus in their blood than horses with active clinical signs of the disease. The inapparent form may become

chronic or acute due to severe stress or the presence of other concurrent diseases. All horses infected with Equine infectious anemia virus (EIAV) remain virus carriers for life.

Etiologic Agent

Classification

EIAV is a member of the *Lentivirus* genus in the Retroviridae family. In 1904, EIA became the first disease of horses proved to be caused by a "filterable virus," one that could survive a special laboratory filtering procedure and remain infectious. EIAV was the first virus shown to be related to the HIV through cross-reaction in tests of blood serum.

Physical, Chemical, and Antigenic Properties

The EIAV genome is 8200 nucleotides long, and comprises three principal genes (*gag*, *pol*, *env*) and three regulatory genes. EIAV is the first persistent virus for which "antigenic drift" was defined, in 1972, with the virus having the ability to change its form sufficiently so that it is no longer vulnerable to existing antibodies.

Resistance to Physical and Chemical Agents

EIAV is readily inactivated by most common disinfectants, including bleach, alcohol, and those containing detergents. The virus is quite labile in the environment, but it has been shown to remain infectious on hypodermic needles for up to four days.

Infectivity for Other Species and Culture Systems

All equids (horses, ponies, mules, donkeys, and zebras) are susceptible to infection by EIAV. *In vitro* culture of the virus is usually carried out on equine monocytes or macrophages, the main target cell for all EIAV strains *in vivo*. Some laboratory-adapted strains of EIAV can be cultivated in fibroblastic equine cells, fetal equine kidney cells, an adherent canine macrophage-like cell line (DH82), canine fetal thymus (Cf2th) cells, or the Fea and FEF feline cell lines that support EIAV replication. There is no evidence that EIAV can infect or cause disease in humans.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Outbreaks of EIA continue to be reported in Asia, Africa, Europe, North America, South America and Australia. The disease is most prevalent in hot, humid areas supporting insect vectors.

EIA is a classic bloodborne infection, although the virus is also present in colostrum, milk, and semen. People (including veterinarians) play an important role in transmission when using blood-contaminated materials on different horses. EIA was the first retrovirus-induced disease proved to be transmitted by insects. The virus is readily transmitted between horses in close proximity by large biting insects, such as horseflies, deerflies, and mosquitoes, but, because of their large mouthparts, horseflies are the most effective natural vectors. The transmission rate depends on the number and habits of the insects, the density of the horse population, the number of times the insect bites the same and other horses, the amount of blood transferred between horses, and the level of virus in the blood of the infected horse from which the initial blood meal was obtained. The rate of transmission cannot be predicted accurately because of these variables. The virus does not replicate in the insects, but can survive there for up to four hours. Vertical transmission (*in utero* or by milk) is also possible. There is also a suggestion that aerosol transmission during very close contact may also be possible, based on experience from an outbreak in Ireland.

Pathogenesis and Pathology

The development of clinical signs of EIA is linked to the increasing levels of circulating virus in the infected animal, with episodes of fever being associated with peaks of viremia.

The pathology of EIA results from viral infection of macrophages, leading to a pro-inflammatory response, with associated fever. This cytokine response downregulates the production of bone-marrow-derived platelets and erythrocytes, resulting in thrombocytopenia, causing hemorrhages and subsequent anemia. In addition, platelets become coated with antibodies and erythrocytes are coated in complement, so the resulting immune responses lead to splenomegaly and hepatomegaly, along with immune-complex vasculitis and glomerulonephritis. In cases of severe disease, enlarged abdominal lymph nodes, liver, and spleen are often observed, along with mucosal and visceral hemorrhages, ventral subcutaneous edema, and thrombosis. Histopathology usually shows an accumulation of macrophages and lymphocytes and macrophages in lymph nodes, the adrenal gland, liver, spleen, and lungs, sometimes accompanied by a nonsuppurative hepatitis, a glomerulonephritis, periventricular leukoencephalitis, meningitis, or encephalitis.

Host Response to Infection

The viremia following EIAV infection stimulates antibody production targeting the surface (gp90), transmembrane (gp45), and major core (p26) proteins. Although p26 is the

most abundant viral protein, it elicits 10- to 100-fold lower antibody levels than gp90 or gp45. The first antibodies are detectable by immunoblot as soon as 7–10 days post infection, and by ELISA by 14 days, whereas antibodies against P26 are detectable by AGID testing by 45 days post infection.

Specific cytotoxic T-lymphocytes can be detected by 14 days post infection, likely being responsible for the majority of the reduction in viremia levels.

Laboratory Diagnosis

EIA is the first retrovirus-induced disease for which a diagnostic test was approved. An effective test for antibodies specific to EIAV was described in 1970 by Leroy Coggins. The agar gel immunodiffusion (AGID) or Coggins test was shown to correlate with horse inoculation test results for EIAV and could therefore be used to identify EIAV carriers. Although other serological tests have been defined and are approved for the diagnosis of EIA, the AGID test is still recognized internationally as the "gold standard" serological test. Several ELISA tests (competitive and non-competitive) are approved for the diagnosis of EIA. These typically detect antibody produced against the p26 core protein antigen and sometimes also the gp45 (viral transmembrane protein) antigens. A positive test result by ELISA may be confirmed using the AGID test as some false positive results have been noted with ELISAs. The results can also be confirmed by the immunoblot technique. PCR tests are commonly used to detect proviral LTR, *gag* or *pol* DNA, but the genetic diversity of EIAV and the often low levels of viremia present challenges for these. In the United States, an EIA APHIS-approved laboratory must be used for testing.

Treatment and Control

There is no effective treatment for EIA. Although supportive therapy may be offered, euthanasia or permanent isolation and quarantine (at least 200 yards away from other horses) are the only realistic outcomes following confirmed infection.

Control relies on regular testing of animals, and on vector control in and around the premises. Standard hygiene practices such as avoiding the reuse of needles are also essential. Although a vaccine has been used on a limited basis in Cuba and China, its broad efficacy has not been conclusively demonstrated. A test and slaughter system in Japan has succeeded in eliminating the disease there since 1984. Elsewhere incentive schemes requiring proof of negative test results prior to shows, sales, and so on, have been used to encourage control. Statutory testing of imported horses is required in some countries. The disease is reportable in many countries, including the United States.

Bovine Immunodeficiency Virus – *Lentivirus* Genus

Disease

Some of the signs potentially linked to infection with Bovine immunodeficiency virus (BIV) include encephalitis, lymphadenopathy, subtle clinical ataxia, multiple secondary infections that fail to resolve after treatment, non-suppurative meningoencephalitis (dullness, stupor, head, or nose pressing postures), possible involvement in bovine paraplegic syndrome, behavioral signs possibly confused with bovine spongiform encephalopathy (BSE), poor seroconversion after vaccination, weight loss, lowered milk yields, and immunodeficiency. Despite some evidence for the above signs, the real significance of BIV infection in the field to cattle health is yet to be clearly established. The majority of infections are inapparent, however. In cattle, although leukocytes generally are the common targets for BIV infection, a specific tropism for B-lymphocytes and for CD8+ T-cells has been suggested.

Etiologic Agent

Classification

BIV, also known as Bovine lentivirus type 1 and Bovine immunodeficiency-like virus, is a member of the *Lentivirus* genus in the Retroviridae family. It was first isolated in 1969 from an eight-year-old cow with emaciation, lymphadenopathy, lymphocytosis, and mild perivascular cuffing in the brain. Further study in 1987 established the similarity of this virus to HIV, confirming its classification as a lentivirus.

Physical, Chemical, and Antigenic Properties

The BIV genome is about 8.9 kb in length, and is the most complex among the lentiviruses, with several regulatory genes in addition to the standard retrovirus genes, The virus is inactivated by heating to 47 °C for 30 minutes, and by low- and high-temperature pasteurization conditions.

Infectivity for Other Species and Culture Systems

Attempts to experimentally infect species other than cattle have been successful with rabbits and sheep, but without producing any clinical signs of disease. *In vitro* culture of the virus is difficult, usually carried out by co-culture of peripheral blood mononuclear cells and fetal bovine lung cells. There is no evidence that BIV can infect or cause disease in humans.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

Serological studies indicate a worldwide distribution of the virus. Seroprevalence levels of 4–8% have been shown in Europe, possibly higher elsewhere. At the herd level, prevalence is much higher. Infections appear to be predominant in dairy cattle, and in warmer climates. Herds infected with BIV are often simultaneously infected with BLV.

Transplacental infection of dairy calves (vertical) has been demonstrated, and is likely the most common method of transmission. Stud bull semen has been shown to contain infected leukocytes. Lactogenic transmission is possible, as infected leukocytes are present in milk. The virus is inactivated in milk following pasteurization. Transmission by way of contact with contaminated blood is also likely, for example, through re-used needles. The higher prevalence in warmer climates suggests a possible role for insect vectors, but this has not been demonstrated.

Pathogenesis and Pathology

In cattle, although leukocytes generally are the common targets for BIV infection, a specific tropism for B-lymphocytes and for CD8+ T-cells has been suggested. Following BIV infection, hypertrophic, or hyperplasial hemal lymph nodes are often observed – slightly movable subcutaneous nodules in the neck and flank, as well as lymphoid follicular hyperplasia and dysplasia in lymph nodes. Atrophy of lymphoid cell compartments with depletion of lymphocytes, and lymphocytic lymphoid folliculitis are also seen. In the brain, encephalitis with mononuclear cell infiltration may be observed, as well as an accumulation of lymphocytes in the pituitary.

Host Response to Infection

BIV infection in cattle usually results in a strong, long-lasting antibody response detectable from five weeks post infection, but this is variable.

Laboratory Diagnosis

Serological assays are most often used to detect infected animals. These include ELISA and Western blotting. PCR tests have also been developed to detect integrated provirus. Virus isolation is notoriously difficult for BIV.

Treatment and Control

Infected animals remain so for life, as the virus integrates into the genome. Screening of breeding animals and AI donors is the only effective means of control. No specific surveillance or screening tools are in place. No vaccine is available.

Jembrana Disease Virus – *Lentivirus* Genus

Disease

Jembrana disease virus (JDV) is closely related to BIV and causes an acute and sometimes fatal disease in Bali (*Bos javanicus*) cattle, but a milder disease in *B. taurus*. Contrary to the chronic disease produced by most lentiviruses, Jembrana disease presents with acute effects. The typical signs of infection are fever, anorexia, diarrhea, and lymphadenopathy, and the fatality rate in Bali cattle is over 15%. The first documented case was identified in the Jembrana district of Bali, Indonesia, in 1964.

Etiologic Agent

Classification

JDV is a member of the *Lentivirus* genus in the Retroviridae family. It shares 74% nucleotide sequence similarity with BIV. The virus was identified in 1992.

Physical, Chemical, and Antigenic Properties

The virus is very similar to BIV, except that the bar-shaped nucleoid typical of other lentiviruses is not seen in JDV. It is hypothesized that JDV originated from BIV. The JDV genome is the smallest of the lentiviruses, being just over 7700 nucleotides in length.

Infectivity for Other Species and Culture Systems

In addition to various types of cattle and buffaloes, pigs have been experimentally infected. As with BIV, *in vitro* culture of the virus is difficult, with attempts having been made using continuous and primary Bali cattle derived cells. There is no evidence that JDV can infect or cause disease in humans.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

So far, the virus has only been found in specific regions of Indonesia. Virus transmission is principally through transfer of infected blood, usually by insects, although the exchange of blood-contaminated materials has also been implicated. The seasonality of disease outbreaks coinciding with the wet season is consistent with an important role for biting arthropods. The virus can also be found in milk, oral fluids, and semen.

Pathogenesis and Pathology

The principal lesions in infected animals are due to the intense lymphoproliferative response. This manifests as lymphadenopathy and splenomegaly, also with vascular damage including mild exudates and hemorrhages. Lymphoid tissues of all organs may be affected, with the peripheral lymph nodes and spleen being enlarged, and B-cell follicles becoming atrophied. Other organs such as liver, lungs, and kidneys are also affected, with a lymphoid infiltrate being found.

Host Response to Infection

Infection with JDV usually results in a strong antibody response. Significant changes in hematology characteristics are also seen, such as a marked leukopenia, mild neutrophilia, and a decrease in circulating lymphocytes and monocytes.

Laboratory Diagnosis

There is great antigenic cross-reactivity between this virus and BIV, but they can be distinguished by some monoclonal antibodies.

Treatment and Control

Infected animals remain so for life, as the virus integrates into the genome. A protective immune response was induced in cattle by vaccination with virus-containing plasma and spleen tissue derived from acutely affected cattle (the virus was inactivated with Triton X-100 and emulsified in either incomplete Freund's adjuvant or a mineral oil adjuvant). Although vaccination suppressed the duration and severity of the disease, it did not completely prevent the development of disease in some animals.

Feline Immunodeficiency Virus – *Lentivirus* Genus

Disease

Feline immunodeficiency virus (FIV) infection is one of the most frequent and significant infections in cats worldwide. It may lead to severe immunodeficiency is some cats, resulting in numerous secondary infections. It was first isolated in 1986 from cats in a California cattery exhibiting degenerative conditions and multiple infections, and was originally named Feline T-lymphotropic virus. Serological evidence shows that the infection dates back to the 1960s.

There are three recognized phases of FIV infection: acute phase, latent phase, and progressive phase. In the acute phase, virus spreads to the lymph nodes, infecting T-cells, and resulting in inflammation of the lymph nodes, leading to fever, depression, and lack of appetite. Following the acute phase, animals progress to the latent phase, lasting for months or years, sometimes never progressing further. During this asymptomatic phase, virus replicates only slowly in lymphocytes, with no significant signs of disease. Some animals will then go on to the progressive phase, developing feline acquired immune deficiency syndrome. They are then very susceptible to secondary infections, which are often fatal.

Etiologic Agent

Classification

FIV is a member of the *Lentivirus* genus in the Retroviridae family. At least six genotypes (A–F) have been identified based on genetic differences, along with various recombinant subtypes. These appear to differ in their geographic distribution and their pathogenicity.

Physical, Chemical, and Antigenic Properties

FIV is morphologically similar to other lentiviruses. Besides the standard retrovirus gene products, additional proteins include Vif (involved in countering host cell cytidine deaminases), OrfA (possibly involved in transcriptional activation, control of splicing, and virion dissemination), and Rev (nuclear export of RNA).

Resistance to Physical and Chemical Agents

As with other retroviruses, FIV survives outside the host only for minutes, and is very susceptible to inactivation with most standard disinfectants.

Infectivity for Other Species and Culture Systems

In addition to domestic cats, FIV infection is also found in tigers, lions, cheetahs, hyenas, panthers, snow leopards, jaguars, pumas, bobcats, and other wild felids. Isolation of FIV is usually carried out from peripheral blood lymphocytes in primary feline T-cells. Laboratory-adapted strains of FIV can be cultured in some feline cell lines. There is no evidence that FIV can infect or cause disease in humans.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

FIV prevalence varies considerably between populations worldwide, ranging from 30% to less than 1%. Based on serological data, the large African wild cats have high

levels of infection, whereas the Asian populations show low infection levels. Genetic analyses suggest that transfer of FIV strains between species is rare. FIV affects all ages of cats, but incidence increases with age, being most prevalent in domestic cats over six years old. More male cats are infected than females. FIV is shed in saliva and transmitted primarily through bites; therefore, outdoor adult male cats taking part in territorial fighting are at the highest risk of infection. Transmission through close contact, for example, in catteries, is highly unlikely. Transmission of FIV has been shown through direct contact of virus with oral, vaginal, or rectal mucosa and through artificial insemination. Vertical transmission from queens to kittens either *in utero* or through passage in the birth canal is also possible. Infected milk also poses a low risk of transmission, as does the use of contaminated blood for transfusions. Infected cats remain so for life, even if asymptomatic.

Coinfection with FeLV occurs regularly, as infection with one retrovirus increases the risk of infection with the other, often resulting in more severe disease.

Pathogenesis and Pathology

FIV primarily infects CD4+ T-cells, but also macrophages and other lymphocytes. Following initial infection of local lymph nodes, the virus spreads to other lymph nodes. As subpopulations of T-cells are gradually depleted, cytokine production is affected, the balance of lymphocytes is altered with an inversion of CD4/CD8 ratios, and cell-mediated immunity becomes impaired. Lymphadenopathy is observed, resulting from lymphoid hyperplasia, and neutropenia also occurs frequently. This process may take years to occur, prior to which the infection is largely asymptomatic. The immune suppression sometimes leads to the development of neoplasia, most frequently lymphoma. Although FIV does not infect neurons, there are more neurovirulent strains that lead to progressive behavioral changes in infected cats.

Host Response to Infection

Following infection with FIV, animals develop a robust antibody response within two to four weeks, as well as cell-mediated responses. Following the peak in virus levels in blood 8–12 weeks after infection, viremia levels drop dramatically as a result of increasing T-cell numbers, but virus replication does continue at low levels.

Laboratory Diagnosis

Routine diagnosis is carried out by serology (ELISA, Western blotting, or IFA) to detect antibodies against FIV. Confirmation of a positive result is often needed, using a different test kit or method. Kittens may have maternal antibodies that are detected by the test (false positive result). Therefore, any positive kitten should be retested after six months of age to rule out detection of maternal antibodies. Although vaccines for FIV are no longer available in the United States and Canada, animals may travel from locations where vaccines are still in use. In addition, the vaccine was removed from the market in the United States in 2015, and cats can have antibodies due to vaccination for more than seven years following the last vaccination. Vaccinated animals will be seropositive, so alternative tests are required to confirm infection. PCR testing is used to detect proviral FIV DNA or viral RNA, but the genetic variability of FIV is a challenge for this method, and false negatives may occur. Virus isolation is very rarely used as a diagnostic tool. An algorithm for diagnostic testing or suspect retroviral infection in cats is shown in Figure 65.6.

Treatment and Control

There is no effective cure for FIV, but infected animals can live normal and healthy lives for many years if managed appropriately. It is recommended that infected cats be housed indoors and neutered to reduce the opportunities for aggressive interactions with other cats. Treatment with antiviral and immunomodulating drugs has been tried, with mixed results. In general, fluid therapy, nutritional support, and antimicrobial therapy for secondary infections are used. Commercial vaccines are available in Europe and the Pacific, with varying efficacy against the diverse strains of FIV, but have not been available in North America since 2017.

Simian Immunodeficiency Virus – *Lentivirus* Genus

Disease

The majority of wild primates from Africa infected with Simian immunodeficiency virus (SIV) do not develop any clinical disease. Most Asian primates, and some wild African chimpanzees, however, do go on to develop a fatal immunosuppressive syndrome analogous to AIDS in humans, termed simian AIDS. The infection may lead to a chronic wasting syndrome with persistent diarrhea, accompanied by a depletion of CD4+ T-cells and lymphadenopathy. A transient skin rash is occasionally observed. Sometimes, a primary encephalopathy also develops. Concurrent opportunistic infections usually follow.

SIV is widely used as an experimental model in nonhuman primates for HIV infection in vaccine and therapeutic research studies.

Etiologic Agent

Classification

SIV is a member of the *Lentivirus* genus in the Retroviridae family, related to HIV-1 and HIV-2. The first strain of SIV was isolated in 1985 from captive rhesus macaques suffering from simian AIDS, and was initially termed simian T-lymphotropic virus III. Since the first complete genome of a human immunodeficiency virus was obtained in 1984, tens of thousands of primate lentivirus genome sequences have been generated, providing insights into their origins and spread. The nomenclature of each lineage of simian lentivirus is based on the host it infects, for example, SIV_Gorilla, or SIVgor for simian immunodeficiency viruses isolated from gorillas. The number of SIVs isolated and characterized from nonhuman primates has increased greatly in recent years following improved methods for virus isolation from wild animal samples, and 40 of these have so far been defined in the HIV Databases at LANL.

Physical, Chemical, and Antigenic Properties

The morphology and physical properties of SIV are similar to the other lentiviruses. In addition to the standard retrovirus genes, accessory proteins present include Nef and Vpr.

Resistance to Physical and Chemical Agents

Standard commercially available disinfectants such as 10% sodium hypochlorite, 70% ethanol are efficacious against SIV.

Infectivity for Other Species and Culture Systems

In general, lentiviruses do not easily cross species boundaries, being constrained by specific virus–host protein interactions. Cross-species transmissions of primate lentiviruses are not so unusual, however, with examples of primate to human, and primate to primate transfers. It is known that baboons have been infected by SIV from the vervet species of African green monkeys. The SIV lineage infecting chimpanzees and gorillas is thought to be the result of a recombination event between SIVs from red-capped mangabeys and greater spot-nosed monkeys. Fighting and predation between different species of primates is rather common, leading to opportunities for cross-infections. SIVs from chimpanzees, gorillas, and sooty mangabeys have historically infected humans. For the isolation and culture of SIV, lymphocyte cultures with stimulated CD4+ cells are used, from primate or human origin.

Host–Virus Relationship

Distribution, Reservoir, and Transmission

In South Africa, SIV prevalence in African green monkeys ranged between 7% in infants/juveniles, 57% in adult males, and 78% in adult females. SIV has not been found in wild primates in Asia or South America. There is a significant prevalence in zoo animals worldwide. In wild African primates, SIV infections are usually non-pathogenic, due to evolutionary adaptation of the hosts to the virus, but infection in Asian or Indian rhesus macaques does results in the development of simian AIDS.

SIV transmission is believed to be mostly horizontal, either through sexual contact or bite wounds, but also occurs vertically from mothers to their offspring. Inadequate veterinary practices whereby contaminated blood or body fluids are shared also accounts for some infections in zoo animals.

Pathogenesis and Pathology

Pathogenic infection with SIV is characterized by a gradual loss of CD4+ T-cells. In response to the host's innate and adaptive immune system reactions, mutations in the virus quickly lead to immune escape variants that become dominant. Continued activation of the immune responses leads to the functional impairment of the immune system. Besides mucosal sites, chronic infection with SIV impacts almost all organ systems in infected individuals, to include the brain, heart, and lungs. The neurological impact of SIV infection likely results from changes in morphology and cytokine secretion of brain macrophages. Another feature frequently observed is a considerable increase in microbial diversity in the gut microbiome. In contrast, in nonpathogenic infections, the host's interferon response is rapidly down-modulated after acute infection.

Host Response to Infection

Both pathogenic SIV infection of macaques and nonpathogenic infection of other primates induce strong innate immune responses, with secretion of type I interferons and upregulation of interferon-stimulated genes. Infected animals also mount a strong antibody response. Animals remain infected for life, with ongoing low levels of viral replication despite the immune responses.

Laboratory Diagnosis

Serological assays are frequently used to identify SIV infection, but some African primate species may harbor the virus and not seroconvert. In addition to ELISA tests,

indirect fluorescent antibody and WB assays are also used. SIV infection can be confirmed following virus isolation. PCR tests are also used to detect proviral DNA or viral RNA, and can be used on fecal samples to enable non-invasive testing of wild animal populations.

Treatment and Control

Due to the many similarities between HIV-1, HIV-2, and SIV infections and disease, much research is being conducted to develop effective therapies and vaccines against these pathogens. Passive transfer of neutralizing antibodies is being explored to provide sterile protection against and post-infection control of SIV infection. Other approaches include the use of RT inhibitors. Among vaccine candidates are a vaccine containing SIV protein-expressing rhesus cytomegalovirus vector, *nef* (accessory gene) deleted live viruses, and deglycosylated live-attenuated SIV vaccines.

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66

Transmissible Spongiform Encephalopathies

Jürgen A. Richt and Nicholas Haley

The transmissible spongiform encephalopathies, or TSEs, are a group of infectious agents that are responsible for progressive, degenerative, and uniformly fatal central nervous system (CNS) disorders in their respective mammalian hosts, including humans (Table 66.1). These diseases, including scrapie of sheep, chronic wasting disease (CWD) of deer, elk and moose, bovine spongiform encephalopathy (BSE) of cattle, and Creutzfeldt-Jakob disease (CJD) of humans, tend to be highly species specific and share similar patterns of clinical disease, pathogenesis, neuropathology, and etiology. TSEs are quite different from the other infectious agents discussed previously, in that the agent itself is comprised solely of a misfolded protein, devoid of nucleic acids and other familiar building blocks found in bacteria, fungi, parasites, and viruses. The misfolded protein in TSEs is the prion protein, capable of forming a highly stable and neurotoxic amyloid; thus, these diseases are commonly referred to as prion diseases. Although misfolded prion proteins are unique among infectious agents due to their independence from nucleic acids (other than the host's nucleic acids that encode them), TSEs are not unique disease forms as they share various pathological processes with a range of other well-known neurodegenerative diseases associated with protein misfolding, including Alzheimer's disease (linked to misfolded β-amyloid and tau proteins) and Parkinson's disease (associated with misfolded α-synuclein). There is increasing justification for classifying these latter neurodegenerative diseases as a subcategory of prion disorders, each involving their own specific misfolded protein(s).

Similar to the abovementioned human protein misfolding disorders, most cases of TSEs in humans are sporadic in nature, with a smaller percentage of cases having a heritable basis. Although there is scant evidence that Alzheimer's and Parkinson's diseases may be transmissible – under certain circumstances using mouse model systems - a very small percentage of human TSEs cases are considered to have a transmissible origin. These include cases of kuru, a disease historically seen exclusively in cannibalistic tribes of Papua-New Guinea, and variant CJD, linked to the consumption of BSE-contaminated beef in the late twentieth century. A small number of cases of human TSEs have also been iatrogenic in nature, through therapeutic interventions such as growth hormone therapy, organ and tissue transplants, and blood transfusions, for example.

In sharp contrast, several TSEs of veterinary importance discussed in this chapter, including scrapie and CWD, are highly transmissible, with horizontal, vertical, and environmental transmission mechanisms. Transmission of some, such as BSE, are primarily iatrogenic in nature, with little evidence of animal-to-animal or environmental transmission. Apart from transmissibility, there are other important distinctions between veterinary prion diseases, including disease pathogenesis, tissue distribution of the prion agent, the potential for distinct prion "strains", and perhaps most importantly their zoonotic potential. In this chapter, following an introduction to the prion protein, its origin, structure, and biochemistry, notable animal prion diseases will be discussed, including our understanding of individual strains, their pathogenesis, transmission and risk of zoonosis, and their diagnosis, prevention, and management.

Etiologic Agent

Initial theories on the nature of the agent responsible for several of the earliest described TSEs, including scrapie and kuru, relied on what was known about the transmissibility and pathogenesis of well-studied infectious agents at

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Table 66.1 Summary of the currently known transmissible spongiform encephalopathies in animal species.

Agent	Natural host range	First identified	Geographic distribution	Transmission	Incubation period	Diagnostic sample choice	Management
Classical scrapie	Sheep, Goats	Eighteenth Century	Worldwide (excluding Australia and New Zealand)	Oral transmission via excreta, placental tissues, carcasses, and environmental contamination	2+ years	Obex and retropharyngeal lymph nodes (RPLN)	Selective breeding for disease resistance
Atypical scrapie	Sheep, Goats	1998	Worldwide, including Australia and New Zealand	Spontaneous	Unknown, likely greater than two years	Brain	Active surveillance
Chronic wasting disease (CWD)	White-tailed deer, mule deer, elk, and related cervid species	1967	United States, Canada, South Korea*, Norway, Sweden, and Finland	Oral transmission via excreta and environmental contamination	12+ months	RPLN and/or obex	Wild Populations: Herd Reduction and selective harvest. Captive Populations: Quarantine and depopulation.
Bovine spongiform encephalopathy (BSE)	Cattle, goats, exotic ungulates, felids, humans	1986	Worldwide, including the United Kingdom, Europe, Japan, and North America	Oral transmission via contaminated meat and bone meal (MBM) and other cattle by-products	2+ years	Obex	Ban on ruminant tissues in feed; active surveillance
Atypical bovine spongiform encephalopathy	Cattle	2004	Worldwide	Spontaneous	Unknown, likely greater than two years	Obex	Active surveillance
Camel prion disease	Dromedary camels	2018	Algeria and Tunisia	Unknown	Unknown	Obex	Active surveillance
Feline spongiform encephalopathy (FSE)	Domestic and exotic felids	1990	Most cases in the United Kingdom or exposed in the United Kingdom	Oral transmission via BSE-contaminated feed	4+ years	Brain	Ban on ruminant tissues in feed
Transmissible mink encephalopathy (TME)	Farmed mink	1947	United States, Europe, and the former USSR	Oral transmission suspected via scrapie- or BSE-contaminated feed	6+ months	Brain	Ban on ruminant tissues in feed

the time. An unconventional virus producing infections with protracted incubation periods was proposed – a lentivirus, perhaps – although growing evidence eventually forced a reevaluation of this theory. Studies that sought to characterize the biochemical nature of the scrapie agent demonstrated a resistance to UV radiation, ionization, formalin fixation, and heat, and its predicted size was just a fraction of that of the smallest known viruses. Experiments using CNS from sheep inoculated into hamsters, enriched for infectivity and treated with an enzyme capable of degrading conventional cellular proteins, soon led to the discovery of an infectious, protease-resistant, proteinaceous particle named the prion protein, PrP^{res} (protease-resistant PrP). The discovery of this protein led to a Nobel Prize for its discoverer, Dr. Stanley Prusiner, as well as a reassessment of both infectious agent categorization and the passage of information in the absence of nucleic acids.

An equally important discovery was that the protein responsible for TSEs was in fact a protein that was encoded by the host. Studies on the nature of the agent itself focused on the variability in incubation periods in mouse models of scrapie. These studies identified a putative gene linked to comparatively short or prolonged incubation periods in mice, named the *Sinc* gene, for its role in scrapie incubation period modulation. The sequence of the protein encoded by the *Sinc* gene and the infectious prion protein sequence were eventually determined to be, for all intents and purposes, identical, and the gene was subsequently renamed *Prnp*, for prion protein. With this important finding, a more harmonized prion theory was developed – one in which a normal cellular prion protein encoded by the host, designated cellular prion protein or PrP^{C}, either spontaneously misfolds or is coerced to misfold by an exogenous, pathogenic prion protein, called PrP^{res}. The sustained conversion of PrP^{C} by PrP^{res} and the subsequent accumulation of PrP^{res} in the form of amyloid in the CNS has been shown to be the pathological basis for each of the documented prion diseases to date.

For most TSEs, natural variations in a species' normal cellular prion gene sequence can profoundly affect the incubation period of prion infection within that species, in some cases leading to a complete resistance to infection. Specific and important variations in the host's prion gene sequence will be further discussed under the individual disease descriptions. Variability in the prion gene sequence between species also forms the basis for what is commonly referred to as the prion "species barrier," whereby prion agents from one host species are incapable of initiating infection, or have limited infectious potential, in another host species. The species barrier is illustrated by the apparently complete resistance of humans to infection with scrapie and CWD, prion diseases from sheep and deer species, respectively, as well as the imperfect transmissibility of BSE to humans as discussed in the sections below.

In the following discussions, specific prion agents responsible for each of their respective diseases are represented as abbreviations, PrP, with the particular agent in superscript, for example, PrP^{Sc} for scrapie, PrP^{CWD} for CWD, and so on. Occasionally in the literature, disease-causing prions of all types may be referred to generically as either PrP^{Sc} (for scrapie prion), PrP^{d} (for disease-causing prion), or PrP^{res} (for protease-resistant prion). When the agent is physically isolated using western blotting or observed *in situ* using immunohistochemistry (IHC), it is often referred to simply as PrP^{res}.

Origin of the Prion Protein, its Biochemistry, Structure, and Strains

As noted above, the prion agent (PrP^{res}) is derived from the normal cellular prion protein – PrP^{C} – which is encoded by the host. Homologues of the prion gene and prion protein are found in all mammalian species (where all reported prion diseases have occurred to date), with orthologues present in avian, piscine, reptilian, and amphibian species. Mammalian PrP^{C} is a ~250 amino acid long protein derived from a single copy gene in humans and other animals, the so-called prion protein gene (PRNP or *Prnp*). The prion protein is cell membrane associated, attached to the cells via a glycosyl phosphatidylinositol (GPI) anchor, and primarily alpha-helical in structure. It is approximately 35 kDa in size and is especially abundant in the CNS within neurons and glial cells. The prion protein is also expressed in the cells of the mononuclear phagocytic system, including macrophages and dendritic-cells. The precise function of the prion protein in cells is uncertain, though it has been proposed that it may be involved in copper metabolism, interactions with the extracellular matrix, olfactory discrimination, apoptosis, and signal transduction. Transgenic animals that had the *Prnp* gene deleted or "knocked-out," including mice and cattle, have been developed. Although these animals have been viable, occasional non-specific behavioral and physiologic abnormalities have been reported.

Biochemically, PrP^{C} is labile and can be inactivated relatively easy by a variety of methods, including protease enzyme digestion and heat. In contrast, PrP^{res} is very resistant to inactivation with protein-degrading enzymes (proteases), heat, ultraviolet light, ionizing radiation, acids, bases, formalin fixation, and disinfectants. Because of this remarkable resistance, effective procedures to inactivate PrP^{res} are limited. Practical inactivation can be achieved using steam autoclaving, alkaline hydrolysis, incineration,

and select chemical treatments described later in this chapter. The resistance of PrP^{res} to conventional inactivation approaches helps explain the potential longevity of PrP^{res} in the environment. It also allows for the transmission of TSEs through contaminated feeds that are cooked or processed, through inadequately autoclaved surgical instruments, or via human/veterinary biologicals derived from tissues or fluids harvested from infected humans or animals.

As a typical host-encoded protein, the normal cellular prion protein, PrP^{C}, is not infectious and does not cause disease. The infectious prion, PrP^{res}, is identical in amino acid sequence and length to PrP^{C} of the host species in which it is replicating, and differs only in its higher order conformation. Although the precise nature of the structural differences between PrP^{C} and PrP^{res} is still a matter of ongoing debate, the central feature thought to be responsible for the physiochemical and infectious transformation of PrP^{res} is a change in the alpha-helical structure of PrP^{C} to one composed of several rigid beta-pleated sheets in PrP^{res}. The misfolded prion protein PrP^{res} has a protease-resistant core that may vary in size between subtypes and strains, although it is typically 27–30 kDa in size and approximately 142 amino acids in length. The tertiary conformational change (alpha-helical structure of PrP^{C} to several rigid beta-pleated sheets in PrP^{res}) is the putative basis for its transformation into an infectious pathogen that propagates and spreads within host cells and causes disease, whereas quaternary structures of PrP^{res} are associated with the formation of a prion amyloid. Experimental evidence suggests that newly formed PrP^{res} represents a physical template for continued conversion of normal PrP^{C}. Even following protease treatment, the 27–30 kDa core of PrP^{res} may serve as that template and remains infectious.

In the case of heritable prion diseases in humans and animals, mutations or insertions in the *Prnp* gene are postulated to cause the resultant altered PrP^{C} to adopt a misfolded, beta-sheet rich tertiary conformation that can then serve as a template for further PrP^{C} – PrP^{res} conversions. Symptomatic onset of these heritable prion disorders in humans typically begin in people younger than 40 years of age. In spontaneous prion disorders, a properly folded PrP^{C} undergoes a spontaneous tertiary conformational change that may serve as a template. The onset of spontaneous prion disorders typically begins in older age groups, both in humans and in animals. Infectious prion disorders, on the other hand, are a result of exogenous transmission of a misfolded prion protein, and disease onset may begin in any age group, though due to its protracted nature, disease is generally seen in adult humans and animals.

Perhaps the most challenging facet of the protein-only prion hypothesis is to explain the identification of distinct prion strains in laboratory species, for example, scrapie strains Nor98, RML, and ME7, derived from affected sheep brain inoculated into mice and hamsters. Despite lacking associated nucleic acids, prion strains demonstrate highly reproducible phenotypes in rodents, including consistent incubation periods, CNS pathology, and cross-species transmissibility patterns. Biochemical variations, including proteinase resistance, guanidine stability, and western blotting characteristics, have also been reported. How can a simple protein be capable of encoding for these very defined phenotypes and biochemical characteristics without the mutations and other genetic variations familiar to bacterial, fungal, parasitic, and viral strains? The most plausible hypothesis relies on the very particular ways in which the abnormal prion protein might misfold. Despite Nor98, RML, and ME7 scrapie strains all carrying the host prion's primary amino acid sequence, variability may exist in each strain's folded tertiary structure such that the misfolded proteins may accumulate faster or slower, in one specific area of the CNS or another, while being more or less capable of coercing alternate species' prion proteins to misfold *in vivo*. Much work remains to identify and fully characterize prion strains, and where known, distinct strains will be mentioned in the discussions below.

Scrapie of Sheep and Goats

Scrapie is considered the prototypical prion disease, with clinical descriptions in sheep appearing in the scientific literature over 200 years ago. Like other prion diseases, it is a chronic, progressive, and uniformly fatal degenerative CNS disease that occurs naturally in sheep and goats. Scrapie has a protracted incubation period, ranging from 18 to 60 months between exposure and the onset of clinical signs. Variability in incubation period may be affected by dose and route of exposure, host *Prnp* genotype, and the type of scrapie strain involved. There is no evidence that scrapie is zoonotic, though experimentally it has been transmitted to various animal species including cattle, deer, mice, and other laboratory rodents. Like other prion diseases, there are no effective treatment options for scrapie, and flocks with reported cases of scrapie are traditionally quarantined and depopulated.

"Classical scrapie," once the most common subtype of the disease in small ruminant producing countries (Australia and New Zealand being the exception), is highly transmissible between sheep and goats with susceptible *Prnp* genotypes. Prevalence in most areas is low, rarely exceeding 0.05%. With the identification of the *Prnp* gene and a better understanding of its relationship to the scrapie incubation period, specific *Prnp* alleles were soon found that could afford complete resistance to classical scrapie

infection in sheep and goats. In the early twenty-first century, breeding programs to select for scrapie-resistant *Prnp* genotypes in sheep and goats have been invaluable in reducing the incidence of scrapie in endemic areas.

"Atypical scrapie," first described in Norwegian sheep in 1998, caused by the Nor98 strain mentioned previously, is a spontaneous and sporadic prion disease occurring in roughly one in a million sheep. Careful examination of archived cases suggested that this scrapie subtype may have been present decades before its initial discovery. Atypical scrapie is not zoonotic nor is it considered naturally transmissible to other sheep or goats. Atypical scrapie has been reported worldwide, including in Australia and New Zealand.

The clinical signs of classical scrapie are progressive and consist of one or more of the following CNS signs: behavioral changes, locomotor problems (incoordination, paresis, proprioceptive limb deficits, and hypermetria), mild head or neck tremors, hyperesthesia, and pruritis, which often results in patchy wool loss from scraping, scratching, or biting and is the origin of the paronym "scrapie." Behavioral changes may include withdrawal from the flock, nervousness, or aggression. A common behavior often used to aid in the clinical diagnosis of classical scrapie consists of upward extension of the head and neck with an accompanying licking, nibbling motion, or teeth grinding in response to rubbing the sheep's rump. Progressive weight loss may also be a feature of scrapie, especially as animals advance through clinical stages. Importantly, it should be emphasized that the particular clinical signs seen in any individual animal may be highly variable.

In contrast to symptoms reported for classical scrapie cases, most atypical scrapie cases have been identified through active surveillance of clinically asymptomatic sheep and fallen stock. In general, advanced cases of atypical scrapie infections may show ataxia and poor body condition; however, pruritis and loss of wool is rarely reported.

Etiologic Agent

Scrapie is caused by an abnormal isoform of the normal sheep prion protein, denoted PrP^{Sc}. There are two subtypes of scrapie, classical scrapie and atypical scrapie, as noted above, which can be differentiated based on biochemical analyses. On western blot analysis, atypical scrapie $PrP^{res/Sc}$ can be identified based on a 5-band profile with a prominent 11–12 kDa unglycosylated lower band. Classical scrapie $PrP^{res/Sc}$ is somewhat more resistant to protease treatment than atypical scrapie $PrP^{res/Sc}$, while having a heavier unglycosylated lower band (19–21 kDa typically).

A number of strains have been isolated from both scrapie subtypes using mouse bioassays, and typically these strains cannot be differentiated without a combination of biochemical analyses, inoculation of rodent models, and brain lesion profiling. The significance of the different scrapie strains isolated in rodents is not yet clear. For example, it remains to be seen whether strains isolated from classical scrapie cases (e.g. ME7 or RML) or atypical cases (e.g. Nor98 and Nor98-like) are found more commonly in specific geographic areas or in specific sheep genotypes; work to better characterize scrapie strain epidemiology is underway.

Pathogenesis

Following oral exposure in susceptible sheep and goats, experiments have tracked the accumulation of PrP^{Sc} in various lymphoid and nervous tissues over time. Initial detection most often occurs in follicular dendritic cells and macrophages within the tonsils and gut-associated lymphoid tissues (GALT), especially in Peyer's patches. PrP^{Sc} multiplies at these sites, slowly accumulating and eventually spreading through the lymphoid vasculature to other lymphoid tissues, including the spleen and peripheral lymph nodes. In the early preclinical period, prior to CNS accumulation, PrP^{Sc} may be detected by western blotting or immunohistochemistry in the retropharyngeal lymph nodes (RPLN) or the third eyelids of sheep. Scrapie prions likely access peripheral nervous system tissues through invasion of nerve endings, including those within lymph nodes or follicles that are distributed throughout the ileum or elsewhere in the gastrointestinal (GI) tract. They then travel retrograde through autonomic nerves or the vagus nerve to the thoracic spinal cord or the dorsal motor nucleus of the vagus (DMNV), respectively. The DMNV, located in an area of the brainstem colloquially referred to as the obex, along with RPLN tissues, are the samples of choice for early and sensitive *postmortem* diagnosis of classical scrapie. Once within the CNS, PrP^{Sc} multiplies and spreads using template-based conversion of PrP^{C}. The increased production and accumulation of PrP^{Sc} within the CNS, based on quantitative assessment by IHC, is directly related to the progressive development of CNS degenerative lesions and the progression of clinical signs.

As the disease progresses, there is no specific humoral or cellular immune response to the accumulating PrP^{Sc} because the protein is identical in its amino acid sequence to the host's PrP^{C}, and therefore is considered a self-antigen. For that reason, there are no available serum antibody tests that allow for *antemortem* diagnosis. Aside from secondary lesions such as hair loss, lesions associated with scrapie (or any other TSE) are found almost exclusively within the CNS. These lesions are often bilateral in distribution and mainly associated with gray matter areas. They consist of three basic changes: (i) spongiform change,

referring to vacuolation within the neuropil of gray matter representing intracytoplasmic vacuolation of nerve cells and processes; (ii) neuronal degeneration/loss, which includes intracytoplasmic vacuolation of neurons, shrunken, dark, angular neurons, rare necrotic neurons, and neuronal loss; and (iii) astrocytosis or the hypertrophy and hyperplasia of astrocytes within the gray matter, representing a nonspecific immune response to the neuronal degeneration. Lesions first appear in the caudal brainstem and progress from there to other regions of the brainstem, cerebellum, and cerebral cortex. PrP^{Sc} can be illustrated in these regions via IHC, even prior to the appearance of spongiform lesions within the CNS, often within neurons and glial cells within the neuropil.

In atypical/Nor98-like scrapie, deposits of PrP^{Sc} are not commonly observed in the DMNV. Obvious vacuolations in the brainstem, generally present in cases of classical scrapie, are also absent in atypical scrapie cases. PrP^{Sc} deposition is primarily observed in the cerebellum, substantia nigra, thalamus, and basal nuclei, making them choice sample regions for the diagnosis of atypical scrapie.

In summary, the pathogenesis of scrapie depends on both lymphoid and neural pathways to reach the CNS. During the initial stages, and subsequent to prion invasion of the CNS, PrP^{Sc} may also localize and accumulate in lymphoid populations in peripheral excretory tissues where it may be shed through various routes. Following neuroinvasion, prion amyloid accumulates in the CNS, eventually producing neurodegeneration and the telltale progressive and irreversible clinical signs.

Transmission

Classical scrapie is spread by direct contact with infected sheep, most likely through ingestion of, or mucosal exposure to, the abnormal PrP^{Sc} protein. Transmission is thought to occur most frequently when naive sheep or goats are exposed to excreta (saliva, urine, or feces) from infected sheep or when infected ewes shed PrP^{Sc} at, or soon after, lambing. The latter transmission likely results from the expulsion of PrP^{Sc}-infected placenta and/or contaminated fetal/uterine fluids. There is at yet no clear evidence of *in utero* transmission; however, experimental evidence points to the additional possibility of ewe to lamb transmission via colostrum and milk. Lambs, interestingly, appear to be more susceptible to transmission than adults, though the basis for this differential susceptibility is not yet clear. There is mixed information available on the role of semen in scrapie transmission, with some evidence of detectable PrP^{Sc} in isolated cases using PrP^{Sc} amplification techniques, though there has been no documented evidence of transmission through natural cover or artificial insemination.

Environmental transmission, through persistent environmental contamination by PrP^{Sc}-laden excreta, placental tissues, or decomposing carcasses, is also an important consideration, making the management of scrapie in the field problematic. In one report, susceptible sheep moved to a pasture previously holding scrapie-positive sheep resulted in reappearance of the disease an entire decade or more after the original flock had been depopulated, highlighting the environmental persistence of the classical scrapie agent. It is likely that environmental transmission occurs most often on pastures with high animal densities and infection rates, especially lambing pastures.

Scrapie is also readily transmissible through experimental inoculation, including through blood transfusions from infected sheep. Further research points to an association of the PrP^{Sc} protein with cellular fractions in blood, including peripheral blood mononuclear cells (PBMCs), B cells, platelets, and plasma cells of clinically affected sheep, based on *in vitro* prion amplification assays discussed later in this chapter. Although certainly possible, the risk of mechanical transmission of scrapie – through blood-contaminated needles or surgical instruments – is likely to be low and much less important than natural routes of transmission. To date there has been no evidence for mechanical or biological transmission of scrapie through blood-feeding insects.

Diagnosis

The conventional diagnosis of classical scrapie relies on the evaluation of brainstem at the level of the obex, and/or RPLN collected *postmortem* using enzyme-linked immunosorbent assay (ELISA), western blotting, or IHC (Figure 66.1). Most commonly, tissues are screened using an automated ELISA process, with positive samples subsequently confirmed by IHC. The IHC examination of certain tissues collected *antemortem*, including third eyelid and rectal lymphoid tissue (RAMALT – recto-anal mucosa associated lymphoid tissue), has been approved by the United States Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS) and the Canadian Food Inspection Agency (CFIA) for live animal testing, though the sensitivity of these tissues for diagnosing scrapie (approximately 85%) are lower than that of obex and RPLN collected *postmortem*.

Experimental PrP^{Sc} amplification techniques, including real-time quaking-induced cyclic amplification (RT-QuIC) and protein misfolding cyclic amplification (PMCA), have shown promise for improved diagnostic sensitivity over conventional ELISA and IHC. These approaches require a substrate (purified recombinant

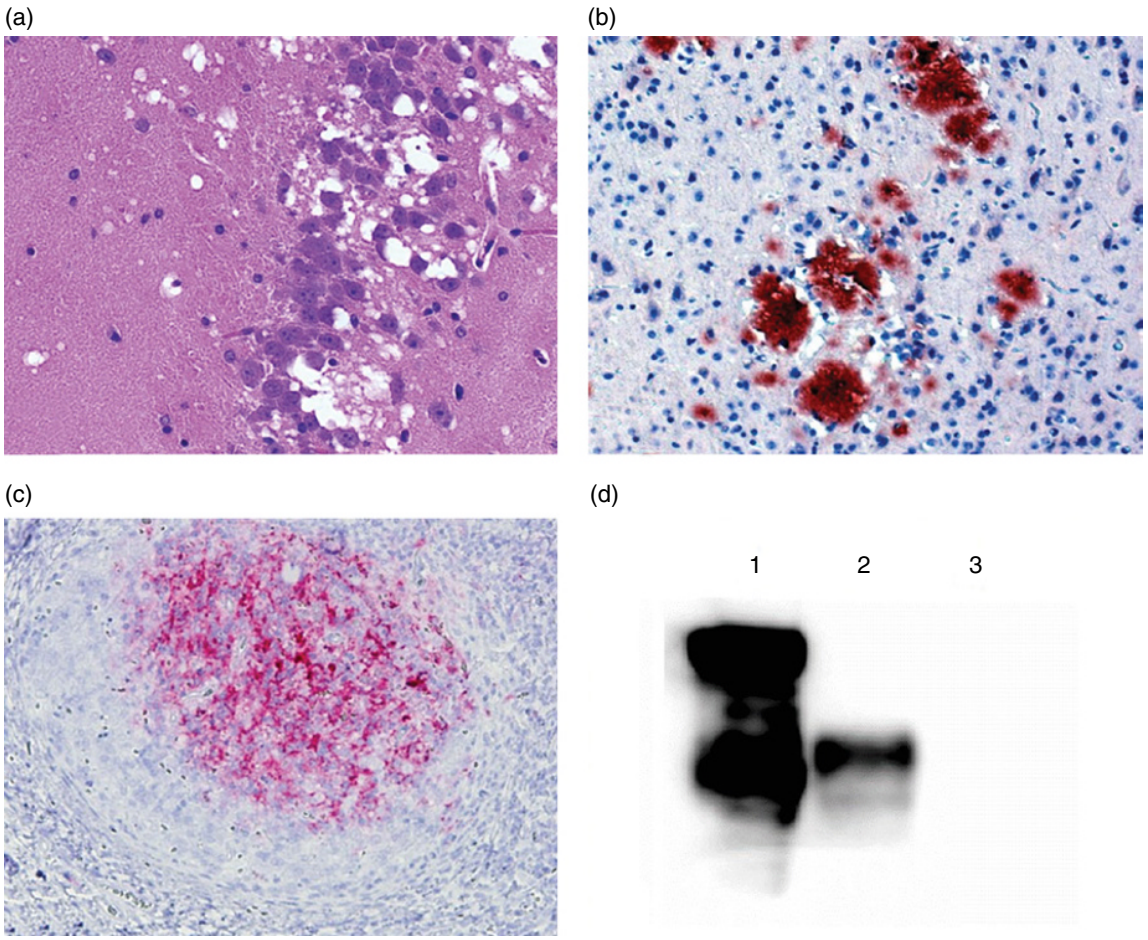


Figure 66.1 Histology and western blot appearance of TSEs and protease-resistant prions (PrP^{res}) in affected animals. (a) Typical spongiform degeneration can be seen in the cerebral gray matter of a sheep with classical scrapie. (b) Florid plaques (accumulations of misfolded prion amyloid labeled using a prion-protein-specific antibody via immunohistochemistry) associated with some TSEs. (c) Immunohistochemically labeled PrP^{res} can be seen in a lymphoid follicle associated with recto-anal mucosa associated lymphoid tissue (RAMALT) in an affected elk. (d) The western blot shows the appearance of the untreated cellular prion protein (lane 1), as well as brain homogenate from an infected animal and an unaffected animal treated with a protease, obliterating the protease-sensitive cellular prion protein in the uninfected animal (lane 3) and leaving only PrP^{res} behind in the infected animal (lane 2). RPLN: retropharyngeal lymph node.

prion protein or crude brain tissue rich in PrP^{C}) along with incubation and fragmentation steps to grow and fragment prion amyloids over successive cycles to amplify very low levels of PrP^{Sc}. The amplification of PrP^{Sc} shares much in common with polymerase chain reaction (PCR) techniques used to amplify low levels of nucleic acids. The PrP^{Sc} amplification techniques can be used with tissues, body fluids, and even environmental samples to detect increasingly low levels of prion protein. These techniques have been in development since the early twenty-first century, and although they have not yet been accepted as official confirmatory diagnostic assays for scrapie or other prion diseases; they are under continuous evaluation by federal regulatory agencies, and may be approved for use in the coming years.

Management and Prevention

Prior to the identification of the *Prnp* gene, and the link between various ovine and caprine prion gene alleles and disease susceptibility, scrapie cases identified through routine surveillance would lead to quarantine of the flock, tracing of connected herds, and eventually depopulation of the index herd. Thus, management of scrapie was initially passive and reactive. With the finding of a definitive link between specific *Prnp* mutations and scrapie susceptibility, scrapie eradication programs based on the *Prnp* genotype of animals were quickly developed in Europe and North America to better aid disease management. Although cases are still reported, the incidence of scrapie is presently rather low, and quarantine and depopulation events in the United States have been infrequent in recent years.

In sheep, natural variations in *Prnp* codons 136, 154, and 171 have been shown to be highly influential in scrapie pathogenesis, with codons 136 and 171 being the most critical to disease resistance. Whereas sheep carrying an allele coding for valine (V) at position 136, arginine at position 154 (R), and glutamine (Q) at position 171 (so-called VRQ sheep) are highly susceptible to scrapie, those that are homozygous for alanine (A) at position 136 and arginine at positions 154 and 171 (ARR/ARR sheep) are considered completely resistant to classical scrapie. Sheep breeding programs that focus on increasing the frequency of ARR alleles in domestic flocks have been invaluable in the effort to eradicate scrapie disease in the European Union and North America, effectively reducing the incidence of classical scrapie in sheep by over 90% in these areas. In goats, genetic variations in codons 146 and 222 have more recently been linked to resistance, and *Prnp* genotype-based scrapie eradication programs have been developed in both the United States and the European Union for goat herds.

CWD of Cervids

CWD of deer and related cervid species shares much in common with sheep scrapie. Although its origins are uncertain, there is some speculation that it may have arisen from scrapie. Research has shown that scrapie can be transmitted via the oral route to white-tailed deer, and CWD can be transmitted to sheep via the intracranial route. No firm evidence is available to confirm a link between scrapie and CWD, however, and it seems equally plausible that CWD arose spontaneously in a deer species just as scrapie is thought to have arisen spontaneously in sheep over 200 years ago. With no definitive links to North American CWD, the apparently independent appearance of CWD in cervid species in Norway in 2016 supports this possibility.

CWD was first identified in northern Colorado and southern Wyoming in the late 1960s and spread slowly and insidiously in this core endemic area for the ensuing decades. In the late 1990s and early 2000s, reports of CWD cases began appearing outside of this core area, including cases in Wisconsin, the Canadian province of Saskatchewan, and South Korea, in part through the movement of subclinically infected farmed deer and elk, and in part through natural migration of deer. Over the past two decades, the endemic range of the disease has continued to expand across North America, with CWD currently reported in free-ranging or farmed deer in 26 states in the United States and three Canadian provinces (Figure 66.2). Since 2016, additional cases in Scandinavia, outside of Norway, have been reported in Sweden and Finland.

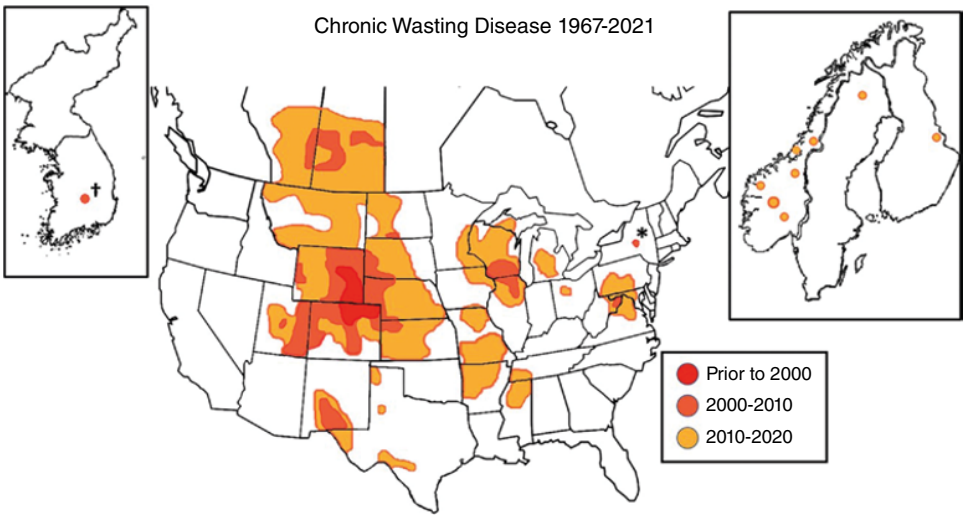


Figure 66.2 Geographic expansion of chronic wasting disease (CWD) in wild and farmed cervids from 1967 to 2021. From 1967 to 2000, chronic wasting disease was endemic in a relatively limited area of the mountain west, including a handful of counties in northern Colorado and southern Wyoming. Between 2000 and 2010, sporadic cases of CWD were reported outside of the initial endemic area, including parts of New Mexico, South Dakota, West Virginia, Wisconsin, and Utah in the United States, and Alberta and Saskatchewan in Canada. Several cases were identified in New York State in 2005 (*), and the outbreak is thought to have been contained with local herd reduction. Cases were also reported in South Korea (†; upper-left inset), where an outbreak occurred on several cervid farms in the early 2000s; that outbreak was contained through depopulation of the affected farms. Since 2010, however, CWD has continued its insidious spread from its original endemic area as well as the new endemic foci in various parts of the United States, Canada, and Scandinavia (since 2016, upper-right inset).

Clinical symptoms of CWD mirror those of scrapie, with affected animals showing behavioral changes including a reduced fear of humans or heightened aggression, along with variable neurological defects, hair loss, and wasting. Increased salivation, bruxism, polydipsia, and polyuria are also common clinical findings in advanced cases. The disease may never be apparent in free-ranging animals, as they are commonly removed from the herd in preclinical stages through predation, hunting, or vehicle collisions ("road kills"). In controlled experimental settings, CWD-infected animals frequently die as a result of aspiration pneumonia, most likely due to a combination of hypersalivation and dysphagia – a common cause of death in humans affected with CJD. The incubation period of CWD may range from 16 months to several years and is strongly influenced by the *Prnp* genotypic background of the cervid host.

A wide range of cervid species have been shown to be susceptible to CWD through either natural or experimental oral transmission, including mule deer, white-tailed deer, North American elk, moose, caribou/reindeer, red deer, sika deer, and muntjac deer. Non-cervid species have been found susceptible to CWD infection through experimental inoculation via intracranial inoculation, for example, certain nonhuman primate species, cattle, swine, raccoon, domestic cats, ferrets, and various laboratory rodents. In contrast, fallow deer have been found to be resistant to natural routes of transmission, though they are susceptible to intracranial inoculation. Based on several epidemiologic studies and extensive research in *Prnp* transgenic mouse models, there has been no evidence to suggest that CWD is zoonotic. Collectively, the disease's continued expansion across North America and now Scandinavia in both farmed and free-ranging cervids, as well the natural and/or experimental susceptibility of cervid and some non-cervid species, makes CWD the most important prion disease identified to date. Effective management of the disease will require a multidisciplinary approach, and continued research into disease pathogenesis, transmission, diagnosis, and prevention is crucial.

Etiologic Agent

The prion agent causing CWD is often referred to as PrP^{CWD}. At present, all cases are considered infectious in origin and transmissible; there have been no spontaneous or sporadic forms of CWD described so far, in contrast to scrapie. There are thought to be at least two distinct strains of CWD, based on differences in incubation periods and pathological profiles in laboratory animals. Differentiating these strains biochemically, using western blot profiles for example, has not yet been possible. There is very little epidemiologic information available for these strains, including data on geographic distribution, species susceptibility, and incubation periods in the natural host. Based on western blotting profiles, there is some evidence that recent isolates of CWD from Norway and other parts of Scandinavia may represent different strains than those present in North America.

Pathogenesis

Like scrapie, the pathogenesis of CWD relies on both lymphoid and neural pathways to reach the CNS. Following oral exposure, PrP^{CWD} can be identified in GI tract-associated lymphoid tissues, RPLNs, tonsil, and mesenteric lymph nodes along the distal GI tract by six weeks post inoculation (PI). Because of the early appearance of PrP^{CWD} in the RPLN, it is one of the preferred tissues for diagnosing CWD in deer *postmortem*. Sympathetic connections associated with the germinal centers in lymphoid tissues serve as a conduit for PrP^{CWD} to reach the CNS, with early accumulation and corresponding spongiform degeneration appearing in the DMNV. The agent continues its spread to other parts of the brain, with widespread PrP^{CWD} accumulation and neurodegeneration coinciding with the onset of clinical disease.

An incomplete picture remains concerning the appearance of PrP^{CWD} in peripheral tissues, integral to horizontal transmission as described below. It is likely that the initial appearance in excretory tissues occurs concurrent with CNS ingress, with progressive accumulation over time in salivary glands, kidneys, GI tract, and others. The effect of *Prnp* genetic profiles of the infected cervid on the kinetics of PrP^{CWD} accumulation in excretory tissues is likely significant. Although it is often argued that animals with more prolonged incubation periods may exhibit a greater duration of environmental shedding, the exact onset of shedding in cervids with specific *Prnp* polymorphisms, discussed in detail below, is unknown.

Transmission

Much work has been done over the past two decades to characterize routes of CWD transmission, relying on studies both in the natural host and gene-edited, transgenic mice, as well as experimental PrP^{CWD} amplification techniques. Several natural transmission routes are likely to be important, including horizontal transmission through excreta, maternal transmission either *in utero* or post parturition, and of course through environmental contamination. Much work remains on characterizing the onset of shedding in CWD-infected animals, and the persistence of CWD prions under various environmental conditions.

Saliva from animals in the clinical and preclinical phases of infection has been shown to carry significant levels of infectivity, approaching those which may be found in CNS tissues. Other excreta, including feces and urine, have lower yet demonstrable levels of infectivity and detectable PrP^{CWD}. As with scrapie, CWD prions have been found in various blood fractions, and have also been reported in the antler velvet of CWD-infected elk. Transmission of CWD from mother to offspring *in utero* seems likely, though the frequency of its occurrence is unknown. Thus far, there have been no reports of infectivity in colostrum or milk, though it is certainly possible based on our current understanding of the pathophysiology of CWD. There has been no evidence for CWD infectivity in semen; however, *in vitro* amplification approaches have identified low levels of CWD prions in semen, and thus sexual transmission of CWD seems plausible. Details on the timing of shedding in CWD-infected animals are not well understood; however, infectious prions have been identified in various forms of excreta from preclinically infected deer and elk. As noted above, it seems likely that *Prnp*-based variations in susceptibility may shape the onset and duration of shedding in affected cervids.

Apart from direct horizontal transmission, environmental contamination and transmission through CWD-infected carcasses is an equally important mechanism of disease spread and makes the disease incredibly difficult to manage – especially in free-ranging cervids. Using highly sensitive amplification techniques, CWD prions have been recovered from water sources in CWD-endemic areas and from soil around mineral licks, and the agent has been shown to bind tightly to soil particles including clay and quartz. CWD prions have even been experimentally recovered from plants cultivated in water containing CWD-infected brain material, although the precise role of plants in CWD transmission in nature is not yet known.

Diagnosis

The diagnosis of CWD requires the examination of obex and RPLN, collected *postmortem*. In white-tailed and mule deer, the RPLN has shown the greatest sensitivity, whereas in elk the obex is commonly relied on for definitive diagnosis. Appropriate *postmortem* tissues in other cervid species, including moose and reindeer, may vary, though in general collection and examination of both obex and RPLN is preferred. As with scrapie, samples are often screened first via ELISA, with positive results confirmed by IHC.

Antemortem testing approaches, using RAMALT or tonsil biopsies for example, are well documented, although to date have not been approved for regulatory testing. The diagnostic sensitivities of these tissues are lower than those of the above-described tissues collected *postmortem*, typically 70–90%. Interestingly, *antemortem* test sensitivity may be significantly affected by the animal's *Prnp* genotypic background, with greater sensitivity reported in the highly susceptible *Prnp* genotypes described in the following section.

Experimental amplification techniques, including RT-QuIC and PMCA, have allowed for much greater sensitivity than conventional ELISA and IHC in samples collected either *antemortem* or *postmortem*. They have additionally allowed for testing samples considered unsuitable by conventional methods, including saliva, nasal swabs, blood, urine, and feces. Although these assays have not yet been approved for regulatory confirmatory testing, continued development and validation foreshadows their eventual acceptance into official diagnostic approaches.

Prevention and Management

Although different alleles for the *Prnp* gene have been reported in various cervid species, none have yet been found to provide complete resistance to natural CWD infection. There are several notable polymorphisms identified in white-tailed deer, mule deer, or elk that are associated with a lowered risk of CWD infection at the herd level and protracted disease incubation periods in individual animals, indicating that some level of resistance is possible. These include the Q95H (glutamine [Q] to histidine [H] at position 95) and G96S (glycine [G] to serine [S] at position 96) polymorphisms in white-tailed deer, a S225F (serine [S] to phenylalanine [F] at position 225) polymorphism in mule deer, and a M132L (methionine [M] to leucine [L] at position 132) polymorphism in elk. Animals heterozygous or homozygous for these amino acid substitutions are underrepresented in naturally occurring cases of CWD, and when found to be infected are often in earlier stages of the disease. When experimentally infected, these animals have significantly longer incubation periods. Notably, these amino acid substitutions exhibit an additive effect with regard to differential susceptibility, whereby prevalence rates may be even lower, and disease course more prolonged, in homozygous animals compared to those that are heterozygous. Although these polymorphisms, and others in related cervid species, have not been found to confer complete resistance to CWD, ongoing genome-wide association studies may identify additional polymorphisms and genes that may contribute to CWD resistance in cervids. Newly identified resistance-associated genes may also be useful for managing prion diseases or protein misfolding disorders in other animal species.

In contrast to classical scrapie, our current understanding of genetic resistance in cervids does not allow for

effective CWD prevention and management; therefore, CWD control must rely on more traditional approaches. In both farmed and wild cervid herds, active surveillance is very important to identify CWD cases early and minimalize horizontal transmission and environmental contamination. Quarantine, trace-outs, and depopulation is the most common approach for managing CWD in farmed herds, whereas herd reduction and dispersal strategies are often relied upon when managing CWD in wild cervid populations. The continued expansion of CWD in both farmed and wild cervid populations suggest that the development of more effective management approaches will be critical for controlling disease spread.

Bovine Spongiform Encephalopathy

BSE or "mad cow disease" was in the late twentieth and early twenty-first centuries the most economically important prion disease of veterinary medicine, and was eventually linked to a new variant of Creutzfeldt-Jakob disease (vCJD) in humans. Clinical symptoms of BSE are typical of those found in other prion diseases, including weight loss and behavioral abnormalities (apprehension, depression, etc.). Progressive neurological dysfunction is also present in advanced cases, and may include hyperesthesia, hyperreflexia, muscle tremors, ataxia, pruritis, and reduced autonomic function (e.g. reduced rumination and bradycardia). Incoordination and inability to rise are important discriminators often used to restrict cattle from conventional slaughterhouse processing. Both classical and atypical (spontaneous) subtypes of BSE have been identified, similar to what has been reported with scrapie, and these forms can be differentiated by western blotting techniques.

Classical BSE emerged as a newly recognized TSE in England in 1986, showing a rapid increase in incidence over the next few years. Soon after, novel TSE diseases were reported in captive zoo ungulates and felids, as well as domestic cats in the United Kingdom. Cases of a vCJD in humans were first identified in 1996, and were distinguished from sporadic cases of CJD based on age of patient at symptomatic onset and other clinical findings. These "zoonotic spillover events" were all ultimately linked to BSE-contaminated beef, sausage, and other by-products entering the food chain.

Soon after the detection of classical BSE in the United Kingdom in 1986, cases of BSE began appearing in other countries across Europe. These cases were ultimately linked to the export of infected animals, BSE-contaminated meat and bone meal (MBM), and possibly other contaminated cattle by-products. Since its initial descriptions, classical BSE has been reported in 26 countries, including cases in Europe, Israel, Japan, and North America, whereas atypical BSE cases have been reported in several European countries, Canada, the United States, Brazil, and Japan.

Wild ungulate and felid TSEs, as well as vCJD cases, indicated that BSE, unlike other known TSEs, has an unusually wide susceptible host range. A handful of BSE cases have also been reported in goats, likely through consumption of contaminated feed. Successful experimental infections have been reported in sheep, pigs, red deer, several nonhuman primates, and mice, although natural susceptibility (through horizontal transmission) in any of these species has not been demonstrated. Because of its wide host range, and particularly with its association with vCJD, BSE is considered a major threat to public health, forming the basis for national surveillance and eradication programs.

Epidemiologic analyses strongly suggest that the primary mode of classical BSE transmission is through the ingestion of feed containing BSE-contaminated MBM. Since a nearly global ban on the use of MBM in ruminant feed, the incidence of the classical form of BSE has decreased sharply, with just a handful of cases reported since 2010. Over the past decade, the majority of the roughly 30 cases of BSE reported worldwide have been of the atypical subtype. There has been no definitive evidence of horizontal transmission of either the classical or atypical forms of BSE. Young cattle appear to be more susceptible to infection with the classical form than adults, and there is an increased risk of BSE in calves born to BSE-infected cows. The mechanism responsible for this increased risk in calves is unknown; however, the possibility of maternal transmission *in utero* or post parturition has not been completely ruled out. There is no strong evidence for environmental transmission of either the classical or atypical subtype.

The initial source of infectious material responsible for the emergence of BSE in the United Kingdom may never be known, though several theories have been provided to explain its emergence (i) BSE arose from the feeding of MBM containing scrapie-contaminated sheep offal to cattle; (ii) BSE arose from a spontaneous or genetic case (or cases) of BSE in cattle that was then rendered and fed as cattle prion-contaminated MBM to other cattle; or (iii) BSE arose from animal feed imported from the Indian subcontinent that had been contaminated with the remains of humans suffering from one of the various human prion diseases. A significant increase in sheep populations throughout the United Kingdom, and a subsequent increase in scrapie incidence supports a link between scrapie and BSE. Regardless of the source, epidemiologic evidence suggests that a change in the rendering process in England in the late 1970s may have allowed for the increased survival of misfolded prion proteins in MBM, allowing for

increasing levels of cattle exposure to the prion agent, and ultimately an epidemic that would not be curtailed until the practice of feeding MBM was halted in the United Kingdom in 1988. Because of the delay between exposure and clinical signs (an incubation period of roughly four to five years), the BSE epidemic peaked in 1992–1993, with over 37 000 cases diagnosed in 1992 alone. Cases of vCJD in humans peaked roughly eight years later, with nearly 230 cases reported worldwide since its discovery.

Etiologic Agent

BSE, like other prion diseases, is caused by a misfolded protein, often denoted as PrP^{BSE}. Several different subtypes, including the classic form and two atypical forms – the L-type (lower molecular weight form, sometimes referred to as bovine amyloidotic spongiform encephalopathy or BASE) and the H-type (higher molecular weight form)-have been described. Each of these forms can be distinguished from one another by evaluating the degree of glycosylation and molecular mass of PrP^{res} on western blot analysis. Atypical L-type BSE is characterized by a slightly lower molecular mass of the unglycosylated PrP^{res} compared to that of classical BSE PrP^{res}, whereas the H-type shows a higher molecular mass for the unglycosylated PrP^{res} band than that of classical BSE.

As with scrapie, most cases of atypical BSE have been detected through active national surveillance programs designed to detect cases of classical BSE, which included sampling of healthy, sick, and downer cattle as recommended by the World Organisation for Animal Health (OIE) guidelines. In general, the atypical BSE cases do not have distinct clinical signs as described for classical BSE. Most importantly, the majority of atypical BSE cases are reported in cattle over 10 years of age. In active surveillance for BSE in France from 2001 to 2007, the estimated incidence of atypical BSE was 0.41 and 0.35 cases per million for H- and L-type forms, respectively. Case incidence in animals over eight years of age were higher, with 1.9 and 1.7 cases per million for H- and L-type forms, respectively. This frequency is similar to that of sporadic CJD, which occurs in roughly one case per million people yearly, suggesting that these atypical BSE cases are likely sporadic and spontaneous in nature.

The PrP^{BSE} agent has been recovered in goats naturally exposed to BSE-contaminated feed. Although no naturally occurring BSE-linked cases have been described in sheep, scrapie strain CH1641 shows some biochemical similarities to BSE on western blot. BSE and CH1641 in experimentally infected sheep can be discriminated using strain-specific antibodies or mouse inoculation studies.

Pathogenesis

Following oral exposure, PrP^{BSE} is first detected in the lymphoid follicles of the distal ileum (Peyer's patches) by approximately six months post infection (PI). The agent may also be recovered from tonsillar tissue between 6 and 14 months PI. Unlike either scrapie or CWD, BSE pathogenesis appears to bypass further lymphoid accumulation; PrP^{BSE} has not been detected to any significant degree in retropharyngeal, mesenteric, or popliteal lymph nodes of naturally infected cattle. The BSE agent is first detected in the CNS and dorsal root ganglia at roughly 32 months PI, followed by the trigeminal ganglia at 36 months PI, likely transported via retrograde axonal transport in peripheral nerve endings innervating gastrointestinal targets. Upon reaching the CNS, PrP^{BSE} progressively accumulates and is associated with degenerative CNS lesions that are generally similar to those reported in other prion diseases. As with other TSEs, there is no specific immunologic response to the PrP^{BSE} agent.

Transmission

As noted previously, the transmission of classical BSE depends on the consumption of BSE-contaminated feeds, for example, rendered MBM derived from BSE-affected cattle. The lack of detectable PrP^{BSE} in broader peripheral lymphoid tissues and excretory organs likely correlates with the absence of horizontal BSE transmission, unlike what is seen with scrapie and CWD. For that reason, a ban on the use of MBM in ruminant feed in the United Kingdom in 1988 essentially broke the transmission chain of BSE. A further ban on specified bovine offal ("offal ban") in 1990, so-called "specified risk material," including skull, CNS, eyes, tonsils, and the distal ileum of the small intestine, from the animal and human food chain further precluded the transmission of BSE to ruminants and other species. Despite these measures, evidence suggested that new foodborne cases continued in animals, and in 1996 the offal feed ban was extended to a total ban on the use of mammalian proteins in feed produced for any livestock. Cases of classical BSE are now rare, outnumbered 10-to-1 by sporadic cases of atypical BSE.

Zoonotic transmission of the BSE prion to humans has been well documented, though it has fortunately been a rare occurrence. Since the initial reports of vCJD in 1996, 229 cases worldwide (as of August 2021) have been reported – the vast majority in the United Kingdom. Interestingly, almost every case of vCJD in humans shared a common *Prnp* genotype – homozygosity for methionine at position 129 of the *Prnp* gene (129M). An alternate allele, carrying a valine at position 129 (129V), was found to

confer a very high level of protection against vCJD infection. The 129 genetic variation in humans is at a position nearly synonymous with the M132L polymorphism in elk, although as noted earlier, elk carrying the 132L gene remain susceptible to CWD.

Prevention and Management

The prevention and management of BSE relies entirely on restricting the use of MBM and other specified risk materials in cattle feed. Although there have been no mutations within the cattle *Prnp* gene found to confer resistance to classical BSE, several insertion/deletion polymorphisms in the promoter region controlling *Prnp* expression levels have been associated with increased disease susceptibility. In addition, an E211K (glutamic acid [E]to lysine[K]) polymorphism at position 211 within the bovine *Prnp* gene may be associated with genetic H-type BSE. This polymorphism is similar to an E200K polymorphism in humans, which is thought to be associated with a high incidence of inherited CJD. Cattle that had the *Prnp* gene "knocked-out" have been developed and shown completely resistant to BSE infection, though their utility given the scarcity of BSE cases in the present day is limited to producing safe products for biomedical use.

Other Notable Prion Diseases

Camel Prion Disease

A novel prion disease of dromedary camels was reported in North Africa in early 2018. Early reports suggest that the camel prion disease shares some commonality with both scrapie and CWD, including the presence of PrP^{res} in lymphoid tissues. Symptoms including weight loss, behavioral abnormalities, and neurological signs were present in roughly 3% of animals submitted to a single slaughterhouse in Algeria, and most importantly were only observed in animals over eight years of age. Details on the suspected origin of the disease, its geographic distribution, its transmissibility between camels and related species, or zoonotic potential are not yet known; however, evidence currently points to an infectious transmission rather than a spontaneous disease. There have been no systemic evaluations of heterogeneity in the camel *Prnp* gene, and thus no studies available to characterize susceptibility and resistance based on *Prnp* variations. The distribution of dromedary camels across Africa, Asia, and Australia, and Bactrian camels in Central Asia, and the dependence of respective human populations on them for transport, meat, milk, medical uses (e.g. urine) and fiber highlight the importance of learning more about this newly discovered prion agent.

Feline Spongiform Encephalopathy

Concurrent with the BSE epidemic in Europe in the late twentieth century, a spongiform encephalopathy was reported in domestic and captive zoo cats, including cheetahs, pumas, and ocelots, called feline spongiform encephalopathy (FSE). The disease is thought to have arisen in cats that had been fed commercial feeds contaminated with BSE-containing tissues. Since the resolution of the BSE epidemic, reports of FSE have been sparse. Behavioral changes and neurological signs were reported in both domestic and zoo cats. The bulk of abnormal prion accumulation appeared in the CNS of affected cats, and there has been no evidence that FSE is horizontally transmissible or zoonotic. Like other prion disorders, the incubation period of FSE is long – estimated at two to nine years in domestic cats and five to eight years in cheetahs.

Transmissible Mink Encephalopathy

In the mid-twentieth century, sporadic reports appeared of a spongiform encephalopathy in farmed mink in the midwestern United States, with the most recent case being in Wisconsin in 1985. There is a hypothesis that outbreaks of transmissible mink encephalopathy (TME) may have been associated with feed contaminated with sheep scrapie, or perhaps cattle prion-contaminated tissues, though a definitive link has never been confirmed. Clinical signs, reported in breeding animals over one year of age, were consistent with other prion diseases. There was no evidence of horizontal or vertical transmission between mink and no evidence that TME is zoonotic.

Prion Disinfection

Due to the remarkable stability of misfolded prion proteins in the environment, disinfection of prion-contaminated carcasses, instruments, surfaces, and environments is incredibly challenging. Initial experiments supporting the hypothesis that prion agents lacked nucleic acids clearly demonstrated their resistance to common decontamination treatments, including alcohol, UV radiation, and autoclaving. Prions have been shown to bind tightly to various substrates, including steel, plastic, and clay, making them even more resistant to decontamination procedures.

For interior surfaces, instruments, and other equipment, a >2% bleach (sodium hypochlorite) solution is often

recommended, with application times greater than one hour at room temperature. Autoclaving at >135 °C for >20 minutes at 30 lb/in^2 may effectively reduce the level of infectivity on instruments and other equipment. Cleaning gross contamination with standard detergents is recommended prior to bleach treatment or autoclaving, though no physical treatment alone or in combination has been found to completely eliminate the risk of prion contamination.

Disinfection and decontamination of environments, including stalls, pastures, and wildlife habitat, is understandably more problematic. Of primary concern is the removal of carcasses, which may either be incinerated, buried in a landfill, or individually destroyed via alkaline hydrolysis in a "digester", a piece of equipment that obliterates organic material using potassium hydroxide, heat, and pressure. The remaining environmental contamination may be reduced by removal of the topsoil through tilling, for example; however, complete removal of environmental sources of prion transmission is likely impossible, especially given that infectious prions may be taken up by plants and groundwater. Subsequent exposure is best prevented by either leaving the property unused through fencing or otherwise preventing access, or restocking with animals with resistant *Prnp* genotypes or non-susceptible animal species.

Summary and Conclusions

Transmissible spongiform encephalopathies are unlike any of the infectious agents discussed previously, and are caused by a misfolded variant of a normal cellular protein. In select cases, these infectious prions may be transmitted horizontally or through the environment from an infected animal to hosts of the same or closely related species. Contaminated environments are likely long-lasting and represent a continued source for new infections. Fortunately, the only TSE reported to be zoonotic, BSE, has been very effectively managed, with a small number of cases reported on a yearly basis worldwide. Genetic management of scrapie has likewise significantly reduced the global incidence of scrapie. At present, CWD poses the greatest threat to animal health among the remaining TSEs, both in North America and now also in Scandinavia.

A better understanding of TSE pathogenesis and transmission, and the role of *Prnp* and prion-associated genes in this process, is important to more effectively manage animal and human prion diseases. The development and implementation of highly sensitive prion amplification techniques will aid in the early and accurate *antemortem* diagnosis of infected animals and humans and identification of contaminated products and environments. Despite numerous investigations, no effective treatment options have been identified for any of the reported TSEs, and there are limited options available for disease management at the herd level. Recent research describes novel approaches of vaccination against prion diseases, which seem to be promising, at least in rodent models. Further research into these key areas – pathogenesis, transmission, detection, treatment, and prevention – will not only benefit veterinary medicine, but human medicine as well, with far-reaching applications for important human protein misfolding disorders such as Alzheimer's and Parkinson's diseases.

Further Reading

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Part V

Control of Infectious Diseases

67

Immune Responses to Infectious Agents

Laurel J. Gershwin

Introduction

The overall premise upon which the immune system functions is that self and non-self can be differentiated, that non-self is a potential danger to the host, and therefore must be eliminated. The ability to determine what is and what is not self is therefore important. Immune responses to infectious agents are initiated by recognition of *pathogen-associated molecular motifs (*PAMPS*)* that are present on or in bacterial, viral, and parasitic pathogens. Recognition occurs upon initial entry of the infectious agent through skin, oral, or respiratory routes. Receptors for these PAMPS are situated on and in sentinel cells that are located at these entry sites. The initial contact between sentinel cells and pathogen gives rise to the production of a variety of molecules that function in an *innate immune response*. In addition, some of the sentinel cells are antigen-presenting dendritic cells that respond differentially to pathogens of different types. These cells initiate an *adaptive immune response* by producing small molecules called cytokines, which can influence the nature of the adaptive immune response. Once the appropriate response has been induced, products of the adaptive immune system act to decrease the spread of the infectious agent within the body. Acquired immune responses include products of both T and B lymphocytes, reflecting the type of immune components required to handle the particular pathogen that is present. The adaptive immune response is characterized as being pathogen specific as well as possessing the ability to "remember" specific pathogens; a process called immunologic memory.

Innate Immunity

As described above, innate immune responses initiated when a pathogen gains entrance to the body involve interaction of PAMPs with sentinel cells that are situated near potential entry sites.

Pattern recognition receptors present on or in sentinel cells serve as ligands for components of bacterial and viral organisms. PAMPS vary by pathogen but consist of substances such as bacterial lipopolysaccharide, flagellar protein, and viral proteins. Sentinel cells include mast cells, dendritic cells, Langerhans cells, and macrophages. Sentinel cell receptors detect these PAMPS. Binding of the PAMPS to their ligands triggers sentinel cell activation and production of pro-inflammatory cytokines: Interleukin 1 (IL-1), tumor necrosis factor α(TNFα), and Interleukin 6 (IL-6). Among other functions, these cytokines trigger the "sickness behavior" that is associated with infection.

Toll-like receptors (TLR) are pattern recognition receptors that are present on sentinel cells and on a variety of other cells. Originally described in *Drosophila*, these molecules and their signaling pathways are important for detection of invading pathogens in fruit flies, mammals, and plants. There are 10 known TLRs in mammals. TLRs and their ligands are listed in Table 67.1. Each TLR recognizes a specific component of a pathogen. For example, TLR1, TLR2, and 6 recognize various microbial components. TLR2 recognizes lipoproteins, lipoteichoic acid from gram-positive bacteria, and lipoarabinomannan from mycobacteria. TLR3 recognizes double-stranded RNA and is therefore important in viral recognition. TLR4 has a role in

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Table 67.1 Mammalian Toll-like receptors bind and become activated by different ligands, which, in turn, are located on different types of organisms or structures.

Receptor	Ligand(s)	Ligand location	Adapter(s)	Location	Cell types
TLR 1	Multiple triacyl lipopeptides	Bacterial lipoprotein	MyD88/MAL	Cell surface	• Monocytes/ macrophages • A subset of dendritic cells • B lymphocytes
TLR 2	Multiple glycolipids	Bacterial peptidoglycans	MyD88/MAL	cell surface	• Monocytes/ macrophages • Neutrophils • Myeloid dendritic cells • Mast cells
	Multiple lipopeptides and proteolipids	Bacterial peptidoglycans			
	Lipoteichoic acid	Gram-positive bacteria			
	HSP70	Host cells			
	Zymosan (Beta-glucan)	Fungi			
	Numerous others				
TLR 3	Double-stranded RNA, poly I:C	Viruses	TRIF	Cell compartment	• Dendritic cells • B lymphocytes
TLR 4	Lipopolysaccharide	Gram-negative bacteria	MyD88/MAL/ TRIF/TRAM	Cell surface	• Monocytes/ macrophages • Neutrophils • Myeloid dendritic cells • Mast cells • B lymphocytes (only in mice) • Intestinal epithelium • Breast cancer cells
	Several heat shock proteins	Bacteria and host cells			
	Fibrinogen	Host cells			
	Heparan sulfate fragments	Host cells			
	Hyaluronic acid fragments	Host cells			
	Nickel				
	Various opioid drugs				
TLR 5	Bacterial flagellin	Bacteria	MyD88	Cell surface	• Monocyte/ macrophages • A subset of dendritic cells • Intestinal epithelium • Breast cancer cells • B lymphocytes
	Profilin	*Toxoplasma gondii*			
TLR 6	Multiple diacyl lipopeptides	Mycoplasma	MyD88/MAL	Cell surface	• Monocytes/ macrophages • Mast cells • B lymphocytes
TLR 7	Imidazoquinoline	Small synthetic compounds	MyD88	Cell compartment	• Monocytes/ macrophages • Plasmacytoid dendritic cells • B lymphocytes
	loxoribine (a guanosine analogue)				
	Bropirimine				
	Resiquimod				
	Single-stranded RNA	RNA viruses			
TLR 8	Small synthetic compounds; single-stranded viral RNA, phagocytized bacterial RNA		MyD88	cell compartment	• Monocytes/ macrophages • A subset of dendritic cells • Mast cells • Intestinal epithelial cells (IECs) [a]

Table 67.1 (Continued)

Receptor	Ligand(s)	Ligand location	Adapter(s)	Location	Cell types
TLR 9	Unmethylated CpG oligodeoxynucleotide DNA	Bacteria, DNA viruses	MyD88	Cell compartment Endoplasmic reticulum	• Monocytes/ macrophages • Plasmacytoid dendritic cells • B lymphocytes
TLR 10	Triacylated lipopeptides		MyD88	Cell surface	• B cells • Intestinal epithelial cells • Monocytes/ macrophages
TLR 11	Profilin Flagellin	*Toxoplasma gondii*[36] Bacteria (*E. coli*, *Salmonella*)[23]	MyD88	Cell compartment[37]	• Monocytes/ macrophages • Liver cells • Kidney • Urinary bladder epithelium
TLR 12	Profilin	*Toxoplasma gondii*[38]	MyD88	Cell compartment	• Neurons • Plasmacytoid dendritic cells • Conventional dendritic cells • Macrophages
TLR 13	Bacterial ribosomal RNA sequence "CGGAAAGACC" (but not the methylated version)	Virus, bacteria	MyD88, TAK-1	Cell compartment	• Monocytes/ macrophages • Conventional dendritic cells

They also have different adapters to respond to activation and are located sometimes at the cell surface and sometimes to internal cell compartments. Furthermore, they are expressed by different types of leukocytes or other cell types.
[a] Only in Crohn's or ulcerative colitis
Source: From Waltenbaugh et al. (2008, p. 17).

transducing the signals of lipopolysaccharide (LPS) from gram-negative bacteria. TLR5 is activated by flagellin, its specific ligand. Thus, TLR5 is important in the response to motile bacteria, but not to those that lack flagella. When the basolateral surface of the intestinal epithelium is exposed to flagellin an inflammatory response occurs. This is the site of expression of TLR5. TLR7 is activated by single-stranded RNA viruses. TLR9, a receptor expressed within the cell, recognizes unmethylated CpG motifs in bacterial DNA.

Recognition of viruses by TLR has been reported for some viruses. TLR4 binds to one of the major surface proteins on respiratory syncytial virus (RSV), an important pathogen of human children and bovine calves. In an experimental mouse model with mutated TLR4, RSV was found to be cleared less efficiently than from mice that have normal TLR4. Other viruses, such as mouse mammary tumor virus, have been shown to bind TLR4 to envelope proteins. In addition, signaling through TLR3 and TLR7 has been shown to induce synthesis of alpha and beta interferons.

Other studies with TLR mutant mice have demonstrated the importance of these receptors in resistance to bacterial infection. TLR4-mutant mice are highly susceptible to infection with the gram-negative bacteria *Salmonella typhimurium*. In contrast, mice defective in TLR2 are highly sensitive to infection with the gram-positive bacteria *Streptococcus pneumoniae*.

Additional Innate Immune Defenses: Antiviral Molecules

Viral infections stimulate the production of a variety of molecules with antiviral effect. The best known of these are the interferons. These molecules were named because of their ability to interfere with viral reproduction in uninfected cells surrounding an infected area. Interferons α and

β are the most studied of these molecules. Together they are referred to as type I interferons to distinguish them from Interferon γ, which is called Type II interferon, also known as immune interferon, as it is a product of T lymphocytes. Many different cell types can be induced to make interferons α and β interferons in response to viral infection. Interferon-induced proteins have also been described. These molecules have various mechanisms that serve to decrease viral propagation in the host. One such example is RSAD2 or viperin, which is made by virus-infected epithelial cells and dampens viral propagation.

Effector Cells of the Innate Immune System

The Neutrophil

The polymorphonuclear leukocyte, the neutrophil, is a bone-marrow-derived end-cell that normally comprises 30–70% percent of the total leukocytes in the peripheral blood of various species. The neutrophil is a granulocytic leukocyte and contains two types of granules: primary or azurophilic granules and secondary or specific granules. Neutrophils spend only about 12 hours in circulation, then go into the tissues, where they survive for an additional two to three days. Within the bone marrow there is a large storage compartment for neutrophils in most species. A bacterial infection within the body initiates a rapid mobilization of this pool, and the neutrophils accumulate at the site of the infectious process. They are attracted by the chemotactic factors C3a and C5a, which are generated subsequent to activation of the complement system. The process of neutrophil accumulation begins when circulating neutrophils adhere to the vascular endothelium (margination), extravasate into tissue spaces, and move toward the focus of injury by chemotaxis (Figure 67.1). Invading microorganisms are ingested by neutrophils in a process called phagocytosis (Figure 67.2a–c).

Phagocytosis of bacteria by neutrophils involves several steps. First, initial recognition and binding occur. This process is made more efficient by the presence of opsonins consisting of immunoglobulin and/or complement components. Opsonization coats the surface of a bacterium, neutralizing the net negative charges, which might otherwise cause the neutrophil and bacterial cell to repel each other (Figure 67.2c). In addition, on the cell membrane of the neutrophil, receptors are present for antibody (Fc receptors) and for complement (CR). These receptors facilitate firm attachment of the opsonized bacterium to the neutrophil. Next, pseudopodia form around the organism and then fuse to form a phagocytic vacuole containing the organism. Some

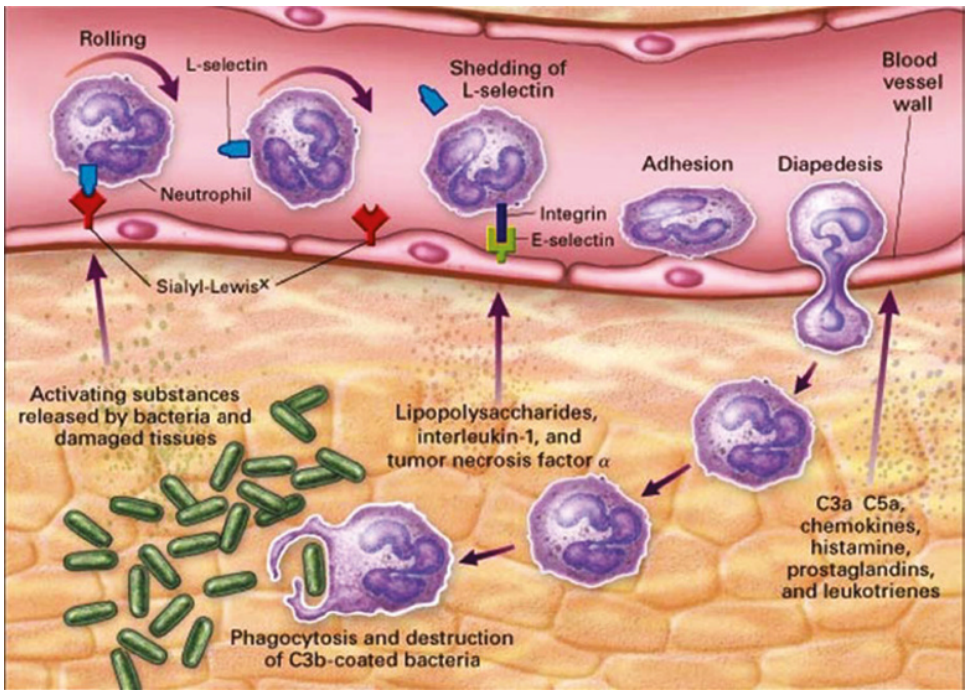


Figure 67.1 The process of neutrophil responses to extravascular bacterial invasion is illustrated: in response to chemotactic stimuli (e.g. C3a, C5a) neutrophils in the circulation begin to express adhesion molecules, L-selectins, which allow them to begin rolling along the endothelium. Integrins bind to E-selectins on endothelium and facilitate adhesion of the neutrophil to the endothelium. Diapedesis occurs, and the neutrophils travel along the chemotactic gradient to the bacteria. At this point, they phagocytose the bacteria for intracellular killing. C3b acts as an opsonin to facilitate the attachment of the neutrophil to the bacteria.

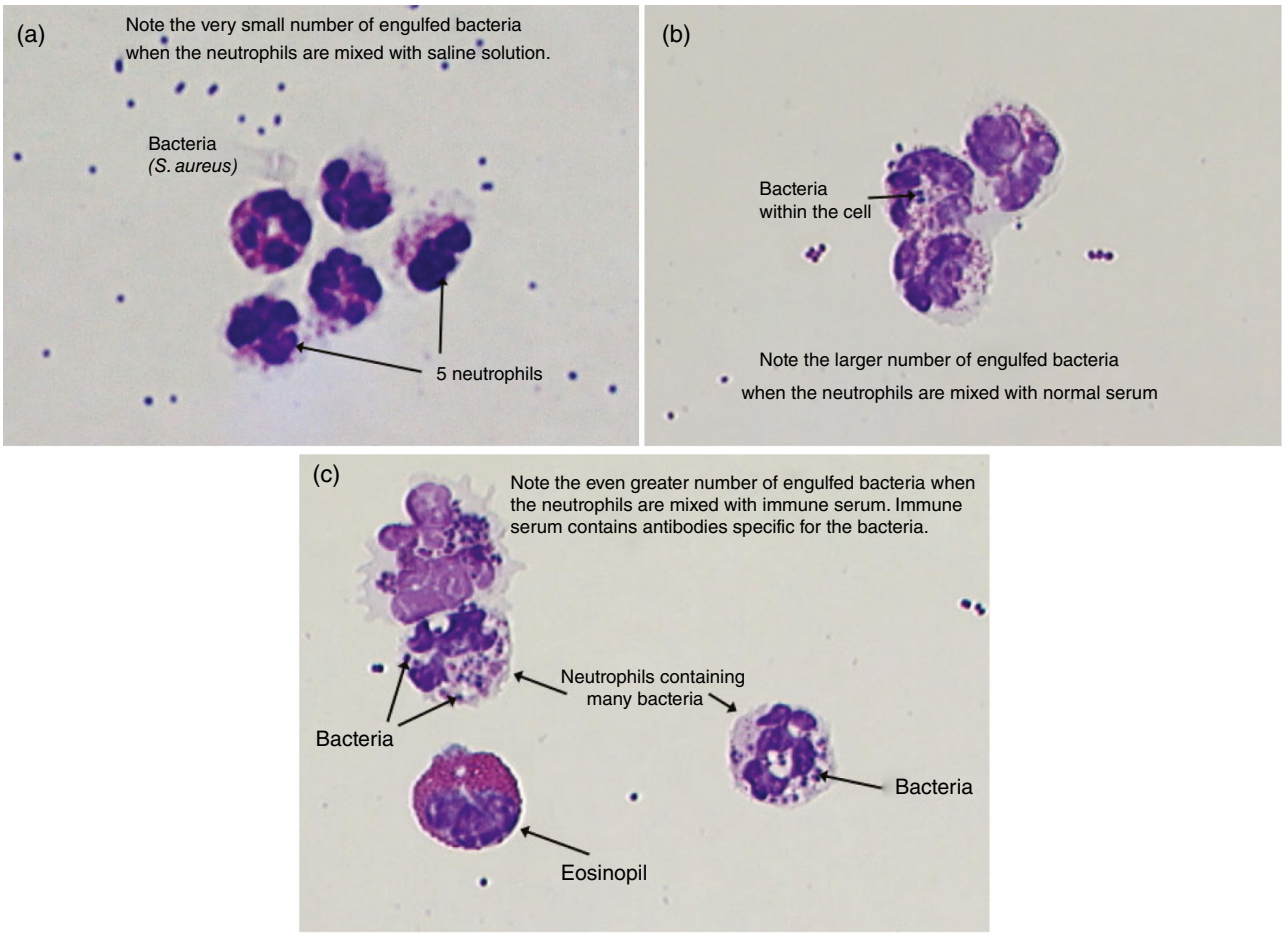


Figure 67.2 (a) Five neutrophils are shown after *in vitro* mixing with the bacterium *Staphylococcus aureus* in a saline environment. Few bacteria are engulfed in the absence of serum-derived opsonins. (b) Neutrophils mixed with *Staphylococcus aureus* in normal serum show engulfment of more bacteria. This illustrates the role of serum opsonins (complement and antibodies) in the phagocytosis process. (c) Neutrophils mixed with *Staphylococcus aureus* in immune serum show enhanced engulfment of bacteria. This illustrates the role of specific antibody as effective opsonins (complement and antibodies) in the phagocytosis process.

organisms are more readily engulfed than others. For example, the presence of a polysaccharide capsule causes an organism to be resistant to phagocytosis. After engulfment, lysosomal granules fuse with the phagosome membrane to form the phagolysosome. The eventual elimination of the engulfed organism occurs within this structure.

Bacterial killing is accomplished by a series of metabolic and enzymatic events. Metabolic activity increases within a neutrophil during phagocytosis. Oxygen consumption increases, and light energy is emitted (chemiluminescence). This metabolic or respiratory burst involves oxidation of glucose by the hexose monophosphate shunt. Bactericidal products are generated. Superoxide radicals are produced and converted to H_2O_2 by superoxide dismutase. Hydrogen peroxide is toxic for bacteria that lack catalase. The enzyme myeloperoxidase, present in azurophil granules, catalyzes the oxidation of halide ions to hypohalite, which is also toxic to microorganisms. Thus, the myeloperoxidase-hydrogen-peroxide-halide system is efficient in bacterial killing. Susceptible organisms are killed within minutes. Inside the primary granules of neutrophils, enzymes released during degranulation act on proteins, lipids, carbohydrates, and nucleic acids to degrade the killed bacterial cells. Some of these enzymes are collagenase, elastase, acid phosphatase, phospholipase, lysozyme, hyaluronidase, acid ribonuclease, and deoxyribonuclease. Lysozyme can cleave glycosyl bonds in the bacterial cell wall, making the cell susceptible to lysis. Also, lysosomes contain cationic peptides (defensins), described above, that form lethal pores in bacteria as well as in fungal cell walls.

The Macrophage

The macrophage is a mononuclear cell derived from the bone marrow. For several days after release from the bone marrow, it circulates as a blood monocyte before going into

the tissues, where it becomes a functional macrophage. Free macrophages are present in many parts of the body and are named accordingly, for example, alveolar macrophages (lung) and peritoneal macrophages. Fixed macrophages line sinus cavities that filter blood. These include the Kupffer cells (liver), Langerhans cells (skin), histiocytes (connective tissue), mesangial cells (kidneys), and sinus-lining cells of the spleen, lymph nodes, and bone marrow. Some of these macrophages are important in antigen processing for induction of an immune response (described later in this chapter).

The macrophage differs from the neutrophil in that it has a longer life span in tissue and can reuse phagolysosomes. In addition, macrophages stimulated by cytokines (e.g., interferon γ) or microbial products (e.g. lipopolysaccharide) result in activation of nitric oxide synthase that catalyzes the production of nitric oxide (NO) from l-arginine. NO is toxic to many bacteria, especially those residing within macrophages (e.g. *Salmonella*, *Listeria*). The macrophage is similar to the neutrophil in that toxic oxygen metabolites are generated for bacterial killing and the lysosomes contain potent hydrolytic enzymes and cationic peptides (defensins). Whereas the neutrophil responds to a stimulus rapidly, the macrophage is not present until later in an infectious process, often after 8–12 hours. In some instances, neutrophils may eliminate an organism before macrophages arrive in any great number. When tissue destruction has occurred as a result of an inflammatory response, macrophages are attracted to the area by products from dying neutrophils and bacteria. They phagocytose the debris and remove it. In some instances, macrophages may engulf particulate material that they are unable to digest. When this occurs, the macrophage may migrate to a mucosal surface such as the respiratory or gastrointestinal tract for elimination from the body.

Unlike the neutrophil, which is a cell of the innate immune system, the macrophage can serve as a link between innate and acquired immunity. After destruction of a pathogen, the macrophage can serve as an antigen-presenting cell (APC) by breaking the pathogen into peptides that are subsequently bound to major histocompatibility complex (MHC) class II molecules on the cell membrane (discussed in Adaptive Immunity below).

Some pathogens have developed mechanisms to elude immune defenses. A group of organisms, often referred to as the facultative intracellular bacteria, have developed such abilities. For example, bacteria in the *Mycobacterium* genus are able to prevent fusion of the lysosomes with the phagosome, thereby preventing the release of destructive enzymes into the vacuole and hence preventing microbe killing. This accounts for the chronic and severe nature of infection with pathogens such as *Mycobacterium* species, *Brucella* species, *Nocardia*, and *Listeria*. Other bacteria that are not capable of surviving within phagosomes have polysaccharide capsules that make them less readily engulfed than their unencapsulated counterparts. Opsonizing antibody or complement components must be present to coat such organisms before phagocytes can engulf them. Thus, innate defenses often require the assistance of products of the acquired immune response.

Innate Lymphoid Cells

During the past decade, a new population of cells has been recognized, innate lymphoid cells (ILC) that are enriched at barrier surfaces and rapidly respond to the presence of invading microbes. Studies have determined that ILCs play an intricate role in regulation of the adaptive immune response. Based on function, ILCs are classified into three groups. Group 1 ILCs express transcription factor T-bet and produce interferon γ and TNF. Their focus is on immunity to intracellular pathogens. Group 2 ILCs consist of cells that express high levels of transcription factor GATA3 and produce the cytokines IL-4, IL-5, IL-9, and IL-13. These cells focus on expelling extracellular parasites. Group 3 ILCs express transcription factor ROR γ t and produce IL-17A and IL-2 upon encountering extracellular bacteria and fungi. Properties of ILCs that differentiate them from traditional T lymphocytes include the absence of antigen-specific receptors.

ILCs are located in lymphoid tissues: ILCs localize in interfollicular regions of lymph nodes, ILCs are found in tertiary lymphoid structures and in Peyer's patches, where they often become situated during fetal life. Other sites for ILCs include the barrier tissues of the gastrointestinal tract, the skin, and the lung. These ILCs initiate their effect on immune responses by rapid production of cytokines.

Natural Killer (NK) Cells

A fourth group of ILCs is the NK cell. These cells provide defense against viruses and intracellular pathogens. They are activated by γ interferon and macrophage-derived cytokines. They have previously been described as large granular lymphocytes, as they are larger and more granular than T and B cells. The granularity is due to the presence of granules containing perforin and granzyme, important implements of target cell killing. As an innate cell, the NK cell does not have a specific receptor for antigen; it uses activating and inhibitory receptors to distinguish healthy from infected cells. Inhibitory receptors are able to bind to self MHC on a cell and by so doing prevent the NK cell from killing the cell. Many viruses downregulate MHC on their

surface, and when the NK cell inhibitory receptors fail to detect and bind the MHC class I molecules, another set of receptors become dominant, the activation receptors. When activating receptors bind to a cell, they signal the NK cell to kill the bound target cell. This mechanism of killing virus-infected cells is operative early in infection before the adaptive response has activated CD8+ T cells available to kill the target cell. NK cells can kill targets using an Fc receptor, in which case they participate in antibody-dependent cellular cytotoxicity (ADCC). The ADCC process uses the specificity of an antibody to recognize epitopes on target cells, thus directing the NK cell to bind a target cell for killing. These targets are often virus-infected cells.

*Invariant natural killer (*iNK*) T cells* belong to the group of non-major histocompatibility complex (MHC)-restricted T cells and therefore have attributes of both innate and adaptive immunity. These cells recognize lipid antigens that are presented by CD1d molecules. They are sensitive to a variety of cytokine signals, having receptors for IL-1, IL-18, IL-5, and IL-3, and are capable of diverse effector functions. Cytokine activation of iNKT cells has also been described; for example, in response to *Salmonella typhimurium*, iNKT cells were activated to secrete interferon γ after TLR-induced production of IL-1 and IL-18 by APCs. Thus, these cells can be activated either by T-cell receptor (TCR) binding or by TLR-induced cytokine production.

One example of iNKT cell effector function is illustrated by the immune response to *Borrelia burgdorferi*, the causative agent of Lyme disease. Membranes of this organism contain lipid antigens that can be presented on CD1d molecules and recognized by iNKT cells. The membrane of this bacteria contains outer surface proteins (OspA and OspB) containing membrane-associated lipoproteins. Activation of iNKT cells in response to *B. burgdorferi* can be either by the CD1d mechanism or by the cytokine activation route.

Effector mechanisms of iNKT cells include stimulation of a strong Th1 response through production of γ interferon and/or cytotoxicity through production of perforins and granzymes.

These iNKT cells are also effective against virus and fungi. That is, iNKT cells have been shown to play a favorable role in response to infection with *Cryptococcus neoformans* and *Aspergillus fumigatus*. Although the role of iNKT cells is less obvious in viral infections, studies with mouse models have demonstrated a role in responses to Influenza virus infection.

Gamma Delta T Cells

Another innate defense mechanism is a cell type called γδ T cells, so named because they display membrane receptors called *γδ* chains. These cells constitute a small percentage of the circulating pool of lymphocytes in nonruminant species. In ruminants, however, *γδ* T cells may represent 30% or more of the circulating lymphocyte pool. In young ruminants, the population of *γδ* T cells is even larger; they are thought to play a major role in neonate protection. In most species, *γδ* T cells are present in the lamina propria underlying mucosal epithelia, strategically beneficial sites for cells involved in host defense. These cells bridge innate and adaptive immunity. They are not MHC restricted and bind to antigens presented by APCs. In bovines, where they represent a large percentage of peripheral blood lymphocytes, they are identified by a marker called WC1, which is a transmembrane glycoprotein that is thought to be a pattern recognition receptor. WC1 can act as a co-receptor to the *γδ* receptor and can also directly bind to bacterial antigens on its own. This allows γδ T cells to recognize a variety of antigens.

Functional studies in mouse models and also data from human beings have demonstrated a role for cells in defense against mycobacterial pathogens. The role of *γδ* T cells in innate defense against *Mycobacterium tuberculosis* appears to be most important early in the infection.

Activation of γδ T cells by antigens from *M. tuberculosis* requires accessory cells, such as alveolar macrophages, which provide co-stimulatory molecules. The major effector function of *γδ* T cells in defense against *M. tuberculosis* is cytokine secretion and cytotoxic effector cell function. These cells produce interferon γ, TNFα, and a small amount of IL-2, as well as several other cytokines.

In Johne's disease, caused by *Mycobacterium paratuberculosis*, ileal accumulation of *γδ* T cells has been described. The majority of studies on the role of *γδ* T cells in response to pathogens have been with mycobacterial agents. However, a role for these cells has been documented in several viral diseases as well as in several parasite infections.

Anatomical and Physiological Aspects of Innate Resistance to Infection

A description of innate immunity would not be complete without mentioning the various anatomical and physiological functions that assist the host in preventing infection. One such function is the mucociliary apparatus, which includes columnar ciliated epithelial cells and mucus-producing cells that line the upper airway. Entry of particulates into the airways is met with mucus production, beating of cilia toward the cranial direction, and eventual expulsion by cough. A genetic mutation (primary ciliary dyskinesis) that causes poor ciliary mobility underscores the importance of this function in keeping the lung pathogen free.

Other features of innate immunity include the presence of a community of commensal bacteria in the gut. By occupying a niche, these organisms make it more difficult for intestinal pathogens to establish themselves in the gut. On occasion, long-term treatment with antibiotics will result in diarrhea; we often attribute this to the killing off of the "good" bacteria.

Adaptive Immunity

The ILCs help to instruct the T helper cells in selection of the appropriate cytokines to produce for modulation of the immune response for optimum pathogen killing. This is demonstrated by the ILC1 cell that secretes interferon γ, the same cytokines that a T helper type 1 cell will make in response to a bacterial infection with facultative intracellular bacteria, such as *Mycobacterium*. For example, production of Interleukin 1, 6, or 7 by a dendritic cell can enhance the differentiation of T helper cells into T helper type 1 cells that make Interferon γ, a cytokine that helps to arm macrophages for elimination of those bacteria that preferentially resist phagosome killing. In contrast, the PAMPS present on a heavily polysaccharide encapsulated bacteria, such as *Streptococcus pneumonia,* would preferentially stimulate the T follicular lymphocytes to facilitate development of a germinal center response with B lymphocytes developing into the plasma cells making opsonizing antibodies; these will enhance effective endocytosis and pathogen killing.

T Lymphocyte Subsets

There are four main categories of CD4+ T cells: Th1 helper cells, Th2 helper cells, Th17 cells, and T follicular helper cells. The first three types are elicited by different kinds of pathogens. They are defined by the types of cytokines that they secrete and are guided in their differentiation by different types of ILCs.

The stimulation of the appropriate CD4+ T cell requires not only the production of cytokines, but also the binding to antigenic peptides presented within the groove of the αβ TCR. In the primary immune response, the cell type that performs this antigen presentation function is the dendritic cell, sometimes referred to a "professional APC" because antigen capture, processing, and presentation are its primary roles. The dendritic cell is the main APC in a primary immune response. When antigen is encountered in a host that has had previous contact with it, there are other cell types that can present antigen to T lymphocytes. These include macrophages and B lymphocytes. It is important to note that all cells that present antigen to CD4+ T lymphocytes express the class II major histocompatibility complex molecule (MHC II) on their surface.

The process of antigen presentation occurs either by the extracellular (phagocytic) pathway or by the endocytic (cytosolic) pathway. Inactivated and killed organisms, once digested and processed in a phagosome, are bound to MHC class II molecules and are brought to the cell surface for presentation to CD4 T (helper) lymphocytes. This occurs after the antigen has been broken down into peptides in endosomes; fusion of the endosomes with other endosomes that contain newly synthesized MHC class II molecules brings them together so that then peptide-containing endosomes can fuse with the MHC-II-containing endosomes. The peptide replaces an invariant chain (Ii) CLIP molecule, which has served as a placeholder, in the antigen-binding site of the MHC molecule. The MHC II with the peptide in the groove is then transported to the cell surface to await recognition by a TCR. Recognition of the antigenic peptide/MHC class II complex by a T cell with the same MHC class II is referred to as MHC restriction and is a characteristic of the adaptive immune response. Production of IL-2 by the T cell occurs after binding with the antigenic peptide and co-stimulatory molecules. IL-2 is a T cell growth factor that facilitates clonal expansion of the participating T cell. These T cells, which are phenotypically CD4+ and functionally called helper T cells, produce additional cytokines to influence the development of B cells, which bear receptors that are specific for the antigen. Under the influence of T-cell-produced Interleukin 4 (IL-4), B cells develop and mature into plasma cells secreting antibodies that have the same specificity for antigen as the B cell receptors on the original B cell. Helper T cells that produce predominantly IL-4 (T helper 2 cells or T_{H2}) facilitate production of IgG_1 and IgE. These antibodies are important for immune defense against helminth parasites.

The T helper cells previously mentioned as being responsive to direction by ILC type 1 cells making Interferon gamma must also recognize the antigenic peptide on MHC II. The cytokines are important in activation of macrophages for killing facultative intracellular bacteria and for supporting other cell-mediated responses. T helper type 1 cells produce interferon γ and IL-6. As noted above, the initial production of interferon gamma by the dendritic cell or ILC-1 cells can prejudice the T helper cell response toward T_{H1}. This response may be initiated through TLR binding to the dendritic cell.

Th17 cells produce IL-17 and require IL-6 for their maintenance; the IL-17 cytokine then stimulate stromal cells to make G-CSF and additional chemokines, whereas IL-6 and 7 stimulates epithelial cells to make antimicrobial peptides. These cytokines also recruit neutrophils to the site of infection. The major targets are extracellular bacteria and some fungi.

Follicular T cells have recently been shown to have an important role in stimulation of naïve B cells in the lymph node germinal centers. These T_{FH} cells produce cytokines like IL-10 that facilitate isotype switching and affinity maturation.

The last group of CD4+ cells to mention is the T regulatory cells, which generally carry the CD25 marker and the transcription factor FoxP3. As regulatory cells, this population downregulates some immune responses, especially those against self antigens.

CD8+ T Lymphocytes

The role of the CD8+ T cells is as effector cells, primarily against intracellular pathogens such as viruses. Recognition of intracellular pathogens is therefore accomplished with a different mechanism than that used by CD4+ cells for extracellular pathogen recognition.

Antigen presentation for intracellular pathogens follows the endocytic pathway through the cytosol. When a virus is replicating in the cytosol, viral proteins are processed and united with MHC class I molecules. The way in which this occurs is not like the process described for MHC II presentation. Unused proteins are continually broken down in the cytosol; they are ubiquitinated and targeted to the proteosome, in which further breakdown occurs to peptides that are between 8 and 15 amino acids in size. When a virus is replicating within the cytosol of a cell, the viral proteins meet this same fate and are subsequently bound to transporter proteins (TAP1 and TAP 2) for movement from the cytoplasm to the endosomes. There they are further shortened and bound to MHC class I molecules. These peptide–MHC complexes are then sent to the surface of the cell, where they are recognized by T cell receptors (TCRs) on the CD8 cytotoxic T cells. This is a very important point as it explains why a vaccine containing a virus that is able to replicate in the cell is more likely to induce a cytotoxic T cell response than a killed vaccine since MHC I recognition is required for cytotoxic T cell activation.

Humoral Immunity (the Antibody Response)

B lymphocytes that have been activated by T follicular helper cells will generate more B cells within germinal centers of the lymph node, with the eventual production of plasma cells that are making antibody with the same specificity as the receptors on the original B cell.

The first antibody to be produced is IgM, and it appears in the circulation 7–10 days after initiation of the immune response. Next, IgG appears but does not rise to very high titers in this primary immune response. Subsequent encounters with antigen result in a secondary or anamnestic response. In the secondary response, the kinetics of antibody appearance in the circulation is more rapid and the quantity of antibody produced is greater. Most importantly, the isotype that predominates in the secondary response is predominantly IgG. The longer half-life of IgG facilitates the maintenance of an antibody titer higher for a longer duration of time (Figure 67.3). Often, evaluation of the immune response (IgG versus IgM) to a disease agent can yield important information as to the chronicity of the exposure. It is a well-accepted diagnostic procedure to obtain acute and convalescent serum samples to be evaluated for antibody titer and isotype. In general, when a disease agent is responsible for clinical signs two to three weeks after the initial appearance of signs, the titer will have increased at least fourfold if the agent was involved in the infectious process. In an initial exposure to a disease agent, IgM is the predominant isotype, whereas a second or tertiary exposure (or vaccination) will elicit mostly IgG.

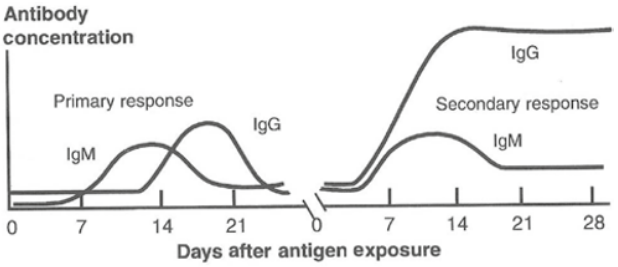


Figure 67.3 The time course of antibody production following exposure to antigen is shown (antibody concentration on the Y axis and time in days on the X axis). In the primary response, IgM appears first, followed by IgG. In the secondary response, there is less IgM and more IgG; the response occurs sooner after antigen introduction, is greater in magnitude, and it lasts longer than the primary response.

Effector Functions of Antibody

Antibodies can neutralize virus, bacteria, and soluble toxins. Some isotypes of antibody (IgG, IgM) can lyse target cells after activating the complement cascade. Antibodies can facilitate phagocytosis by acting as opsonins to help phagocytes adhere to microbes. The antibody response that is important in defense against bacterial disease depends on the pathogenic mechanisms involved, the site of the infectious process, and the isotype of the antibody elicited. In a disease caused by an extracellular toxin, such as tetanus, antitoxin antibodies are important to neutralize and bind the toxin before it can bind to cellular sites and initiate clinical signs. This mechanism is important in other diseases including anthrax and botulism – all toxin-mediated diseases. In some instances, when a non-immune host is at risk of developing a toxin-mediated disease, immediate administration of antitoxin (a solution containing antibodies to the toxin) is required to prevent the disease.

In order to eliminate infectious agents, antibodies serve as opsonins as well as initiating the complement cascade (activation through the classical pathway). Opsonins lead to increased uptake by phagocytic cells, whereas complement activation leads to initiation of inflammation and generation of compounds that are harmful to the infectious agent (e.g. membrane attack complex).

The IgE response is generally limited to parasitic infections and hypersensitivity reactions to various environmental allergens, such as pollens and grasses. Occasionally, IgE is elicited in response to vaccination against some bacterial and viral pathogens. When this occurs, very serious adverse responses, such as anaphylactic shock, can result. Often there is a hereditary predisposition toward IgE production, and such atopic individuals are at increased risk of experiencing such a vaccine reaction. For immunity to parasite infections, IgE may assist in the phenomenon of "self-cure," in which large numbers of nematodes are purged from the gut by mast-cell-mediator-induced smooth muscle contraction. Alternatively, some infestations are controlled by antibody-dependent cellular cytotoxicity (ADCC), in which IgE binds to eosinophils by low-affinity Fc receptors and facilitates release of major basic protein and other caustic enzymes on the parasite cuticle surface.

Production of IgA antibodies is a very desirable response to infectious agents that affect mucosal surfaces. As secretory IgA (SIgA) is protected by a secretory component from digestion in the gut by proteolytic enzymes, it is the most efficient active antibody in the environment of the gastrointestinal lumen. There it can neutralize viruses and bacteria to prevent their respective attachment to cellular receptors. Similarly, SIgA is effective within the secretions of the respiratory tract. Before a virus can infect a cell, it must first bind to a cell surface protein that acts as a receptor for the infectious agent. Thus, binding of the infectious agent by antibody can inhibit binding to the receptor, and thus lower the infectivity of the agent. For example, Influenza virus expresses a hemagglutinin that binds to certain glycoproteins expressed on epithelial cells in the respiratory tract. Binding of antibodies to the hemagglutinin prevents entry of the virus into these cells, and disease is prevented.

Antibodies are most effective against viruses that undergo a viremic phase, when there are numerous virus particles in the extracellular environment. For example, viruses such as influenza viruses are neutralized by antibodies specific for the major surface antigens (hemagglutinin and neuraminidase). Other viruses, such as Herpes virus, remain closely associated with cells and do not present much opportunity for antibody-mediated inactivation. The IgA isotype is especially effective on mucosal surfaces and functions to neutralize viruses before entry into the body. Secretory IgA is an extremely effective defense against respiratory and gastrointestinal viruses, as well as viruses that cause systemic disease but enter via the oral route. Virus neutralization occurs because the antibody binds to surface determinants of the virus and prevents the virus from binding to the cellular receptors to which it must attach in order to initiate the infectious process.

The relative importance of antibody and cell-mediated immune response against a pathogen depends upon the pathogenesis of the disease. For example, bacteria that produce potent exotoxins, such as *Clostridium tetani*, require antibodies to neutralize the toxin. Heavily encapsulated bacteria, such as *Klebsiella pneumonia*, require opsonizing antibodies for effective removal of the bacteria and ultimate killing by phagocytes. In contrast, bacteria that are capable of living within phagocytes, such as *Listeria monocytogenes*, are not effectively killed by an antibody, and require the T_{H1} response for effective elimination. Similarly, viral infections that produce viremia, such as West Nile virus, are handled well by an appropriate antibody response, compared with herpes viruses that are cell associated and require a cell-mediated immune response for effective control.

Cell-Mediated Immunity

Cell-mediated immune responses mediated by T cells involve two different mechanisms: macrophage activation and cytotoxic T cell activity.

Killing of Facultative Intracellular Bacteria by Activated Macrophages

As previously mentioned, as a mechanism of immune evasion some bacteria have the ability to prevent phagosome–lysosome fusion. Bacteria in this category include *Brucella*, *Mycobacterium*, *Listeria*, *Salmonella*, and *Rhodococcus equi*. Infection by one of these organisms often results in death of the macrophage. In the presence of CD4 T_{H1} lymphocytes that produce interferon γ, the macrophage is armed and able to circumvent the bacteria and to kill. The cytokine induces lysosomal fusion and increases macrophage bactericidal activity. Thus, macrophages are activated following the production of interferon γ by these T_{H1} cells, and the "arming" of macrophages results in destruction of the infectious agent that the macrophage had previously been unable to destroy.

Killing of Virus-Infected Cells by Cytotoxic T Cells

Pathogens, such as viruses, that live and grow inside of cells are best contained by elimination of the cell in which they are reproducing. As previously described, viral proteins made in the cytosol associate with MHC I molecules on the cell membrane for presentation to T cells. Cytotoxic T cells (CD8) recognize an antigenic peptide that is held in the groove formed by the chains of the MHC class I

molecule on the cell surface. All nucleated cells have MHC class I on their surface and are therefore able to bind and present antigen from inside the cell in this manner. Recognition of the combination of MHC class I and the antigenic determinant by TCRs on the cytotoxic T cells stimulates release of perforins that make small pores in the cell membrane. This allows destructive enzymes called granzymes to enter the cytoplasm. In addition, tumor necrosis factor α is produced. Death of the cell can also be facilitated by the interaction of the Fas–Fas ligand system, stimulating apoptosis. One CD8 T cell can repeatedly kill infected target cells, programming one to die, and then moving on to the next cell to kill it. The cytotoxic T cell is an efficient way to decrease viral progeny in an infected host.

Effector Cells Can Use Antibody to Bind Target Cells

Antibody-dependent cellular cytotoxicity (ADCC) occurs when antibody binds to a cell that has receptors for the Fc portion of immunoglobulin G or E. The Fc receptor for the gamma chain is CD21, and the low-affinity IgE receptor is CD23. These molecules are present on several types of effector cells, including neutrophils, macrophages, NK cells, and eosinophils. The attachment of antibody to a cell that previously had no receptor for antigen renders it antigen-specific and capable of binding antigen. Besides NK cells, eosinophils and macrophages can also become involved in ADCC. ADCC is an effective method of killing cells infected with microorganisms (viral, bacterial, or fungal) as well as parasites. In the case of parasites, the eosinophil releases granules containing major basic protein, and other caustic enzymes rendering the cuticle of the parasite permeable.

Evaluation of Immune Responses to Infectious Agents

The use of serological assays to evaluate exposure to or infection with bacterial and viral pathogens has been a mainstay for control of infectious diseases. In addition, for some infections that are not characterized by antibody production, such as those with mycobacterial species, the *in vivo* skin testing for cell-mediated responses has been of even greater use.

For determination of recent infection status for many bacterial and viral agents, serum samples are obtained during acute illness and again 2 to 3 weeks later. These acute and convalescent samples are then assayed for antibody titer. When the titer has increased at least fourfold (two dilutions), seroconversion has occurred, and the disease agent for which the titers are specific is confirmed as having stimulated a recent response.

Antibody-Based Serology

For decades now the technique of enzyme linked immunoassay (ELISA) has been used both for quantitative and qualitative measures of immune responses to a variety of pathogens. Detection of pathogens has also utilized ELISA technology. These assays can take the form of a highly quantitative assay performed in a microtiter plate or can be adapted to quick-format qualitative assays that enable a doctor to perform an animal-side assay and obtain a quick result. These assays are generally more sensitive than assays based on precipitin formation or complement fixation. Depending on the disease to be diagnosed, ELISA can be designed to detect antigen (as in Feline leukemia virus and Canine parvovirus infections) or antibody (as in Feline immunodeficiency virus). ELISA has the advantage that the format is easily adaptable to either a quick (positive or negative) read-out or a titer determination. Numerous test kits are available for the veterinarian to evaluate the Feline leukemia virus (FeLV) infection status of a cat. Some use a solid phase format (Figure 67.4a), whereas others use a well-based assay (Figure 67.4b).

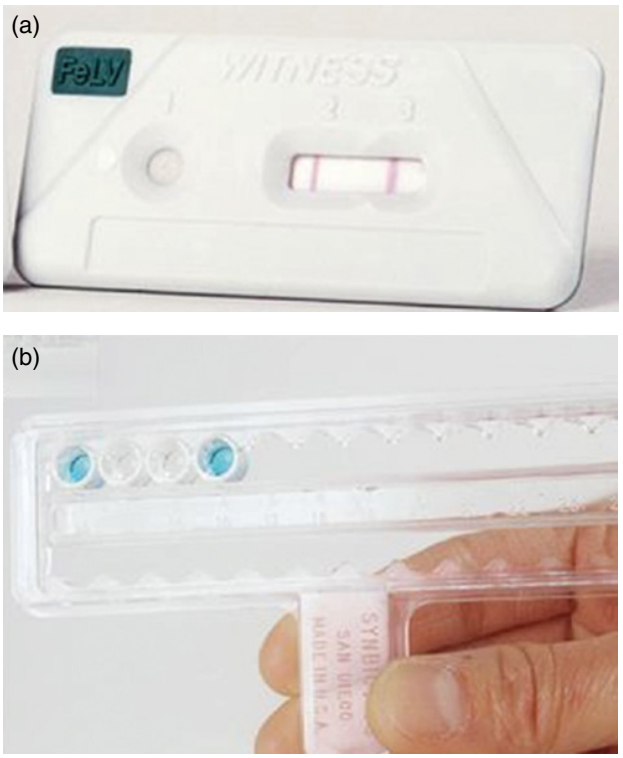


Figure 67.4 (a) Example of a positive Witness lateral flow assay for Feline leukemia virus (FeLV). This test detects the FeLV viral antigen in the sample (blood or serum). The second line shows that the test is valid because the positive control works correctly. (b) FeLV is tested for using an ELISA. The presence of color confirms the presence of viral antigen in the well.

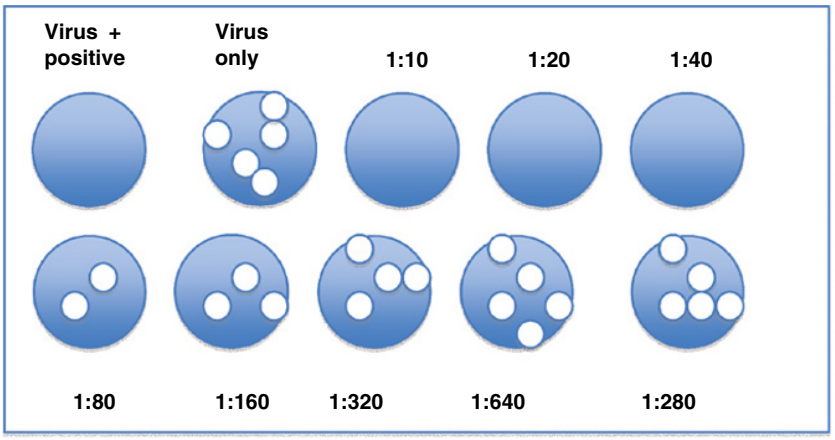


Figure 67.5 Serum virus neutralization test. The diagram shows how the serum viral neutralization assay works. The blue circles represent a confluent cell culture. The positive control contains cells with virus that has been mixed with a serum positive for the antibodies. The next well has been infected with virus to visualize the CPE (cytopathic effect) induced by viral infection. Test serum is mixed with virus and added to the cells. Addition of gradually more dilute serum in successive wells shows how much the serum can be diluted and still protect the cells from viral infection. A titer is determined by visualizing the last well that is protected from viral CPE.

The lateral flow technology that is used in many rapid test kits for antibody detection is also adapted to detection of antigen, and this technology has wide application in both veterinary and human medicine. For example, detection of antibodies for the fungal agent of Valley fever (*Coccidioides*) is easily performed with a lateral flow kit; and similarly, detection of antigens from the fungal pathogen *Cryptococcus neoformans* can also be accomplished using lateral flow technology.

When using antibody as an indication of potential protection, it is important to remember that the ELISA detects binding antibody, which is not always equivalent to effective protective neutralizing antibody. The serum virus neutralization (SVN) assay uses serum as a source of antibody, incubated with virus, which is then tested for ability to infect an appropriate cell culture. When neutralizing antibody is present in a sample, it will prevent viral entry into the cells and subsequent cell infection and death. Thus, a high SVN titer is more meaningful than a high ELISA titer for prediction of protection (Figure 67.5). However, assays for neutralizing antibody are time consuming and therefore expensive. The serum neutralizing test for a virus, such as Canine distemper virus (CDV), is performed by inoculation of tissue culture cells with CDV, either alone or mixed with dilutions of the patient's serum. The presence of neutralizing antibody in the serum will prevent the virus from infecting the cells. This is evident when the cells are observed for cytopathic effect.

For determination of antibody titers to those viruses that have hemagglutinins, the hemagglutination inhibition test (HI) can be used to determine protective antibody titers to the virus. For example, Canine parvovirus will agglutinate red blood cells, but if pre-incubated with serum containing hemagglutinin-specific antibodies, hemagglutination will not occur. The last dilution of serum that still inhibits the hemagglutination is the endpoint that determines the titer.

Recently there have been at least two different test kits developed for in-house detection of titers against both CDV and Canine parvovirus (CPV). Both products have been evaluated in comparison with the "gold standard" assays (serum neutralization for CDV and the HI test for CPV). These test kits are useful for making decisions on whether or not a dog is sufficiently protected from infection or needs a booster vaccination. The format of these assays is solid phase ELISA.

For many years, a gel diffusion test has been used for detection of antibodies to Equine infectious anemia virus (Coggins test). Several commercially available ELISA kits are available. The comparison of these testing formats is a good illustration of differences in sensitivity, because the gel diffusion test is very dependent on the concentration of antigen and antibody for a positive test result, whereas the ELISA, a primary binding assay, is more sensitive and less dependent on concentration effects. The ultimate effect is that a positive Coggins test, when read by an approved laboratory technician, is thought to be diagnostic, whereas a positive ELISA needs to be confirmed by a Coggins test, so as to prevent false positive readings. The gel diffusion test can also be used for detecting antibody against other pathogens, such as *A. fumigatus*, which stimulates a strong IgG/precipitating antibody response in infected dogs (Figure 67.6).

Indirect immunofluorescence is still used as a diagnostic test, and although the requirement for a microscope equipped for immunofluorescence limits its use outside

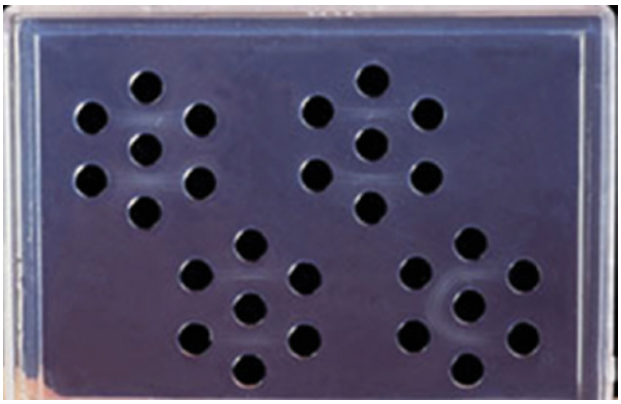


Figure 67.6 Agar gel immunodiffusion and precipitation. Detection of antibodies against *Aspergillus fumigatus*: center well contains antigen. Sera are distributed clockwise with positive sera at 12 and 6 locations. The presence of a line of identity with positive controls indicates the presence of antibodies binding to the antigens in the center well. This is apparent in the lower right test.

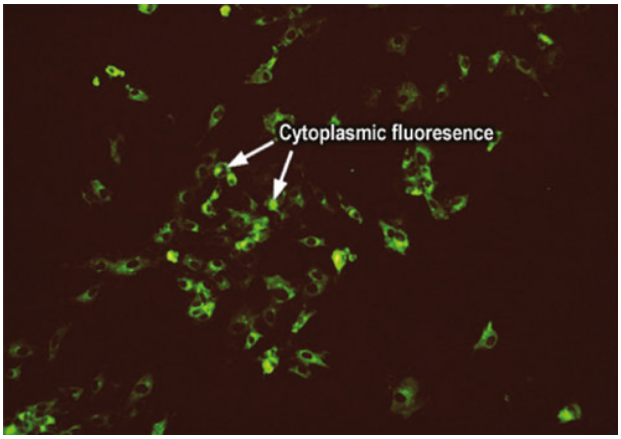


Figure 67.7 Indirect immunofluorescence. Showing antibody specific for Feline corona virus (FIP). Cells were infected with Feline corona virus, and the fixed slide was incubated with dilutions of cat serum followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-feline IgG.

diagnostic laboratories, it is a sensitive assay for evaluation of antibody titers to a number of viral pathogens. Although detection of antibody against Feline coronavirus is not specific for the clinical syndrome of feline infectious peritonitis, an indirect immunofluorescence assay is sometimes used to determine the titer of antibody in a feline infectious peritonitis (FIP) suspect patient (Figure 67.7).

Direct immunofluorescence has been used for many years to detect the presence of virus in cells and in organ sections at necropsy. For example, a conjunctival swab from a CDV-infected dog can be used to demonstrate the presence of virus (Figure 67.8). With the introduction of RT-PCR techniques, this technique is less commonly applied than previously. For detection of pathogens in tissue, direct immunofluorescence has been replaced in many

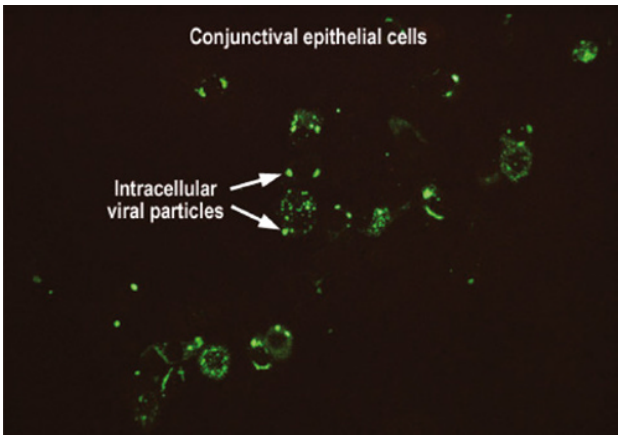


Figure 67.8 Direct immunofluorescence is using antibody against Canine distemper virus on a conjunctival swab from a CDV-infected dog shows the presence of cells containing the virus.

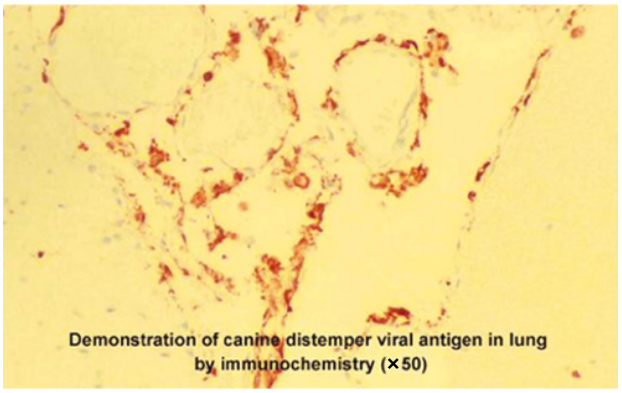


Figure 67.9 Canine distemper virus (CDV) is shown in a lung section from a CDV-infected dog, using an enzyme conjugated antisera against CDV; this shows how immunohistochemistry can be used to reveal viral antigen in tissue.

laboratories with immunohistochemical techniques, such as immunoperoxidase staining. The ability to perform the latter technique on fixed tissues makes it a more logical choice for necropsy specimens. For example, CDV is readily identified in lung tissue from an infected dog using anti-CDV sera conjugated with horseradish peroxidase (Figure 67.9).

The principle of agglutination has long been a standard method for demonstrating antibody responses to bacteria. These assays depend upon the ability of antibodies to cross-link the cells to form a lattice of agglutinated cells as opposed to a pellet that would result from the settling of unagglutinated cells. Currently there are additional options for detection of antibodies; most use ELISA-based techniques. *Brucella canis* antibody titers have historically been determined using the tube agglutination test as seen in Figure 67.10. Soluble antigens can be made particulate for use in a passive agglutination test by covalently linking them to latex particles. Antibodies to *Toxoplasma gondii* can be identified and titered using this method in a microtiter plate format (Figure 67.11).

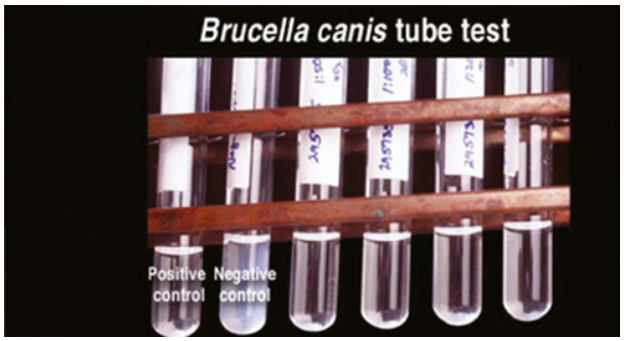


Figure 67.10 In this tube agglutination test for antibodies against *Brucella canis*, the first tube (positive control) shows a 4+ agglutination of the bacteria by the antibodies (note clear supernatant and lattice formation on tube bottom). The negative control shows a lack of agglutination with cloudy supernatant and lack of lattice formation on the tube bottom. Successive dilutions of patient serum: 1:50, 1:100, 1:200 show strong agglutination.

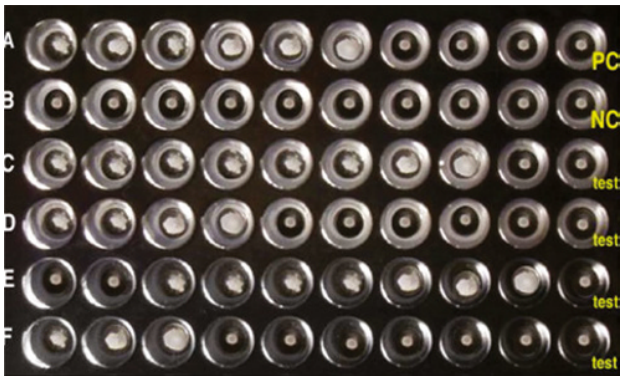


Figure 67.11 *Toxoplasma gondii* passive agglutination antibody titration in a microtiter plate format.

Cell-Mediated Immunity-Based Diagnostics

For those pathogens that induce a strong T helper 1 type immune response with production of associated cytokines (such as interferon γ), an intradermal skin test with antigen from the pathogen can often be used to demonstrate exposure or infection. Recently, *in vitro* correlates have been used for some diseases. *Mycobacterium bovis* infection can be diagnosed with intradermal injection of tuberculin. Within 48 to 72 hours of antigen injection, erythema and induration at the site are apparent in infected patients. Infection with *Mycobacterium avium* subspecies *pseudotuberculosis* (Johne's disease agent) can be detected by *in vitro* incubation of patient lymphocytes with antigen and subsequent analysis of interferon γ levels in the culture supernatant. Infection by additional organisms that are among the group called facultative intracellular pathogens can be detected using similar testing strategies.

Evaluation of cytotoxic T cell responses has been problematic in domestic animal species and in humans; only in syngeneic mouse strains is it easy to evaluate virus-infected target cell killing by effector T lymphocytes. This is because MHC restriction (discussed above) requires that targets have the same MHC type as effectors; and that targets with non-compatible MHC will be attacked by heterologous T cells regardless of infection status. Nonetheless, there have been studies carried out in veterinary species in which autologous target cells were infected with virus and T cell killing evaluated using either dye or radioactive chromium incorporation as a read-out.

Another way to examine T cell responses to pathogens is to perform *in vitro* cultivation of the animal's T lymphocytes with inactivated antigens of the pathogen. The culture supernatant is then assayed for production of cytokines by ELISA or Luminex assay, and the demonstration of Th1 cytokines (such as γ Interferon) is indicative of specific T cell responses. The lymphocyte stimulation assay for detection of activation by antigen or mitogens (phytohemagglutinin, concanavalin A, or LPS) was originally performed using tritiated thymidine incorporation as a measure of T cell activation (DNA synthesis in multiplying cells). Currently, nonradioactive renditions of this assay utilize a colorimetric detection with MTT (3-[4,5-dimethylthiazol-67 -yl]-67,5-diphenyl-tetrazolium bromide). This type of assay can also be useful for detection of immunocompromised patients, when performed with mitogens, for measurement of T cell responsiveness in comparison to normal controls.

Recent Innovations in Vaccinology

Knowledge of the nature of the protective immune response against a pathogen is the first requirement for development of an effective vaccine to prevent the disease. During the past few decades, and within recent years, immunologists have provided doctors and veterinarians with new and improved methods for stimulation of protective immunity, particularly to viral pathogens. During the years 2020 and 2021, the COVID 19 pandemic had a dramatic effect on the human population. Ultimately several vaccines became available; several were viral vectored (adenovirus as in AstraZenica, Johnson & Johnson) vaccines, and two were RNA vaccines (Pfizer and Moderna). In fact, these "new" vaccine modalities had been experimentally applied for other pathogens in a variety of laboratory and veterinary species during the past decade. Commercial use of safe and effective canary pox vectored CDV vaccines has been in place for multiple years; RNA vaccines for rabies and influenza have

been studied experimentally prior to the application of this technology to COVID-19. These advances in vaccinology are based on knowledge of the sequence of viral antigens and thus enable scientists to make an accurate and prompt response to the threat of new pathogens.

Summary

The immune system is composed of innate and adaptive responses that recognize pathogens or their component parts and respond to induce the most effective response to that particular pathogen. Early initiation of the immune response involves interaction of pathogen-associated molecular patterns with sentinel cells. Cytokines, cell receptors, and ligands work in concert to stimulate the appropriate effector mechanisms. We can detect these responses using diagnostic assays, many of which are currently available in rapid simple kits that can be used on-site. The knowledge thus obtained facilitates the most appropriate and timely treatment of affected patients. New technologies promise to provide improved vaccines for the prevention of infectious diseases of animals and humans.

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68

Laboratory Diagnosis

D. Scott McVey, Bruce Brodersen, Duan Loy, and John Dustin Loy

Detection and Identification of Microbial Pathogens

Bacteria and Fungi

A key decision made early in the microbiological diagnostic workup is whether the patient's clinical signs indicate a possible infectious etiology. This decision is important because drugs used to treat conditions with noninfectious etiologies – corticosteroids, for example – are often contraindicated for treatment of conditions with an infectious one, for which antibiotics are appropriate.

One of the first major goals of the microbiology laboratory is to isolate or detect clinically significant microorganisms from an affected site and, if more than one type of microorganism is present, to isolate them in approximately the same ratio as occurs *in vivo*. Whether an isolate is "clinically significant" or not depends upon the circumstances of isolation. For example, the isolation of large numbers of a particular microorganism from a normally sterile site in the presence of an inflammatory cytology would be interpreted as significant.

Attention must be given to the site cultured as well as to the method of obtaining the sample for culture. The determination of significance is greatly simplified if the sample is obtained from a normally sterile site. It may be unrealistic to expect meaningful answers with a sample obtained from the alimentary canal unless one is looking for the presence or absence of a specific microorganism, for example, *Salmonella* or *Campylobacter* species.

Specimen Collection

Attention must be paid to how the diagnostic specimens are collected; if not, interpretation of the results may be difficult. Most infectious processes arise following the contamination of a compromised surface or site by microorganisms that are also a part of the flora occurring on a contiguous mucosal surface. In other words, microorganisms isolated from an affected site are often similar (if not identical) to those found as part of the normal flora of the patient.

Transport of Specimens

The sooner the specimen is processed in the microbiology laboratory, the greater the probability of obtaining useful outcomes. Realistically, the time between sample collection and processing may range from minutes to hours to days. Drying (all microorganisms) and exposure to a noxious atmosphere (oxygen for obligate anaerobes) are the major factors that compromise specimens and lead to inaccurate diagnosis. For this reason, it is important that the specimen be kept moist and, if conditions warrant (see below), air excluded. Moistness is maintained by placing the sample in a transport (holding) medium composed of a balanced salt solution usually in a gelled matrix. However, some tests such as for virus isolation or polymerase chain reaction (PCR) (described below) require a liquid medium; therefore, gel-based matrices may be contraindicated for collection and transport for these tests. Because this medium does not contain any nutrient material, microorganisms in the sample multiply poorly if at all (and relative numbers and ratios are thereby preserved) but remain viable for some time, generally for at least 24–48 hours. Swabs should always be placed in transport medium, regardless of the time elapsed between processing and collection. The type of swab is also important as certain types of swabs may inhibit PCR testing. In addition, flocked swabs may provide increased recovery of organisms as they release more cells and bacteria into the transport media after collection. Fluids that may contain

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anaerobic bacteria (e.g. exudate from draining tracts, peritoneal and pleural effusions, abscess material) should be inoculated onto appropriate media immediately. If this material is contained in a syringe, then the air should be expelled, and a sterile stopper placed over the needle. If a swab is used to collect the sample, it should be placed in an anaerobic transport medium. There are now "universal" transport media that provide recovery of both aerobic and anaerobic pathogens. If a syringe containing the specimen cannot be processed immediately, the syringe should be emptied into an anaerobic transport medium and held at room temperature. It is best not to refrigerate samples suspected to contain anaerobes because some bacterial species do not tolerate reduced temperatures.

Demonstration of an Infectious Agent

The presence of an infectious agent may be accomplished by examination of stained smears made from a portion of the clinical sample, culture techniques, molecular/immunologic methods, or a combination of these methods.

Direct Smears

Information obtained from examination of a stained smear is valuable because it may be the first indication (and sometimes the only indication) that an infectious agent is present. Also, what is observed (shape, staining characteristics) will help guide the choice of therapy 24 hours before culture results are available. At least 10^4 microorganisms/ml or gram of material must be present in order to be readily detected microscopically; therefore, the sensitivity of this approach is reduced compared to methods that facilitate organism replication.

As is the case with a sample obtained from a normally sterile site, the presence of bacteria in bladder urine is a significant finding. However, interpretation of the results of analysis of urine samples obtained by catheter or by "catch" is difficult because of the confounding presence of flora flushed from the distal urethra. Finding bacteria by direct smear in concentrated (the preferred) or unconcentrated urine obtained by percutaneous aspiration of bladder urine is a significant finding. Demonstration of one bacteria cell per oil field in a drop of unconcentrated urine (which has been allowed to dry and then stained) represents about 10^5–10^6 bacteria/ml of urine.

Two types of stains are commonly available, the Gram stain and Romanowsky-type stains such as Wright's or Giemsa. Each type of stain has advantages and disadvantages. The Gram stain is useful in that the shape and the Gram-staining characteristics of the agent may be observed. The disadvantage of the Gram stain is that the cellular content of the sample is not readily discernible. On the other hand, a Romanowsky-type stain gives the observer a feeling for the cellular nature of the sample and whether or not an infectious agent is present. Cytological evaluation of the sample is very important in assessing the significance of the microorganism observed and subsequently isolated by culture. Additionally, stains such as a modified acid fast stain may provide additional information for organisms, such as mycobacteria, which are not as readily stained using other methods.

Culture Techniques

Media are inoculated with a portion of the specimen. Inoculation should be performed in a semiquantitative fashion (especially samples of bladder urine obtained by catheter or catch) to enable estimation of the relative abundance of organisms in the sample.

Determination of the relative numbers of microorganisms in a sample greatly helps interpretation of significance. Colonies of microorganisms growing on all four quadrants of a petri plate indicate that there are large numbers of microorganisms in the sample, as this indicates there is growth remaining after streaking, which is effectively a serial dilution. If a sample yielded one or two colonies growing on the plate, the significance of these colonies and thus the question as to the infectious etiology of the condition would be in doubt. However, this is pathogen dependent, as with some organisms any growth is considered significant. Enrichment prior to plating of a sample obtained from a normally sterile site should usually not be necessary because one microorganism may replicate to numbers that equal many thousands in a very short period of time. Enrichment culture procedures may lead to the proliferation of contaminating microorganisms. Occasionally samples with extremely limited numbers of organisms (i.e. blood cultures) or those that are fastidious (mycoplasmas) require enrichment prior to growth on solid media.

Determination of clinical significance is aided by the cytology of the sample obtained from the affected site. Isolation (demonstration) of numerous microorganisms from a normally sterile site without the presence of inflammatory cells should be suspect. One exception to this rule is cryptococcal infection wherein the sample may contain a large number of yeast cells but very few inflammatory cells. The isolation or demonstration of a "significant number" of microorganisms from a normally sterile site without evidence of an inflammatory response can be explained by contaminated collection devices; contamination of the collection device from a contiguous, normally nonsterile site; contamination of the medium inoculation device in the microbiology laboratory; or contamination of the medium before inoculation. Collection devices (e.g. catheters) sterilized by liquid disinfectants quite often become

contaminated by microorganisms that can survive in such environments (*Pseudomonas*, for example).

Media plates may be streaked in any fashion as long as individual isolated colonies are produced after incubation. Assessing relative numbers is very subjective. Relative numbers of microorganisms may be reported by noting how much growth occurs on the surface of the plate. Obviously, growth of one colony (presumably from one bacterium) versus growth of colonies over the whole plate would be viewed differently with respect to clinical significance. Determination of the actual numbers of bacteria present is mostly used when analyzing urine obtained by "catch" or catheter because of the problem of contamination of the sample by bacteria in the distal urethra. In this instance, disposable calibrated loops containing 0.001 or 0.01 ml of urine are used to inoculate appropriate media. Greater than 10^5 bacteria/ml of urine obtained by catheter or "free catch" is considered significant (i.e. the bacteria are more likely to be coming from the bladder rather than the distal urethra).

Traditionally, bacteria and fungi are identified based on a combination of colony morphology, cellular morphology, observed growth characteristics on various media, as well as metabolic and biochemical testing utilizing various defined media. Such approaches can be successful but have limited discretionary power and often require days to weeks for some pathogens. More recent spectrometric, chromatographical, and biochemical approaches have been applied with greater efficiency and power of diagnostic discrimination (see below).

Aerobic Bacteria

The standard medium inoculated for the isolation of facultative microorganisms is a blood agar plate (usually sheep blood suspended in a growth medium in a semisolid matrix containing agarose). Many laboratories include selective media such as MacConkey agar and those containing nalidixic acid as well. MacConkey agar is useful because enteric microorganisms (members of the family Enterobacteriaceae, e.g. *Escherichia coli*, *Klebsiella*, and *Enterobacter*) grow very well, as does the nonenteric *Pseudomonas*. Most other nonenteric gram-negative rods and all gram-positive microorganisms do not grow well on this medium. Assessing growth on MacConkey agar will facilitate detection of enteric organisms (Figure 68.1). Media containing nalidixic acid can suppress growth of gram-negative organisms, many of which can grow rapidly, allowing for isolation of these organisms in mixed culture samples.

Anaerobic Bacteria

Anaerobic bacteria will grow on blood agar that is specially prepared to eliminate oxygen (pre-reduced). Some fastidious anaerobes also require more enriched media such as

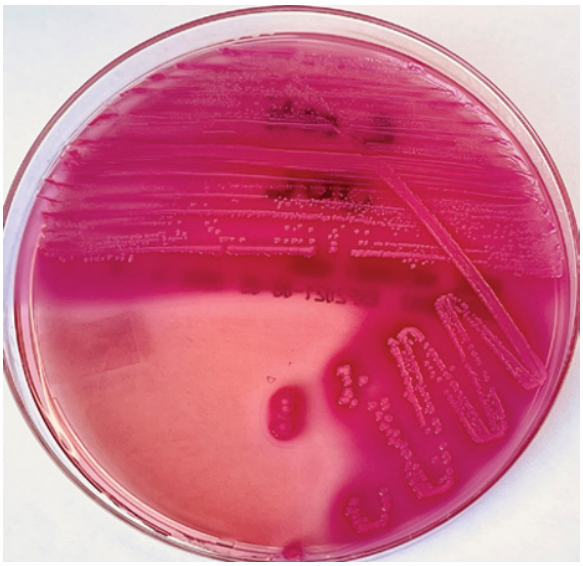


Figure 68.1 *Escherichia coli* on blood agar plate (hemolytic, a) and MacConkey agar (b). Purple color indicates acid production (lactose fermentation).

Brucella agar, which is supplemented with Vitamin K and hemin. Selective media for organisms such as *Bacteroides* and *Clostridium* are also available and may be useful in samples that may have mixed growth, such as abscesses. After anaerobic plates are inoculated, they should be placed in a closed, anaerobic environment, which can be either jars containing anaerobic packs or environmental chambers from whose atmosphere oxygen has been removed. Processing specimens for anaerobic culture is time-consuming and expensive. The most common sites that contain anaerobic bacteria are deep tissue wounds; draining tracts; abscesses; pleural, pericardial, and peritoneal effusions; pyometra; osteomyelitis; and pneumonic lungs. Anaerobic culture of sites that contain a population of anaerobic bacteria as part of the normal flora is often unrewarding (e.g. feces, vagina, distal urethra, and oral cavity) unless the diagnostician is looking for a specific species or type of anaerobic organism. Anaerobic culture of the urinary tract is not routinely performed because the recovery of these microorganisms from this site is extremely rare.

MALDI-TOF/Biochemical Testing

Rapid approaches to biochemical or phenotypic testing are now readily available. These commercially available systems include phenotypic microarrays that can assess metabolism of numerous substrates at a time, in addition to the more common strip-based tests, which include dozens of biochemical tests in an easy-to-use platform, that is usually combined with a database. The main disadvantage of

these systems is that it takes hours or days once inoculated to interpret as they require metabolic activity, and there is frequently phenotypic overlap among the species.

A recent innovation has revolutionized clinical microbiology. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a robust approach to microbial identification. Although the technology has been used for decades to characterize complex macromolecules in chemistry labs, only recently has this technique been applied to organism identification. The technique uses a smear of colonial growth taken from solid media, which is then placed on a stainless steel target in a thin film. The organism is then treated with a matrix, usually HCCA (α-Cyano-4-hydroxycinnamic acid), which crystallizes on the microbial film, enabling a laser to disrupt microbial cells and proteins into ionizable fragments. This soft ionization process occurs inside a vacuum contained in a flight tube, where the application of an electromagnetic charge allows measurement of the time of flight of the ions. Each bacterial species generates a unique composition of ion fragments, which once separated by mass and charge in the flight tube impact a detector that records the mass-to-charge ratio of the ions, generating a unique fingerprint that can be compared to a database of known organisms. As the ionized proteins contained in the cells are mostly ribosomal in origin, the MALDI fingerprint tends to have high agreement with sequencing-based identification approaches such as 16S rDNA (see the following section). MALDI-TOF has become the standard in many labs as it is fast, reproducible, cost efficient, and eliminates the need for dozens of biochemical tests to be maintained in inventory. MALDI identifications can be generated from a single colony with results in a few minutes that are repeatable between labs, allowing for database entries to be shared. Many commercial systems have thousands of database entries that include yeasts and molds in addition to human and veterinary bacterial pathogens, plant pathogens, and environmental bacteria. In addition, methods have been developed that enable discrimination of specific peaks contained in the MALDI spectra that can be used to characterize isolates to the subspecies level.

Molecular/Immunologic Methods

Sometimes it is important to determine the presence or absence of a particular microorganism as quickly as possible so that appropriate control measures can be initiated. This is especially true when infectious agents are suspected that pose a threat to other animals, including human caregivers (e.g. *Salmonella* and *Leptospira*). Likewise, some infectious agents take so long to isolate in culture that formulation of a rational therapeutic strategy is difficult (e.g. some fungal agents and *Mycobacterium*). Still others are hard to detect because they are difficult to culture (e.g. *Leptospira* and *Rickettsiae*) or have not been cultured in artificial media (e.g. *Clostridium piliformis* and *Mycobacterium leprae*). In these instances, other diagnostic techniques are available.

Immunologically based techniques make use of antibodies specific for the microorganism in question. These antibodies are usually immobilized on a solid support and are used to trap the agent. The presence of the trapped agent is then detected with a specific antibody that has been labeled in some way (usually with a color reagent). Some kits making use of this approach are commercially available (e.g. *Salmonella*). Molecular techniques utilizing DNA probes specific for a segment of DNA that is unique to the microorganism in question, or the PCR using specific DNA primers, have been designed for a number of agents (described below). PCR-based assays are available and routinely used directly on nucleic acid extracted directly from clinical samples for the detection of bacterial pathogens or from pathogen isolates for further characterization. The DNA probes usually contain a fluorescently labeled tag to enable real-time detection of signal from the target pathogen (real-time PCR; see Section 68.2.3.5). For example, real-time fecal PCR testing is routinely used to test feces for *Mycobacterium avium* ssp. *paratuberculosis* (Johne's disease), urine samples for the presence of *Leptospira* spp., or blood samples for *Anaplasma* spp. The cycle threshold value of the target can then be used to estimate relative pathogen abundance in clinical samples. PCR-based methods are also routinely used to detect difficult isolate pathogens such as *Mycoplasma*. Other PCR-based assays are routinely used for strain typing purposes, which are especially valuable when the presence of certain virulence factors alters the clinical picture. Multiplexed PCR assays (multiple targets in one test) are available that enable rapid detection of *E. coli* fimbrial or toxin genes, or *Clostridium perfringens* toxins, that can provide information to help determine if the isolate is more likely to be pathogenic or associated with clinical findings. PCR can also be useful to amplify conserved or housekeeping genes from a bacterium or fungus if identification using the common tools proves insufficient. These use "universal" oligonucleotide primers that target 16S or ITS ribosomal genes that contain variable regions that can be used to assist in identification. These genes can be amplified using targeted PCR with these universal primers followed by sequencing and bioinformatics analysis to identify bacteria. It is important to note that the utility of these approaches varies, as ribosomal or other housekeeping loci may not provide sufficient resolution to the species level for some genera.

Virus Detection and Identification

General Considerations

The specific etiologic diagnosis of viral diseases traditionally has been both tedious and time-consuming, but modern technologies such as PCR have increased the utility of this approach. Prompt and accurate diagnosis of viral diseases is therefore essential to determine an effective course of disease prevention and control. The implementation of next-generation sequencing approaches has led to the discovery of numerous additional viruses associated with different clinical diseases that have been unable to be isolated in culture. These tools are becoming more available and may become more applied to diagnostics as access increases and cost decreases.

Proper methods of collecting and processing clinical specimens and a complete history are vital to the successful isolation of viruses. Tissues that are extensively autolyzed or poorly stored usually do not yield infectious viruses because of the susceptibility of most viruses to detrimental environmental conditions. Rapid viral isolation and/or identification should be attempted in the following conditions:

1) During outbreaks of vesicular disease in livestock (e.g. foot-and-mouth disease in cattle, pigs, sheep, or goats).
2) During outbreaks of disease in large animal populations such as feedlots, poultry houses, or catteries where many animals are at risk and prompt, accurate diagnosis is critical to the institution of control methods (such as vaccination).
3) In instances of potentially zoonotic diseases such as rabies, West Nile fever, and equine encephalomyelitis, particularly when human exposure has occurred.
4) In determining the etiology of new disease, or in defining uncharacterized aspects of existing ones.

Tissues for virus isolation and identification should be collected from recently dead animals whenever possible (Table 68.1). Collection of appropriate specimens during the acute phase of the disease, and inclusion of additional submissions from similarly affected animals, enhances the

Table 68.1 Suggested specimens from mammalian species for virus isolation and identification.

Type of illness or infection	Common name or associated virus	Other infections	Clinical specimens to collect	Diagnostic identification tests
Respiratory	Adenovirus (bovine, porcine, canine, equine)		Nasal and ocular secretions, feces, lung, brain, tonsil	VI (CPE), HA, CF, FA, VN, PCR
	Infectious canine hepatitis (*Adenovirus*)		Spleen, liver, lymph nodes, kidney, blood	VI (CPE), HA, FA, VN, PCR
	Canine distemper virus (*Morbillivirus*)	Central nervous system (CNS), enteric	Spleen, liver, lymph nodes, kidney, brain, blood, nasal secretion	VI (CPE), IHC, FA, PCR
	Bovine viral diarrhea (mucosal disease) (*Pestivirus*)	Genital, abortions, enteric	Nasal sections, oral lesions, lung, spleen, mesenteric lymph nodes, intestinal mucosa, vaginal secretions, fetal tissues, unclotted blood, serum, ear notches	VI (CPE and virus interference), FA, VN, IHC, PCR, ELISA
	Infectious bovine rhinotracheitis (*Herpesvirus*)	CNS, genital, abortions	Nasal and ocular secretions, lung, tracheal swab, tracheal segment, brain, vaginal secretions, serum, aborted fetus, liver, spleen, kidney	VI (CPE), FA, VN, PCR
	Feline rhinotracheitis (*Herpesvirus*)		Nasal and pharyngeal secretions, conjunctival membranes, liver, lung, spleen, kidney, salivary gland, brain	VI (CPE and inclusions), FA, PCR
	Equine rhinopneumonitis (*Herpesvirus*)	Genital, abortions	Placenta, fetus, lung, nasal secretions, lymph nodes	FA, VI (ECE and CPE), VN, PCR
	Influenza (equine, porcine, canine) (*Orthomyxovirus*)		Nasal and ocular secretions, lung, tracheal swab, serum	VI, PCR, ELISA, HI
	Influenza (equine, porcine) (*Orthomyxovirus*)		Nasal and ocular secretions, lung, tracheal swab	VI (ECE), HA, HI

Table 68.1 (Continued)

Type of illness or infection	Common name or associated virus	Other infections	Clinical specimens to collect	Diagnostic identification tests
	Parainfluenza (bovine, equine, porcine, ovine, canine) (*Paramyxovirus*)		Nasal and ocular secretions, lung, tracheal swab	VI (ECE), HA, HI, VN, PCR
	Bovine respiratory syncytial virus (*Pneumovirus*)		Trachea, lung, nasal secretions, clotted blood	VI (CPE), FA, ELISA, PCR
	Bovine herpesvirus 4 (Movar strain, DN599 strain)	Abortions	Trachea, lung, nasal secretions, fetus, clotted blood	VI (CPE), FA, VN, PCR
	Reovirus (bovine, equine, canine, feline)		Feces, intestinal mucosa, nasal and pharyngeal secretions	VI, HA, HI, PCR, EM
	African horse sickness (*Orbivirus)*		Whole blood in anticoagulant, lesion material, nasal and pharyngeal secretions, serum	VI (CPE), ELISA, FA, VN, EM, PCR
	Malignant catarrhal fever (*Herpesvirus*)	CNS, Enteric, Ocular	Whole blood in anticoagulant, lymph nodes, spleen, lung	VI (CPE), ELISA, FA, VN, EM, PCR
	Pseudorabies (*Herpesvirus*)	CNS, genital, abortions	Nasal secretions, tonsil, lung, brain (midbrain, pons, medulla), spinal cord (sheep and cattle), spleen (swine), vaginal secretion, serum	VI (CPE and rabbits), VN, ELISA, FA, PCR
	Canine herpesvirus	Abortion	Kidney, liver, lung, spleen, nasal oropharyngeal, and vaginal secretions	VI (CPE and inclusions), FA, VN, PCR
	Porcine inclusion body rhinitis (*Cytomegalovirus*)		Turbinate, nasal mucosa	VI (CPE), FA, VN, PCR, EM,
	Equine rhinovirus (*Picornavirus*)		Nasal secretions, feces	VI (CPE), FN, PCR
	Maedi-Visna, ovine progressive pneumonia (*Retrovirus*, *Lentivirus*)	CNS	CSF, whole blood, salivary glands, lung, mediastinal lymph nodes, choroid plexus, spleen, serum	VI (CPE), VN, AGID, ELISA
	Bovine rhinovirus (*Picornavirus*)		Nasal secretions	VI (CPE), VN
	Rift Valley fever (bovine, ovine) (*Phlebovirus*)		Whole blood in anticoagulant, fetus, liver, spleen, kidney, brain	VI (CPE and mice), VN, CF, FA, PCR
Enteric	Bovine enterovirus		Feces, oropharyngeal swab	VI (CPE), VN, PCR
	Porcine transmissible gastroenteritis, porcine epidemic diarrhea, porcine delta coronavirus (*Coronavirus*)		Feces, nasal secretions, jejunum, ileum, serum	VI (newborn pigs), FA, EM, PCR, ELISA
	Neonatal diarrheas			
	1. Rotaviruses		Feces, small intestine	VI (CPE with trypsin), ELISA, FA, EM, PCR
	2. Parvoviruses	Abortion	Feces, intestinal mucosa, regional lymph nodes, brain, heart	VI (CPE), FA, EM, HA, HI, VN, PCR, IHC
	3. Coronaviruses		Feces, small intestine	VI (CPE with trypsin), FA, EM, PCR
	Picornavirus SMEDI (enterovirus)		Feces, intestine, brain, tonsil, liver	VI (CPE, VN, EM) PCR
	Polioencephalomyelitis (Teschen, Talfan), (Enterovirus)	CNS	Brain, intestine, feces	VI (CPE, VN), PCR
	Rinderpest (morbillivirus)		Blood in anticoagulant, spleen, mesenteric lymph nodes	VI (CPE and cattle), AGID, CF, VN

(*Continued*)

Table 68.1 (Continued)

Type of illness or infection	Common name or associated virus	Other infections	Clinical specimens to collect	Diagnostic identification tests
	Peste des petits ruminants (*Morbillivirus*)		Blood in anticoagulant, spleen, mesenteric lymph nodes	VI (CPE and goats), AGID
CNS	Rabies (*Lyssavirus*)		Brain, salivary gland	VI (mice), FA, PCR
	Equine encephalomyelitis (VEE, EEE, WEE) (*Alphavirus*)		Whole blood, brain, cerebrospinal fluid, nasal and pharyngeal secretions, pancreas	VI (ECE and mice), VN, CF
	West Nile Virus (Equine)		Brain, spinal cord, cerebrospinal fluid, serum	VI, VN, ELISA, IHC, PCR
	Louping ill encephalomyelitis (*Flavivirus*)		Whole blood, brain, cerebrospinal fluid	VI (ECE, and CPE)
	Hemagglutinating encephalomyelitis virus (*Coronavirus*)		Brain, spinal cord, tonsil, blood	VI, PCR
Skin and mucosa	Foot-and-mouth disease (Bovine, Ovine, Swine) (*Aphthovirus*)		Vesicular fluid, epithelial tissue from rupture vesicles, esophageal or pharyngeal fluid, anticoagulant blood, serum	VI, VN, ELISA, PCR
	Senecavirus		Vesicular fluid, epithelial tissue from rupture vesicles, oral fluids	VI (CPE), PCR, FA
	Vesicular stomatitis virus (Bovine, Equine, Ovine, Swine) (*Vesiculovirus*)		Tissue, vesicular fluid, lesion swabs, serum	VI, VN, CF, PCR, ELISA
	Swine vesicular disease virus (*Picornavirus*)		Tissue, vesicular fluid, lesion swabs, serum	VI, ELISA, PCR
	Bovine mammillitis virus (Herpesvirus 2)		Affected tissue, serum	VI, VN
	Papillomavirus (Bovine, Equine, Canine)		Affected tissue	IHC, EM, PCR
	Pseudocowpox (*Parapoxvirus*)		Affected tissue, serum	VI, IFA, EM

VI, virus isolation; CPE, cytopathic effect; HA, hemagglutinin; CF, complement fixation; FA, fluorescent antibody; VN, virus neutralization; ECE, embryonating chicken eggs; HI, hemagglutination inhibition; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; AGID, agar gel immunodiffusion; IHC, Immunohistochemistry.

likelihood of isolating viruses. The following factors should be considered in selecting clinical specimens: (i) type of disease (e.g. respiratory – lung or trachea, or vesicular – vesicle or skin biopsy), (ii) the age and species of the host, (iii) the nature of the lesions in affected animals, and (iv) the size of carcasses or tissues (can be shipped on ice?). The following is a systematic approach for the rapid laboratory diagnosis of a viral-caused disease in animals:

1) Examination (gross and histological) of the diseased animal/tissues as a presumptive diagnosis for a viral etiology.
2) Detection of viral-specific antibodies (ideally using acute and convalescent sera to demonstrate responding antibodies) during clinical disease.
3) Immunohistochemical staining of tissue sections with virus-specific antibodies to detect individual viral antigens in the tissue.
4) Examination of feces, plasma, or serum by immunoassays that detect specific viral antigens (e.g. the rotavirus in feces, feline leukemia virus in serum, and bovine respiratory syncytial virus in lung).
5) Examination of positive- or negative-stained specimens by electron microscopy (EM) to identify the morphology of viruses. This diagnostic procedure is limited by the concentration of viral particles required for detection ($>10^5$/ml).
6) Isolation or amplification of infectious viruses in cell cultures and identification of the virus propagated from clinical submission. Alternatively, detection of

virus-specific RNA or DNA may be an acceptable and more efficient approach.

Many viral diseases do not kill the host, but the host may serve as a reservoir of viruses and disseminate the virus to other contact animals. Serological assays can sometimes be used to determine which animals have been exposed to specific viruses and which animals may be susceptible to infection.

Isolation of a virus from an animal does not necessarily implicate that virus as the causative agent of any disease that is occurring in that animal. It is very important to confirm that the isolated virus produces a similar disease in the same or related species, which may even involve the inoculation of susceptible or nonimmune animals. When two or more viruses are isolated from a specimen, a clear interpretation of the role of each isolate in the disease process is also necessary. Finally, it must be remembered that vaccine strains of attenuated viruses can also be reisolated from vaccinated animals and can be confused with true field strains.

Isolation of Virus from Clinical Specimens

Cultivation in Tissue Culture

Viruses are isolated from clinical specimens by inoculating susceptible primary or continuous cell cultures derived from the host or related species, embryonated eggs, or laboratory animals. Specimens submitted for viral isolation should be placed in virus transport media (e.g. a balanced salt solution containing antibiotics) in sealed containers for safety in handling. They should be clearly identified by appropriate labeling and submitted on ice (4 °C) or frozen (−20 °C). Attention to aseptic technique during specimen collection procedures will improve the probability of successful virus isolation.

Specimens should be collected from live animals during acute phases of the disease. Depending on the specific disease process, excretions or secretions, swabs of body orifices or liquids (lymph or blood), and tissue collected by biopsy are all suitable specimens for viral isolation. In the laboratory, tissue specimens are processed as a 10% or 20% (w/v) homogenate in a balanced salt solution along with antibiotics. Heavily contaminated specimens may be filtered to remove other microorganisms. Virus isolation should be conducted in cell cultures free of contaminating agents such as the noncytopathic Bovine viral diarrhea virus or *Mycoplasma*. The cell culture medium may contain low concentrations of broad-spectrum antibiotics.

To isolate the virus, tissue homogenates are placed onto cellular monolayers and absorbed for one hour or longer at 35–37 °C, and the inoculum is left on or removed and fresh media added. Inoculated and uninfected cell cultures are observed for 7–10 days. Viral cytopathic effect (CPE) in cells is usually evident between 24 hours and 72 hours for most cytopathic viruses (Figure 68.2). However, for most clinical material containing low concentrations of viruses, several (>3) blind cell passages are recommended.

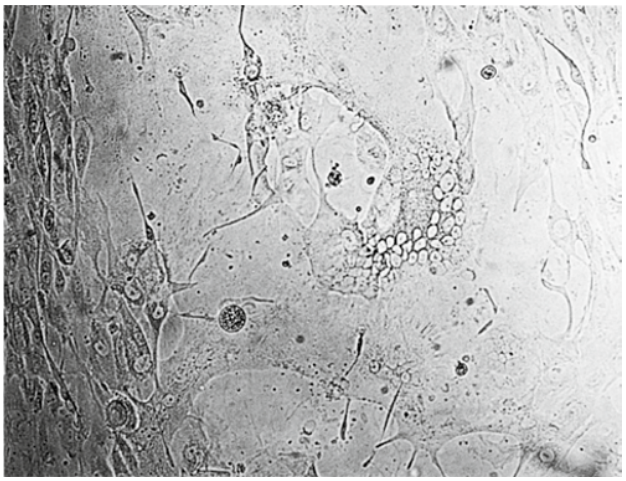


Figure 68.2 Cytopathic effect CPE (syncytial) on bovine fetal kidney cells by the herpesvirus of malignant catarrhal fever herpesvirus (×200).

After a virus has been demonstrated at limiting dilution to replicate in a cell by CPE or other parameters, the infectious virus is released from cells by three cycles of freeze–thaw or sonication, followed by centrifugation and storage to maintain maximum infectivity. Each virus isolated should be identified as to species of origin, morphological type, passage level, and host cell used for propagation.

Embryonated Eggs

A number of mammalian viruses and many avian viral pathogens can be isolated in embryonating chicken eggs (ECE). A key to successful viral isolation in ECEs is the route of inoculation (Figure 68.3). Candled ECE that die within 24 hours after inoculation are considered traumatic deaths. Subsequent deaths of inoculated ECE are placed at 4 °C for several hours (to avoid hemorrhages) prior to collection of fluids or visual examination of the embryos and egg membranes. Embryos that are stunted, deformed, edematous, or hemorrhagic, and membranes that contain lesions (i.e. pocks) should be homogenized in a sterile balanced saline as a 10% (w/v) suspension and repassaged in ECEs or cell cultures.

Animal Inoculation

The inoculation of susceptible laboratory animals remains a useful procedure for the identification of some viral pathogens, particularly those that are highly fastidious and difficult to propagate in other systems.



Figure 68.3 Inoculating an embryonated chicken egg (10–12 days old). For chorioallantoic membrane inoculation, a hole is first drilled through the egg shell and shell membrane; the shell over the air sac is then perforated, causing air to enter between the shell membrane and the chorioallantoic membrane, creating an artificial air sac, where the sample is deposited. The sample comes in contact with the chorionic epithelium. Yolk sac inoculation is usually carried out in younger (six days old) embryos, in which the yolk sac is larger. *Source:* Courtesy of Dr. Bill Wilson, USDA ARS.

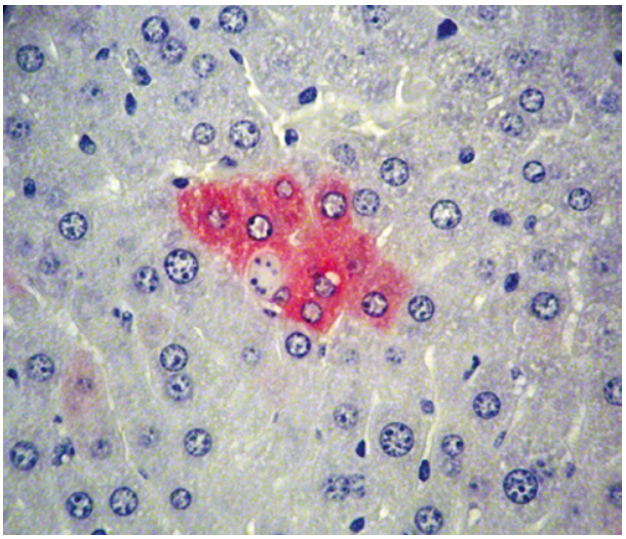


Figure 68.4 Immunohistochemistry assay demonstrating the presence of Rift Valley fever virus in the liver of an infected mouse. A Rift Valley fever virus antigen-specific antibody reacts specifically to viral antigen in the tissue (red color). *Source:* Courtesy of Dr. Barbara Drolet, USDA ARS.

Identification of Viruses or Viral Antigens in Clinical Specimens

Electron Microscopy

EM can be used to rapidly identify the morphology and size of any virus present in a specimen or isolated in cell culture of ECEs. Tentative diagnosis of viral diseases can be made by EM on thin sections of affected tissues and cell-free homogenates of clinical specimens. The use of EM for diagnosis is limited, however, because the method is not very sensitive ($>10^5$ virus particles/ml are required to see a single viral particle on a 200-mesh grid), and viruses from different species have similar morphology and size.

Immune Electron Microscopy

Immune electron microscopy (IEM) enhances detection of viruses in tissues, cells, or fecal specimens by reacting specific immune sera with viruses. In IEM, a specific antibody to a virus, preferably polyclonal, is mixed with the virus for one hour to produce antigen–antibody complexes. These immune complexes are centrifuged at $1000 \times g$ onto Formvar-coated grids, then stained with 4% phosphotungstic acid (PTA), pH 7.0, and examined by EM. The reaction of viral fluids with specific acute or convalescent serum as viewed by EM determines if a virus is associated with a specific disease. This procedure has been used successfully for viruses associated with infectious diarrhea.

Immunofluorescence

Immunofluorescence is a visible fluorescence accentuated by ultraviolet light as a specific antibody covalently bound to a fluorochrome (e.g. fluorescein isothiocyanate and rhodamine) that combines with a fixed antigen. This technique provides a sensitive and rapid method for detecting and identifying specific viruses in either tissues or cell cultures (Figure 68.4). Immunofluorescence is detectable by either a direct or indirect procedure. The direct immunofluorescence test employs a virus-specific antibody labeled with fluorescein that combines with a specific viral antigen located in cells or tissues. The indirect test requires the use of a fluorescein-labeled antiserum to a virus-specific immunoglobulin.

Immunohistochemical staining uses the same approach, except that the virus-specific antibody is either directly or indirectly labeled with an enzyme. The presence of the enzyme is determined by addition of its substrate, and the reaction is detected by a color change. The advantage of immunohistochemical staining over immunofluorescence is that a fluorescent microscope is not required, and the use of an enzyme-enhancement step greatly increases the sensitivity of the procedure.

Nucleic Acid Hybridization

Molecular hybridization techniques have led to the synthetic production of viral DNA probes that are highly specific for individual viruses. These probes are labeled with various detection systems that allow identification of the presence of individual viruses, either in tissues or in extracts of them.

Polymerase Chain Reaction

The relatively recent development of PCR has revolutionized the rapid diagnosis of many viral diseases. The importance of the procedure lies in its ability to amplify small amounts of viral DNA or RNA – even from contaminated specimens – and simultaneously evaluate vast numbers of samples for large-scale testing. Furthermore, technical developments

such as real-time PCR allow the quantitation of template that is present in a sample, which is reflective of the viral load. The PCR assay is based on the cyclic synthesis of a DNA segment limited by two specific oligonucleotides that are used as primers to specifically amplify portions of the viral genome. Real-time PCR assays frequently also include a third segment, a "probe," which is an additional oligonucleotide segment that has been modified with a fluorescent dye (fluorophore) and a quencher dye. When amplification occurs, the polymerase cleaves the probe and quencher from the probe as it incorporates the oligonucleotides into the amplified segment. This separation of the fluorophore from the quencher generates a signal that can be detected in real time by the thermocycler. Properly run, the PCR assay is both sensitive and specific, although the identification of viral nucleic acid does not prove that an infectious agent was present, so PCR-positive samples often should be subjected to traditional virus isolation procedures. Improvements and modifications of the real-time PCR methods have enabled the use of multiple fluorescent dyes simultaneously so that assays can be multiplexed (multiple assays in a single tube). This has led to the development of many syndromic panels, where differentials can be multiplexed in a single assay for clinical syndromes to detect multiple agents that are associated with the same clinical disease.

Next-Generation Sequencing

Next-generation sequencing (NGS), also called deep sequencing or massive parallel sequencing, is an emerging method that is being explored in diagnostic laboratories. Several technologies and chemistries are available that enable both non-targeted and targeted sequencing from complex samples, such as clinical specimens. The technologies vary in their capacities for read length, accuracy, and run times. NGS is being applied for novel pathogen discovery (non-targeted sequencing) or in sequencing whole genomes (WGS) of organisms to look at strain variation or relatedness. These approaches require significant bioinformatics analysis and interpretation for diagnostic use. For example, human public health laboratories have recently widely implemented these methods as a replacement for pulsed field gel electrophoresis (PFGE) to enable outbreak investigations and epidemiological studies of foodborne pathogen outbreaks as these methods can detect individual mutations in a pathogen genome. As the cost of these technologies goes down, these approaches may see more widespread use in veterinary diagnostic labs in the future in a variety of applications.

Serological Detection of Viruses

Most viruses usually elicit an immune response in the host; thus, the detection of a humoral (antibody) or cellular response is often used to determine prior infection of an animal with a viral pathogen. Serological assays measure humoral immunity in animals, and assays for measuring cellular immunity to viruses are used infrequently in veterinary diagnostic medicine.

Viruses have certain antigens that are type or group specific and that in part determine the serological assay used. Serological diagnosis of virus infections typically requires the collection of paired samples: an acute (at or prior to the onset of clinical signs) and a convalescent serum (10–28 days later). A fourfold or greater rise in antibody titer (the reciprocal of the serum dilution) indicates recent or ongoing viral infection. Antibody titers in single serum samples are more difficult to interpret, although the presence of antibodies is indicative of prior exposure (or resulting from the passive transfer of maternal antibodies in young animals), which is especially important in chronic diseases with a carrier state such as infections with bovine leukemia virus in cattle, equine infectious anemia, and the equine arteritis virus infection of stallions.

Serology can help to rapidly establish a diagnosis when viral isolation procedures are negative. Serology can also be used to definitively rule out the absence of specific viruses in a given disease outbreak, whereas a negative viral isolation cannot.

Serum Virus Neutralization Test

Most viruses produce a visible CPE in cell cultures. CPE is used to determine the presence of protective or virus-neutralizing antibodies in a serum. To quantify neutralizing antibodies, serum from an animal is serially diluted and mixed with a known amount of virus (generally 50–300 infectious doses of virus – TCID50) for one hour at 37 °C prior to inoculation of a volume of the mixture into animals, ECEs, or cell cultures. The serum neutralization (SN) test is very specific and highly sensitive, but it is time-consuming and expensive. The SN can be used to confirm recent infection of animals if paired sera are evaluated.

Hemagglutination Inhibition Test

Viruses that possess a hemagglutinin (HA) protein will agglutinate erythrocytes; a property that has been used to quantify the amount (titrate) of these viruses present in a sample. The hemagglutination inhibition (HI) test can be used to identify or type a virus through the inhibition of hemagglutination by virus-specific antiserum.

Hemadsorption Inhibition Test

The hemadsorption inhibition test is based on the ability of certain virus-infected cells (monolayers) to attract specific erythrocytes to their surface. The presence of hemadsorbing erythrocyte clusters on a cellular monolayer indicates that viral protein (hemagglutinin) has accumulated on the surface of the cell membrane. The hemadsorption phenomenon can be inhibited by pretreatment of virus-infected cells

for 30 minutes, usually at ambient temperature with twofold dilutions of antisera followed by the addition of 0.05–0.5% erythrocytes. Antibody (Ab) can be quantified by comparing the observed washed virus-infected cell monolayers that contain adhered clumped erythrocytes on the surface of the cell (Ab negative) with the cell monolayers that contain free-floating erythrocytes (Ab positive).

Complement Fixation

Complement fixation (CF) tests employ the complement cascade in reactions with viral antigens that fix complement – usually from guinea pig serum – when combined with specific, antiviral antibodies. Although CF has been used in early test tube assays to detect viruses (e.g. leukemia viruses), virus-infected cells, or virus-specific antibodies, the complexity of the assay and the time required have led to its replacement by simpler procedures.

Immunodiffusion

The immunodiffusion procedure is routinely used as a diagnostic tool to monitor the spread of specific viral pathogens in various animal diseases (e.g. bluetongue, equine infectious anemia, bovine leukosis, caprine arthritis, encephalitis, and infectious bursal disease). The basis of the test is the ability of certain soluble viral antigens to diffuse in a semisolid medium (agar) with the formation of a precipitin line with specific antisera.

Radioimmunoassay

The radioimmunoassay (RIA) is an exquisitely sensitive method for quantifying antigens or antibodies when one component is radio-labeled. Although RIA has an advantage for detecting minute amounts of antibodies, the need for a scintillation counter to measure radioactivity and very pure reagents limits use of this assay to appropriately equipped diagnostic laboratories.

ELISA for Antibody Detection

The enzyme-linked immunosorbent assay (ELISA) is a rapid, highly sensitive immunoassay adapted to measure viral antigens or antibodies (see Chapter 2). ELISAs have been developed for numerous avian viral pathogens (e.g. the Avian laryngotracheitis virus, Avian encephalitis virus, Newcastle disease virus, Infectious bronchitis virus, and reoviruses), and increasingly are being developed to detect viruses that infect other species of domestic animals.

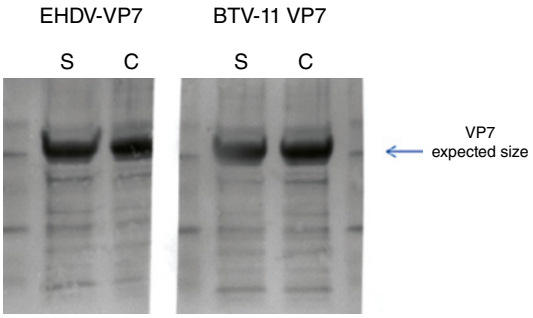


Figure 68.5 Western immunoblot demonstrating antibody binding to specific protein antigens separated by gel electrophoresis chromatography. Baculovirus-expressed EHDV-2 or BTV-11 VP-7 from cell supernatant (S) or cell pellet (C). Membrane blotted with 1:2000 monoclonal antibody [EHDV(4F4.H1) or BTV(1AA4.E4)] and 1:4000 goat α-mouse HRP. *Source:* Courtesy of Dr. Chris Lehiy, USDA ARS.

ELISAs are highly specific and sensitive immunoassays in which the specificity of the reaction can be enhanced by increasing the level of purification of the antigen or antibody employed. The ELISA can detect nanogram levels of IgG-, IgM-, and IgA-type antibodies. ELISAs can be made quantitative if appropriate standard curves are developed. Numerous commercially available assays for avian and mammalian viruses provide qualitative information on antibodies to various viruses, whereas others detect the viruses themselves in clinical specimens. The blocking ELISA evaluates the ability of a test serum to displace the binding of a virus protein-specific antibody to its antigen.

Western Immunoblot Assay

The western immunoblot assay can detect antibodies to a full range of viral proteins as revealed on a strip of nitrocellulose paper as discrete bands by electrophoresis. When a serum sample is applied to the nitrocellulose strip, antibodies from animals infected with a specific virus bind to the specific viral proteins at the appropriate positions. These bands become dark and distinct when the nitrocellulose paper is treated with a reagent (Figure 68.5). Because it provides a full viral antibody profile of the serum sample, this test is the most specific viral diagnostic test currently available.

Further Reading

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Antimicrobial Chemotherapy and Antimicrobial Resistance

Michael D. Apley

Introduction

Consideration of antimicrobials for use in veterinary medicine involves the four basic areas of clinical pharmacology:

- Can I do some good? (efficacy)
- Can I do some harm? (toxicity, selection for resistance)
- Can I get it into the animal? (a practical regimen)
- What does it cost?

In a text dedicated to microbiology, it is appropriate to focus on the efficacy and resistance selection aspects of antimicrobial therapy. When considering the larger context of clinical use, an antimicrobial capable of inhibiting the growth of a pathogen *in vivo*, or killing it, is of clinical use only if toxicity is negligible or manageable, if the regimen allows long-term clinical use without untenable selection for resistance, if the regimen is practical, and if the economics of treatment allow use.

This chapter is intended to introduce the reader to the basic pharmacology of antibacterial and antifungal agents by evaluating different approaches to classifying these agents, describing the key characteristics of different classes, evaluating potential clinical efficacy through antimicrobial susceptibility testing, and describing lessons learned in our recent efforts to preserve the clinical efficacy of these valuable tools.

Resistance mechanisms and selection for these mechanisms are also emphasized in many of the antibiotic and antifungal sections of this chapter. The reader is encouraged to note the consistent pattern that resistance to one antibiotic does not always occur in isolation, but is rather often associated with resistance to other antibiotics due to mechanistic relationships or association in mobile genetic elements.

Classifying Antimicrobials

The main reason to classify antimicrobials is to guide us in selecting a reasonable, or perhaps the optimal, antimicrobial for efficacy against an infectious disease. The clinical efficacy of any antimicrobial depends on a wide array of characteristics of the antimicrobial, the patient, the pathogen(s), and the combined effects of the disease process, coupled with how the antimicrobial is presented to the pathogen(s).

There are certain characteristics of antimicrobials that may be absolutely required for efficacy in diseases. For example, the ability to penetrate into the central nervous system for treatment of thrombotic meningoencephalitis (TME) in cattle, or the prostate in the case of canine prostatitis. However, presence at the site of infection does not alone ensure efficacy, and hence the need to consider multiple aspects of the patient–antimicrobial–pathogen interaction when evaluating the potential for changing the course of disease.

Spectrum

A general classification of an antimicrobial as "broad" or "narrow" spectrum does not meaningfully contribute to evaluation of clinical utility. A definition may be whether an antibiotic is active against both gram (+) and (−) bacteria, or just (+) or (−). Classification as narrow or broad spectrum ignores the nuances of specific inclusions or exclusions within the four-quadrant matrix described by efficacy in the aerobic/anaerobic and gram (+)/gram (−) quadrants. This classification also ignores the changing spectrums encountered in clinical use due to constantly developing acquired resistance profiles.

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The classification of narrow versus broad spectrum has been shown to depend on the classifier's view of his or her particular pathogen population of concern, and what may be a narrow spectrum drug to one clinician may address a wide array of pathogens of concern for another and therefore be considered broad spectrum. Another problem with spectrum classifications is that narrow spectrum classification is sometimes used as a proxy for minimizing antimicrobial resistance selection pressure during therapy. This leads to a false sense of security as changes in the resistome of a bacterial population are the result of interaction between the antimicrobial and all exposed bacteria, whether pathogens or commensals. Resistance selection pressure is the product of antibiotic exposure and the underlying resistant bacterial populations advantaged by this exposure.

Considering the inconsistent definitions of broad and narrow spectrum, an inconsistent relationship with resistance selection pressure, and the need to consider local changes in the spectrum due to resistance development, it is time to put these terms behind us and focus on understanding the typical pathogen inclusions in the activity range of each antibiotic class and class member, as well as current resistance trends.

Bactericidal versus Bacteriostatic

A bactericidal antibiotic may be classified as such if it meets the standard of at least a three log reduction in the bacterial population over 18–24 hours in a static concentration culture. It is possible for an antibiotic to display both bacteriostatic and bactericidal characteristics against the same pathogen isolate *in vitro*, depending on the concentration. It is also possible for members of antibiotic groups typically considered bacteriostatic to have bactericidal activity against some pathogens, for example, florfenicol displaying *in vitro* bactericidal activity against bovine respiratory disease pathogens such as *Mannheimia haemolytica*, or the killing characteristics of azithromycin compared to other macrolides.

A meta-analysis of human trials found that bactericidal and bacteriostatic antibiotics were most typically not found to be therapeutically different, and that when a difference was demonstrated, the superiority of one classification or the other was evenly distributed. When confronting the dogma that a bactericidal antimicrobial is required for a disease, it first should be determined whether this recommendation is based on actual clinical trial data, or on the assumption that a more rapid rate of killing will be more beneficial.

Pharmacodynamics

For antimicrobials, the science of pharmacodynamics is best described as how the antimicrobial best interacts with bacteria. The primary consideration in pharmacodynamic modeling is the best shape of the plasma concentration curve in relation to the minimum inhibitory concentration (MIC) of the pathogen. As used in creating antimicrobial susceptibility testing interpretive criteria, the three possible pharmacodynamic (PD) targets include the time for which the plasma concentration remains above the pathogen MIC (T > MIC), the ratio of the peak plasma concentration to the organism MIC (C_{max}/MIC), and the area under the plasma concentration curve divided by the pathogen MIC (AUC/MIC). These relationships must be evaluated in the context of plasma-free drug concentrations.

Mechanism of Action

Although few clinicians would consciously consider the mechanism of action in selecting an antibacterial or antifungal agent, their knowledge and experience related to efficacy and toxicity are based on observations that depend on these fundamental interactions. Spectrum, rate of bacterial killing, and pharmacodynamic characteristics are all determined by the relationship of the mechanism of action of the antimicrobial with the physiological characteristics of exposed bacterial and fungal populations. Acquired resistance is related to bacterial or fungal mutations that alter the targets for antimicrobial mechanisms of action. The toxicity of an antimicrobial is related to how the mechanism of action and other characteristics of the drug interact with the tissues of the patient.

The mechanism of action also serves as a robust framework on which to base an initial classification of different antibiotic classes and will be utilized as such in this chapter. For classification purposes in this chapter, four main mechanism classifications will be utilized:

1) Inhibition of cell wall synthesis
2) Damage to cell membrane function
3) Inhibition of nucleic acid synthesis or function
4) Inhibition of protein synthesis

Inhibition of Cell Wall Synthesis

Antibiotics that interfere with cell wall synthesis include the β-lactams (penicillins, cephalosporins, monobactams, and carbapenems), bacitracin, and vancomycin. The bacterial cell wall is a thick envelope that gives shape to the cell. This tough wall outside the cell membrane is a major difference between bacterial and mammalian cells. In gram-positive bacteria, it consists largely of a thick layer of peptidoglycan, which gives the cell rigidity and maintains

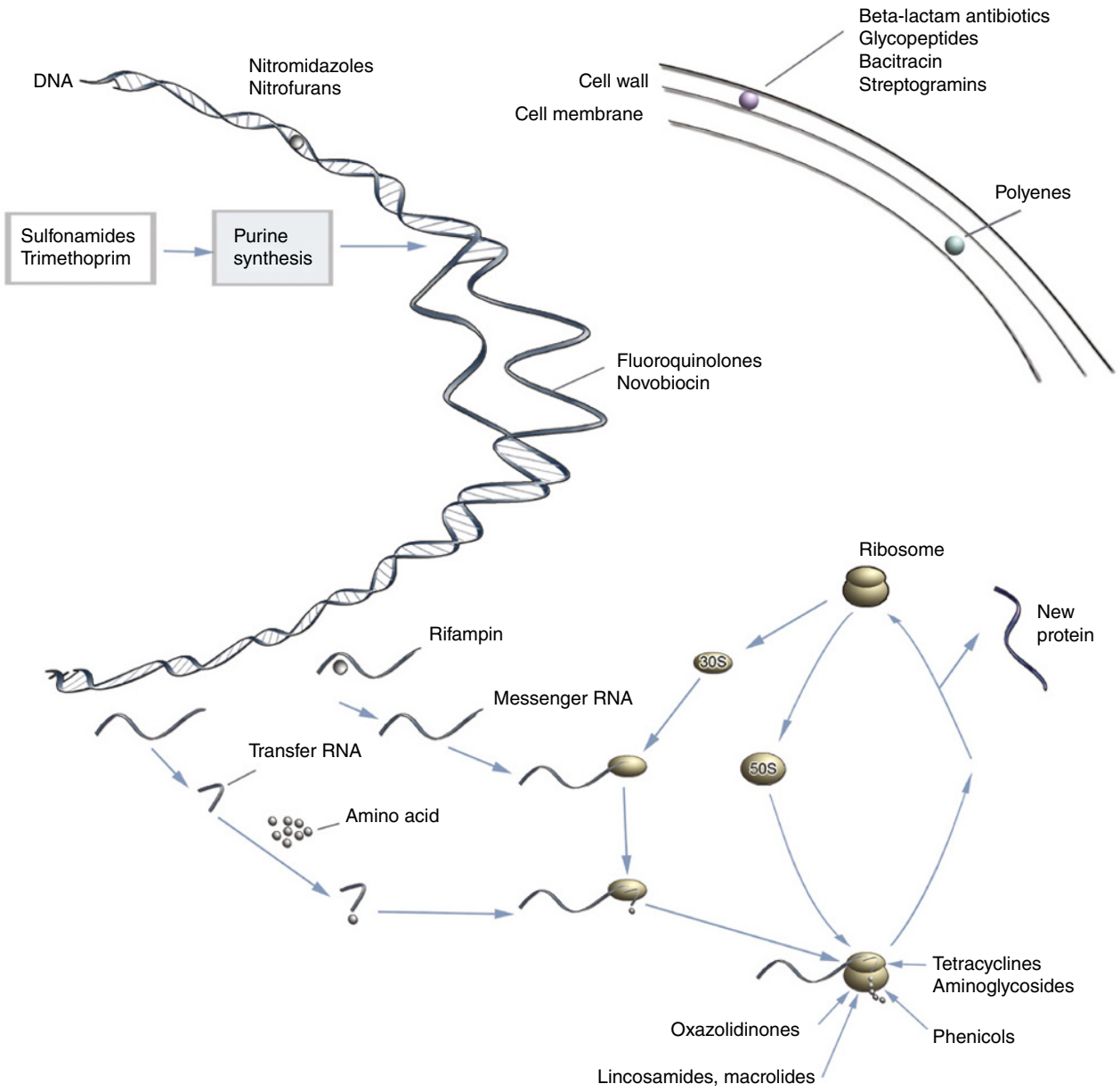


Figure 69.1 Mechanisms of action of antibacterial drugs. *Source:* Courtesy of Dr. John F. Prescott, University of Guelph, Canada.

a high internal osmotic pressure of about 20 atm. In gram-negative bacteria, this layer is thinner but with the addition of an outer lipid membrane, and the internal osmotic pressure is correspondingly lower. Peptidoglycan consists of a polysaccharide chain made up of a repeating disaccharide backbone of alternating *N*-acetylglucosamine-*N*-acetylmuramic acid in β-1,4 linkage, a tetra peptide attached to the *N*-acetylmuramic acid, and a peptide bridge from one tetrapeptide to another, so that the disaccharide backbone is cross-linked both within and between layers. The cross-linkage between transpeptides gives the cell wall remarkable strength. Several enzymes are involved in transpeptidation reactions.

The effect of β-lactam antibiotics is to prevent the final cross-linking in the cell wall, inhibiting division and creating weak points. Among the targets of these drugs are penicillin-binding proteins (PBPs), of which there are three to eight in bacteria; many of these PBPs are transpeptidase enzymes. They are responsible for the formation and remodeling of the cell wall during growth and division. Different PBPs have different affinities for drugs, explaining the variation in the spectrum of action of different

β-lactam antibiotics. Degradative mechanisms are also involved in cell wall production. These are carried out by autolysins, and some penicillins act partly by decreasing the normal inhibition of the autolysins.

The action of β-lactam antibiotics is thus to block peptidoglycan synthesis, which severely weakens the cell wall, and to promote the action of the autolysins, which lyse the cell. β-Lactams are active only against actively growing cells. The greater activity of some β-lactams against gram-positive bacteria is the result of the greater quantity of peptidoglycan and higher osmotic pressure in gram-positive bacteria, the impermeability of some gram-negative bacteria because of their lipopolysaccharide and lipid exterior, and the presence of β-lactamase enzymes in many gram-negative organisms. The remarkable activity of some of the newest penicillins and cephalosporins against gram-negatives is the result of not only their improved ability to enter gram-negative cells and bind PBPs but also of their ability to resist a variety of β-lactamase enzymes normally found in the periplasmic space of gram-negative bacteria. β-lactamase-inhibiting drugs with no intrinsic antibacterial activity, such as clavulanic acid, sulbactam, relebactam, vaborbactam, and avibactam are combined with amoxicillin piperacillin, imipenem/cilistatin, meropenem, and ceftazidime, respectively, to expand the spectrum of activity of these latter compounds by neutralizing enzymes that might otherwise degrade them. Of these potentiated compounds, amoxicillin/clavulanic acid would be most widely used in veterinary medicine with some use of piperacillin/tazobactam in refractive small animal infections.

Bacitracin as well as the glycopeptides inhibit the early stages in peptidoglycan synthesis. They are active only against gram-positive bacteria.

Beta Lactams: Penicillins

Sir Alexander Fleming's observation that colonies of staphylococci lysed on a plate that had become contaminated with a *Penicillium* fungus was the discovery that led to the development of modern antibiotics. In 1940, Chain, Florey, and their associates succeeded in producing therapeutic quantities of penicillin from *Penicillium notatum*. Almost a decade later, penicillin G became widely available for clinical use. In the years that followed, this antibiotic was found to have certain limitations: its relative instability in stomach acid, its susceptibility to inactivation by penicillinase, and its relative inactivity against most gram-negative bacteria. Isolation of the active moiety, 6-aminopenicillanic acid, in the penicillin molecule has resulted in the design and development of semisynthetic penicillins that overcome some of these limitations.

The development of the cephalosporin family, which shares with penicillin the β-lactam ring, has led to a remarkable array of drugs with improved ability to penetrate different gram-negative bacterial species and to resist β-lactamase enzymes. In recent years, other naturally occurring β-lactam antibiotics have been described that lack the bicyclic ring of the classical β-lactam penicillins and cephalosporins. Many of these new drugs have potent antibacterial activity and are highly resistant to β-lactamase enzymes.

Clinically important penicillins can be divided into five groups:

1) *Benzyl penicillins*: Injectable penicillins, highest activity against gram-positive organisms, but susceptible to acid hydrolysis and β-lactamase inactivation (e.g. penicillin G).
2) *Orally absorbed penicillins*: Spectrum similar to benzyl penicillins (e.g. penicillin V).
3) *Antistaphylococcal isoxazolyl penicillins*: Relatively resistant to staphylococcal β-lactamases (e.g. cloxacillin and methicillin).
4) *Extended-spectrum penicillins*: Aminopenicillins (e.g. amoxicillin and ampicillin).
5) *Antipseudomonal penicillins*: Carboxy- and ureidopenicillins (e.g. carbenicillin, piperacillin, and ticarcillin).

Antimicrobial Activity

The penicillins are bactericidal with a pharmacodynamic target of time > MIC, with suggested values of 25–50% of the dosing interval above the MIC for some gram-positive bacteria such as *Streptococcus* spp., and up to 100% of the dosing interval above the MIC for gram-negative infections advocated by some.

Penicillin G is the most active of the penicillins against gram-positive aerobic bacteria such as non-β-lactamase-producing coagulase-positive staphylococci, β-hemolytic streptococci, *Bacillus anthracis* and other gram-positive rods, *Corynebacteria*, *Erysipelothrix*, *Listeria*, and against most anaerobes. It is moderately active against the more fastidious gram-negative aerobes such as *Haemophilus*, *Pasteurella*, and some *Actinobacillus*, but it is inactive against members of the order Enterobacterales, and the genera *Bordetella* and *Pseudomonas*. The penicillinase-resistant isoxazolyl penicillins (oxacillin, cloxacillin, methicillin, and nafcillin) are resistant to coagulase-positive staphylococcal penicillinase, but are less active than penicillin G against other penicillin-sensitive gram-positive bacteria. Most gram-negative bacteria are resistant to them. Ampicillin and amoxicillin are slightly less active than penicillin G against gram-positive and anaerobic bacteria and are also inactivated by penicillinase produced by coagulase-positive

staphylococci. They have considerably greater activity against gram-negative bacteria. They are ineffective against *Pseudomonas aeruginosa*. Carbenicillin and ticarcillin resemble ampicillin in spectrum of activity, with the notable difference of having activity against *P. aeruginosa*. Temocillin is highly resistant to *β*-lactamases including extended-spectrum cephalosporinases, and has broad activity against members of the order Enterobacterales, including otherwise resistant isolates. *Mycoplasma* spp. and mycobacteria are resistant to penicillins. Methicillin-resistant staphylococci are considered resistant to all *β*-lactam antibiotics, although with the advent of newer β-lactams in recent years this dogmatic statement needs to be reconsidered.

Resistance

In gram-positive bacteria (particularly coagulase-positive staphylococci), resistance is mainly through production of extracellular *β*-lactamase (penicillinase) enzymes that break the *β*-lactam ring, with affinity for penicillins based on penicillinase structure. However, in some gram-positive pathogens such as *Enterococcus faecium*, penicillinases are rare and resistance is largely due to alterations in PBPs. As of 2018, over 2770 uniquely structured β-lactamase enzymes had been identified.

Resistance in gram-negative bacteria results, in part, from a wide variety of *β*-lactamase enzymes and also from low bacterial permeability or the lack of PBP receptors. Most or all gram-negative bacteria express low levels of species-specific chromosomally mediated *β*-lactamase enzymes within the periplasmic space.

Plasmid-mediated *β*-lactamase production is widespread among common gram-negative bacteria. The enzymes are constitutively expressed and cause high-level resistance. TEM-type *β*-lactamases readily hydrolyze penicillin G and ampicillin rather than methicillin, cloxacillin, or carbenicillin. OXA-type *β*-lactamases hydrolyze isoxazolyl penicillins (oxacillin, cloxacillin, and related compounds). SHV-type *β*-lactamases are found particularly in *Klebsiella pneumoniae* but may be found in other members of the order Enterobacterales.

A major advance was the discovery of broad-spectrum inhibitors of *β*-lactamase enzymes (e.g. clavulanic acid and sulbactam). These drugs have weak antibacterial activity but show extraordinary synergism when administered with penicillin G, ampicillin, amoxicillin, or ticarcillin because they irreversibly bind the *β*-lactamase enzymes of resistant bacteria, although not all β-lactamases are inhibited by each inhibitor.

Absorption, Distribution, and Excretion

The penicillins are organic acids that are generally available as the sodium or potassium salt of the free acid. Acid hydrolysis limits the systemic availability of penicillin G and the extended-spectrum penicillins. Penicillin V, the isoxazolyl penicillins, and the aminobenzylpenicillins (e.g. ampicillin, amoxicillin) are relatively stable in acid and can be administered orally.

The penicillins are predominantly ionized in the blood plasma, have relatively small apparent volumes of distribution, and have short half-lives (0.5–1.2 hours) in all species of domestic animals. Exceptions to the short apparent half-lives are when flip-flop pharmacokinetics are displayed by procaine penicillin G or benzathine penicillin G. Although mixtures of procaine and benzathine penicillin G result in prolonged plasma concentrations, the contribution of the benzathine fraction is a long, low plasma concentration tail for which efficacy might be expected for only the most susceptible pathogens. When considered for food animals, the increased residue potential for this combined formulation must be considered in relation to the limited potential for increased efficacy compared to a formulation containing only procaine penicillin G.

After absorption, penicillins are widely distributed in body fluids. Because of their high degree of ionization and low solubility in lipid, they achieve only low intracellular concentrations and do not penetrate well into transcellular fluids. The relatively poor diffusibility of penicillins across cell membranes is reflected in their low milk-to-plasma concentration ratios after systemic administration (0.3). Ampicillin and amoxicillin, in addition to having a wider spectrum of antimicrobial activity, penetrate cellular barriers more readily than penicillin G and have somewhat longer elimination half-lives. Ampicillin is available as a trihydrate salt for parenteral injection.

Penetration into cerebrospinal fluid (CSF) is usually poor but is enhanced by inflammation. In addition, active removal of penicillin from CSF is diminished by inflammation. The penicillins are eliminated almost entirely by renal excretion, which results in very high concentrations in the urine. The renal excretion mechanisms include glomerular filtration and mainly proximal tubular secretion.

Adverse Effects

Penicillins are remarkably free of toxic effects, even at doses grossly in excess of those recommended. The major adverse effect is acute anaphylaxis; milder hypersensitivity reactions (urticaria, fever, angioneurotic edema) are more common. Penicillin G has been linked to Coombs-positive hemolytic anemia in horses, and also rarely with abortions in sows. All penicillins are cross-sensitizing and cross-reacting. Anaphylactic reactions are less common after oral penicillin administration than after parenteral administration. Many of the acute toxicities reported in animals are the toxic effects of the potassium or procaine with which

the penicillin is combined, for example, the "K pen squirts" in horses following IV administration of K penicillin G with the resulting propulsive effect on gut motility. The use of penicillin and ampicillin in guinea pigs invariably causes fatal *Clostridium difficile* colitis, and use of ampicillin in rabbits causes fatal *C. difficile* or *Clostridium spiroforme* colitis.

Beta Lactams: Cephalosporins

Cephalosporins are natural or semisynthetic products of the fungi *Cephalosporium* spp.; the related cephamycins are derived from the actinomycetes type of bacteria. The nucleus of the semisynthetic cephalosporins, 7-aminocephalosporanic acid, bears a close structural resemblance to that of the penicillins, which accounts for the common mechanism of action and other properties shared by these two classes of drugs. Attachment of various R groups to the cephalosporanic acid nucleus has resulted in compounds with low toxicity and high therapeutic activity. Though not an ideal description, the classification of cephalosporins as belonging to five generations relates to their increasing spectrum of activity against gram-negative bacteria because of improved penetration of cells and their progressive resistance to the β-lactamases of gram-negative bacteria.

Antimicrobial Activity

As for the penicillins, cephalosporins are considered bactericidal with a pharmacodynamic target of time > MIC with similar target values. The first-generation cephalosporins (e.g. cephalothin, cephalexin, cephaloridine, and cefadroxil) have a similar spectrum of activity as ampicillin, with the notable difference that β-lactamase-producing staphylococci are often susceptible, although resistance may invalidate this generalization. They are active against a variety of gram-positive bacteria such as coagulase-positive staphylococci, many streptococci, corynebacteria, and gram-positive anaerobes (*Clostridium*). Among gram-negative bacteria, *Haemophilus* and *Pasteurella* are considered within the spectrum, as are some *Escherichia coli*, *Klebsiella*, *Proteus*, and *Salmonella*. *Enterobacter, Enterococcus* spp., and *P. aeruginosa* are resistant. Many anaerobic bacteria, except members of the *Bacteroides fragilis* group, are susceptible. The second-generation cephalosporins (e.g. cefoxitin and cefuroxime) have increased resistance to gram-negative β-lactamases and thus broader activity against gram-negative bacteria as well as against bacteria susceptible to the first-generation drugs. They are active against some strains of *Enterobacter* and against cephalothin-resistant *E. coli*, *Klebsiella*, and *Proteus*. Some *B. fragilis* are susceptible. Like the first-generation cephalosporins, these drugs are not active against *P. aeruginosa, Enterococcus* spp., or *Serratia*. The third-generation cephalosporins (e.g. cefotaxime, cefpodoxime, ceftiofur, and cefoperazone) are characterized by reduced activity against gram-positive bacteria, modest activity against *P. aeruginosa*, and greatly enhanced activity against members of the order Enterobacterales. Some third-generation cephalosporins (e.g. ceftazidime) are highly active against *P. aeruginosa* at the expense of activity against members of the order Enterobacterales. Cefotaxime is considered the third-generation cephalosporin most active against anaerobes.

Fourth-generation cephalosporins (e.g. cefepime, cefpirome, cefquinome) are stable to hydrolysis by many β-lactamases. This generation has enhanced activity against *Pseudomonas* and the order Enterobacterales. Efficacy is also retained for *Streptococcus* and *Staphylococcus* spp. The fifth-generation cephalosporins (e.g. ceftobiprole, ceftaroline) again make strides against resistant pathogens with enhanced activity against *P. aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), and also vancomycin-resistant enterococci (VRE). There is also activity against a wide array of gram-negative and gram-positive bacteria.

The most recent advance in cephalosporins is the siderophore cephalosporins (e.g. cefiderocol). This group takes advantage of gram-negative bacteria iron uptake systems by conjugating iron binding groups to the cephalosporin nucleus, thereby bypassing the outer membrane barrier.

Resistance

Plasmid-mediated resistance to first-, second-, and third-generation drugs has been described in gram-negative bacteria. Emergence of resistance in *Enterobacter*, *Serratia*, and *P. aeruginosa* during treatment with third-generation drugs results from derepression of inducible, chromosomal β-lactamase enzymes, which in turn results in broad-spectrum resistance to β-lactam antibiotics. Plasmid-mediated resistance to third-generation cephalosporins is increasingly reported. It can involve either TEM- or SHV-β-lactamases or other β-lactamase families including CTX-M1, which hydrolyzes cefotaxime. Collectively these are referred to as extended-spectrum beta lactamases (ESBLs). The early ESBLs were predominantly variations of SHV or TEM B-lactamases and were still typically susceptible to B-lactamase inhibitor combinations. However, variants resistant to these combinations, referred to as "inhibitor resistant" TEM enzymes (IRTs), soon appeared,. Most recently, CTX-M β-lactamases have dominated in multidrug resistant gram-negative pathogens. A concerning development is that of pathogens containing multiple β-lactamase enzymes, presenting challenges to B-lactamase inhibitor combinations. The

evolution of β-lactamases is a fascinating example of pathogen evolution in response to antibiotic pressure. The reader is referred to recent reviews of β-lactamases listed at the end of this chapter for a deeper understanding of this evolution.

Absorption, Distribution, and Excretion

Cephalosporins are water-soluble drugs. Of the first-generation cephalosporins, cephalexin and cefadroxil are relatively acid stable and sufficiently well absorbed from the intestine to be administered orally to dogs and cats, but not to herbivores. Other first-generation cephalosporins must be administered parenterally although they are often painful on intramuscular injection and irritating on intravenous injection. Second- and third-generation cephalosporins are sometimes available for oral use and could be given to dogs and cats by this route rather than parenterally. Following absorption from injection sites, cephalosporins are widely distributed into tissue and body fluids. Some third-generation cephalosporins penetrate CSF moderately well, and because of their high activity against gram-negative bacteria have particular potential application in the treatment of meningitis.

Like the penicillins, many cephalosporins have short half-lives, and most are excreted unchanged in urine. Exceptions to the short half-life generalization include ceftiofur sodium and cefovecin, both of which display prolonged apparent elimination half-lives due to plasma protein binding; even longer apparent elimination half-lives are obtained for ceftiofur with the hydrochloride and crystalline free acid formulations, contributing to flip-flop pharmacokinetics.

Adverse Effects

Cephalosporins are relatively nontoxic antibiotics in humans. Allergic reactions occur in 5–10% of human patients who are hypersensitive to penicillin. Intravenous and intramuscular injections of some drugs are an irritant. Gastrointestinal microbiome alterations may lead to effects such as anorexia, diarrhea, and vomiting in some patients. Cephalexin-associated seizures have been reported in epileptic dogs otherwise controlled with phenobarbital.

Beta Lactams: Monobactams

Members of the monobactams include aztreonam, tigemonam, and carumonam. These compounds possess the β-lactam ring but not the thiazolidine ring of the other β-lactams. Activity is limited to gram-negative bacteria, leading to substitution of aztreonam for aminoglycosides in combination therapy regimens. Aztreonam is considered β-lactamase stable in most cases, but may still be hydrolyzed by some of these enzymes.

Beta Lactams: Carbapenems

Also referred to as the "thienamycins," this group includes imipenem, meropenem, ertapenem, and doripenem. Imipenem is extensively metabolized in the brush border proximal renal tubular cells to produce a toxic metabolite. Therefore, imipenem is administered with a dehydropeptidase 1 inhibitor, cilistatin, for clinical use. Commercial products containing imipenem/cilistatin combined with the B-lactamase inhibitor relebactam, and meropenem combined with vaborbactam, have been approved to address carbapenem-resistant pathogens.

There are no veterinary carbapenem labels; however, extralabel use in small animals has been reported for imipenem/cilistatin and meropenem. The carbapenems have what is arguably one of the widest spectrums of activity of any antibiotic. They are active against gram-positive and gram-negative aerobes and anaerobes.

However, the adaptability of the β-lactamase strategy has again been demonstrated. Plasmid-associated carbapenemases in *Klebsiella pneumoniae* (KPCs) were discovered at the onset of the twenty-first century. The New Delhi metallo-β-lactamase-1 (NDM-1) enzyme was identified in 2009 and has spread around the world. Another carbapenemase, OXA-48, is found in multidrug resistant Enterobacterales, where it can hydrolyze carbapenems and extended-spectrum cephalosporins. It appears that vaborbactam is the only β-lactamase inhibitor with activity against this particular β-lactamase.

Bacitracin

Bacitracin is a cyclic polypeptide that inhibits peptidoglycan formation in the bacterial cell wall by binding to the pyrophosphate carrier and preventing the dephosphorylation reaction required for regeneration. Considered bactericidal, its activity is limited to gram-positive bacteria. Bacitracin is limited to topical use due to nephrotoxicity when administered parenterally. It is approved as a feed drug in several food animal species, where the poor absorption limits activity to the gastrointestinal tract and avoids systemic toxicity.

Glycopeptides

Vancomycin, teicoplanin and avoparcin are examples of the glycopeptide group. Lipoglycopeptide semisynthetic derivatives include dalbavancin, oritavancin, and

telavancin. The mannopeptimycins are a recent subclass of glycopeptides not related to vancomycin. Glycopeptides interfere with cell wall peptidoglycan synthesis, attaching to a different binding site for inhibition than penicillins and cephalosporins.

With the exception of avoparcin, to the author's knowledge this class has not been approved for use in veterinary medicine. Avoparcin was approved as a growth promoting in-feed drug for food animals in the European Union (EU), but approval was withdrawn due to association with selection for glycopeptide resistance in enterococci of animal origin. Extralabel use of the glycopeptides in food animals is prohibited in some jurisdictions, including the United States.

Glycopeptide activity is primarily against gram-positive, aerobic, cocci, and bacilli. Poor penetration limits gram-negative activity. Vancomycin is an important therapy for methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *Enterococcus* spp. Resistance to vancomycin requires a combination of three key genes: vanA or vanB, vanH, and vanX.

Toxicity for glycopeptides includes nephrotoxicity and the potential release of large quantities of histamine if vancomycin is administered intravenously at a rapid rate.

Damage to Cell Membrane Function

Antibiotics that damage cell membrane function include the polymyxins, ionophores, and the antifungal polyenes (amphotericin, nystatin) and imidazoles (fluconazole, itraconazole, ketoconazole, miconazole), discussed under antifungal drugs. The cell membrane lies beneath the cell wall, enclosing the cytoplasm. It controls the passage of materials into or out of the cell. If its functionality is impaired, cellular contents (proteins, nucleotides, ions) can leak from the cell and result in cell damage and death.

Polymyxins

Included in this group are polymyxin B (included in many topical formulations) and Polymyxin E (colistin). The structure of the polymyxins is such that they have well-defined separate hydrophilic and hydrophobic sectors. Polymyxins act by binding to membrane phospholipids, which results in structural disorganization, permeability damage, and cell lysis. The polymyxins are selectively toxic to gram-negative bacteria because of the presence of certain phospholipids in the cell membrane and because the outer surface of the outer membrane of gram-negative bacteria consists mainly of lipopolysaccharide. Parenteral use is associated with nephrotoxic, neurotoxic, and neuromuscular blocking effects. The major clinical applications are limited to the oral treatment of gram-negative bacterial infections. However, colistin has recently become more frequently used for systemic therapy in humans due to the absence of alternative treatments for some multidrug-resistant bacterial pathogens, although the recent advent of potentiated carbapenems and potentiated ceftazidime may reduce the need for colistin use. One application for polymyxin B in veterinary medicine is the use of a low, carefully controlled systemic dose to bind endotoxin in cases of equine colic.

Ionophores

The ionophores are polyether, carboxylic antibiotics. Members of the group include monensin, lasalocid, laidlomycin, narasin, and salinomycin. They function by forming complexes with cell wall sodium, resulting in the export of potassium in exchange for the import of hydrogen ions, which leads to a lethal drop in intracellular pH. Ionophores are effective against gram-positive bacteria, thus shifting the bacterial population in the rumen to species that produce more propionic acid, thereby increasing the efficiency of glucose production. The spectrum also includes *Campylobacter* spp., *Serpulina hyodysenteriae*, *Toxoplasma*, and coccidia. Action as a coccidiostat is a primary reason for the use of the ionophores in food animals, especially cattle and chickens.

The ionophores also decrease the formation of 3-methylindole from tryptophan in the rumen, reducing the incidence of pulmonary edema from the ingestion of lush forages (fog fever) if fed far enough in advance. Ionophores have also been demonstrated to reduce methane production by ruminants.

Toxicity is due to disruption of cell membranes in muscle, with primary effects on cardiac muscle in cattle and horses, and skeletal muscle in sheep. This toxicity is dose dependent, with horses being the most susceptible. Concurrent feeding of drugs that inhibit hepatic metabolism may result in toxicity due to reduced metabolism of the ionophore.

To the author's knowledge, there are no documented instances of the non-medically important ionophores co-selecting for resistance to medically important antibiotics.

Inhibition of Nucleic Acid Function

Examples of drugs that inhibit nucleic acid function are nitroimidazoles, nitrofurans, nalidixic acid, the fluoroquinolones, novobiocin, rifampin, sulfonamides, trimethoprim, and 5-flucytosine. Because the mechanisms of nucleic acid synthesis, replication, and transcription are

similar in all cells, some drugs affecting nucleic acid function have poor selective toxicity. Most act by binding to DNA to inhibit its replication or transcription. Drugs with greater selective toxicity are the sulfonamides and trimethoprim, which inhibit the synthesis of folic acid.

Nitroimidazoles

Nitroimidazoles (metronidazole, dimetridazole, ipronidazole, ronidazole, tinidazole) possess antiprotozoal and antibacterial properties. Activity within bacterial cells is due to the unidentified reduced products of the drug, which are seen only in anaerobes or microaerophiles. Nitroimidazoles cause extensive DNA strand breakage either by inhibiting the DNA repair enzyme, DNase 1, or by forming complexes with the nucleotide bases that the enzyme does not recognize. Nitroimidazoles are bactericidal to anaerobic gram-negative and many anaerobic gram-positive bacteria and are active against protozoa such as *Tritrichomonas fetus*, *Giardia lamblia*, and *Histomonas meleagridis*. Chromosomal resistance may cause slight increases in the MIC, but as is the case for nitrofurans, plasmid-encoded resistance is rare. Nitroimidazoles are generally well absorbed after oral administration, but parenteral injection is highly irritating. They are well distributed throughout body tissues and fluids, including brain and CSF. Excretion is through urine. The most serious potential hazard is the controversial report of carcinogenicity in laboratory animals. For this reason, these drugs are not used in food animals. Acute central nervous system (CNS) toxicity has been reported in dogs and cats with high doses. Inappetence may be noted in horses. Nausea and vomiting has been reported in dogs.

Nitrofurans

Like the nitroimidazoles, the nitrofurans are antiprotozoal but have wider antibacterial activity; they are most active under anaerobic conditions. The nitrofurans are synthetic 5-nitrofuraldehyde derivatives with broad antimicrobial activity and include furazolidone, nitrofurantoin, and nitrofurazone. After entry into the cell, bacterial nitroreductases produce uncharacterized unstable reduction products, which differ with each type of nitrofuran. Furazolidone and nitrofurazone have essentially zero oral bioavailability. Nitrofurantoin is well absorbed orally but so rapidly excreted through the kidneys that it is effective only for treating urinary tract infections. These products cause strand breakage in bacterial DNA. Toxicity and low tissue concentrations limit their use to the local treatment of infections and to the treatment of urinary tract infections.

The nitrofurans are mutagenic and procarcinogenic, resulting in prohibition for use in food animals in many jurisdictions, including the United States. Other reported toxicities include inappetence, neurotoxicity, and in poultry, cardiotoxicity.

Fluoroquinolones

Discussion of the fluoroquinolones requires some clarification of terminology. First came the quinolones. The first 4-quinolone, nalidixic acid, had a narrow gram-negative spectrum with rapid development of resistance and a significant toxicity profile. Adding a fluorine molecule at carbon 6 extended the spectrum to include some gram-positive organisms, while adding a piperazine ring at carbon 7 extended the spectrum to include *Pseudomonas* spp. The fluoroquinolones also possess improved toxicity and pharmacokinetic profiles as compared to the 4-quinolones. Chemical alterations continue to improve this class of completely synthetic antimicrobials; additions of alkyl chains on the para position and a nitrogen molecule at position one of the piperazine ring increase the lipid solubility and volume of distribution of enrofloxacin. Four generations have been proposed for the fluoroquinolones.

Antimicrobial Activity

First-generation fluoroquinolones (e.g. flumequine) are of minimal consequence in veterinary medicine. Second-generation fluoroquinolones labeled for veterinary use include enrofloxacin, orbifloxacin, marbofloxacin, danofloxacin, and ibafloxacin. Human-labeled examples include norfloxacin and ciprofloxacin. This class shares a characteristic spectrum of strong activity against gram-negative aerobes, more limited gram-positive activity (variable against *Streptococcus* spp.), and very limited activity against anaerobes. Activity is also displayed against *Mycoplasma, Campylobacter, Leptospira,* and *Rickettsia*. This spectrum is quite similar to the aminoglycosides, resulting in an early slang term for fluoroquinolones of "the oral aminoglycosides," although many other characteristics, such as toxicity, are quite different.

Third-generation fluoroquinolones include pradofloxacin in veterinary medicine. Human-labeled examples include levofloxacin, grepafloxacin, and sparfloxacin, along with many others. This generation has enhanced activity against *Streptococcus* and *Enterococcus* spp. and anaerobes. Fourth-generation fluoroquinolones display extensive improvements in anaerobic activity. Delafloxacin is a fourth-generation fluoroquinolone with human approval and displays increased cellular penetration because it is mildly acidic in character as compared to the characteristic zwitterion nature of other fluoroquinolones.

Other fourth-generation fluoroquinolones include trovafloxacin, gatifloxacin, moxifloxacin, and clinafloxacin.

The fluoroquinolone mechanism of action is selective inhibition of bacterial DNA synthesis by inhibiting DNA gyrase (topoisomerase II) and DNA topoisomerase IV. DNA gyrase is involved in packing (supercoiling) DNA into bacterial cells, whereas topoisomerase IV is involved in relaxing supercoiled DNA. Fluoroquinolones are bactericidal drugs.

Fluoroquinolone efficacy is best characterized by the free drug plasma concentration area under the curve (AUC) to MIC ratio. This target ratio for efficacy was initially characterized as 125, but more recent work has demonstrated that ratios as low as 30 may be sufficient depending on patient characteristics, the nature of the disease, and the desired effect on the pathogen population. When specifically considering resistance, a peak concentration of at least 8–10 × the pathogen MIC is associated with reduced resistance selection (the mutant prevention concentration, MPC), where first-mutation, low-level resistant bacteria will still be inhibited by this concentration. This value forms the top of the mutant selection window (MSW), with the bottom formed by the MIC of the target organism. It should be noted that the evidence for the MIC forming the bottom of the MSW is minimal to nonexistent, although it is used by convention. Following the MSW approach, the goal is for plasma-free drug concentrations resulting from a fluoroquinolone regimen to spend as little time as possible in the MSW, thereby minimizing the selection pressure for first-mutation resistant pathogens.

Absorption, Distribution, and Excretion

The fluoroquinolones are rapidly and highly absorbed after oral administration and have half-lives varying from 4 to 12 hours. Elimination is primarily through the kidney by both renal tubular excretion and glomerular filtration, with urine concentrations reaching 100 to 300 times plasma concentrations. They are widely distributed in tissues, and may be concentrated, for example, in the prostate. Penetration into cerebrospinal fluid is about half that of serum, which makes these drugs useful for treating meningitis. A de-ethylation reaction in the liver biotransforms enrofloxacin to ciprofloxacin, with varying degrees of biotransformation in different veterinary species.

Resistance

Chromosomally mediated resistance has been demonstrated in target pathogens with single-step nucleotide mutations in target enzymes to high-level resistance, such as *Campylobacter jejuni, Pseudomonas aeruginosa,* and *S. aureus*. Chromosomal resistance development in other organisms, such as *Streptococcus pneumoniae*, consists of two mutations in target enzymes with the first mutation resulting in an initial low-level resistance state (often an MIC of 0.5–1 μg/ml). Resistance can also result from decreased permeability of the cell wall as well as from acquisition of or enhanced activity of an efflux pump that actively transports fluoroquinolones from the cell. The AcrAB-tolC efflux system is chromosomally mediated and can export fluoroquinolones as well as chloramphenicol, β-lactams, aminoglycosides, tetracyclines, and multiple other compounds; this efflux system is also associated with biofilm formation in *E. coli* and *Salmonella* Typhimurium and supporting topoisomerase mutations. Plasmid-mediated efflux pumps include *Qep*A and *Oqx*AB, the latter encoding for resistance to ciprofloxacin, norfloxacin, tetracycline, tigecycline, chloramphenicol, nitrofurantoin, trimethoprim, and detergents and disinfectants.

Plasmid-mediated fluoroquinolone resistance involves multiple mechanisms, including the *qnr* gene family, which encodes for proteins that may bind to and protect the target enzymes for fluoroquinolones. The *qnr* genes are carried on plasmids that also encode for resistance due to carbapenemases, extended-spectrum β-lactamases (ESBLs), and AmpC β-lactamases. The *qnr* gene family encodes for low-level resistance; however the presence of this gene family supports the selection of high-level resistance mutants under fluoroquinolone selection pressure.

Also associated with plasmids, AAC(6′)-Ib-cr is an aminoglycoside acetyltransferase that inactivates both aminoglycosides (amikacin, tobramycin, kanamycin) and fluoroquinolones (e.g. ciprofloxacin and norfloxacin).

Adverse Effects

The toxicity of the fluoroquinolones includes regimen-dependent articular cartilage damage in rapidly growing puppies and foals, and regimen-dependent enrofloxacin retinal degeneration in cats. Enrofloxacin has been reported to lower the seizure threshold in epileptic dogs with seizures controlled by phenobarbital. The US label for pradofloxacin (labeled for cats only) states that "pradofloxacin has been shown to cause bone marrow suppression in dogs. Dogs may be particularly sensitive to this effect, potentially resulting in severe thrombocytopenia and neutropenia." Pradofloxacin is labeled for dogs in the EU. The strong affinity of some fluoroquinolones for the cytochrome P-450 isozyme IA-2 may lead to drug interactions; for example ciprofloxacin may increase theophylline concentrations by 2–5 μg/ml in humans, leading to toxicity.

Rifamycins: Rifampin and Ansamycins

Rifampin, which has particular activity against gram-positive bacteria and mycobacteria, inhibits bacterial

DNA-dependent ribonucleic acid (RNA) polymerase. Rifampin prevents initiation of transcription and is considered bactericidal. Resistance develops rapidly in mycobacteria as the result of chromosomal mutation, so that this drug is rarely used on its own but rather is used in combination with other antimicrobial drugs. Rifampin is commonly used in combination with a macrolide for treatment of *Rhodococcus equi* in foals.

Rifampin is a potent microsomal enzyme inducer. It creates clinically significant increases in the excretion of steroid drugs, ketoconazole, barbiturates, digitoxin, and oral anticoagulants. Clinical hepatitis may occur with prolonged therapy in dogs. Rifampin may function as a hapten and has caused immune-complex disease in humans with prolonged, intermittent therapy.

Sulfonamides and Diaminopyrimidines

Sulfonamides are synthetic drugs with broad antibacterial and antiprotozoal properties. They interfere with the biosynthesis of folic acid and prevent the formation of purine nucleotides. Sulfonamides are functional analogues of para-aminobenzoic acid (PABA) and compete with it for the same enzyme, tetrahydropteroate synthetase, forming nonfunctional folic acid analogues and inhibiting bacterial growth. Selective toxicity of sulfonamides occurs because mammalian cells have lost their ability to synthesize folic acid but rather absorb it from the intestine, whereas bacteria must synthesize it. In the bacterial cell, preformed folic acid is progressively exhausted by several bacterial divisions.

Diaminopyrimidines (trimethoprim, ormetoprim) affect folic acid synthesis by interfering with the enzyme dihydrofolate reductase. One example is trimethoprim, which is selectively toxic to bacteria rather than to mammalian cells because of its greater affinity for the bacterial enzyme. The enzyme inhibits the conversion of dihydrofolate to tetrahydrofolate, producing with sulfonamides a sequential blockade of folic acid synthesis.

Sulfonamides

The sulfonamides constitute a series of weak organic acids that enter most tissues and body fluids. They are available in a wide variety of preparations for either oral or parenteral use. Their use in non-potential forms is limited. because of widespread resistance, difficulties in administration, and the existence of better alternatives. Certain individual sulfonamides are combined with trimethoprim in fixed-ratio (5:1) combination preparations that have the advantage of both synergistic and bactericidal effects.

Individual sulfonamides are derivatives of sulfanilamide, which contains the structural prerequisites for antibacterial activity. The various derivatives differ in physicochemical and pharmacokinetic properties and in degree of antimicrobial activity. The sodium salts of sulfonamides are readily soluble in water, and parenteral preparations are available for intravenous administration (sulfadimethoxine is common); the pH values of these solutions are high, resulting in significant pain and tissue damage if injected intramuscularly or subcutaneously. Certain sulfonamide molecules are designed for low solubility (e.g. phthalylsulfathiazole) so that they will be slowly absorbed; these are intended for use in the treatment of enteric infections.

Antimicrobial Activity

Sulfonamides exert a bacteriostatic effect against both gram-positive and gram-negative bacteria and can also inhibit other microorganisms (some protozoa), although resistance has developed through many years of use. Many anaerobes are considered within the spectrum.

Resistance

Resistance to sulfonamides in pathogenic and nonpathogenic bacteria isolated from animals is widespread. This situation reflects their extensive use in human and veterinary medicine since the late 1930s. Sulfonamide resistance may occur as a result of mutation causing overproduction of PABA or as a result of a structural change in the dihydrofolic-acid-synthesizing enzyme with a lowered affinity for sulfonamides. Most often, sulfonamide resistance is plasmid mediated.

Absorption, Distribution, and Excretion

Most sulfonamides are rapidly absorbed from the gastrointestinal tract and distributed widely to all tissues and body fluids, including synovial and CSF. The variation in degree of ionization and lipid solubility of the large number of individual sulfonamides influences absorption, determines the capacity to penetrate cell membranes, and can affect the rate of elimination. They are bound to plasma proteins to a variable extent. In addition to differences among sulfonamides in the extent of binding, there is variation among species in binding of individual sulfonamides. Extensive (80%) protein binding serves to increase half-life.

Sulfonamides are excreted by a combination of renal excretion and biotransformation processes in the liver. This combination of elimination processes contributes to the species variation in the half-life of individual sulfonamides. Although a large number of sulfonamide preparations are available for use in veterinary medicine, many of these are different dosage forms of sulfamethazine. This sulfonamide is most widely used in food-producing animals and can achieve effective plasma concentrations (within the

range 50–150 μg/ml) when administered either orally or parenterally. Prolonged-release oral dosage forms of sulfamethazine are available.

Adverse Effects

The sulfonamides can produce a wide variety of side effects, some of which may have an allergic basis whereas others are due to direct toxicity. The more common adverse effects are urinary tract disturbances (crystalluria, hematuria, or even obstruction) and hematopoietic disorders (thrombocytopenia and leukopenia). Sulfaquinoxaline is thought to have an antagonistic effect on vitamin K. Hemorrhagic diathesis has been reported; treatment of puppies with sulfaquinoxaline for coccidiosis has resulted in hemorrhage due to hypothrombinemia. Some adverse effects are associated with particular sulfonamides. Sulfadiazine and sulfasalazine given for long periods to dogs to control chronic hemorrhagic colitis have caused *keratoconjunctivitis sicca*. Crystalluria has been addressed in the past by using combinations of sulfas ("triple sulfa" combinations) due to independent solubility displayed by the individual sulfas.

Diaminopyrimidine–Sulfonamide Combinations

The diaminopyrimidines include trimethoprim, pyrimethamine, and ormetoprim (a diaminobenzylpyrimidine). Trimethoprim is combined with a sulfadiazine or sulfadoxine (veterinary labels) and sulfamethoxazole (human label). Ormetoprim is combined with sulfadimethoxine (veterinary label), and pyrimethamine is combined with sulfadoxine for treatment of malaria in humans. These combinations produce a bactericidal effect against a wide range of bacteria, with some important exceptions, and also inhibit certain other microorganisms. Veterinary preparations contain trimethoprim combined with sulfadiazine or sulfadoxine in the 1:5 ratio.

Antimicrobial Activity

Trimethoprim–sulfonamide combinations have a wide spectrum of activity, usually bactericidal, against many gram-positive and gram-negative aerobic bacteria, including members of the order Enterobacterales. The combination is active against a large proportion of anaerobic bacteria, at least under *in vitro* conditions. *Mycoplasma* and *P. aeruginosa* are resistant. According to the Clinical and Laboratory Standards Institute, trimethoprim-sulfamethoxazole may appear active *in vitro* against the *Enterococci*, but are not effective clinically and should not be reported as susceptible. Also, *in vitro* testing of the potentiated sulfonamides against the anaerobes may appear promising but *in vitro* activity may be limited due to conditions at the site of infection.

Synergism occurs when the microorganisms are sensitive to both drugs in the combination. When bacteria are resistant to sulfonamides, synergism may still be obtained in up to 40% of cases, even when bacteria are only moderately susceptible to trimethoprim. Because of differences between the trimethoprim and sulfonamide in the distribution pattern and processes of elimination, the concentration ratios of the two drugs will differ considerably in tissues and urine from the ratio in the plasma. This variation is not important because the synergistic interaction occurs over a wide range of concentration ratios of the two drugs.

Resistance

Resistance to sulfonamides is due to structural alteration in the dihydrofolic-acid-synthesizing enzyme (dihydropteroate synthetase), whereas resistance to trimethoprim usually results from plasmid-encoded synthesis of a resistant dihydrofolate reductase enzyme. Bacterial resistance to the combination has progressively developed with use of these preparations in animals.

Absorption, Distribution, and Excretion

Trimethoprim is a lipid-soluble organic base that is approximately 60% bound to plasma proteins and 60% ionized in the plasma. This combination of physicochemical properties enables the drug to distribute widely, to penetrate cellular barriers by nonionic diffusion, and to attain effective concentrations in most body fluids and tissues, including brain and CSF. Hepatic metabolism is the principal process for elimination of trimethoprim. The half-life and fraction of the dose that is excreted unchanged in urine vary widely among different species. The drug is well absorbed following oral administration in dogs, cats, and horses or from injection sites in these and other species. In cattle, the elimination half-life changes with the start of rumen function and the associated change in liver metabolism at approximately six weeks of age. The extensive first pass metabolism encountered in functional ruminants results in an apparent half-life for trimethoprim in the 30 minute range.

Adverse Effects

Keratoconjunctivitis sicca in dogs may be irreversible after long-term therapy with sulfasalazine or sulfadiazine, including the trimethoprim/sulfadiazine combination. Other adverse effects reported in dogs include bone marrow depression, hypersensitivity reactions (skin), focal retinitis, fever, vomiting, nonseptic polyarthritis (many of the reported cases are in Dobermans), hepatic necrosis, and

immune-mediated thrombocytopenia. Trimethoprim-sulfamethoxazole has been shown to significantly decrease neutrophil counts by approximately 50% in dogs in the third week of approximately 25–30 mg/kg PO, BID (by about 50%). Neutrophil counts returned to normal approximately one week after discontinuation of therapy. For these reasons, clinicians differ as to whether the potentiated sulfas should be used in dogs.

Some sulfonamides have induced mild thyroid dysfunction in humans and severe dysfunction in rats. It is possible to cause clinical hypothyroidism in dogs with long-term sulfonamide therapy. Trimethoprim-sulfadiazine had no significant effect on T_3 and T_4 in dogs when given at 15 mg/kg PO Q12h for four weeks. However, trimethoprim-sulfamethoxazole at 30 mg/kg PO Q12H for six weeks in dogs significantly decreased T_3 and T_4. The condition will resolve slowly (1–12 weeks) with cessation of the drug.

Inhibition of Protein Synthesis

Examples of drugs that inhibit protein synthesis are tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline, tigecycline); aminoglycosides (amikacin, gentamicin, kanamycin, neomycin, streptomycin, tobramycin, and others); aminocyclitols (spectinomycin); chloramphenicol; lincosamides (clindamycin, lincomycin); and macrolides (azithromycin, clarithromycin, erythromycin, tylosin, tiamulin, and others). Because of the marked differences in ribosomal structure, composition, and function between prokaryotic and eukaryotic cells, many important antibacterial drugs selectively inhibit bacterial protein synthesis. Antibiotics affecting protein synthesis can be divided into those affecting the 30S ribosome (tetracyclines, aminoglycosides, aminocyclitols) and those affecting the 50S ribosome (chloramphenicol, macrolides, lincosamides).

Tetracyclines

The tetracyclines have been characterized by generations to recognize significant advances in spectrum. The founding compound of the first generation of tetracyclines was chlortetracycline, the *Streptomyces* strain producing chlortetracycline having been isolated from a soil sample submitted from a dormant timothy hay field on the University of Missouri campus in 1943. Oxytetracycline soon followed, and then one of the first of many semisynthetic antibiotics, tetracycline in 1954, produced by removing the C5 chlorine group from chlortetracycline.

The first member of the second-generation tetracyclines was methacycline, with the more commonly recognized doxycycline and minocycline being developed in 1967 and 1971, respectively. The first third-generation (both semisynthetic and full synthetic compounds) tetracycline was tigecycline (a glycylcycline), approved by the Food and Drug Administration (FDA) for human use in 2006. Other third-generation human labels to follow in the United States were omadacycline (an aminomethylcycline) and eravacycline (a fluorocycline), both approved for human indications in 2018.

Antimicrobial Activity

Tetracyclines interfere with protein synthesis by inhibiting the binding of aminoacyl tRNA to the recognition site. The various tetracyclines have similar antimicrobial activity but differ in pharmacological characteristics. Efficacy is considered to be best correlated with the AUC/MIC ratio, utilizing a target ratio of 25–40.

Tetracyclines are active against gram-positive and gram-negative bacteria, including rickettsiae and chlamydiae, some mycoplasmas, and protozoa such as *Theileria*. Tetracyclines have good activity against many gram-positive bacteria, the more fastidious nonenteric bacteria such as *Actinobacillus*, *Bordetella*, *Brucella*, *Haemophilus*, some *Pasteurella*, and many anaerobic bacteria, but their activity against these bacteria and against members of the order Enterobacterales is limited by acquired resistance. *Pseudomonas aeruginosa* is considered intrinsically resistant, except in urinary tract infections, where, because of their high concentrations, tetracyclines may be drugs of choice and effective.

Second-generation tetracyclines as a group have increased potency and initially had less acquired resistance than did the first generation. The third-generation tetracyclines share the characteristic of being less susceptible to efflux pumps and ribosomal protection proteins, although still susceptible to enzymatic degradation in some cases.

Absorption, Distribution, and Excretion

Oxytetracycline is well absorbed after intramuscular or subcutaneous administration, but absorption of chlortetracycline, oxytetracycline, or tetracycline after oral administration in food animals is much less, typically less than 20% bioavailability. Antimicrobial susceptibility testing for the tetracyclines used in food animals is based on systemic administration, and the results will overestimate the efficacy potential of these products when orally administered.

Doxycycline has a higher bioavailability of 20–40% in some food animals and in the 50–60% range for dogs. Minocycline has a reported oral bioavailability of 50–60% in dogs and cats. The oral bioavailability of chlortetracycline, oxytetracycline, and tetracycline is adversely affected by the presence of food in the stomach (over 60% decrease in bioavailability for tetracycline), but this effect is minimal for doxycycline.

The tetracyclines distribute well to most tissues with volumes of distribution in the range of 0.8–1.0 l/kg. Distribution to the CNS is variable among the tetracyclines, with minocycline expected to have superior concentrations due to extensive lipid solubility.

Tetracycline, chlortetracycline, and oxytetracycline are excreted primarily in the urine as the unchanged, original compound. They also exhibit extensive enterohepatic recirculation, which has the effect of prolonging their apparent elimination half-times. Bile concentrations may reach 20 times that of plasma.

Doxycycline and minocycline are more lipophilic than the other tetracyclines. Minocycline is thought to undergo significant metabolism. Doxycycline is primarily eliminated through the bile and diffusion into the intestinal tract. The renal component of doxycycline elimination is minimal, making it the tetracycline of choice for patients with renal impairment. Doxycycline is approximately 90% and 99% protein bound in dogs and cats, respectively, both of which will limit distribution to tissues by decreasing the diffusion gradient based on free drug in the plasma. Doxycycline concentrations in the prostate are approximately 15% that of plasma.

Resistance

Widespread resistance to the tetracyclines has considerably reduced their usefulness, which is largely limited to intracellular bacterial pathogens of different types. Such resistance is high level and usually plasmid-, transposon-, and conjugative transposon-mediated. Forty-six tetracycline resistance genes have been identified: 30 encode for energy-dependent efflux mechanisms, 12 for ribosomal protection proteins, 3 for enzymatic degradation, and 1 encodes for resistance in which the mechanism is unknown. Resistance is typically complete across all first-generation tetracyclines, variable for second-generation tetracyclines according to each mechanism, and rarely affects third-generation tetracyclines at this time. Ribosomal protection protein mosaic genes have been identified that combine portions of two or three different previously identified genes into one functional gene.

Additional Effects beyond Antibacterial Activity

High-dose intravenous oxytetracycline has been advocated for use in flexor tendon contraction deformities in neonatal foals. *In vitro* work has shown that collagen gel contraction by equine myofibroblasts was inhibited by oxytetracycline *in vitro*. The evidence for this effect in calves is not substantial. The well-demonstrated anti-inflammatory effects of the tetracyclines have been demonstrated to be mediated by suppression of neutrophil chemotaxis, inhibition of T-lymphocyte activation and phospholipase A2, inhibition of mitogen-induced human lymphocytic proliferation, and inhibition of metalloproteinases.

Adverse Effects

Tetracyclines are generally safe antibiotics with a reasonably high therapeutic index. The main adverse effects are associated with their severely irritant nature, with disturbances in gastrointestinal flora, with their ability to bind calcium (cardiovascular effects, teeth or bone deposition), and with the toxic effects of degradation products on liver and kidney cells. In horses, there is the potential to produce broad-spectrum suppression of the normal intestinal flora and fatal superinfection with *Salmonella* spp. or *Clostridioides difficile*. However, intravenous oxytetracycline is still used in horses due to low cost. Doxycycline is used in horses via the oral route, but intravenous administration has been reported to result in cardiovascular toxicity.

Phenicols: Chloramphenicol and Florfenicol

Chloramphenicol was the original member of this class but has been prohibited from use in food animals due to potential aplastic anemia when used for therapy in humans. Thiamphenicol was synthesized from chloramphenicol by substituting the nitro group of chloramphenicol with a sulfonylmethyl group. This compound does not cause aplastic anemia in humans. Florfenicol is a fluorinated derivative of thiamphenicol; it has approvals for use in food animals. They are stable, lipid-soluble, neutral compounds.

Antimicrobial Activity

Chloramphenicol and florfenicol are active against gram-positive and gram-negative bacteria, including chlamydiae and rickettsiae, and some mycoplasmas. They act by binding to the 50S subunit of bacterial ribosomes. Amino acids are not able to transfer to growing polypeptides due to prevention of the binding of aminoacyl tRNA to the ribosome. This binding is reversible. *P. aeruginosa* is considered intrinsically resistant to the phenicols by the Clinical and Laboratory Standards Institute.

These drugs have been considered bacteriostatic for the majority of pathogens except at extremely high concentrations or for extremely susceptible isolates, where they may be bactericidal. Florfenicol displays bactericidal activity against *M. haemolytica* and *Pasteurella multocida*. The pharmacodynamic target of AUC/MIC is commonly used for this group.

Most gram-positive and many gram-negative pathogenic aerobic and anaerobic bacteria are susceptible, though

resistance is increasing in members of the order Enterobacterales. Florfenicol is considered to be more active than chloramphenicol against many pathogens.

Resistance

Most resistance to chloramphenicol is the result of plasmid-encoded acetylase enzymes (CATS), consisting of two genetically unrelated families. There is also a mechanism of membrane-associated transporters that export the phenicols from the bacterial cell. The genetic determinants for these resistance mechanisms have been demonstrated to spread by integrative and conjugative genetic elements as well as plasmids, gene cassettes, and transposons. In addition to these mechanisms, multidrug transporters may also export the phenicols, as well as mutations that result in target site alterations and reduction in expression of outer membrane proteins.

Absorption, Distribution, and Excretion

In dogs, cats, and preruminants, chloramphenicol is well absorbed from the intestine; in ruminants, the drug is inactivated after oral administration. Because of its low molecular weight, lipid solubility, and modest plasma protein binding, the drug is well distributed in most tissues and fluids, including the CSF and aqueous humor.

The half-life of chloramphenicol varies widely in animals from a low of one hour in horses to five or six hours in cats. In neonates, the half-life is considerably longer; the glucuronyl transferase activity in foals appears to reach adult levels in one week. The drug is mainly eliminated by glucuronide conjugation in the liver.

Adverse Effects

The fatal aplastic anemia seen in 1 in 25 000–40 000 humans treated with chloramphenicol does not occur in animals, although prolonged high dosing may cause reversible abnormalities in bone marrow activity. The potential for non-dose-related fatal aplastic anemia in humans has led to prohibition of chloramphenicol for use in food animals in most countries because of fear of the presence of drug residues in meat products. Florfenicol does not have this effect and so is used in food animals.

Chloramphenicol reduces the activity of glucuronide conjugation and oxidative pathways in the liver. It may dramatically prolong the activity of barbiturates. The classic example is administration of chloramphenicol to a dog receiving phenobarbital for control of seizures. The dog will lapse into a phenobarbital-induced comatose state until the drug can be eliminated. Also, chloramphenicol may affect the metabolism of propofol, benzodiazepines, and methadone. Florfenicol has the potential to cause serious gastrointestinal disturbances in horses.

Concerns of chloramphenicol-related immune suppression have been published in the literature, but subsequent trials have demonstrated no effect on the ability of animals (dogs in one study) to respond to vaccinations while undergoing chloramphenicol therapy. Chloramphenicol was demonstrated to have no effect on response to distemper vaccine in beagle pups; this was confirmed by disease challenge. Studies have also been done related to florfenicol and vaccination. A study was done where cattle were vaccinated with a modified-live infectious bovine rhinotracheitis (IBR) vaccine: half were treated with florfenicol, and then all were challenged with IBR. No effect of florfenicol on the immune response was demonstrated. In trout, florfenicol administration at the time of vaccination for *Aeromonas salmonicida* and *Vibrio anguillarum* had no effect on antibody production or circulating leukocyte levels.

Aminoglycosides

The aminoglycoside antibiotics are polar organic bases. Their polarity largely accounts for the similar pharmacokinetic properties that are shared by all members of the group. Chemically, they consist of a hexose nucleus to which amino sugars are attached by glycosidic linkages. All are potentially ototoxic and nephrotoxic. The newer aminoglycosides are more resistant to plasmid-mediated enzymatic degradation and are less toxic than the older compounds. The general order in relation to potency, spectrum of activity, and stability to plasmid mediated resistance is amikacin > tobramycin > gentamicin > neomycin = kanamycin > streptomycin. This activity mirrors the chronology of introduction of the drugs, with streptomycin being the oldest of the aminoglycosides (1944).

A next-generation aminoglycoside, plazomicin, has been recently approved for human use in complicated urinary tract infections and nephritis in the United States (2018).

Antimicrobial Activity

The aminoglycosides are bactericidal. The mode of action of streptomycin is best understood. Streptomycin has a variety of complex effects in the bacterial cell: (i) it binds to a specific receptor protein in the 30S ribosomal subunit, distorting the codon–anticodon interactions at the recognition site and causing misreading of the genetic code so that faulty proteins are produced; (ii) it binds to "initiating" ribosomes to prevent the formation of 70S ribosomes; and (iii) it inhibits the elongation reaction of protein synthesis. The other aminoglycosides act similarly to streptomycin in causing mistranslation of the genetic code and in irreversible inhibition of initiation, although the extent and type

often differ. They have multiple binding sites on the ribosome, whereas streptomycin has only one, and can also inhibit the translocation step in protein synthesis.

Aminoglycosides are particularly active against gram-negative bacteria as well as against mycobacteria and some *Mycoplasma*. Anaerobic bacteria are usually resistant. As a general rule, gram-positive bacteria are resistant to older aminoglycosides (streptomycin, neomycin) but may be inhibited by newer drugs (gentamicin, amikacin). All anaerobes and *E. faecium* and *faecalis* are considered intrinsically resistant by the Clinical and Laboratory Standards Institute.

A particularly useful property is the activity of newer aminoglycosides against *Pseudomonas aeruginosa*. Their bactericidal action on aerobic gram-negative bacilli is markedly influenced by pH; they are most active in an alkaline environment. Increased local acidity secondary to tissue damage may account for the failure of an aminoglycoside to kill usually susceptible microorganisms at infection sites or in abscess cavities. Combinations of aminoglycosides with penicillins are often synergistic; the concurrent administration of the newer β-lactam antibiotics with gentamicin or tobramycin has been used to treat serious gram-negative infections, for example, those caused by *P. aeruginosa*.

Resistance

Resistance among the aminoglycoside group is marked by less routine cross-resistance across the entire group as compared to other antibiotic groups; mechanisms frequently differ between aminoglycoside molecules and bacterial species. This should not be interpreted as indicating that cross-resistance does not happen, but rather that cross-resistance must be understood and interpreted for each resistance mechanism. There are a wide variety of mechanisms. Bacteria may decrease intracellular concentrations by either reduced drug uptake or by active efflux. Five families of efflux systems have been identified. *P. aeruginosa* can display adaptive resistance by overproducing a characteristic efflux system.

There are three major classes of aminoglycoside-modifying enzymes that have been identified. As discussed under fluoroquinolones, one of particular concern is AAC(6′)-Ib-cr, a plasmid-associated aminoglycoside acetyltransferase that inactivates both aminoglycosides (amikacin, tobramycin, kanamycin) and fluoroquinolones (e.g. ciprofloxacin and norfloxacin).

As of this writing, 11 different target modifying genes have been identified that encode methylation of the ribosomal target, resulting in high-level resistance. These genes may be found on mobile genetic elements that also carry genes for resistance to other antibiotic groups such as the fluroquinolones, extended-spectrum β-lactams, and the carbapenems.

Absorption, Distribution, and Excretion

Aminoglycosides are poorly absorbed from the gastrointestinal tract, bind to a low extent to plasma proteins, and have limited capacity to enter cells and to penetrate cellular barriers. They do not readily achieve therapeutic concentrations in transcellular fluids, particularly cerebrospinal and ocular fluid. Poor diffusibility is because of their low degree of lipid solubility. Their apparent volumes of distribution are relatively small, and their half-lives are short (two hours) in domestic animals. Even though these drugs have a small volume of distribution, they exhibit selective binding to the renal cortex and cochlear and vestibular tissues of the ear due to high concentration of phospholipids in their cellular matrix; the anionic nature of these phospholipids attract the cationic aminoglycosides.

Elimination takes place entirely by renal excretion (glomerular filtration), and unchanged drug is rapidly excreted in urine. Impaired renal function decreases their rate of excretion and makes adjustment of the maintenance dosage necessary to prevent accumulation with attendant toxicity. In humans, the half-lives have increased to as much as 24 hours in patients with end-stage renal failure.

Aminoglycosides are dosed once daily in veterinary medicine if given by the parenteral route. Efficacy depends on the peak concentration in relation to the target pathogen MIC, and toxicity is minimized by allowing the plasma concentration to fall below a critical threshold prior to the next dose (reported as 2 μg/ml for gentamicin and 6 μg/ml for amikacin in humans). In veterinary medicine, this is most often achieved by an intravenous bolus, whereas in human medicine the dose may be administered over a matter of minutes (e.g. 30 minutes) through an infusion pump. Oral administration through the water, for example, in swine, does not typically create concerns about toxicity due to the poor oral bioavailability of the aminoglycosides.

Adverse Effects

All aminoglycosides can cause varying degrees of ototoxicity and nephrotoxicity. The tendency to produce vestibular or cochlear damage varies with the drug: neomycin, amikacin, and kanamycin are most often associated with cochlear damage (hearing) and gentamicin, streptomycin, and tobramycin are most often associated with vestibular damage (balance). Cats are very susceptible to the vestibular effects of the aminoglycosides, whereas dogs tend to present with initial auditory symptoms.

Nephrotoxicity (acute tubular necrosis) occurs in association with prolonged therapy and excessive trough concentrations of the aminoglycoside (particularly gentamicin) in plasma. The aminoglycosides can also produce neuromuscular blockage of the nondepolarizing type, which causes flaccid paralysis and apnea. This is most likely to occur in

association with anesthesia, and has occurred when gentamicin was included in a saline flush of the abdomen and directly exposed to the diaphragm.

The nature of binding to renal tissues creates an extended withdrawal time requirement when used in an extralabel manner in food animals where no residue tolerance or MRL and no withdrawal time have been established. For example, in the United States, current testing protocols are capable of detecting residues in the kidneys of cattle (any of which would be violative due to no established tolerance in cattle) up to 18 months post administration. As tissue testing methods continue to evolve, it is likely that this time period will be extended even more.

Aminocyclitols

Spectinomycin is an aminocyclitol antibiotic with a spectrum of activity and mechanism of action similar to that of kanamycin but without the toxic effects of the aminoglycosides. It is normally bacteriostatic and is not particularly active on a weight basis. Its activity against gram-negative bacteria is unpredictable because of naturally resistant strains. Chromosomal resistance develops readily, but it does not cross-react with aminoglycosides. Plasmid resistance is uncommon but often extends to streptomycin. The drug has most of the pharmacokinetic properties of aminoglycosides but appears to penetrate CSF better. It has been used in agricultural practice to treat salmonellosis and *Mycoplasma* infections.

Macrolides

The term *macrolide* means "large lactone"; all macrolides are considered to be macrocyclic lactones. The macrolides most commonly used consist of 14-, 15-, or 16-member ring structures. The original macrolide, first approved for human use in 1952, is the 14-member ring compound erythromycin. Fourteen-member ring semisynthetic compounds include roxithromycin, clarithromycin, dirithromycin, the ketolide telithromycin, and the fluoroketolide solithromycin. Telithromycin was withdrawn from the market in the United States due to an adverse event profile that included liver failure and cases of myasthenia gravis, linked to a pyridine moiety unique to telithromycin that acted as a cholinergic receptor antagonist.

Fifteen-member ring macrolides include the semisynthetic derivatives of erythromycin, the azalides, including azithromycin and gamithromycin. The semisynthetic triamilides are thus called because they contain three polar amine groups, hence are "tri-basic." Tulathromycin is a triamilide that is widely used in cattle and swine. Gamithromycin is labeled for cattle in the United States and other countries. Azithromycin is a staple of macrolide therapy in human medicine, and is also used in an extralabel manner in companion animals.

The founding member of the 16-member ring macrolides is tylosin, which is widely used in veterinary medicine. Tylosin is composed of four separate compounds, of which tylosin A is usually 80–90% of the mix. Tylosin was patented in the United States in 1960. Human-labeled 16-member ring macrolides include spiramycin, josamycin, midecamycin, and rikamycin. Veterinary-labeled compounds include tildipirosin, tylvalosin, and tilmicosin.

Fidaxomicin (tiacumin b) is a naturally occurring 18-membered macrocyclic lactone ring product that is often referred to as a macrolide, but it is not related to erythromycin and has a different mechanism of action: it prohibits the initial separation of the DNA strands by inhibiting RNA polymerase. It has a very narrow spectrum of activity focused on *Clostridioides difficile*.

Macrolide antibiotics have action and pharmacokinetic properties similar to the lincosamides. Like the lincosamides, they are lipid-soluble, basic drugs that are concentrated in tissue compared to serum and penetrate cells well.

Antimicrobial Activity

Macrolide antibiotics are typically considered bacteriostatic, with activity particularly against gram-positive bacteria and *Mycoplasma*, although significant activity is present against the Pasteurellaceae involved in swine and bovine respiratory disease. Some compounds, such as azithromycin, display notable gram-negative aerobic activity. Azithromycin also displays bactericidal activity against some pathogens. The pharmacodynamic parameter most closely associated with efficacy is AUC/MIC. This ratio is quite low compared to other drugs because the macrolides often do not exceed the pathogen MIC in the plasma, yet clinical efficacy is demonstrated due to their extensive distribution characteristics. AUC/MIC ratios as low as 5–10 for the entire AUC have been correlated with efficacy for macrolides in the treatment of bovine respiratory disease.

The macrolides bind to the 50S ribosome in competition with chloramphenicol and inhibit the translocation step of protein synthesis. Rather than nonspecific inhibitors of protein synthesis, the macrolides have been shown to function as specific modulators of translation; many bacterial proteins are still synthesized depending on the specific macrolide. This translation modulation occurs in the nascent peptide exit tunnel (NPET) by interaction with macrolide-arrest motifs (MAMs) in the forming protein. It is these MAMs that govern the interaction of a specific macrolide with a specific protein, by blocking the peptide transferase center. In addition to arresting ribosomal translation events, the macrolides have also demonstrated the

ability to induce translation errors and stimulate ribosomal frameshifting.

Bactericidal activity is thought to be related to a longer period of binding to the ribosome, with longer binding times associated with depletion of components of the gene expression pathway such that synthesis will not resume once binding has ceased.

Most macrolides used in veterinary medicine include gram-positive bacteria, *Mycoplasma* spp., and some gram-negative fastidious bacteria in their spectrums. Tulathromycin, gamithromycin, tildipirosin, and tilmicosin are used extensively in food animal medicine due to their activity against *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma* spp. (tilmicosin is the exception for *Mycoplasma* spp. with very limited activity against *Mycoplasma bovis*). Erythromycin has an antibacterial spectrum similar to penicillin G, but it includes activity against penicillinase-producing coagulase-positive staphylococci, *Campylobacter*, *Leptospira*, *Bordetella*, rickettsiae, chlamydiae, some mycoplasmas, and atypical mycobacteria. It may be bactericidal at high concentrations. Tylosin and spiramycin are less active than erythromycin against bacteria but more active against a broad range of *Mycoplasma*. Azithromycin and clarithromycin are particularly active against nontuberculous mycobacteria. The activity of these two drugs against a variety of intracellular bacteria depends not only on their intrinsic activity but also on the often remarkable concentration of the drugs in cells, including macrophages. Azithromycin may concentrate 200–500-fold inside macrophages compared to its serum concentrations.

Generalization of macrolide spectrums may mislead the clinician, and the specific spectrum characteristics of each macrolide must be considered. Azithromycin and clarithromycin are examples where a wider array of pathogens may be included in the spectrum. The Clinical and Laboratory Standards Institute (CLSI) reports that the order Enterobacterales is intrinsically resistant to the macrolides, with the exception of *Salmonella* spp. and *Shigella* spp. for azithromycin.

Additional Effects beyond Antibacterial Activity

Some macrolides are noted for their anti-inflammatory effects. The anti-inflammatory and immunomodulating effects of the macrolides have been extensively reviewed, including inhibiting cytokine production, inducing regulatory functions for macrophages, inhibiting neutrophil influx, and altering cell phagocytosis. Erythromycin, azithromycin, clarithromycin, and roxithromycin inhibit mucus secretion in some human inflammatory pulmonary diseases by inhibiting neutrophil infiltration into the airways, thought to be due to inhibition of cytokines such as interleukins and leukotrienes.

Some 14- and 16-member ring macrolides have demonstrated intestinal prokinetic activity through stimulation activity on motilin receptors. Erythromycin has demonstrated this effect in horses, as well as erythromycin, tylosin, and tilmicosin in cattle.

Resistance

Three mechanisms of resistance are described for the macrolides: ribosome target modification by methylation and mutation of ribosomal proteins, decreased intracellular concentrations due to efflux pumps, and enzyme modification of the macrolide molecule. The latter mechanism is considered least important. The erm methyltransferase genes confer the "MLSb" resistance phenotype, which displays cross-resistance between macrolides, lincosamides, and the group b component of streptogramins.

Target site alteration is constitutive or inducible; alteration of the ribosomal binding site by erm methyltransferases may be chromosomally or plasmid mediated. The genes for efflux pumps are transmissible by transposons. Macrolide deactivating enzyme genes are associated with plasmids.

Absorption, Distribution, and Excretion

Erythromycin stearate and estolate are well absorbed after oral administration, but the base is not. Intramuscular injection of erythromycin is very irritating. The absorption of tylosin from the intestine varies with the formulation. These drugs are well distributed through body tissues and fluids, except the CSF. Tissue concentrations often exceed serum concentrations, notably for azithromycin and clarithromycin, which are, therefore, often dosed once a day or less frequently. In the case of spiramycin, such tissue concentration is extreme and is associated with tissue binding. The clinical importance of these tissue concentrations is uncertain, and these concentrations should not be compared to MICs for pathogens.

Excretion is primarily through the bile, with only minimal renal contributions (3–5% for erythromycin). Apparent elimination half-times for some of the veterinary macrolides may be quite long. Observed plasma $T_{1/2}$ values for tulathromycin and tildipirosin in cattle after subcutaneous injection are 92 and 210 hours, respectively. Even intravenous administration of tulathromycin in cattle results in a mean $T_{1/2}$ of 65 hours.

Adverse Effects

Macrolides are generally safe drugs, though painful on injection. Their potential for causing irreversible diarrhea in adult horses means that they should be avoided in this

species. The drugs should not be given orally to non-ruminant herbivores because of their potential for disturbing the rumen flora. Tilmicosin is administered orally to swine but may be fatal if administered parenterally. Tilmicosin can cause fatal cardiac effects in humans through the mechanism of calcium channel blockade; safe injection practices are an absolute requirement for the use of this drug in cattle.

Lincosamides

Lincomycin, pirlimycin, and clindamycin are all used in veterinary medicine. The drugs bind the 50S ribosomal subunits at binding sites that overlap with those of chloramphenicol and the macrolides. They inhibit the peptidyl transferase reaction. Lincomycin is less active on a weight basis than clindamycin. Lincosamides are active against gram-positive aerobic and all anaerobic bacteria, and against mycoplasmas, but most gram-negative aerobes are resistant. Clindamycin is more active than lincomycin against anaerobes and may be bactericidal.

Chromosomal stepwise resistance may develop, and plasmid-mediated resistance is common. Cross-resistance between lincosamides is complete and commonly occurs also with macrolides. Lincomycin is readily absorbed after oral or intramuscular administration. Food delays and reduces absorption. The absorption of different clindamycin compounds is variable. The lincosamides are widely distributed in body tissues and fluids, including the prostate and milk, but CSF concentrations are low. They penetrate intracellularly because of their lipophilic properties. Most excretion is through the liver. The major adverse effect of lincosamides is their ability to cause fatal *C. difficile* typhlocolitis in horses, rabbits, guinea pigs, and hamsters. In rabbits, fatal diarrhea may also result from proliferation of *C. spiroforme*. Oral lincosamides at low concentrations produce severe ruminal disturbances in adult ruminants.

Pleuromutilins (Diterpenes)

The semisynthetic plueromutilins include tiamulin used in veterinary medicine, retapamulin used topically in humans, and the first pleuromutilin to be used systemically in humans, lefamulin. As for the macrolides and lincosamides, the pleuromutilins also bind to the 50S ribosomal subunit where they inhibit protein synthesis, and are generally considered bacteriostatic.

The spectrum of tiamulin is similar to the macrolides, with the exception of improved gram-negative anaerobic and *Mycoplasma* spp. activity. The spectrum also includes *Actinomyces pleuropneumoniae* and *Brachyspira hyodysenteriae*. Activity against gram-negative aerobes is considered limited.

Tiamulin exhibits approximately 85% oral bioavailability in swine. Approximately 75% is excreted in the feces after hepatic metabolism and biliary excretion.

Tiamulin should not be fed to non-ruminant herbivores due to significant disruptions in enteric flora.

Antimicrobial Susceptibility Testing

The use of antimicrobial drugs to successfully treat infections depends on the interaction between the antimicrobial and the pathogen at the site of infection. There are multiple nuances to this interaction, for example bound versus free drug concentrations, the effect of the size of the pathogen inoculum, pH effects, oxygen status, and the growth state of the pathogen. It is not possible to accurately predict and mimic all these infection site characteristics in the laboratory; instead, antimicrobial susceptibility testing (AST) is designed to evaluate the interaction between the pathogen (or suspected pathogen) and the antimicrobial under standardized, repeatable conditions. These *in vitro* results are then related to whether the antimicrobial will likely affect the disease outcome through a regimented set of evaluations. It is not as simple as measuring the most convenient concentration of the antimicrobial *in vivo* and comparing it to the *in vitro* MIC of the pathogen.

There are two primary groups that establish interpretive criteria and testing standards for AST of human isolates, the Clinical and Laboratory Standards Institute (CLSI) in the United States and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). In veterinary medicine, the predominant source of breakpoints is the CLSI Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee. This chapter refers to the methods for creating standardized veterinary testing methods and interpretive criteria as established by the CLSI.

For the student of susceptibility testing, there are several key CLSI documents. CLSI VET01 provides performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, including the most current updated tables for performance standards for antimicrobial disk and dilution susceptibility testing for bacteria isolated from animals. CLSI VET01S is a supplement to VET01 that provides the latest updated tables from VET01S with all CLSI-approved interpretive criteria categorized by pathogen, antimicrobial, disease (in some cases), and animal species; it may be accessed for free on the CLSI website. CLSI VET02 describes the development of quality control ranges, breakpoints, and interpretive categories for antimicrobial agents used in veterinary medicine. CLSI

VET09 is an educational document entitled "Understanding Susceptibility Data as a Component of Antimicrobial Stewardship in Veterinary Settings," written specifically to support veterinarians in utilizing AST results.

The first step in developing CLSI-approved interpretive criteria is to establish quality control (QC) ranges for both broth dilution and disk diffusion testing. QC testing is established by testing well-characterized isolates, such as those from the American Type Culture Collection (ATCC) in multiple laboratories with multiple lots of media in order to define the limits of normal testing variation for an antimicrobial agent. Laboratories may then use these organisms and ranges in the future to establish whether their AST procedures are in control for that drug and organism. These QC ranges are established using the standardized test procedures (incubation conditions, incubation atmosphere, pathogen inoculum, etc.) that will be used in laboratories for AST of clinical isolates.

To establish interpretive criteria, the initial work is done with broth dilution testing. There are three components to consider: wild type isolate distribution (wild type cutoff, COwt), pharmacokinetic/pharmacodynamic (PK/PD) analysis (pharmacodynamic cutoff, COpd), and correlation of MIC values with clinical outcomes demonstrated in clinical trials where isolates of pathogens were subjected to AST and then correlated to clinical outcomes (clinical cutoff, COcl).

Wild type isolate distribution consists of collecting a minimum of 100 isolates of the pathogen(s) and subjecting each isolate to both broth dilution and disk diffusion AST. The broth dilution testing consists of establishing the MIC, which is the lowest concentration at which bacterial growth is inhibited over 18–24 hours. Wild type isolates, those that likely lack acquired or mutational resistance mechanisms, are typically normally distributed with closely related (and low) MIC values. If there is a second population (with higher MIC values) separated from the first, this second population likely contains resistance mechanisms and is termed the "non-wild type" population.

The MIC values selected to classify an isolate as susceptible, intermediate, or resistant are referred to as breakpoints. For this portion of interpretive criteria development, the susceptible breakpoint may be set at the top of the MIC dilution range for the wild type population, the resistant breakpoint may be set at the bottom of the MIC dilution range for the non-wild type population, and the intermediate breakpoint as a single MIC or MIC range between the two populations. Setting the susceptible breakpoint through this process is essentially the same as developing an epidemiological cutoff (ECOFF).

PK/PD analysis involves first establishing the non-protein bound (free) drug pharmacokinetic values in the target animal species for the regimen being considered. These values are then evaluated in association with the correct pharmacodynamic target for the antimicrobial. Possible PD targets include the time for which the plasma concentration remains above the pathogen MIC (T > MIC), the ratio of the peak plasma concentration to the organism MIC (Cmax/MIC), and the area under the plasma concentration curve divided by the pathogen MIC (AUC/MIC). These PK/PD targets have been developed by studying these relationships with *in vitro* and *in vivo* studies. The susceptible breakpoint set by this method is the highest MIC that meets the PK/PD criteria for that antimicrobial and pathogen.

The third component of setting interpretive criteria involves correlating the MIC values of pathogens isolated from clinical trials with clinical success or failure. In the author's experience, this is seldom a clear, definitive separation in populations, but the overall distributions help to inform the final selection of the interpretive criteria.

The final selection of interpretive criteria involves evaluating all three components and their respective breakpoint values. CLSI VET02 has a flowchart to prioritize the relative weight of each method in consideration of the individual method values. Once the broth dilution breakpoints have been set, the disk diffusion zone diameter data are correlated to the broth dilution breakpoints through the error rate bounding process.

The process described above is typical for sponsor-supported breakpoints, where a drug sponsor brings their drug development data to the CLSI VAST. However, there are multiple breakpoints developed by the CLSI VAST that are not supported by sponsor-generated data. These "generic breakpoints" are typically based on wild type isolate distributions and PK/PD modeling based on plasma concentrations, lacking the component of directly correlating pathogen MIC values with clinical outcome. The regimens used for the PK/PD component are listed in the tables in VET01S.

It is not possible to incorporate into AST all possible components that affect therapeutic outcome. However, AST is still able to provide reasonable guidance to the clinician in many cases. To effectively use AST results as a clinician, there are some general considerations of AST that are appropriate to review:

- The concentrations used in AST are not intended to replicate the drug–pathogen interaction at the site of infection. AST is based on *in vitro* methods that are designed to be highly repeatable across labs and that have defined QC procedures. Comparing CLSI interpretive criteria to concentrations such as tissue homogenates is a process without clinical confirmation of the relationship that is quite likely to lead to erroneous conclusions.
- The interaction between the pathogen and the antimicrobial is only part of the determination of the clinical

outcome of the patient. There will be clinical failures with pathogens characterized as susceptible, and clinical successes with pathogens characterized as resistant. To discount AST because of one of these occurrences displays a fundamental misunderstanding of the AST process and of the interaction between antimicrobials and infectious disease.

- Breakpoints are developed for a specific animal species, disease (for sponsor supported breakpoints), pathogen(s), and antimicrobial regimen (dose, route, duration, frequency). When any of these factors are changed, the breakpoint may not be correlated with the ability of the antimicrobial to make a difference in the disease being evaluated. An antimicrobial does not have a breakpoint in and of itself; an antimicrobial in combination with a specific animal species, disease, pathogen, and regimen has a breakpoint. For example, applying the bovine respiratory disease breakpoint for an antimicrobial in relation to *M. haemolytica* to AST of an *E. coli* from enteric disease is quite likely to give misleading guidance in antimicrobial selection. The diagnostician or clinician is responsible for knowing when the AST breakpoints being used are directly applicable to a specific animal species–antimicrobial regimen–disease–pathogen combination, and when the breakpoints are developed for another situation and must be interpreted with caution.
- AST will not contribute to clinical decision-making if the isolate being tested is not representative of the pathogen population causing the disease.

Factors Considered in the Clinical Use of Antimicrobials

Pharmacokinetics

The pharmacokinetic properties of an antimicrobial are the foundation for regimen design. The abbreviation ADME stands for absorption, distribution, metabolism, and excretion, each component ruling in and ruling out selected antimicrobials for specific applications.

Absorption from administration routes other than intravenous are characterized by bioavailability, which is determined by comparing the area under the plasma concentration curve (AUC) for intravenous administration to the other route under consideration for a designated period post administration. The oral bioavailability of the aminoglycosides in adult animals is typically 5% or less, relegating oral administration to enteric diseases only. In contrast, fluoroquinolones have a near-100% oral bioavailability in some species.

Bioavailability from intramuscular and subcutaneous administration may be influenced by the chosen injection site, as demonstrated in cattle for oxytetracycline. The salt of the antimicrobial molecule also influences the shape of the plasma concentration curve. Maximum drug exposure is indicted by the maximum plasma concentration (C_{max}), the time of maximum exposure by the time of maximum concentration (T_{max}), and the extent of overall exposure by the AUC. The rate of absorption of penicillin G has been altered by moving from the rapidly absorbed Na and K salts to the more slowly absorbed procaine salt, and finally to the benzathine bridge between two penicillin G molecules. The latter two molecular forms result in "flip-flop" pharmacokinetics, where the absorption of the compound is slow and prolonged due to the need to disassociate prior to absorption, causing the rate of release from the injection site to determine the shape of the plasma elimination curve rather than the speed of clearance of the antimicrobial from the plasma. The rate and extent of absorption from the gastrointestinal tract may also be affected by modifying the dosage form of the antimicrobial; for example, the pressure at which sulfonamide boluses are compressed has a dramatic effect on absorption when administered to a ruminant animal. Topical administration may also be possible, for example, in intramammary administration, external ear infections, dermal infections, and intrauterine administration. In these cases, much higher local concentrations of the antimicrobial may be achieved with less systemic exposure of the animal to the antimicrobial.

Distribution of antimicrobials from the central plasma compartment is measured by the volume of distribution (VD). Although there are multiple methods of calculating the VD, they are all based on the same concept: if the drug is not being accounted for in the plasma at steady state, then it must be outside the plasma in perivascular fluid or other tissue compartments. The aminoglycosides and B-lactams are examples of polar, water-soluble antimicrobials whose distribution is limited to body water, resulting in low volumes of distribution and very limited penetration into sites such as the eye, central nervous system, and the prostate. In contrast, macrolides, fluoroquinolones, and the tetracyclines are lipid-soluble compounds with much higher volumes of distribution; these antimicrobials are said to concentrate in tissues, but these concentrations are not described as to the proportion that is available to interact with pathogens, and although whole tissue homogenate concentrations have been compared directly to pathogen MIC values in the past, this would not be acceptable therapeutic reasoning today. High plasma protein binding also serves to limit the distribution of a drug beyond plasma, an example being the highly lipid-soluble doxycycline, which nevertheless has a low apparent VD because it is highly protein bound, and the

bound fraction is not free to distribute to tissues; when the free drug fraction is considered, the VD is much higher.

Metabolism is responsible for much of the active substance in some antimicrobials. In the case of ceftiofur, the parent drug is rapidly metabolized to desfuroylceftiofur, which is largely the same in activity, except for *Staphylococcus* spp., where the MICs are several dilutions higher for the metabolite. In contrast, activity after intramammary administration for this antimicrobial is primarily due to the parent compound. In the case of enrofloxacin, rapid metabolism to ciprofloxacin occurs to a varying extent in veterinary species. Metabolism may also be a primary mechanism for excretion, an example being chloramphenicol, where the primary elimination product is the result of conjugation with glucuronic acid in the liver.

Excretion may be primarily renal or hepatic. In the case of renal excretion, this may be metabolites or active compound. For β-lactams, renal excretion is almost entirely an active compound, which results in very high urine concentrations. Fluroquinolones are also excreted renally as the active compound. The activity of fluoroquinolones may be affected by urine pH because they are zwitterions, with low pH hindering their entry into the bacterial cell, which increases the pathogen MIC by 1–2-fold at a pH of 6 and up to 4-fold at a pH of 5, which would be a rare urine pH value. This effect is likely overcome by the huge fluoroquinolone urine concentration. An interesting situation is the excretion of doxycycline. For other tetracyclines such as tetracycline, oxytetracycline, and chlortetracycline, excretion is primarily through urine as the unchanged compound, also exhibiting significant enterohepatic recirculation. However, for doxycycline excretion is primarily through the bile and diffusion into the intestinal tract, making it the tetracycline of choice for patients with renal impairment.

Protein binding of a drug may have significant effects on the apparent pharmacokinetics. In general, serum protein binding of drugs up to 90% is of little clinical importance. There are examples of highly protein bound antimicrobials where the apparent elimination half-time is dramatically increased. For example, the prolonged duration of exposure of cefovecin in companion animals is due to high protein binding serving as a drug reservoir in the plasma. Sulfa drugs also vary greatly as to their plasma protein binding, resulting in large differences in apparent $T_{1/2}$. Outside the plasma, aminoglycosides and polymyxin bind extensively to intracellular constituents and thus are inactivated by pus.

Pharmacodynamics

The concept of pharmacodynamics was introduced at the beginning of this chapter with specific examples given within the discussions of some antimicrobial groups.

Examples of each pharmacodynamic category are presented below.

C_{max}/MIC – Aminoglycosides
Time > MIC – β-lactams
AUC/MIC – Fluoroquinolones (peak concentrations are also important), tetracyclines, macrolides, lincosamides, florfenicol (although not well established)

We are coming to understand the limitations of applying pharmacodynamic target values for an antibiotic against one pathogen in a specific model (e.g. *S. aureus* in a mouse thigh infection model) to a disparate infection such as *M. haemolytica* in bovine respiratory disease. Another limitation to consider is applying a plasma-concentration-based pharmacodynamic target to concentrations determined in other fluids such as cerebrospinal fluid, pulmonary epithelial lining fluid, nasal secretions, or urine. Clinical confirmation of efficacy is necessary to confirm these extrapolations.

It is important to note that the preferable pharmacodynamic target is suggested for each antimicrobial group based on *in vitro* and *in vivo* studies, and that the line between one or the other target being superior may be quite fine. The AUC may be considered as a measure of total exposure, with C_{max}/MIC indicating that peak concentration is the most important, and T > MIC indicating that duration of exposure is the most important. AUC/MIC suggests that the shape of the plasma exposure curve is not as important. In the case of fluoroquinolones, although total exposure is important for efficacy, peak concentrations and elimination half-time influence selection for first-mutation mutants (see the discussion on the MSW in Fluoroquinolone section), and therefore the shape of the curve does matter for resistance selection.

It is also important to evaluate over what duration the AUC is reported for AUC/MIC targets. Many of the ratios are AUC24, where the AUC was calculated over a 24 hour segment, often with repeated administrations. In the case of single administration antimicrobials, extrapolating the AUC/MIC ratio from a 24-hour segment in a multiple administration study to a longer duration resulting from a single administration, where the shape of the concentration curve might be quite different, should be carefully scrutinized and confirmed with efficacy data. The context of the AUC/MIC target ratio being cited should be fully understood before using that target to evaluate other regimens.

Other pathogen-interaction properties of antimicrobials may play a role in the regimen required for clinical efficacy. The post-antibiotic effect (PAE) is the continued suppression of bacterial growth after a short exposure of a bacterial culture to the antimicrobial, followed by removal. The post-antibiotic sub-MIC effect (PA SME) is when bacterial growth is still inhibited in the presence of sub-MIC concentrations. Both of these effects may extend the period

before redosing is required, and may explain why the plasma concentration does not need to stay above the MIC of the pathogen for the entire dosing interval for some T > MIC antimicrobial group–pathogen combinations. In addition, the post-antibiotic leukocyte effect (PALE) may increase the susceptibility of antimicrobial-exposed bacteria to the killing mechanisms of leukocytes.

An inoculum effect may also occur at the site of infection. AST is performed at a fixed inoculum of approximately 5×10^5 cells per milliliter of broth. When these bacterial concentrations are increased, decreased antimicrobial activity may be noted. This has been reported for β-lactams in the presence of β-lactamase-producing bacteria, but also in non-β-lactamase-producing strains. The clinical relevance of this finding is debated, although a few animal studies suggest that this effect may transfer to *in vivo* settings.

Other Antimicrobial Regimen Considerations

Duration of Therapy

The optimal duration of antimicrobial therapy remains elusive for most diseases. Although it is axiomatic that a drug must be present for a sufficient time at the site of infection, the variables affecting the time of treatment have not been well defined. It is clear that many of our antimicrobial therapy durations are arbitrary, based on 7-, 10-, or 14-day intervals by convention. In light of these arbitrary durations, it is hard to understand the admonition to "take the antibiotic for the full duration of the prescribed regimen to suppress selection for resistant organisms." Simple consideration of the concept of the resistance implications of prolonged selection pressure on bacterial populations invalidates the opposing concept of taking an antimicrobial for 10–12 days beyond symptomatic resolution of symptoms in order to complete an arbitrary regimen.

It is understood that a clinician must select a duration, and in the absence of research-based guidelines these durations will be selected based on clinical experience and dogmatic guidance. More evidence is coming forward that supports shorter antimicrobial regimens. For example, in human female uncomplicated bladder infections, studies have demonstrated that a single dose of a β-lactam is as effective as more prolonged regimens in acute cases. Similar work has failed to demonstrate a significant outcome difference in shorter and longer durations of therapy for community-acquired pneumonia in humans, although many of these studies were not analyzed as noninferiority studies.

Rather than perpetuate dogmatic duration guidelines here, it is hoped that the reader will find encouragement to carefully scrutinize any duration recommendations they may offer, and to make the disease-specific duration of therapy a component of their research careers. This is one of the most important challenges in preserving the antimicrobials currently in use.

Use of Antibacterial Combinations

Combinations of drugs sometimes succeed dramatically where individual drugs fail. An outstanding early historical example was the use of penicillin–streptomycin combinations in enterococcal endocarditis in humans. However, early studies of the outcome of combination treatment of pneumococcal meningitis in humans showed the serious adverse clinical effects of mixing bacteriostatic and bactericidal drugs. The importance of antagonistic interactions between drugs is greatest in those infections or patients where immune defenses are poor (meningitis, endocarditis, or chronic osteomyelitis). If a bacteriostatic drug is mixed with a bactericidal drug, then the former may neutralize the latter, which may be crucial for clearance of infection from certain sites or infections (meningitis, endocarditis, chronic osteomyelitis). However, this is far from a universal rule. In other patients or diseases, because of the complexity of the host–bacterial–antimicrobial interaction, it is harder to detect either synergistic or antagonistic effects clinically, and it is likely that antagonistic effects of drugs are "laboratory artifacts" with no clinical meaning.

A drug combination is additive if the combined effect of several drugs is the sum of their independent activities measured separately, synergistic if the combined effect is significantly greater than their independent effects, and antagonistic if it is significantly less than their independent effects. Synergism and antagonism are not absolute characteristics; such interactions are often difficult to predict, vary with bacterial species and strains, and may occur only over a narrow range of concentrations. No single *in vitro* method detects all such interactions. The methods used to determine *in vitro* interactions are generally time-consuming and are not often available in the laboratory.

Antimicrobial combinations may be synergistic if they involve the following mechanisms: (i) sequential inhibition of successive steps in metabolism (e.g. trimethoprim–sulfonamide combinations); (ii) sequential inhibition of cell wall synthesis (e.g. vancomycin–penicillin and mecillinam–ampicillin); (iii) facilitation of drug entry of one antibiotic by another (e.g. *β*-lactam–aminoglycoside and polymyxin–sulfonamide); (iv) inhibition of inactivating enzymes (e.g. ampicillin–clavulanic acid); and (v) prevention of emergence of resistant populations (e.g. macrolide–rifampin combination against *R. equi*). The combination of penicillin and gentamicin is a classic staple of equine antimicrobial therapy.

As suggested earlier, to some extent, antagonism between antibiotic combinations is a laboratory artifact that depends on the method of measurement and is thus, with some exceptions, unimportant clinically. The antagonistic effects of some combinations are, however, detected clinically. Antagonism may occur if antimicrobial combinations involve the following mechanisms: (i) inhibition of bactericidal activity (e.g. bacteriostatic and bactericidal drugs used to treat meningitis, where, depending on the time–dose relation, bactericidal effects are prevented); (ii) competition for drug binding sites (e.g. macrolide–chloramphenicol combinations, which are of unclear clinical significance); (iii) inhibition of cell permeability mechanisms (e.g. chloramphenicol or tetracycline–aminoglycoside combinations, which are of unclear clinical significance); and (iv) derepression of resistance enzymes (e.g. new third-generation cephalosporin antibiotics with older β-lactam drugs).

Antifungal Chemotherapy

The susceptibility of fungi to different drugs is often, but not always, predictable. Fungal drug susceptibility testing is technically complex, and simple methods paralleling the disk diffusion antibacterial susceptibility test are not generally available.

Antifungal Agents for Systemic Use

Historically, the major antifungal drug for systemic use, amphotericin B, had the disadvantages of toxicity and intravenous administration, but it did have the advantage of fungicidal activity. The development of the imidazoles (ketoconazole, itraconazole, and fluconazole) was a major advance in systemic fungal therapy because of their oral administration, relative lack of toxicity, and effectiveness.

Griseofulvin

Griseofulvin is a fungistatic antimicrobial that inhibits mitosis and is active only against dermatophytes (ringworm fungi). Resistance in some dermatophytes has been reported to develop during treatment. Griseofulvin is effective against ringworm fungi only if administered orally. The drug is incorporated into keratin in the basal cells of the epidermis and reaches the superficial dead and parasitized keratinized epithelium through progressive maturation of the basal cells.

Polyenes

Amphotericin B is the only polyene antimicrobial used systemically. Nystatin and natamycin are only used topically, with the poor oral bioavailability of nystatin also making it useful for enteric infections. The polyene antifungals bind ergosterol, the principal sterol of the fungal membrane, causing leakage of the cell contents. They are generally fungicidal antimicrobials with a wide range of activity. Amphotericin B is active against *Blastomyces dermatitidis*, *Candida* spp., *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Sporothrix schenckii*. Strains of filamentous fungi, though commonly susceptible, vary from extreme susceptibility to resistance. Amphotericin B must be administered intravenously. Renal toxicity is an inevitable side effect of such treatment and must be monitored; the effect is reversible if the drug is stopped. Amphotericin is still an important drug available for treating systemic mycoses caused by dimorphic fungi and by yeasts. The drug is given by slow intravenous injection. Infusion-related reactions are frequently reported in humans receiving amphotericin B, which has been attributed to amphotericin B initiating signaling pathways that lead to the release of pro-inflammatory cytokines.

Flucytosine

5-flucytosine (5-FC) is deaminated in the fungal cell to 5-fluorouracil, which is incorporated into messenger RNA to produce garbled codons and faulty proteins. It has a narrow spectrum of activity, which includes many *Candida* and most *C. neoformans*, but most filamentous fungi are resistant. Resistance develops readily during treatment. Therefore, 5-FC is often used only in combination with other drugs, usually amphotericin. It is highly bioavailable and may be administered orally.

Imidazoles

Imidazoles interfere with the biosynthesis of ergosterol and bind fungal cell membrane phospholipids to cause leakage of cell contents. In the United States, one imidazole is approved for systemic use (ketoconazole), and five triazoles (itraconazole, fluconazole, voriconazole, posaconazole, and isavuconazole). The imidazoles have varied spectrums of activity, but in general are fungistatic against a wide range of yeasts, dimorphic fungi, and dermatophytes; they also have some antibacterial and antiprotozoal activity. Ketoconazole, itraconazole, and fluconazole are more active than miconazole and are the drugs favored for systemic administration because they can be given orally rather than intravenously. In humans, the imidazoles may cause electrocardiogram abnormalities as well as hepatotoxicity and gastrointestinal side effects. Azoles are substrates and inhibitors of cytochrome (CYP) isoenzymes, and some inhibit P-glycoprotein. Potential drug interactions due to alterations in metabolism must be carefully considered. Side effects vary from compound to compound, the individual toxicity profile of each

imidazole should be understood prior to use. They appear to be an effective treatment for many systemic fungal infections in dogs and cats, but there has been little experience with their use in other animal species. They have the disadvantage of fungistatic action; prolonged treatment may be necessary in serious infections to prevent the relapses that have occurred, and this is expensive.

Echinocandins

Anidulafungin, caspofungin, and micafungin are members of this class. They work by inhibiting the synthesis of a cell wall component and are currently available for IV injection only. The spectrum includes *Candida* spp. and *Aspergillus* spp.

Allylamines

Terbinafine is topically and orally administered with a spectrum that includes yeasts and dermatophytes. It is used to treat dermatophyte toenail infections in humans. Terbinafine functions as a non-competitive squalene epoxidase inhibitor, interfering with fungal membrane formation. There are rare reports of liver failure in humans, and drug interactions are possible through terbinafine inhibition of CYP2D6.

Antifungal Agents for Topical Use

Many chemicals have antifungal properties and are used for topical treatment of fungal infections of the skin and sometimes of the mucosal surfaces. These include phenolic antiseptics such as hexachlorophene; iodides; quaternary ammonium antiseptics; 8-hydroxyquinoline; salicylamide; propionic, salicylic, and undecanoic acids; and chlorphenesin. Among the more effective topical broad-spectrum antifungal drugs are the polyenes natamycin and nystatin, the allylamine terbinafine, and the imidazoles clotrimazole, ketoconazole, enilconazole, and miconazole.

Resistance to Antibacterial Drugs

The most fundamental concept about antimicrobial resistance is that resistance occurs through the interaction of an antimicrobial regimen, or the summation of regimens, with a bacterial population. Consideration of either component alone is an oversimplification of resistance selection, ignoring the incredible complexity of antimicrobial resistance selection. Almost as fundamental is the understanding that not just inappropriate antimicrobial use selects for antimicrobial resistance, all antimicrobial use has the potential to do so.

At the time of this writing, the truly effective antimicrobial era is only 86 years old, a human lifetime, dating back to the advent of the sulfonamides in 1935. (Although penicillin G was discovered in 1928, clinical use came about in 1942 after much additional development work.) During this antimicrobial era lifetime, medical professions have learned hard lessons in the survival capabilities of bacterial populations, with the first reports of pathogen resistance often coming five to seven years after the release of a new antimicrobial. The period of the late 1940s through the 1980s was a euphoric period during which antimicrobial resistance was addressed by the constant development of new antimicrobial classes and new generations of existing classes. There seemed to almost always be a solution to an antimicrobial-resistant pathogen. Then the discovery of novel classes fell off, and resources for development of new generations of existing classes were redirected to more profitable pursuits, such as the statins for control of high cholesterol. By the turn of the century, we found ourselves reverting back to the systemic use of previously unthinkable substances, such as colistin, when all other options were lost for some human pathogens. Recent focus on new antimicrobial development has yielded more options, but the economic viability of many of the small companies developing these compounds is tenuous at best, with bankruptcy occurring even when a new antimicrobial is developed and sold. According to the PEW Charitable Trust 2021 Global Antibiotics Pipeline report, there are 43 new antibiotics in development globally, with approximately 1 in 4 being of a novel drug class or mechanism of action. Over 95% of these antimicrobials are being developed by small companies, 70% of them in the pre-revenue stage. The economic status of the development process has been addressed by post-market incentives for successful antimicrobials in Sweden and the United Kingdom, with bills for similar initiatives in the US Congress as of this writing.

The signals from applying selective pressure to large populations with short generation times are not just coming from bacteria. Resistance development in fungi, internal and external animal parasites, and crop pests (both insects and weeds) display similar trends.

The use of antimicrobial drugs does not induce resistance in bacteria but rather eliminates the susceptible bacteria and leaves the resistant bacteria present in the population. The exposure of animals and humans to antimicrobials is the basis of selection for the evolution and spread of resistance genes and resistant bacteria. The genetic processes involved in the development of resistance in bacteria are precisely those involved in the evolution of bacteria generally; it is a natural, Darwinian,

selection for "fitness." In fact, antimicrobials and antimicrobial resistance are as old as bacteria, both having developed is an arms race for dominance in competition for resources. Understanding the types of resistance and how bacterial populations respond to selection pressure is a start in our ever more pressing struggle to maintain the efficacy of these valuable resources.

Resistance to antimicrobial drugs can be classified as constitutive or acquired.

Constitutive Resistance

Microorganisms may be resistant to certain antibiotics because the cellular mechanisms required for antibiotic susceptibility are absent from the cell. Bacteria in the genus *Mycoplasma*, for example, are resistant to benzyl penicillin G because they lack a cell wall; similarly *E. coli* is resistant to penicillin G largely because the drug fails to penetrate into the cell. There are key antimicrobial and pathogen intrinsic resistance relationships that a clinician must know. Many of these are available in tables in the previously described VET01S from the Clinical and Laboratory Standards Institute, available without charge on the CLSI website.

Acquired Resistance

Acquired, genetically based resistance can arise because of mutation or, more importantly, through horizontal gene transfer ("transferable drug resistance") – the acquisition of genetic material from other bacteria by different means (bacteriophages, plasmids, transformation, or transposons). Mutations, especially in the chromosome, tend to produce changes in bacterial cell structures, whereas horizontal gene transfer tends to encode the synthesis of enzymes that modify antibiotics. Mutation-based resistance is often a gradual, stepwise process, whereas transferable resistance is often high-level, all-or-none, resistance.

Important mechanisms of acquired resistance include (i) enzymatic inactivation of antibiotics, (ii) failure of bacterial permeability, (iii) alteration in target receptors, (iv) development of bypass mechanisms in metabolic pathways, (v) development of enzymes with low drug affinity, and (vi) removal of antimicrobial drugs from the cell through efflux pumps, or combinations of these mechanisms.

Mutation to Resistance

Mutation to resistance is a minor problem relative to horizontal gene transfer. Mutations to antibiotic resistance are spontaneous events involving changes in DNA sequences uninfluenced by the presence of antibiotics. Such mutations, especially in the chromosome, may lead to other changes that leave the cell at a disadvantage so that, in the absence of antibiotic selection, these mutants may gradually be lost. Mutation to antibiotic resistance can be dramatic, as in the case of single-step mutation to streptomycin resistance where MIC increases 1000-fold, or gradual, as in the case of chromosomal resistance to penicillin, where a series of mutational events may gradually increase the MIC of the organisms. These differences occur because, when antibiotics affect one target site, chromosomal mutation is a single-step process, whereas when several targets are affected, mutation to resistance is a multistep process.

The rate of mutation differs for, and is characteristic of, each antibiotic. Sometimes, as discussed earlier, antibiotics are used in combination to overcome the possibility of mutation to resistance, because the chance of mutation to resistance to two antibiotics is the product of the chances of mutation for each antibiotic alone. In veterinary medicine, mutational resistance has limited the use of streptomycin, novobiocin, rifampin, and, to a lesser extent, erythromycin. Interestingly, for fluoroquinolones, there are differences between bacterial species in the importance of single-step mutations in the development of resistance. For *C. jejuni* and *P. aeruginosa*, a single nucleotide change at a particular site in the *gyrA* DNA gyrase gene can lead to clinical resistance, whereas in *E. coli*, a single nucleotide change may lead to only a slight reduction in susceptibility.

A chromosomal mutation resulting in multiple antibiotic resistance has been described for clinically relevant bacteria. The region involved, the Mar (multiple antibiotic resistance) locus, controls efflux systems, resulting in resistance to a variety of drugs without modification of the drugs.

Horizontal Drug Resistance Transfer

Horizontal gene transfer as a cause of antibiotic resistance is of major importance in veterinary and human medicine. Unlike chromosomal mutation to resistance, which occurs in individual bacteria, transfer of genetic material produces "infectious" resistance. The three ways in which bacteria can acquire foreign resistance genes are by transformation (uptake of naked DNA), by bacterial viruses (transduction), and by conjugation (mating by means of plasmids) (Figure 69.2). Depending on the type of horizontal gene transfer, this may result in transfer of resistance to several or even many antibiotics at one time, which can spread to and between bacteria from the original source. The extrachromosomal DNA molecules responsible for most infectious antibiotic resistance are usually plasmids, called R plasmids.

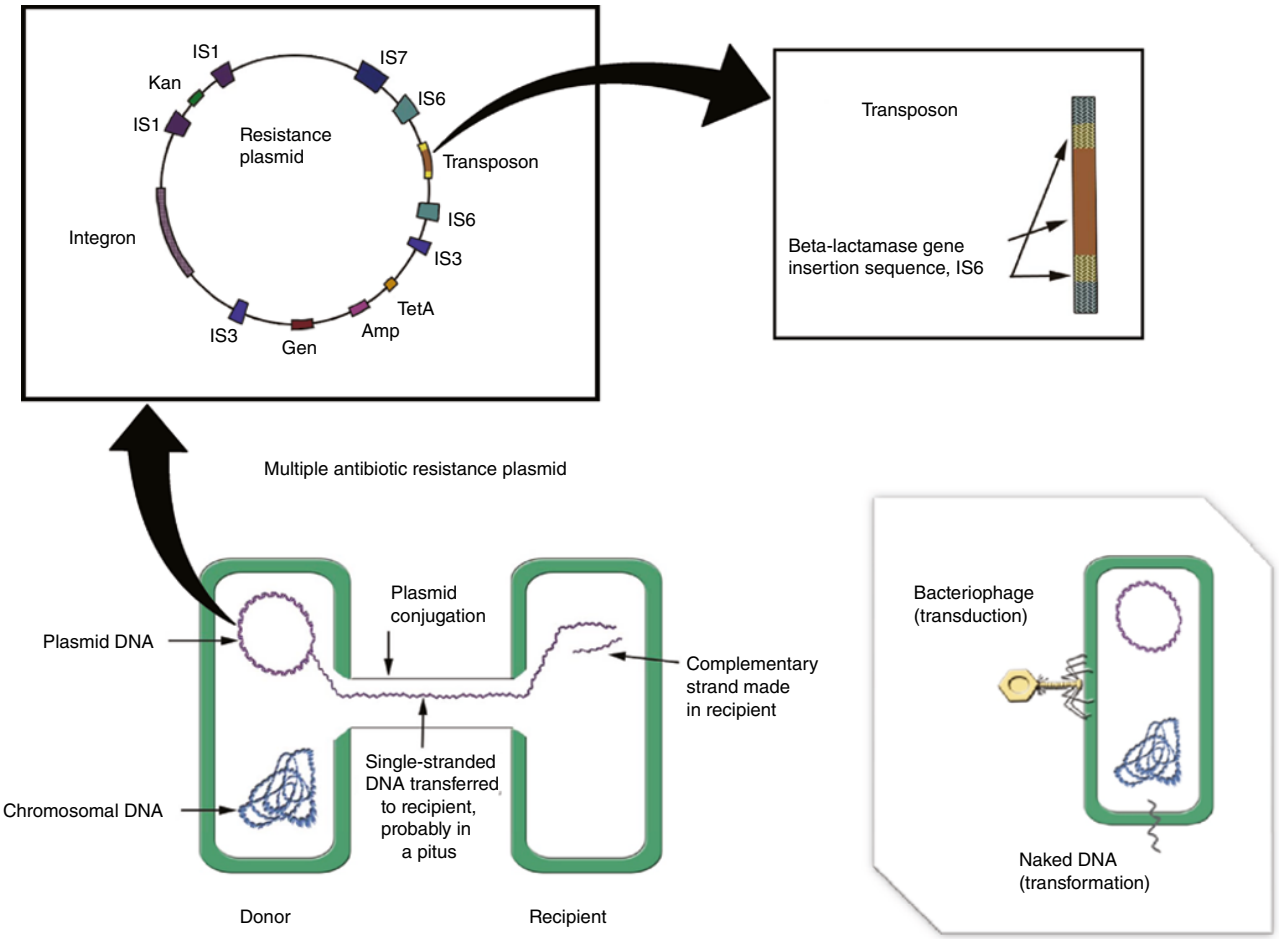


Figure 69.2 Mechanisms of horizontal gene transfer of resistance in bacteria. *Source:* Courtesy of Dr. John F. Prescott, University of Guelph, Canada.

The development of resistance genes including those associated with horizontal gene transfer is often an enormously complex combination of different mobile genetic elements, but also includes mutation and recombination within these elements, all of which confer a selective advantage ("fitness") on bacteria possessing these elements and genes in surviving exposure to the antibiotics (and sometimes concurrently to disinfectants and heavy metals) to which they may be exposed. To add further complexity, these same transferable elements may also acquire other determinants that enhance their fitness, such as the ability to form biofilms in the case of hospital-associated *Enterococcus* species.

Plasmids

Plasmids that carry DNA responsible for resistance can reproduce themselves within a cell and spread to other cells by conjugation. Plasmids themselves are commonly partly constructed from transposons and integrons.

Conjugation refers to plasmid-mediated transfer of resistance (Figure 69.2). In this common process of gene transfer, a donor bacterium synthesizes a sex pilus, which attaches to a recipient bacterium in a mating process, resulting in transfer of copies of plasmid genes to the recipients. The donor retains copies of the plasmid, but the recipient has now become a potential donor. Conjugation can occur not only between species of the same genera but also across genera and families, so that similar plasmids can be found in a wide range of unrelated bacteria. The large intestine is a site where bacterial conjugation through resistance plasmids commonly occurs.

Much plasmid-mediated resistance, and indeed some chromosomally mediated resistance, is associated with transposons (Figure 69.2). The simplest form of transposon is a resistance gene flanked on either side by an insertion sequence, the smallest form of mobile genetic element. These short DNA sequences, also called "jumping genes,"

can transpose from plasmid to plasmid, from plasmid to chromosome, or from chromosome to plasmid. A transposon copy usually remains at the original site. The frequency of transposition is characteristic of the particular transposon and bacterium. The importance of transposition as a key element in resistance transfer is that it is independent of the recombination process of the bacterial cell; homology with the interacting DNA is not required. Because of transposons, plasmids from diverse sources often possess identical antibiotic inactivating genes. Due to the widespread nature of insertion sequences in many bacteria, there is essentially no gene in bacterial genomes (including genes that encode resistance) that cannot be mobilized and moved to other bacterial genomes as a transposon. In addition, because of the DNA homology between insertion sequences, DNA recombination events frequently modify resistance plasmids that can combine to add DNA with additional resistance genes. Plasmids may also carry integrons encoding for resistance). Conjugative transposons may cause bacteria to conjugate, perpetuating the mechanism of spreading.

A well-known example of a resistance gene spread by plasmids is the $bla_{\text{CMY-2}}$ gene encoding resistance to third-generation cephalosporins. This gene, which likely originated from the chromosome of a *Citrobacter* spp., has spread among related plasmids within the order Enterobacterales, for example, to *Salmonella* serovars and to *E. coli*. The plasmids encoding $bla_{\text{CMY-2}}$ are relatively "promiscuous," so that the resistance gene has disseminated rapidly among commensal and pathogenic members of the Enterobacterales.

Bacteriophage

Bacterial viruses (bacteriophages) can transfer resistance genes originating in the chromosome or in plasmids between bacteria in a process known as transduction (Figure 69.2). An example is transfer of β-lactamase genes from penicillin resistant to previously penicillin-susceptible staphylococci. The importance of transduction in the spread of antimicrobial resistance may have been underestimated, although the narrow specificity of bacteriophages has likely limited their role.

Transformation

Transformation is a special type of horizontal gene transfer that occurs in some bacterial pathogens (Figure 69.2). Transformation describes a process by which DNA released by dying bacteria is taken up from the environment by other usually closely related bacteria and then recombines during DNA replication to produce novel forms of existing genes. The best-known example of this is the emergence of penicillin resistance in *S. pneumoniae*, an important human pathogen, through the development of novel PBPs.

Other Genetic Elements Associated with Resistance

Integrons

Integrons are increasingly recognized as widespread genetic elements associated with multiple drug resistance in gram-negative enteric bacteria. An integron is a gene capture and dissemination system, consisting of an integrase gene and a site-specific integration site ("gene capture") into which the integrase can insert antimicrobial drug resistance "cassettes" ("gene dissemination"). Over 9 types of integrons and over 60 different gene cassettes have been identified in gram-negative enteric bacteria, with some integrons containing as many as 7 different resistance gene cassettes. In addition, some bacteria may carry cassettes in their genomes that have not incorporated into integrons but are capable of doing so. Integrons (with or without resistance gene cassettes) may be found in the chromosome, in plasmids, or in transposons.

Genomic Resistance Islands

Genomic resistance islands are large chromosomal regions that contain multiple resistance genes, mostly associated with different integrons, and which can be moved between bacteria by plasmids. A well-known example is the *Salmonella* genomic island 1 associated with multidrug resistance in *Salmonella enterica* serovar Typhimurium DT104, but also found in strains of other multidrug resistant serovars, as well as in other Enterobacterales.

Clinical Importance of Antimicrobial Drug Resistance

Acquired drug resistance has become a major problem in some pathogenic bacteria of veterinary importance and has been described as a crisis in human medicine. It is common in many bacterial species, although notably some bacteria, particularly gram-positive bacteria such as many streptococci and corynebacteria, have remained highly susceptible to commonly used drugs. As a result, diseases produced by these bacteria have in many cases declined considerably in importance, to be replaced by infections produced by bacteria that have the ability to evolve more readily.

Acquired resistance to penicillins is frequent in coagulase-positive staphylococci, and recently methicillin-resistant *S. aureus,* and in dogs methicillin-resistant *S. pseudintermedius*. Acquired multiple antibiotic resistance to many common antibiotics seriously limits their use in members of the order Enterobacterales such as

Salmonella and *E. coli*. In animals, acquired resistance is increasingly observed in nonenteric bacteria such as *Bordetella*, *H. somni, M. haemolytica*, and *P. multocida*, and has been identified in virtually every pathogenic bacterial genus as well as in the normal flora.

There is to some extent a causal relationship between antimicrobial drug use and the development of resistance, but its importance varies with different pathogens and essentially reflects their ability to adapt to changing circumstances, that is, to evolve, as well as to colonize different animal species.

The development of resistance is particularly notable among enteric bacteria in animals. The intestine is a major site of transfer of antibiotic resistance because of the vast numbers of bacteria present and their ability to transfer resistance genes horizontally, the use of oral antimicrobial drugs, and because of the opportunities for spread of these bacteria through focally contaminated environments. Within the intestine, "promiscuous" plasmids may transfer resistance between bacteria within families of bacteria, for example, between members of the Enterobacterales such as from *E. coli* to *Salmonella* or *Proteus*, or even between bacteria belonging to unrelated taxonomic families such as *E. coli* to *Bacteroides*. The emergence and spread of drug resistance in the Enterobacterales and other opportunist infections is increasingly well documented in individual companion animals such as dogs, and may reflect in some cases infections acquired from their owners through antibiotic use in human medicine.

Where multiple drug resistance genes are present on a plasmid or an integron, the use of any antimicrobial to which resistance is encoded by one of the genes will help maintain the entire collection of resistance genes. Plasmids may gradually accumulate resistance genes through acquisition by their integrons of resistance cassettes or through acquisition of transposon-mediated resistance genes.

Intestinal *E. coli*

Extensive study of antimicrobial resistance in intestinal *E. coli* in food animals has provided considerable information on the mechanisms and ecology of antimicrobial resistance. These studies have shown the relationship between the extent of resistance and the degree of antimicrobial use. For example, resistance in *E. coli* from adult ruminants at pasture is slight, whereas it is pronounced in intensively reared young animals where antibiotic use is common and the intestinal flora is immature. As a result of plasmid-mediated resistance, these *E. coli* may be resistant to many clinically useful drugs. Among enterotoxigenic *E. coli* from farm animals, plasmid-mediated resistance to tetracyclines, sulfonamides, and streptomycin is now practically universal. Antibiotic-resistance-encoding plasmids in enterotoxigenic *E. coli* in swine and calves may also include genes for virulence determinants such as toxin production or adhesins.

Within the intestine, R plasmids are found in *E. coli* and in the more dominant anaerobic flora of the large bowel. Within a short time of treating an animal with an antibiotic, the *E. coli* and much of the anaerobe population become resistant to that antibiotic, principally because of selection of resistant strains but also because of enhanced transfer of R plasmids. In the absence of antimicrobials, conditions in the large bowel seem generally to prevent the transfer of R factors. Short-term oral use of antimicrobials is followed by high levels of *E. coli* resistance, which fall once the antimicrobials are removed, because the majority of R plasmid bearing *E. coli* are not good intestinal colonizers. However, the continuous presence of antimicrobials is associated with extensive resistance, which persists long after the antimicrobial is removed, because resistant *E. coli* that are good intestinal colonizers have been selected.

Salmonella

Multiple antimicrobial resistance is a major problem in selected serovars and in individual strains within serovars of *Salmonella*, including serovar Typhimurium. It is often the result of a chromosomally incorporated genomic island containing integrons. Among *S*. Typhimurium strains, clones of certain phage types such as DT104 are characterized by the presence of a multiple-antimicrobial-resistant genomic island, which has spread to other serovars. The extent of resistance can be marked among calf isolates because of the extensive use of antimicrobials in some types of calf rearing and because the nature of salmonellosis in calves apparently provides an opportunity for the development and spread of resistant *Salmonella*. Multiply resistant and more virulent clones of *S*. Typhimurium have a history of emerging to spread within some cattle populations, and to spread from cattle into other species including humans, and then to decline in importance. For example, the multiply resistant clone DT 104 disseminated widely through cattle on a global basis before declining after a decade. This clone had a region containing florfenicol- and tetracycline-resistance-encoding genes flanked by two integrons carrying a β-lactamase and streptomycin resistance gene cassette. This cluster of genes formed part of the larger, distinctive *Salmonella* genomic island 1, which may have been a plasmid integrated into the chromosome. This island or its variants has also been identified in other *Salmonella* serovars, such as *S*. Agona and *S*. Newport. *Salmonella* carrying these multidrug resistance genes have caused serious infections in people.

Hospital-Acquired Resistant Infections

Acquired antimicrobial resistance in resident hospital bacteria is a major problem in human hospitals and is increasingly recognized in veterinary hospitals. In human medicine, hospital-associated infections with multiresistant bacteria belonging to the so-called ESKAPE group can become a major problem. The ESKAPE bacteria are *E. faecium*, *S. aureus*, *Klebsiella* species, *Acinetobacter baumannii*, *Pseudomonas. aeruginosa*, and *Enterobacter* species. Resistance is by no means restricted to hospital-acquired infections, but hospitals are inevitably a breeding ground for the development and selection of resistance in bacterial pathogens. There is a causal relationship between antimicrobial use in hospitals and the development of resistance in bacteria.

Colonization of patients by resistant opportunist bacteria is hard to prevent because of shared air spaces and environment, utensils, and veterinary staff. In addition, patients with serious and sometimes immunosuppression-associated illnesses may be repeatedly treated with numerous antimicrobial drugs, thus removing the normal colonization resistance of the body, including the large intestine, provided by the normal microbial flora. Under such circumstances, these patients can readily be colonized by bacteria that are either intrinsically resistant to many antimicrobial drugs (ESKAPE group) or that have acquired resistance.

Public Health Aspects of Antimicrobial Resistance in Animal Pathogens

The use of antimicrobial agents in animals can result in antimicrobial-resistant bacteria and their resistance genes reaching the human population through a variety of routes. The scale of the contribution via these routes has not been determined and indeed, because of the complexity of resistance in bacterial pathogens, is hard to determine.

Most antimicrobial resistance in human pathogens stems from antimicrobial use in human medicine. However, antimicrobial-resistant bacteria of animal origin, such as *Enterococcus* spp. *E. coli* and *Salmonella*, can colonize the intestines of people. Heavily exposed humans (e.g. farmers who use feed containing antimicrobials, slaughterhouse workers, cooks, and other food handlers) often have a higher incidence of resistant *E. coli* in their feces than the general population. Pathogenic bacterial species from the intestines of animals may cause zoonotic infections in humans (e.g. *Salmonella* and *C. jejuni*), and these infections may be harder to treat because of acquired resistance. The nonpathogenic bacteria of animals acquired by humans are also a potential source of resistance genes for human pathogenic bacteria other than the zoonotic bacteria. These bacteria may reach humans not only through the food chain but also through environmental contamination by resistant bacteria of animal origin. As stated above, although all of these routes are possible, the extent of the contribution to human resistance problems through these routes remains unquantified.

The topic of the contribution of antimicrobial use in food animals, particularly for growth promotion and disease prophylactic purposes, to resistance in human pathogens has been the subject of repeated review over many years, but the last 15 years have seen the topic subjected to the most intense scrutiny. The alarming increase in resistance in human pathogens, particularly those causing "community-acquired" infections rather than hospital-acquired infections, has led to intensive searches for the root cause of this challenge as resistance has left the hospital to enter the community. The evolution of antimicrobial resistance in all sectors of use has led to a total reappraisal of the use of all antimicrobial drugs in all circumstances.

When the 2019 Centers for Disease Control and Prevention (CDC) report on antibiotic resistance threats in the United States is considered in relation to potential zoonotic pathogens, it is difficult to cite use in veterinary medicine for resistance in human pathogens such as carbapenem-resistant *Acinetobacter* (no carbapenems are labeled for use in food animals in the United States), *Candida auris*, *C. difficile*, *Neisseria gonorrhoeae*, *P. aeruginosa*, *S. pneumoniae*, and tuberculosis. Methicillin-resistant *S. aureus* has been shown to move both ways between people and their pets; however, the main methicillin-resistant *Staphylococcus* spp. affecting dogs and cats is *Staphylococcus pseudintermedius*, which only occasionally affects humans.

VRE is listed as a serious threat by the CDC, having a long history in both Europe and the United States, as well as the rest of the world. In Europe, vancomycin-resistant *E. faecium* was being selected in intensively reared farm animals by the use of avoparcin, a glycopeptide antimicrobial drug used as a growth promoter and disease prophylactic. These VREs were almost universal in the intestines of treated animals and were found in a small proportion of healthy humans in Europe. In contrast, they were not present in healthy humans in the United States, where the drug was not used in food animals. Although avoparcin was banned from use in animals in the EU in 1997 and VRE intestinal colonization rates declined in some areas of Europe, VRE rates in *E. faecium* in Europe increased from

4.7% in 2001 to 20.3% in 2014. During the same period, VRE rates among *E. faecium* in the United States increased from 60.0% to 80.7%, without any use of glycopeptides in food animals. Although certainly not absolving animal use of avoparcin from contribution to human resistance issues., these rates do emphasize there is much more to the puzzle of human resistance than just animal contribution.

Legislative and regulatory action has been taken around the world to address the relationship between the use of antimicrobials in animals and resistance in human pathogens. The growth promotors avoparcin, bacitracin, spiramycin, and tylosin were banned in Europe by late 1999. A similar ban has occurred in Australia. In the United States, there has also been extensive reexamination of the use of antimicrobials in animals, including actions taken toward existing antimicrobial approvals. One notable decision by the Center for Veterinary Medicine of the US Food and Drug Administration (FDA CVM), through a Notice of Opportunity for a Hearing (NOAH) process concluded in 2006, was to reverse the approval of a fluoroquinolone for treatment of *E. coli* septicemia in chickens because of the rapid development of fluoroquinolone resistance in *C. jejuni*. It was estimated through a quantitative risk assessment that as many as 14 000 people annually treated for campylobacteriosis had their treatment affected (approximately one additional day of diarrhea) because of infection with resistant bacteria acquired from fluoroquinolone-treated chickens. In 2012, the FDA CVM finalized GFI #209, a "judicious use" guidance document that promoted two principles, the first that use of antibiotics in animals be limited to prevention, treatment, and control of disease (i.e. not for growth promotion) and that all use in feed or water be under veterinary oversight (i.e. not available over the counter). These actions were finalized with the advent of the revised antimicrobial drug labels on January 1, 2017. Currently, as outlined in GFI 263, the FDA CVM is pursuing the voluntary transition of all remaining over-the-counter veterinary antimicrobials to prescription status.

Changes have also occurred in the approval processes for antimicrobials in the United States. In 2003, the FDA CVM enacted Guidance for Industry (GFI) #152 for the evaluation of the impact of potential resistance selection as part of the approval process for new antimicrobial drugs. The document takes into account the relative importance of different antimicrobial drug classes in human medicine and analyzes the risks of production of resistance and its acquisition by humans, depending on the proposed usage of the drug. In 2013, a second guidance for industry document (#159) was enacted that evaluates potential antimicrobial residues in food of animal origin in relation to effects on the human intestinal flora.

It seems likely that antimicrobial drugs important in human medicine will be removed from use as growth promoters or long-term disease prophylactics throughout the world. Some of these removals have been based on clear evidence of the effect of such uses on human health, whereas others are rooted in the precautionary principle. As of this writing, consideration is being given to removing some critically important classes from all animal uses in the EU.

The problem of antimicrobial resistance embodies two classic dilemmas, these being short term versus long term and individual versus community. How do we work together to balance the benefits of using antimicrobials with their diminishing efficacy through selection for resistance? How do we balance benefits to the individual with the need to preserve these societal resources? As contentious as these discussions can become, the answers will come through research and actions that support antimicrobial stewardship.

Addressing Antimicrobial Resistance through Antimicrobial Stewardship

There are multiple definitions of antimicrobial stewardship. In 2018, the American Veterinary Medical Association deliberated and adopted the following antimicrobial stewardship definition and core principles:

> Antimicrobial Stewardship for Veterinarians Defined
>
> Antimicrobial stewardship refers to the actions veterinarians take individually and as a profession to preserve the effectiveness and availability of antimicrobial drugs through conscientious oversight and responsible medical decision-making while safeguarding animal, public, and environmental health.
>
> Core Principles of Antimicrobial Stewardship in Veterinary Medicine
>
> Antimicrobial stewardship involves maintaining animal health and welfare by implementing a variety of preventive and management strategies to prevent common diseases; using an evidence-based approach in making decisions to use antimicrobial drugs; and then using antimicrobials judiciously, sparingly, and with continual evaluation of the outcomes of therapy, respecting the client's available resources.

In addition, multiple countries, veterinary and producer organizations, food marketing entities, and food animal processors have instigated or completed the development of policies or guidelines related to antimicrobial stewardship. Governments have instituted voluntary and mandatory antimicrobial reduction strategies in both human and veterinary applications. The warning of Alexander Fleming in his 1945 Nobel Prize acceptance speech has come to pass, and we now find ourselves back to where we started for some cases of the most antimicrobial-resistant pathogen and disease combinations. The tension between human and antimicrobial uses of antimicrobials can only increase, with hard decisions ahead.

As antimicrobial stewardship has evolved, it has become apparent that human behavior is perhaps as important as the sciences of microbiology, epidemiology, and pharmacology. If you are reading this chapter and this book, you have an interest in microbiology and how we interact with the microbial sphere as a society. You may rest assured that you have chosen a career track that is essential to maintaining the efficacy of antimicrobials, and with them the practice of modern medicine.

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Vaccines

D. Scott McVey, Jishu Shi, and Donald L. Reynolds

Introduction

Vaccines are complex preparations that are used to elicit immune responses to prevent or minimize disease produced by infectious agents. Vaccines can be composed of the infectious agent itself (either live or killed), a portion of the agent that induces a protective immune response (toxoid, DNA/RNA, or subunit vaccine), or a product of the agent (such as a membrane extraction). Products containing a killed bacterial agent are bacterins. Products that have inactivated or inactive toxins (via chemical inactivation or naturally occurring variations) are called toxoids.

How Vaccines Work

To be effective, vaccines should stimulate an immune response that ideally prevents infection but, at the very least, should interfere with the development of severe disease. These outcomes are the result of regulated and coordinated immune responses that display a maturation of the specific immune response to a given pathogen. And, importantly, immunologic memory is a critical element of effective responses to vaccines.

The first step in immune responses is the initial cellular assimilation and processing of the vaccine antigen – accomplished by macrophages and dendritic cells. Inflammatory processes activated by infected tissues also activate these antigen-processing phagocytic cells. Such cells have increased phagocytic and enzymatic activity. This increase in activity is associated with the destruction of infectious agents. This biological inactivation (stopping further replication and/or production of toxic materials) and structural degradation of the antigens result in antigen processing, facilitating the initiation and stimulation of the adaptive immune response. This cellular activation is also associated with secretion of multiple factors that recruit lymphocytes (and other leukocytes) to further facilitate local tissue responses and coordinate activation of the immune response. These cellular responses begin at the site of antigen delivery and proceed (via lymphatic drainage) to local secondary lymphoid tissues.

Antigens acted upon by antigen-processing cells via the exogenous pathway elicit antibodies. Thus, extracellular bacteria (live or killed), inactivated viral particles, portions (subunits) of a virus, and products are processed by the exogenous pathway. The predominant immune response in these types of responses is antibody. Antibodies function immunologically by binding to epitopes on the surface of the infectious agent and/or one of its products. By binding to the surface of an infectious agent, antibodies interfere with attachment to host target cells by stearic interference and/or by changing the charge or hydrophobicity of the surface of the agent (enhancing phagocytosis by multiple cell types) and trigger the complement cascade, damaging surface membranes (causing cellular lysis or disruption of physiological processes) and by enhancing phagocytosis (opsonization). Antibodies that bind to products of infectious agents (like toxins) can block the attachment of the toxins to receptors on cellular targets and/or change the configuration of the product, resulting in a change in binding affinity.

Some infectious agents replicate within cells and may be shielded from soluble proteins like antibodies and complement cascade components. If the agent multiplies within a mononuclear phagocyte, then antigens are processed by way of the exogenous and/or endogenous pathways and thus activate multiple protective cellular responses. These exogenous and endogenous pathways refer to mechanisms of antigen processing by antigen presenting cells of the immune system (see Chapter 67).

These responses are often referred to as cell-mediated immunity (CMI). These cellular immune responses result in the generation of "activated" macrophages and/or specific

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cytotoxic T lymphocytes as well as numerous regulatory lymphocytes. Cytotoxic T lymphocytes recognize affected and infected host cells (e.g. cells infected with viruses or bacteria). This recognition stimulates these lymphocytes to secrete substances that kill the affected host cell, halting the replication of the infectious agent. If the affected host cell contained an infectious agent, that agent would now be either destroyed or liberated from the cell and be in contact with other host immune effectors (e.g. antibodies, complements, and activated macrophages). More information regarding the cellular nature and protective mechanisms is included in Chapter 67.

Infectious agents are foreign antigens and are generally recognized by the immune system and induce immune responses (inherent immunogenicity). However, immune responses are not always protective and may actually contribute to the pathogenesis of an infectious disease and/or enhance the severity of the disease. Some experimental and some registered vaccines may produce antibodies that do not completely protect animals from the disease for which they are intended. In fact, some antibody responses may exacerbate or enhance disease under some circumstances (antibody-dependent enhancement [ADE]). This phenomenon has been observed with some inactivated vaccines (discussed in the next sections of this chapter) for the porcine respiratory and reproductive syndrome virus. The mechanisms responsible for ADE are not fully understood.

It is essential that vaccine development work characterize immune responses of experimental vaccines and always ensure both efficacy and safety. In general, the immune responses to vaccines should resemble convalescent responses to the specific infectious disease (at least in part). Correlation of protection in controlled challenge of immunity studies (or field efficacy) with characterizations of immune response parameters is very important. This correlation of immunity with surrogates of immunity forms the basis for testing quality-assured and quality-controlled manufacturing processes – producing pure, safe, and potent vaccines for veterinary medicine.

Adjuvants

Adjuvants are used to influence the types of immune response elicited by an antigen. To date, numerous compounds of organic, inorganic, synthetic, and natural origin demonstrate an ability to stimulate immune responses and have potent adjuvant properties. These substances can be broadly divided in two different groups: immunostimulants (saponins, Toll-like receptor agonists, cytokines) and delivery agents (emulsions, microparticles, mineral salts). The immune response is influenced at various stages, depending upon the adjuvant. Some adjuvants function as a critical component of the formulation so that the antigen is delivered to the antigen-processing cells to maximize the immune response. Examples include water/oil emulsions, minerals/salts (bentonite and aluminum), pro-inflammatory cytokines, and inert particles (microspheres). Other adjuvants direct activity to the processing step in the initiation of the immune response. Examples include "immune-stimulating complexes" (ISCOMs) composed of cholesterol–phospholipid structures that contain the immunogen, and liposomes (lipid vesicles). "Targeting" various components by using various cytokines as adjuvants can influence immune responses. For example, IL-1 activates T lymphocytes, IL-12 and INF-γ influence the helper T lymphocyte subset selection, and granulocyte macrophage colony-stimulating factors activate macrophages and increase the efficiency of antigen processing.

Adjuvants may also contribute to the phenomenon of trained immunity. Trained immunity is the concept of the innate immune response (covered elsewhere in this text) being enhanced, or strengthened, by previous encounters with infectious agents (i.e. pathogens). This concept may play an important role in future vaccine and vaccine adjuvant development.

Vaccine Administration Routes

The administration route of a vaccine may be defined as the path by which a vaccine is delivered to the host. The administration route is an important consideration when developing a vaccine and/or vaccine program. When determining the most appropriate vaccine administration route, points to consider include the type of immune response desired, the host (i.e. species), characteristics and properties of the immunogen being delivered, administration logistics (e.g. facilities and equipment), cost/financial considerations, and health and safety concerns of the host (i.e. immune status, adverse reactions, etc.) and those delivering the vaccine (i.e. vaccinators).

Vaccine administration routes are often classified as either by location on the host or method of delivery. Parenteral (meaning outside the intestinal tract) administration is typically used to mean a vaccine that is injected. Common parenteral administration routes include subcutaneous (SQ or sub-Q), intramuscular (IM), intravenous (IV), intraperitoneal (IP), and intradermal (ID). Other vaccine administration routes include intraocular (IO, eye-drop), intranasal (IN), spraying (course droplet spray for upper respiratory, fine droplet spay for lower respiratory), oral (either feed or water) and *in ovo*. *In ovo* vaccination is used extensively in the poultry industry. The egg embryos

are injected with vaccine into their amnionic cavity at the time the eggs are transferred from the incubator to the hatching unit.

Viral Vaccines

Immunization of animals with viral vaccines is critical to prevent many viral diseases. The basis of an effective vaccine is its ability to induce an immune response or responses capable of eliciting protection to subsequent field exposure to pathogenic viruses. A multitude of vaccine preparations have been developed and used over the years, with variable success. The success of a potential vaccine is primarily dependent on safety and efficacy; however, economics also determine vaccine design, development, and ultimate production on a commercial basis.

Various approaches to vaccination have been employed over the years. These include (i) administering a live virulent virus in an anatomical site such that the target tissue or tissues are not infected, (ii) administering a live virulent virus to animals at a time of relative resistance to disease expression, (iii) concurrent administration of a live virulent virus and immune serum, which no longer is acceptable for obvious reasons, (iv) use of live avirulent viral strains (e.g. attenuated or "modified-live" viruses), and (v) use of inactivated viruses. In recent years, additional approaches to vaccine development have become available. These include subunit, synthetic peptide, and recombinant products. Regardless of the vaccine type, the desired result is to induce immune responses specific for viral antigens expressed on the virion surface or on the surface of infected cells, so that the immunized host is immune when exposed to the virulent virus. The rational development of an efficacious viral vaccine requires an understanding of viral pathogenesis, of protective immune responses induced following infection, and of their protein specificities. The latter point is of obvious importance for developing recombinant and synthetic peptide vaccines.

Concerning pathogenesis, the following three general types of viral infections occur:

1) Infections that are confined to the mucosal surfaces of the respiratory or gastrointestinal tracts. In such instances, local immunity in the form of a secretory antibody (e.g. IgA) is important. The role of CMI is less well characterized in such infections.
2) Infections that begin at mucosal surfaces but then cause a systemic infection with viremia, and subsequent infection of distant target tissues. In these infections, both immunity at the mucosal surface and systemic immunity are important.
3) Infections that gain direct entry into the host's circulation via insect bite (arthropod-borne viruses), inadvertent inoculation, or a traumatic break in an epithelial surface. In such infections, systemic immunity is the primary line of defense.

These mechanisms of viral infection and subsequent dissemination must be considered in vaccine development. Modified-live (attenuated) and inactivated (killed) virus vaccines dominate the veterinary vaccine market (Table 70.1). In recent years, there have been new experimental and licensed

Table 70.1 Types of viral vaccines.

1) Live viral vaccines
   a) Attenuation for low virulence of viruses that produce natural diseases.
   b) Host-range mutants – use of different viral strains infecting different host species that are related antigenically to the virus strain that produces a natural disease in the original host.
   c) Recombinant heterologous viral vector vaccines – construction of an infectious viral recombinant that expresses protective antigen(s) of another virus that produces a natural disease. Construction of a recombinant virus with insertion of genes with known antiviral activities or with known immunoregulatory functions.
   d) Recombinant homologous viral strains attenuated by targeted mutations on deletions of genes coding for specific virulence factors that produce a natural disease.
   e) Nonreplicating recombinant viral vector vaccines capable of replicating to high titer *in vitro* but unable to grow efficiently *in vivo*.
2) Inactivated (nonreplicating) viral vaccines
   a) Chemically inactivated viral vaccines
   b) Physically inactivated viral vaccines (mechanical shearing)
   c) Purified native viral antigens using immunoaffinity chromatography
   d) Cloned viral protein subunit vaccines produced in eukaryotic or prokaryotic cells by recombinant DNA technology
   e) Synthetic viral polypeptide vaccines representing immunologically identified domains of viral surface antigens.
   f) Direct injection of plasmid DNA encoding viral protective antigens into tissues *in vivo*.
   g) RNA vaccines – mRNA-like constructs
   h) Use of anti-idiotypic antibodies as antigens to induce an antiviral antibody response.

vaccines introduced to veterinary medicine. The regulatory descriptions and definitions for all veterinary biologicals are managed by the Center for Veterinary Biologics, USDA Animal and Plant Health Inspection Service (Figure 70.1).

Live-Attenuated Viral Vaccines

The attenuated viral vaccines include artificially attenuated (modified-live) strains of viruses or naturally occurring viruses with reduced virulence for the host. The origin of these naturally attenuated isolates may be the natural host, or a closely related virus isolated from a different host; for example, the cowpox virus was initially used to vaccinate humans against smallpox. The major requirements of such an approach are that it induce adequate immunity and that the attenuation (lack of virulence) of the vaccine strain be stable. The majority of vaccines currently used today in veterinary medicine are attenuated viruses. The most common approach to viral attenuation is the development of host-range mutants. Other approaches include development of temperature-sensitive and cold-adapted mutants (missense mutations), deletion mutants, and recombinant viruses.

Virus-Serum-Toxin Act
21 USC 151-159 et. seq.

CHAPTER 5. VIRUSES, SERUMS, TOXINS, ANTITOXINS, AND ANALOGOUS PRODUCTS

151. Preparation and sale of worthless or harmful products for domestic products for animals prohibited, preparation to be in compliance with rules at licensed establishments
152. Importation regulated and prohibited
153. Inspection of imports: denial or entry and destruction
154. Regulations for preparation and sale; licenses
154a. Special licenses for special circumstances; expedited procedure; conditions; exemptions; criteria
155. Permits for importation
156. Licenses condition on permitting inspection; suspension of licenses
157. Same; inspection daytime or nighttime
158. Offenses; punishment
159. Corporation; commerce; trade
(37 Stat. 832-833; as amended December 23. 1985, Pub. L. 99-198, 99 Stat.1654-1655; 21 U.S.C. 151-159).

151. Preparation and safe of worthless or harmful products for domestic animals prohibited; preparation to be in compliance with rules at licensed establishments.

It shall be unlawful of any person, firm or corporation to prepare, sell, barter, or exchange in the District of Columbia, or in the Territories or in any place under the jurisdiction of the United States, or to ship or deliver for shipment in or from the United States, the District of Columbia, any territory of the United States, or any place under the jurisdiction of the united States, any worthless, contaminated, dangerous, or harmful virus, serum, toxin, or analogous product intended for use in the treatment of domestic animals, and no person, firm, or corporation shall prepare, sell, barter, exchange, or ship as aforesaid any virus, serum, toxin, or analogous product manufactured within the United States and intended for use in the treatment of domestic animals, unless and until the said virus, serum, toxin, or analogous product shall have been prepared, under and in compliance with regulations prescribed by the Secretary of Agriculture, at an establishment holding an unsuspended and unrevoked license issued by the Secretary of Agriculture as herein after authorized.



United States Department of Agriculture
Marketing and Regulatory Programs
Animal and Plant Health Inspection Service
Veterinary Services
Center for Veterinary Biologics
1920 Dayton Avenue
Ames. IA 50010
PHONE (515) 337-6100
FAX(515) 337-6120

Veterinary Biological Products

Licensess and Permittees Prepared
April 1, 2020

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Figure 70.1 (a) Virus-Serum-Toxin act, (b) the USDA Animal and Plant Health Inspection Service (APHIS) Center for Veterinary Biologics, and (c) catalog of licensed products. *Source:* U.S. Department of Agriculture (USDA), https://www.aphis.usda.gov/animal_health/vet_biologics/publications/currentprodcodebook.pdf.

Host-range mutants are developed by serial passage in a host system different from the natural host to be vaccinated, usually laboratory animals, embryonated chicken eggs, or, increasingly, cell cultures. Upon serial passage in these systems, viruses often lose their virulence for the natural host due to accumulation of mutations in the viral genome that result in changes in virus-specified proteins. The basis of attenuation of many modified-live virus vaccines, however, is poorly characterized, and the possibility of reversion to virulence after growth in the natural host is always a concern.

Conditional lethal mutants have been generated with the intent that such viruses would exhibit limited replication in the host and so serve as vaccines. Temperature-sensitive mutants are typically created by mutagenesis and phenotypically selected on the basis of temperature. Cold-adapted mutants are generated by propagation at successively lower temperatures, the end product being incapable of replication at normal body temperatures. The cold-adapted mutants typically acquire multiple mutations in genes encoding virulence and are relatively more stable than temperature-sensitive mutants.

A unique approach to the expression of cloned viral genes is the use of heterologous viral expression vectors. The vaccinia virus has widely been used for many years as an infectious vaccine expression vector because (i) it has been widely used as a human vaccine and (ii) at least 22 kilobases of the vaccinia genome can be deleted without loss of infectivity. The latter attribute provides ample space in which to insert foreign cloned genes and has the potential to permit insertion of multiple foreign viral genes to design multivalent and multiviral vaccines. A major potential advantage of such infectious vaccine vectors is the potential for induction of both humoral and cellular immunity by inserting expressed viral proteins into the host cell membrane in context with histocompatibility antigens.

Construction of deletion mutants is another potential mechanism of virus attenuation; thus, the selective deletion of genes that express factors for virulence, persistence, or immunosuppression can often be accomplished without compromising viral replication. This approach requires a thorough understanding of the virus and the pathogenesis of the respective disease. A deletant vaccine has been developed to prevent pseudorabies in swine. The thymidine kinase gene is deleted in the pseudorabies vaccine strain, which is able to induce an immune response without producing disease. Furthermore, the genes encoding the virus glycoproteins gpI, gpIII, or gpx are deleted in the vaccine strain. The presence of an antibody to these specific virus antigens can be used to differentiate field-strain infected animals from vaccinated animals, an important advance in defining the epidemiology and control of this disease. These vaccines are referred to as DIVA (differentiation of infected from vaccinated animals) vaccines.

Nonreplicating recombinant viral vectors that are not capable of replication *in vivo* but that can express foreign proteins during abortive infections can induce humoral and cellular immunity in immunized hosts. Experimental studies show that dogs or cats are resistant to wild-type rabies viral challenge when inoculated with avian pox-rabies glycoprotein recombinant viruses. There are effective commercial recombinant canarypox-vectored vaccines for canine distemper (hemagglutinin and fusion protein antigens) and feline leukemia, and rabies (glycoprotein G) vaccine for cats. This type of viral vaccine has the distinct advantage of being safe even in the immunosuppressed host.

There are advantages and disadvantages to all vaccines, including the attenuated viral vaccines. Table 70.2 lists the general characteristics of live attenuated as compared to killed viral vaccines. A major advantage of live viral vaccines is their ability to replicate within the host and thereby elicit both humoral and cellular immune responses. In the case of viral infections that attack primarily the mucosal surfaces of the respiratory and gastrointestinal tracts, administering attenuated viruses by nasal and/or oral routes stimulates local immunity. Therefore, local routes of administration are often more effective than systemic administration. Economic considerations also favor attenuated vaccines due to the lower cost of production and because adjuvants, immunopotentiating agents, and multiple immunizations are typically not required.

Although attenuated viral vaccines continue to be the most commonly used veterinary vaccines, they have some serious disadvantages. With some vaccines, attenuation may result in complete or partial loss of immunogenicity. Thus, modified-live vaccine strains of viruses may require a compromise between loss of virulence of the virus and loss of its immunogenicity because some viruses exhibit reduced immunogenicity as they become attenuated. Furthermore, accurate assessment of viral attenuation can often be difficult because experimental reproduction of clinical disease is difficult with certain viruses. In such instances, a vaccine virus considered to be attenuated may not provide complete protection or it may produce clinical disease under special circumstances, involving stress, physiological imbalance, or concurrent infections with other organisms. If any of these circumstances occur, the vaccine is removed from the market and improved. Viruses that have a wide host range are also problematic. Virus attenuated for one animal species may retain virulence for more susceptible species. If the vaccinated animals shed

Table 70.2 Relative advantages and disadvantages of live versus inactivated virus. vaccines.

Criterion	Live	Inactivated
Immunity	Long	Short
Adjuvant	No	Yes
Safety	Variable	Usually very safe
Complications (potential)	Reversion to virulence, spread to susceptible animals	Sensitization
Potential contamination	Possible	Minimal
Interference	Possible	Minimal
Cost	Minimal	Significant
Immunomodulation	Not required	Required
Vaccine marker	Possibly genetic marker	Serological marker
Stability	Poor	Good
CMI induction	Yes	No
Local secretory immunity	Yes	No
Reassortment/ recombination	Possible	No
Persistent	Yes	No

the attenuated virus in the environment, transmission to susceptible species may occur.

A major concern with the use of attenuated viruses is reversion to virulence. This phenomenon has plagued vaccine development and licensing over the years. Reversion to virulence is a more serious possibility with those viruses that enjoy a wide host range or that are biologically transmitted by arthropod vectors. Although the virus may appear stable in the host for which the vaccine was intended, reversion to virulence may occur in the vector or in another species. Vaccination of pregnant animals may also be problematic, as attenuated viruses may be pathogenic for the developing fetus. Vaccinating animals with reduced immunologic responsiveness can result in expression of clinical disease and death.

Additional negative features of attenuated virus vaccines include (i) the potential for reassortment (viruses with segmented genomes) or recombination between vaccine strains or with wild-type viruses to create new viral strains, (ii) lack of a vaccine marker for serological differentiation of vaccine and wild-type virus exposure, (iii) development of persistent infections, (iv) poor stability of vaccine viruses, especially in hot tropical areas, and (v) replication interference between viruses in multivalent vaccines. Viruses that exhibit continued antigenic drift present another dilemma because new isolates must be continually attenuated and tested for safety and efficacy.

Inactivated Virus Vaccines

Many inactivated vaccines have been developed for use in veterinary medicine. Virus inactivation has most commonly employed formalin, β-propiolactone, acetyl ethyleneimine, or binary ethyleneimine. Additional methods include ultraviolet light, gamma irradiation, psoralen compounds, and ozone gas. The primary advantage of inactivated virus vaccines is safety – many potential disadvantages of live virus vaccines are eliminated as no virus replication occurs. Viruses for inactivation have been produced in laboratory animals, embryonated chicken eggs, and, most commonly today, in cell cultures. From an economic viewpoint, viruses that grow to high titer in cell cultures and exhibit first-order inactivation kinetics provide the best candidates for vaccine preparation. Adjuvants are typically required to induce good immunity with killed viral products, and multiple doses are usually required to provide an active immunity. Some highly effective adjuvants can lead to undesired consequences that include severe local tissue reactions, reduced weight gain in growing animals, systemic hypersensitivity, and even death. With the continued development of better adjuvants and ISCOMs, inactivated vaccines will become more effective. Inactivated vaccines are also relatively stable under adverse environmental conditions, and their potential for strain interference in multivalent preparations is reduced compared to attenuated vaccines.

There are, however, certain disadvantages associated with the use of inactivated virus vaccines. Some inactivating agents are toxic, and some are carcinogenic. Also, inactivating agents may be present at very low concentrations in the final product. Unlike a live virus, an inactivated vaccine virus is not quantitatively amplified, so it requires adjuvant and multiple inoculations. Furthermore, such preparations do not induce strong cellular immunity, because inducing such responses requires that the antigen be presented in association with histocompatibility antigens on cell surfaces (processed via the endogenous pathways; see the preceding text). Nor are such vaccines associated with the development of local secretory immunity due to the usual parenteral route of vaccination.

The success of viral inactivation depends on the characteristics of the inactivant and virus. Although most viruses can be successfully inactivated, the retention of critical antigenic integrity is variable. Antigens responsible for inducing protective immunity must be preserved. A possible complication of inactivated vaccine use is the potential for animal sensitization such that an exacerbated clinical disease is experienced upon exposure to a virulent field virus. This sensitization is not well understood, but it is often immunologically precipitated by atypical immune responses such as incomplete antibody responses that lack effective avidity, antibody responses to non-neutralizing epitopes, preferential stimulation of IgE (antibodies that mediate type I hypersensitivities), or other aberrant stimulation of immune-mediated inflammation.

Development of new subunit vaccines is currently an area of extensive research; it includes purification of viral subunits, recombinant technology, and peptide synthesis. The basis of a subunit vaccine is an immunogenic protein (or peptide sequences) capable of eliciting protective immunity. Such proteins would typically be found on the virion surface and contain epitopes capable of inducing neutralizing antibodies. Vaccines can be prepared by disruption of the virus followed by protein purification. The potential cost of such preparations has sometimes precluded their commercial development, but recent biotechnological advances have offered alternatives via recombinant DNA and peptide synthesis technologies.

The approach to developing recombinant vaccines involves insertion of DNA that contains the desired viral genomic coding sequences into an appropriate expression vector. The viral or complementary DNA (cDNA) is inserted into a plasmid or bacteriophage followed by infection of susceptible prokaryotic cells such as *Escherichia coli*, yeast cells, or mammalian cells. Multiple strategies have been used to construct vaccine expression vectors, and most include a strong promoter (constitutive or inducible). Following infection of the cell with the plasmid, the cloned cDNA can be expressed, and the desired gene product purified for vaccine use. Over the last 15 years, attention has also been focused on the inoculation of plasmid DNA encoding viral antigens directly into animals. Such DNA vaccines offer the potential advantage of having viral proteins and glycoproteins expressed on the surface of transfected cells and inducing immunity without interference from passively acquired viral antibodies.

Synthetic peptide vaccines have also been developed. As with cloned viral vaccines, the successful development of synthetic peptide vaccines requires extensive knowledge of the viral proteins involved in inducing protective immunity. Two basic approaches are available for determining critical peptide sequences: (i) indirectly, from nucleotide sequences derived from cloned viral genes, and (ii) directly, by sequencing purified peptides. Immunologically based peptide and epitope mapping to determine the regions involved in protective immunity facilitate the latter approach. An additional approach to determining critical peptide sequences is based upon the projected tertiary structure of the viral protein, with the areas that demonstrate hydrophilic characteristics serving as candidate sequences. One major drawback of synthesizing peptide vaccines is the potential for critical epitopes to be formed by the tertiary structure (an epitope formed by juxtaposition of two separated peptide sequences). Such epitopes confound attempts to deduce the peptide sequence and realize such complex configurations in the synthetic product.

Anti-idiotypic antibodies have also been used as immunogens to stimulate the production of viral neutralizing antibodies. The advantage of this type of immunogen may overcome viral variability problems by inducing broadly neutralizing antibodies. However, this type of vaccine induces only antibody responses in most cases. Production and formulation technologies have not been developed for these vaccines.

Nucleic Acid Vaccines

DNA vaccines are those in which the gene encoding the antigen in question is inserted into a plasmid vector that has a strong promoter (e.g. cytomegalovirus immediate/early promoter and SV40 early promoter) that will result in expression of the target gene, a termination sequence (polyA tail), and a number of cytidine–phosphate–guanosine (CpG) motifs. The function of the CpG motif (a motif common in bacterial genomes) is to direct the antigen-processing cell to secrete lymphokines that favor T_{H1} lymphocytes. The construct is administered in any number of ways (intramuscular, intradermally, upon a mucosal

surface), but intramuscularly is the most common. Myocytes that become transfected serve as antigen-presenting cells and express antigens in the context of MHC-I (turn on CD8 T lymphocytes). It is unclear how an antigen is expressed in the context of MHC-II (for CD4 T lymphocytes). Possibilities include MHC-II antigen-presenting cells (macrophages/dendritic cells/B lymphocytes) becoming transfected, or transfected myocytes transferring the plasmid construct to MHC-II antigen-presenting cells.

DNA vaccines have been experimentally successful in eliciting protective immune responses (both humoral and cellular) to a variety of bacterial, viral, and protozoal microorganisms. Commercial DNA vaccines are now available for vaccinating horses against the West Nile virus and as an oral therapeutic vaccine to treat malignant melanomas in dogs.

More recently, RNA vaccines have been developed where the individual constructs are in essence mRNA molecules that encode for rapid expression of viral antigens inducing a focused and protective immune response. This approach has been experimentally and clinically successful for immunity to coronaviruses such as the SARS-CoV-2 vaccines available for humans in early 2021. The specific RNA segments selected generally encode for surface antigens that drive effective neutralization and clearance of the virus, thus preventing infection and significantly reducing transmission. In addition, the process of immunization mimics viral infection, and the vaccine-derived immune responses are very similar to convalescent cellular immune responses. The mRNA vaccine avoids the need to utilize inactivated whole-virus particles or exposure to live-attenuated virus.

Toxoids, Bacterins, and Bacterial Vaccines

As with viral vaccines, the basis of an effective toxoid, bacterin, or bacterial vaccine is the ability to induce an immune response or responses capable of eliciting protection from field exposure to the pathogenic microorganism. Most of the principles outlined above with respect to viral vaccines apply to products designed to induce protective immunity to bacterial agents. The development of an efficacious product depends on understanding the pathogenesis of the bacterial disease that is to be prevented. However, in general, it is more difficult to develop a vaccine to a bacteria or fungal pathogen than it is to a virus. Most pathogenic bacteria have multiple virulence factors, and neutralization of one such factor may only partially reduce the virulence of the infection. Vaccines to bacterial infections often do not prevent infection. In contrast, many attenuated viral vaccines will prevent infection.

In general terms, diseases produced by bacteria can be grouped into three categories: (i) those that result from association with a bacterial toxin, (ii) those that result from the sequelae of extracellular multiplication of the bacterial agent, and (iii) those that result from the sequelae of intracellular multiplication. In addition, bacterial diseases may result from a combination of these factors.

Toxoids

Bacterial toxins are of two kinds: exotoxins and endotoxins. Endotoxins are strictly defined as the lipopolysaccharide portion of the gram-negative cell wall (it is the lipid A portion that is specifically responsible for the "toxic" manifestations). Muramyl dipeptide, which is present in gram-positive cell walls and to a lesser extent in gram-negative, also has "toxic" properties. We have used quotation marks around "toxic" because both endotoxin and muramyl dipeptide elicit their "toxic" activities by inducing the production of a variety of cytokines by host cells. It is the degree of vigor of the host response that defines the toxicity. Exotoxins are proteins that interact with host cells (usually after binding to a specific receptor), resulting in deregulation of host cell function without undue harm to the cell, interference of the normal physiology of the host cell(s), or death of the host cell.

Antibodies elicited to various epitopes on toxins that result in neutralization are sometimes called antitoxins. As mentioned previously, an antibody may block interaction between a toxin and its cellular receptor or change the configuration of the toxin so that it no longer has an effect on the host cell. Antibodies to exotoxins have been shown to be efficacious in preventing disease. Antibodies to endotoxins have had mixed results in preventing disease.

Toxoids are toxins without toxic activity that can elicit an immune response, that is, antibodies (see above for explanation). Toxoids can be produced by chemical inactivation of the native toxin or by manipulations of the gene encoding the toxin so that the toxin is inactivated. For example, in the case of A–B heat labile toxins (see Chapter 6), where the A subunit is responsible for the toxic activity of the toxin and the B subunits are responsible for binding of the toxin to the host, the gene encoding the A subunit can be eliminated, and a toxoid produced that is composed of B subunits. An antibody to lipopolysaccharide (endotoxin) is elicited by immunization with mutants (called "rough" mutants) that produce very little of the O-repeat unit of the lipopolysaccharide (see Chapter 6).

The main advantage of toxoids is that they are safer than the disease-associated toxin. Toxoids administered parenterally elicit antibodies (IgM and IgG) that interfere with toxin–host cell interactions that are not at a mucosal surface. On the other hand, the administration of toxoids on a mucosal surface elicits antibodies (sIgM and sIgA) that interfere with toxin–host cell interaction at the mucosal surface. The main disadvantage of toxoids used for

immunization by way of a mucosal surface is their extremely short half-life; thus, they are unable to stimulate an immune response that is sufficient for protection.

Bacterins

Bacterins are killed pathogenic bacteria. They are usually produced by chemical killing of the infectious agent, with the aim of preserving bacterial structures expressing epitopes important in eliciting a protective immune response. The immune response to bacterins is an antibody response similar to that elicited by toxoids as described above in the toxoids section.

The advantage of bacterins is that they are safe. If administered parenterally, the antibody elicited will be effective if the bacterin is made from a pathogen that has an extracellular life cycle. If the bacterin is administered by way of a mucosal surface, the antibody elicited (sIgM and sIgA) will interfere with interactions between pathogens and host cells. The disadvantage is that the main immune response may be antibodies, so that only antibody-mediated protection will occur. Thus, bacterins and toxoids administered parenterally are not as effective against intracellular pathogens. Bacterins placed on a mucosal surface have extremely short half-lives, a serious disadvantage. Another disadvantage is that the pathogen is usually grown *in vitro*, and epitopes expressed *in vivo* may not be expressed. This can result in a product that elicits antibodies with inappropriate specificities or incomplete profiles.

Bacterial Vaccines

Bacterial vaccines are composed of attenuated versions of the pathogen; that is, they are live but reduced in virulence. Attenuation may be accomplished in a number of ways: selection of a naturally occurring attenuated strain, repeated passage on artificial media, or elimination of a virulence trait by mutation of the gene encoding the trait.

The major advantages of bacterial vaccines are directly related to their ability to replicate and express some residual virulence. Live vaccines not only have longer half-lives than their inactivated counterparts (regardless of location) but they will also express epitopes that may only be expressed *in vivo*, thus eliciting antibodies to epitopes that the pathogen will also express following infection. Another advantage is that live vaccines will elicit antibody and cellular immunity. A major disadvantage is that live vaccines may produce disease, for example, through reversion to the virulent phenotype. Also, if the vaccinated host has reduced resistance or if used in alternative host species, then the vaccine may produce disease.

Although there are several good examples of successful use of attenuated bacterial vaccines, there have also been recent developments to reduce the residual virulence and overall safety of these vaccines. Attenuated strains of *Salmonella* have been developed that will undergo autolysis after limited replication, thereby drastically reducing the potential for persistent infection with the vaccine as well as reducing any risks of reversion to virulence. These strains can not only be used for immunization against salmonellosis, but can also be expression vehicles for multiple and combined antigens. This system could be delivered at mucosal surfaces or parenterally by injection.

Special Considerations for Agricultural Uses of Vaccines

Vaccine development for agricultural use (food animals) differs from that for human and companion animals. In humans, vaccine safety for each individual is of paramount importance, whereas disease control measures for farm animals must be cost-effective and vaccines with less than ideal safety and efficacy could be approved for use. Whereas it is common for human vaccines to be marketed at more than a hundred dollars per dose, the price for vaccines used in livestock industry is usually no more than a couple dollars per dose. Consequently, financial resources for animal vaccine development are significantly less than those for human vaccines.

Summary

As described in this chapter, there are numerous types of vaccine in use in all sectors of veterinary medicine. An exhaustive discussion of all examples is beyond the scope of this chapter. Figure 70.1 provides a link to and a brief descriptive overview of the definitions and regulatory framework for veterinary biologicals.

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Disinfection and Sterilization

John Dustin Loy, D. Scott McVey, and M.M. Chengappa

The concepts of sterilization and disinfection are of great importance to veterinarians. The sterilization of dressings, surgical instruments, and syringes is a common procedure as is the disinfection of kennels, infected premises, laboratories, and contaminated footwear. As many infectious agents can be transmitted on inanimate objects that require disinfection, and sterility is required for many surgical procedures, an understanding of the principles of sterilization, disinfection, and antisepsis is necessary.

Sterilization: This is the process whereby all viable microorganisms are eliminated or destroyed. Sterilization includes the destruction of microbial spores, the most resistant form of microbial life.
Disinfection: Disinfection involves the destruction or significant reduction of pathogenic organisms associated with inanimate objects or surfaces, usually by physical or chemical means. All disinfectants are effective against the vegetative (growing) forms of organisms to varying degrees, but not necessarily against their spores.
Antisepsis: Antisepsis involves the inactivation and destruction by chemical means of microbes usually directly associated with an animal or tissue. Antiseptic agents may be bactericidal or bacteriostatic. Bacteriostatic implies the inhibition of multiplication or growth, which, unlike the bactericidal effect, may be reversible. For ordinary purposes, the terms *disinfectant* and *antiseptic* are often used synonymously.

Sterilization

Sterilization indicates that a product, device, or solution is completely free of microbial forms of life, including spores and viruses. Sterility can be achieved through numerous avenues including dry or moist heat (autoclave), gases, or radiation. The most common way of achieving sterility in veterinary clinics or laboratories is through the use of a steam autoclave (see Section 71.2.2).

Complete incineration or complete alkaline digestion can be used alone, or in combination, to achieve sterilization. These methods may also be combined with autoclaving in some high-risk situations, as in biocontainment laboratories. In all situations where disinfection or sterilization procedures are utilized, strict adherence to safe working practices is required to protect workers and the environment from the extreme temperatures and harsh, dangerous chemicals that may be associated with the process.

Physical Methods

Boiling

Boiling water or steam (common instrument, sterilizer) at 100 °C is widely used in veterinary practice for preparing syringes, needles, and instruments for minor surgery. This process kills vegetative forms of microorganisms and viruses in five minutes. Many spores are also killed at 100° C as well, but many of the more resistant spores, for example, *Clostridium* sp. and the common *Bacillus* sp., can survive boiling for several hours. Thus, although boiling kills many pathogenic organisms, it is not sufficient for sterilization.

Steam under Pressure (Autoclaving)

The most resistant spores are killed by maintaining a temperature of 121 °C for 15 minutes. This temperature is obtained at sea level by steam at a pressure of 15 pounds per square inch in excess of atmosphere pressure. The autoclave, which is a metal cylinder, is an essential piece of equipment in microbiology laboratories and in operating

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rooms. Many veterinarians have small autoclaves for sterilization of instruments, dressings, solutions, and surgical packs available for clinic use.

Steam autoclaves function by removal of air and injection of steam into a chamber, where it reaches a saturation point, "saturated steam." Pressure is then increased to allow the steam to reach sufficient temperature. In order to obtain a temperature of 121 °C, all air must be removed. In the large modern, high-vacuum autoclaves, 98% of the air is removed using a vacuum pump. Air is removed in two stages; in downward displacement autoclaves, steam is admitted at the top of the chamber and residual air driven out at the bottom. The effectiveness of steam sterilization is attributable to the fact that when it condenses on the item being sterilized, it liberates a large amount of latent heat. The shrinkage resulting from condensation – a thin film of moisture on the surface of the autoclave load – draws in fresh steam and thus more heat. As this process continues, steam penetrates and surrounds the various items, producing a high uniform temperature. Therefore, the time and conditions for sterilization vary on the types of material and objects to be sterilized.

For pre-cleaned items such as surgery packs or surgical equipment, typically 20 minutes at 121 °C is likely sufficient for sterilization. For other materials such as biohazardous waste, 30–60 minutes at 121 °C at 15–17 psi may be required to achieve adequate sterilization. Autoclave manufacturers typically have preset options for different types of materials programmed into the sterilizer. The "gravity" settings on autoclaves allow for air to be evacuated through a gravity purge, which is typically sufficient for clean/porous items that steam can easily penetrate. A "vacuum" setting uses pressure and vacuum to evacuate the air from the chamber by force and is typically used for hard or solid waste items. Finally, a "liquids" cycle is often used that does not use vacuum and allows for liquids, such as media or liquid waste, to be sterilized without the risk of the liquid being forced from their vessels by the vacuum action.

To decontaminate and sterilize laboratory waste with high microbial burdens (such as biohazardous waste or microbial cultures), higher temperatures and times may be required; please see your autoclave manufacturer's manual for guidance regarding the proper sterilization of these materials.

One key to autoclave operation is verification of proper sterilization. Verification is typically performed through routine inclusion of both chemical (autoclave tape) and biological indicators (BIs) into the autoclave load. One rapid and convenient to do this is through inclusion of ampules containing microbial spores (typically *Geobacillus stearothermophilus*) which are included in the sterilization cycle and then can be incubated and checked for growth to ensure sterility. BIs should be included on a routine basis to ensure that proper autoclave function and that sterilization is occurring.

The following points should be kept in mind when using smaller autoclaves for clinic use:

1) Air and condensate must be removed for effective sterilization.
2) The required temperature of 121 °C must be maintained.
3) All mechanisms, such as gauges and timers, must be in proper working order.
4) Packs should be properly prepared. They should not be too large, nor should the wrappings be impervious. Volumes of fluid should not be too large. The load should be arranged to allow for penetration of steam.
5) The use of chemical and BIs should be routinely utilized to ensure proper functioning.

Steaming (Tyndallization)

This process has been replaced largely by filtration. It involves placing the media or solutions in flowing steam for one hour on each of three successive days. The period between steaming allows the spores to germinate and thus to be killed at the next exposure. Tyndallization requires a medium or solution that supports germination of the spores in the intervals between steaming. Bacteriologic media qualify in this respect.

Pasteurization

This process originally involved the heating of milk to the point that all human pathogens are killed. Since the introduction of the Pasteurized Milk Ordinance by the US Public Health Service in 1924, this has greatly reduced the incidence of zoonotic brucellosis and tuberculosis infections in humans. The occasional outbreaks of Group A streptococcal infections that can be spread by milk have also been eliminated by this process.

In the slow method of pasteurization, milk is held at 62.78 °C for 30 minutes, and in the flash method, the temperature is 71.67 °C for 15 seconds. A few heat-resistant vegetative bacteria (thermoduric) and spores may survive. The milk is rapidly cooled after pasteurization to discourage growth of any remaining viable organisms.

Ultraviolet Light

Direct sunlight kills unprotected vegetative organisms fairly rapidly, but spores are resistant to the bactericidal activity of sunlight, which is caused by the ultraviolet wavelength of the spectrum. Glass is impervious to this radiation. Sunshine is no doubt of great importance to the in the destruction of pathogenic organisms contaminating fields, pastures, and other areas often used by the livestock.

Ultraviolet light is a non-ionizing or low-energy radiation at an approximate wavelength of 2500 Å. Ultraviolet light from mercury lamps is widely used in biosafety cabinets, operating theaters, and animal quarters in other areas to reduce airborne infections. It acts mostly efficiently at temperatures from 27 to 40 °C. Ultraviolet radiation acts by causing errors in replication of DNA. The mutagenic activity of this radiation on living cells is well known. However, UV light lamps have significant disadvantages in that they require frequent changing in order to maintain sufficient output to ensure antimicrobial activity, and this activity can be difficult to assess. The antimicrobial activity is also affected by the distance from the UV source; therefore, proper exposure time and distance must be assessed depending on the environment.

Ionizing Radiation

This form of radiation has an energy content much higher than that of ultraviolet light and consequently has a strong disinfectant action. Gamma rays emitted from cobalt-60 are used commercially to sterilize syringes, needles, surgical sutures, dressings, bone grafts, plastic arterial prostheses, catheters, plastic petri dishes, and other heat-sensitive items. Radiation can also be emitted by electron beams that accelerate electrons and have the advantages of being ultra-short cycle and nonradioactive. Unfortunately, when ionizing radiation is used on foods, flavors may be affected, and when it is used on such items as drugs, hormones, and enzymes, the effect of gamma rays may be reduced.

Filtration

Filtration has been used for decades to sterilize bacteriologic media, serum, injection solutions, and other materials containing heat-sensitive substances. A pore size of 0.45 µm or less removes most or all bacteria from solutions. Membrane filters composed of cellulose esters and plastic polymers have largely supplanted other types of filters. The porosity of these membrane filters ranges from 0.1 to 10 µm. The core sizes are used for clarification before using smaller pore sizes for sterilization. Filters commonly used to remove bacteria cannot remove viruses or mycoplasmas. Air filters with a porosity of about 0.3 µm, commonly referred to as high efficiency particulate air (HEPA) filters, are used to filter air in laboratory rooms, biosafety cabinets, and operating theaters. These are commonly used when aerosols of infectious or contaminated materials may be generated, or when sterile surgical conditions are required.

Chemical Agents

Chemical agents act more selectively on microorganisms than do the physical methods such as heat and radiation. The following paragraphs discuss antiseptics and disinfectants that are used by veterinarians and microbiology laboratorians. The goal is generally to select the best disinfectant for a particular situation or task.

1) **What types of organisms are killed?** Spores are highly resistant compared with vegetative forms, and only a few disinfectants, halogens, hypochlorites, and ethylene oxide are effective in the concentrations typically used. Mycobacteria are more resistant than most other vegetative organisms. Many fungal agents are resistant to disinfection to some extent. Phenolic and alcoholic compounds are recommended for these organisms. Generally speaking, viruses are more resistant than vegetative bacteria. Halogens, oxidizing agents, and formalin are active against many viruses. Combinations of alcohol and newer quaternary ammonium disinfectants are very effective against many viruses given sufficient contact time.
2) **Inhibition of organism growth**. Organisms should be killed rather than merely inhibited. The quaternary ammonium compounds employed in high dilutions are bacteriostatic rather than bactericidal. Other disinfectant classes, such as aldehydes, oxides, and chlorine, are clearly bactericidal under most circumstances.
3) **Rate of action.** The rate of action differs greatly among the various chemical agents. Some act rapidly, whereas others are completely effective only after some minutes or even hours of contact time. Disinfectants often have an optimum pH range and are all more active at higher temperatures. Growing and multiplying cells are more readily killed than those in the resting or stationary state. All disinfectants are to some extent inhibited in their activity by the presence of organic matter, such as that found in feces, plus, exudates, discharges, and blood. Mercury salts, halogens, and quaternary ammonium compounds are especially inhibited by organic matter. In the disinfection of premises, stables, and kennels, the power of penetration of dirt, grease, and organic matter is important. Soaps and surface-active agents assist penetration.
4) **Side effects**. Other limiting considerations are toxicity, generation of irritating vapors, undesirable staining properties, and destructive effect on instruments and fabric surfaces. Anesthetics or disinfectants used on or around animals should be relatively nontoxic to tissues.
5) **Additional considerations.** Disinfectants should be reasonable in price and maintain their potency for long periods of time. They should be soluble in water and stable in aqueous solution.

Disinfectants are chemical compounds capable of killing most microbial forms. Disinfectants vary in their ability to

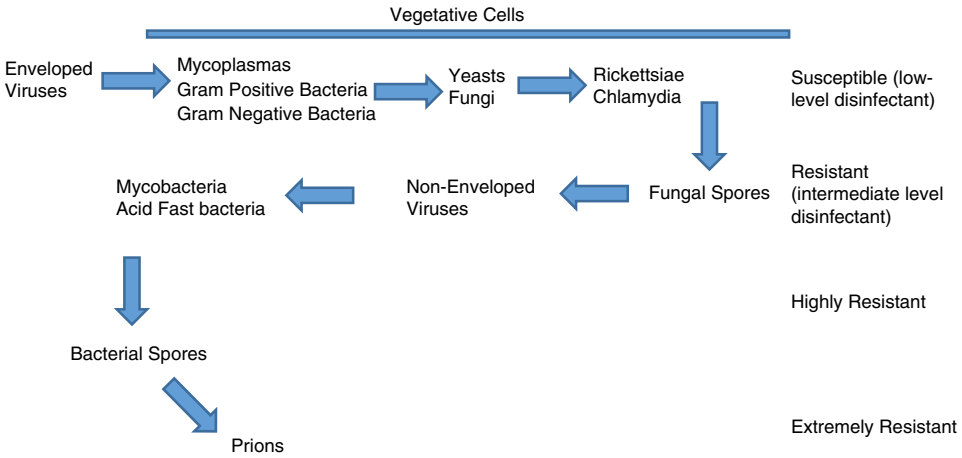


Figure 71.1 Relative resistance of microbial agents to sterilization and disinfection.

kill different microbial forms that may be more resistant including spores, mycobacteria, and some viruses and fungi. Chemical compounds are classified as germicides (capable of killing microbes) or sporicides (capable of killing microbes and spores). See Figure 71.1.

Disinfectants are classified as antimicrobial pesticides. They are regulated by the United States Environmental Protection Agency (US EPA) and are classified as sporicides, disinfectants, and sanitizers. Other regulations include those by the Food and Drug Agency (FDA), which classifies and regulates liquid chemical germicides as (i) high-level disinfectant (sterilant), (ii) Intermediate-level disinfectant, and (iii) low-level disinfectants. For most general laboratory disinfectants, a broad-spectrum, intermediate-level disinfectant is typically recommended.

The use of disinfectants is typically recommended to reduce environmentally mediated transmission, or laboratory-associated infections (LAIs). LAIs or vertical transmission of disease require a sufficiently virulent pathogen, sufficient dose, a mechanism for transmission to occur, a portal of entry into a host, and insufficient immune response in a host resulting in infection or disease. These conditions also hold for transmission of an environmental pathogen to and between animals, and all the conditions must be satisfied for environmental transmission of a pathogen. Disinfectants typically function by reducing the concentration of pathogens or potential pathogens within the environment, such as on laboratory surfaces, other surfaces such as clinical examination areas, or animal-holding areas such as kennels, pens, or bedding areas to reduce the risk of environmentally mediated transmission.

The efficacy of disinfection is affected by several factors. These include the forms of organisms present (i.e. vegetative cells vs. spores) and the concentration of organisms. Organic matter (dirt, manure, blood) inactivates disinfectants, so their presence impedes activity. The type of surface or material to be disinfected (i.e. porous, or non-porous, metal vs. plastic, etc.) as well as the environmental temperature and contact time must also be considered when selecting and utilizing a disinfectant. Product labels typically indicate the required contact time and types of disinfection potential for each product. Following the label indications to ensure adequate contact time and chemical concentrations is critical to maintain disinfectant efficacy.

Additional Disinfectants

Salts. Organic mercury compounds, such as mercurochrome and thimerosal, are less toxic than other mercuric compounds but they have only slight effects on activity.

Alkaline agents. Lye (sodium hydroxide) solutions are used in veterinary medicine for disinfecting stables and premises, as indicated later in this section.

Hydrogen peroxide. This oxidizing agent, available as a 3% aqueous solution, is useful for cleaning and disinfecting wounds, and there is a mouthwash in humans and animals with septic stomatitis.

Soaps lack bactericidal activity, but they are effective in the mechanical removal of organisms. However, the active washing appears to bring the resident flora to the surface of the skin of the hands, resulting in increased bacterial counts; therefore, the germicides hexachlorophene and triclosan are added to enhance antimicrobial properties.

Detergents

Detergent concentrates in the cell membrane and probably act by disrupting the normal function of the membrane.

Anionic detergents. These include the natural soaps. They are used as cleaning agents, and because they have little vector cell activity, they are combined with phenolic compounds.

Table 71.1 Common disinfectants.

Disinfectants	Example	Activity level
Aldehydes	Formaldehyde, glutaraldehyde	Intermediate- to high-level disinfection. Sterilization at prolonged contact times
Hypochlorites	Sodium hypochlorite	Intermediate- to high-level disinfection
Alcohols	70%	Intermediate-level disinfection
Phenolics	TekTrol®, One Stroke Environ®, Pine-Sol®	Low- or intermediate-level disinfection
Iodophors	Povidone-iodine	Low- or intermediate-level disinfection
Quaternary ammonium compounds	Roccal®, DiQuat®	Low-level disinfection
Alkalis	Sodium hydroxide, calcium oxide, ammonium hydroxide	Intermediate- to high-level disinfection
Acids	Acetic acid, citric acid	Low-level disinfection
Biguanides	Chlorhexidine	Low- or intermediate-level disinfection
Oxidizing agents	Hydrogen peroxide, Virkon® S	Intermediate- to high-level disinfection

Cationic detergents. These include many quaternary ammonium compounds such as Zephiran®. Because of their surface-active properties, are excellent cleansing agents for skins, burns, wounds, and inanimate objects. These are more active against gram-positive and gram-negative organisms. In high dilutions, they are bacteriostatic, but spores, viruses, mycobacteria and often *Pseudomonas* sp. are relatively resistant. This class of detergents is neutralized by soap anionic detergents.

Biguanide compounds. Chlorhexidine is the most commonly used disinfectant in this group. It is bactericidal but viruses, spores, and mycobacteria are relatively resistant. It is combined with quaternary ammonium compounds to increase its germicidal activity; this combination is called Nolvasan® and is widely used by veterinarians for preoperative treatment of skin, surgical scrubs, obstetric procedures, and milking in dairy operations.

Aldehydes. Glutaraldehyde (Cidex®) is the most widely used compound in this group. It has one of the widest spectrums of any disinfectant as it is bactericidal, virucidal, fungicidal, and sporicidal. A 2% aqueous solution buffered to pH 7.5–8.5 is useful for disinfecting surgical instruments and equipment, although steam sterilization is preferable. Spores are inactivated within three hours, and vegetative bacteria are destroyed in 10 minutes.

Resistance to Disinfectants

Some general rules broadly apply to types of pathogens and their resistance to disinfectants (see Figure 71.1). There are numerous disinfectants available on the market with different classes of chemicals. These chemical compounds have a spectrum of activity that varies depending on the class of organism, and label indications should be examined and followed for the pathogen of interest (Table 71.1). In general, the most susceptible pathogens are vegetative bacterial organisms and mycoplasmas (except mycobacteria or other acid-fast organisms) and enveloped viruses. Non-enveloped viruses and mycobacteria can be resistant to certain types or classes of disinfectants. For these agents, disinfectants with higher activities should be utilized, such as halogens (chlorine or iodine compounds) or aldehydes. Other resistant pathogens are bacterial spores, and disinfectants with sporicidal claims should be used to inactivate them. The most resistant are prions, which are resistant to a wide variety of chemicals and require high degrees of heat treatment and alkaline conditions to be inactivated (Table 71.1).

Acknowledgment

Materials from Dr. G.R. Carter, M.M. Chengappa, and A.W. Roberts. *Essential of Veterinary Microbiology, 5th Edition,* Williams and Wilkins.

Further Reading

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Epidemiology of Infectious Diseases

Natalia Cernicchiaro, Ana R.S. Oliveira, and Lee W. Cohnstaedt

Introduction

Infectious diseases remain the most significant health issue worldwide despite advances in preventive medicine, research, and detection. The rapid expansion and unpredictability of exotic and emerging pathogens can cause massive political, social, and economic devastation, as demonstrated in 2019 by the pandemic COVID-19 disease.

Infectious diseases are conditions caused by pathogenic microorganisms, such as bacteria, viruses, parasites, or fungi, that impair normal functioning and can be manifested by clinical signs. These diseases are considered highly dynamic, nonlinear systems as their responses are not proportional to changes in the population. Within these systems, populations are heterogeneous, comprising susceptible and infectious individuals, and for transmission of the infectious agent to occur, there must be an opportunity for these two types of individuals to make contact, directly or indirectly. Also, infection usually spreads exponentially until susceptible populations develop immunity or control measures are implemented.

Infection does not necessarily imply disease, as it may not lead to the development of clinical signs, which determines the occurrence of disease. Although hosts can become infected upon exposure to disease agents, infection may not lead to disease if either host or environmental conditions are not favorable to its development. *Disease,* however, *implies infection*, as diseases are always considered in terms of their etiologic agent (e.g. *Leptospira* spp. is the causative agent of leptospirosis).

Infections can be classified based on the size of the infectious agent as **microparasitic** (e.g. *Mycobacterium tuberculosis* as the causative agent of tuberculosis) or **macroparasitic** (e.g. *Toxocara canis* as the causative agent of toxocariasis). Microparasitic infections refer to infectious agents that cannot be counted singularly due to their small size. They are usually simple organisms, such as viruses and bacteria, that multiply rapidly and have a short duration of infection. They usually have high pathogenicity, low prevalence (or the number of existing disease cases in a population [at a certain point or period]), and persistence of infection requires new pools of susceptible individuals. Macroparasitic infections are those whose agents are larger in size, and their more complex life cycles involve various hosts; they do not usually lead to immunity as the pathogenicity is low, but prevalence is high, and agents largely depend on external determinants, such as weather, to cause disease.

Epidemiologists use the *epidemiological triad* to describe the causative nature of infectious diseases, as well as the communicability of the infectious agent (Figure 72.1). It portrays the three most important determinants of disease – agent, host, and environment – which may be represented as the vertices of a triangle, or a Venn diagram, with pathogen transmission occurring when there is overlap of all determinants, potentially leading to disease (Figure 72.1).

Agent factors include characteristics of the life cycle of the infectious organism and the **minimal infective dose** (the lowest dose required for transmission of the agent). The life cycle of an agent is the set of all stages required for its development from infection of a host to the infection of a second host. The **infectivity** of an infectious agent is determined by its ability to infect a susceptible host and to survive and multiply in that host, whereas **infectiousness** is related to how easy it is for the microorganism to be transmitted from one host to the other. The **pathogenicity** of an agent refers to its ability to induce pathological changes in a newly infected host and depends on host specificities. The **virulence** of an agent consists of its ability to multiply within the host at the same time it induces clinical signs, whereas **antigenicity** refers to its ability to be recognized by the antibodies that are produced following an immune response in the host.

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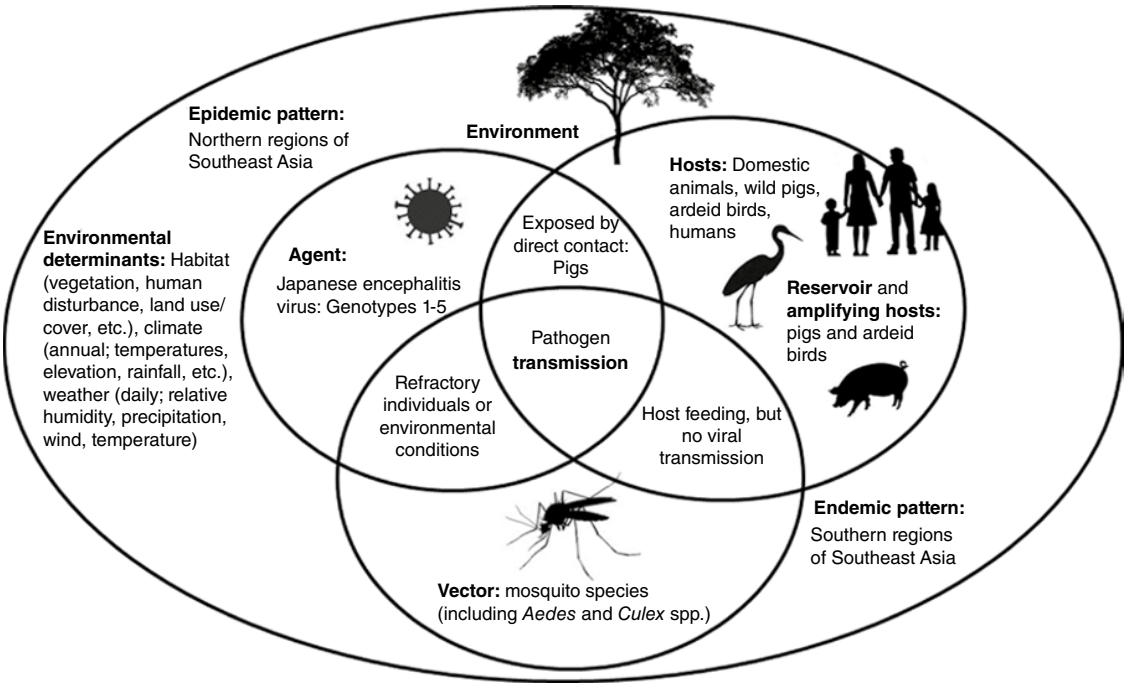


Figure 72.1 Representation of the epidemiological triad for Japanese encephalitis.

Host factors include heterogeneity and immunity. **Heterogeneity** relates to the innate resistance of vertebrate hosts to becoming infected, granted that the susceptibility to infection and to disease among individuals of a certain population differs regardless of their immunity, and is likely related to genetic factors. **Immunity** factors are those related to the immune response of hosts toward the infective agent. Other host factors contributing to their heterogeneity and immunity include age, sex, breed, species, and physiological state, among others. Infected hosts can be **amplifying** (those increasing the concentration of the agent), **reservoir** (those carrying the agent), or **dead-end** hosts (those neither amplifying nor carrying the agent). When the host recovers from an infection but is still able to infect other hosts (i.e. remains infectious), it is referred to as an **infectious carrier**.

Environmental factors that may affect the transmission of infectious agents include the size of the particles that contain the agent and biotic and abiotic factors outside the hosts, including weather, climatic characteristics, geographic location, specific habitats, altitude, precipitation, temperature, and land coverage.

A glossary of common terms is included in this chapter (Table 72.1). Note that concepts currently used developed from different scientific areas and evolved from those established in the nineteenth and twentieth centuries. It is also critical to recognize that using any single definition carries the risk of oversimplification, and there may be different appropriate definitions depending on the context. Likewise, not all infectious organisms may fit specific categories or definitions, and therefore there are exceptions that may not be mentioned in this chapter. Terms related to measures of disease frequency (e.g. prevalence, incidence, case fatality rate), which can be useful when describing the frequency and severity of an infectious disease, were also included and defined. The glossary presented here defines the most common terms used contemporarily in the field of epidemiology as it pertains to infectious diseases.

Development of Disease

Infectiousness and disease dynamics dictate the timeline of infection and the occurrence of disease within the host (Figure 72.2). These can vary based on individual host, environmental, and genetic (of agent, host, or both) factors or those related to the infection dose.

After **infection** of the susceptible host by the infectious agent, the **latent period** – the time period from infection to the development of infectiousness – follows. When infectiousness is established, the host animal is then able to pass on the infection to another host (**period of infectiousness** or **infectious period**), after which it either recovers from infection by developing immunity, being treated (becoming **noninfectious**) or by being removed from the population (following death, culling, selling or transfer to

Table 72.1 Common terms used in epidemiology of infectious diseases and their definition.

Term	Definition
Agent	Usually refers to an infectious microorganism or pathogen (virus, bacterium, parasite) [but it can also include chemical or physical elements].
Antigenicity	Ability of an infectious agent to induce an immune response in a host.
Basic reproductive number (R_0 ["R naught"])	A parameter representing the ease with which an infectious agent spreads within a population. Average expected number of new infectious hosts per infectious host during the period of infectiousness in a susceptible population at the beginning of an epidemic.
Carrier animals	Non-diseased animals that remain infectious after the recovery from the disease and may also be shedding the infectious agent.
Case fatality rate	Proportion of deaths resulting from disease cases over a specific time frame.
Curvilinear age pattern	Host-related pattern of disease incidence in which the risk is higher in adults, rather than young or older people (or animals).
Cyclical pattern	Incidence pattern in which the disease frequency fluctuates over a period of several years.
Determinant	Any factor that when altered produces a change in the frequency or characteristics of the disease. Also known as exposure, risk factor, independent, predictor or explanatory variable, as factors suspected of producing the outcome of interest.
Disease	A condition of the living animal, plant body or of one of its parts that impairs normal functioning and is typically manifested by distinguishing clinical signs and symptoms.
Disease communicability	Ability of an infectious agent to be transmitted from one individual to another.
Disease outbreak	The occurrence of disease cases beyond the number that would be normally expected within a geographical area or season.
Endemic disease	Disease that is present throughout the year in a given geographic area or that reoccurs frequently in that same area.
Environment	It refers to extrinsic factors that can influence the causative agent and the opportunity for exposure (e.g. weather, socioeconomic factors).
Epidemic disease	Disease spreading at a particular time in a given region and season and occurring in excess of what would be expected.
Epidemiology	Study of the distribution and determinants of health-related states or events in specified populations and the application of this study for the control of health problems.
Health	A state of complete physical, mental, and social well-being, and not merely the absence of disease. The condition of being sound in body, mind, and spirit.
Herd immunity	Indirect protection, or herd effect, occurs when the risk of infection in a population is reduced because a proportion of individuals within that population is immune to that specific disease.
Host	It refers to the individuals (animals, humans) that can get the disease.
Horizontal transmission	Mode of transmission occurring among animals, directly, indirectly or via airborne transmission, and regardless of generational relatedness.
Inapparent cases or silent infections	Situations in which an infected host does not develop clinical signs of the disease but is still able to infect other hosts.
Incidence	Rate (associated with the rapidity of change in a dynamic process) of new cases of disease in the population.
Incidence rate (incidence density)	The proportion of individuals that develop new cases of disease (or a condition of interest) in a defined population over a specified period of time (measures "speed").
Incidence risk (cumulative incidence risk)	Proportion of at-risk population that developed the disease of interest.
Incubation period	Time period during which the development of disease symptoms occurs.
Infection	Ability of the infectious agent (e.g. microorganism) to invade the host, multiply and elicit an immune response.
Infectious disease	Disease caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; diseases can be spread, directly or indirectly, from one host to another.

Table 72.1 (Continued)

Term	Definition
Infectiousness	The relative ease with which a microorganism is transmitted from one host to the other.
Infectivity	Ability of an infectious agent to infect a susceptible host and to survive and multiply in that host.
Latent disease	Disease situations with no apparent infection or clinical signs in the host despite the presence of genetic material of the infectious agent in that host.
Latent period	Period from infection to the development of infectiousness.
Linear age pattern	Host-related pattern of disease incidence in which disease risk increases with age.
Mode of transmission	The means by which an infectious agent is transmitted from one susceptible individual to another.
Multi-nodal age pattern	Host-related pattern of disease incidence in which disease risk is higher in different age groups.
Non-diseased period	Period between the disappearance of disease symptoms and host recovery.
Noninfectious period	Period following the recovery of the host by developing immunity, being treated, or being removed from the population (death, culling, or selling).
Pandemic	Epidemic occurring worldwide.
Pathogenicity (of the infectious agent)	Ability of the infectious agent to induce symptomatic disease in the host.
Period of infectiousness or infectious period	Period during which the host animal is able to pass on the infection to another host.
Persistent or chronic disease	Disease that persists for years or for life.
Preclinical disease	Disease with clinical signs yet to appear.
Prevalence	Relates to the number of existing cases of the disease of interest in the population (at a certain point or period).
Reservoir	Animal, arthropod, or substance in which an infectious agent lives and multiplies, on which it depends for its survival, and where it reproduces itself, so it can be transmitted to a susceptible host.
Route of infection	Route through which an infectious agent enters the organism of a susceptible individual.
Seasonal pattern	Incidence pattern in which a disease occurs following weather trends.
Secular pattern	Incidence pattern in which the disease follows gradual changes in frequency over longer periods (years).
Sporadic pattern	Incidence pattern in which a disease occurs in an irregular and infrequent fashion.
Symptomatic period	Period following the incubation period, when the host presents the symptoms of the disease.
Transmission probability	Probability of a susceptible host becoming infected with an infectious agent after contact with an infective source.
U-shaped age pattern	Host-related pattern of disease incidence in which there is a higher risk of disease in younger and older age.
Vector	An organism or vehicle that does not cause disease itself but which transmits the causative agent from one host to another.
Vertical transmission	Mode of transmission that occurs when animals transmit the infection to the next generation, *in utero* or via colostrum.
Virulence	Ability of an infectious agent to multiply within the host and induce clinical signs.

another location). Depending on the infectious agent, the infectious period varies in length (Figure 72.2).

Within the timeline of disease, infection of the host is followed by the **incubation period** (defined as the time period occurring between host infection and the detection of the agent), in which disease symptoms occur and the **symptomatic period** starts. The **communicable period** (not depicted in Figure 72.2) is the period in which the agent may be transferred from one host to another vertebrate host or invertebrate vector, and the **extrinsic incubation period** corresponds to the period between infection of a biological vector and its ability to transmit the agent to

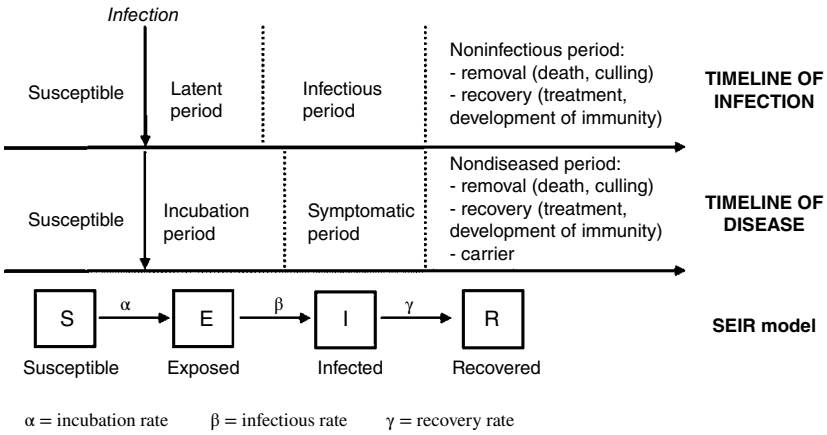


Figure 72.2 Timelines of infection and occurrence of disease and their relationship to the Susceptible-Exposed-Infectious-Recovered (SEIR) compartmental model.

a vertebrate host, an essential factor for the occurrence of disease in susceptible animals. After the symptomatic period, the **non-diseased period** and the **recovery** of the host (or removal from the population) follow (Figure 72.2).

Taking the example of mastitis in dairy cattle due to *Mycoplasma bovis* infection, although the incubation period is generally short (two to six days), it may last several weeks if infection persists in the herd. After that, clinical signs start to develop (symptomatic period) and include changes in milk quality, decrease in milk yields in a short period (three to five days), failure to respond to antibiotic treatment, atrophy of the affected udder quarters, and finally, agalactia (no milk production). The period of infectiousness may also precede the symptomatic period in cows, which is important in terms of disease management. Cows that do not recover from infection are usually culled.

Modes of Disease Transmission

The mode of transmission is the way in which an infectious agent is transmitted from one susceptible individual to another. Transmission may be **horizontal** (*direct, indirect*, or *airborne*), when transmission takes place among animals, irrespective of generational relatedness. **Vertical** transmission pertains to the transmission of an infectious agent from mother to offspring, transovarially via utero or egg, transplacentally (within the host or vector), or perinatally/colostrally via colostrum or milk (within the host). This form of transmission may be relevant to overwintering mechanisms of infectious agents, as it has been described for the Japanese encephalitis virus (Figure 72.1 depicts its main epidemiological features).

Direct transmission occurs when the infectious agent is transferred directly to the susceptible individual with no intermediate biological or mechanical element being involved. Transmission may occur via direct contact, for instance, through scratches or bites (e.g. Feline immunodeficiency virus [FIV] in cats), sexual intercourse (e.g. *Tritrichomonas foetus* in cattle), or via droplets (airborne or waterborne transmission) or blood (liquids) projected or sprayed onto the conjunctival or mucous membranes (eye, nose, or mouth droplets by sneezing or coughing) within distances of 1 m or less (e.g. Influenza virus). Contaminative transmission is another form of direct transmission, in which the infectious agent survives in the environment for long periods of time, and infection occurs when susceptible individuals become exposed to the agent, as occurs with anthrax. The probability of direct contact depends primarily on the frequency of contacts among individuals (susceptible and infectious) within the population, as well as the probability of transmission when a successful contact occurs.

Indirect transmission occurs through an intermediate medium (inanimate or animate object) by which the agent is passed from one susceptible individual to another. The period of time from contamination of the object to transmission to the new susceptible individual varies based on the agent, the object itself, and the individual. Inanimate objects (i.e. vehicles) are usually contaminated fomites, such as bedding materials, tools or equipment, soil, water or food, blood, sera, tissues, and other biological materials. Exposure may be single, multiple, or continuous. *Staphylococcus aureus* contamination in dairy milking machines is an example of indirect transmission via fomites. Animate objects (i.e. arthropod **vectors**) consist of invertebrate vectors, such as mosquitoes, flies, or ticks, that transmit the infectious agent either mechanically or biologically. Reservoir animals are also an example of indirect transmission by which the agent may be transmitted by another animal species, as occurs in rabies transmission (from bats to dogs).

Vector-borne mechanical transmission implies the mechanical transfer of the agent from vector to susceptible host (e.g. the agent is transported or carried by arthropods, such as flies, without any development or multiplication

stage taking place in the vector). **Vector-borne biological transmission** occurs when the infectious agent goes through a development or multiplication stage in the vector for it to become infective, as occurs with *Borrelia burgdorferi*, the causative agent of Lyme disease in dogs, which requires the infection of *Ixodes* ticks, or mosquito-borne pathogens, such as the West Nile virus. Transmission within the vector (in a single generation or multiple generations of that vector) can be transovarial or transstadial (passage from one developmental stage of an organism to another, e.g. from larvae to nymph or from nymph to adult).

Within the epidemiological triad framework, the role of the vector has been identified and can be incorporated as a fourth element (Figure 72.1). Because vectors modify the interactions occurring between host, infectious agent, and the environment, their role must be considered within the complexities of disease transmission. Vectors have an equal role as the host and the infectious agent within the environment, and they may represent a key point to control the pathogen, serving as a mitigation strategy that halts the spread of disease and limits agent transmission. Japanese encephalitis is a vector-borne disease whose understanding and management efforts focus on the vector as a central part of its epidemiology (Figure 72.1). The complex transmission dynamics of the Japanese encephalitis virus requires the use of this new conceptual tool to better address and intervene in all issues revolving around virus transmission and spread.

Airborne transmission refers to the dissemination of small (1–100 μm) airborne particles (i.e. aerosols) that contain the microorganisms that cause the disease and are generally transmitted via the respiratory route (e.g. Newcastle disease in poultry transmitted via nasal, mouth, or ocular discharges from infected birds).

Different infectious agents are transmitted in different ways, and their potential for spreading and causing disease depends on the characteristics of such organisms, including the rate of growth and route of infection. The **route of infection** refers to the specific route by which an infectious agent enters the organism of a susceptible individual and includes the oral, respiratory, urogenital, anal, conjunctival, and skin routes. More than one route may serve as an entry point for an infectious agent.

The ability of an infectious agent to be transmitted from one individual to another (e.g. person to person) is referred to as *disease communicability*. Hence, a **communicable** or **transmissible** disease is caused by an infectious agent (or the results of its toxic products) that is transmitted from an infected individual (or reservoir animal) to a susceptible host through physical contact (touch, sexual, fecal–oral, droplets), contact with contaminated surfaces or objects, food, blood or water; bites from insects or animals that can transmit the disease; and via air. Examples of communicable diseases include hepatitis A, B, and C; influenza; measles; cholera, *Salmonella,* and other foodborne illnesses; and tuberculosis, among others. A **noncommunicable** or **nontransmissible** disease, on the other hand, is not transmitted from one individual to the other, although it can also be caused by infectious (but also noninfectious) agents. Infectious agents include any microorganism originating from the environment or the individual's own flora. Noninfectious causes of disease refer to metabolic, immunologic, or nutritional imbalances. Congenital defects and environmental toxins are also considered noncommunicable sources of disease. Whereas noncommunicable diseases in veterinary medicine do not increase the probability of disease in a herd when one individual is affected, communicable diseases do, as a diseased animal may constitute a source of infection to the other individuals within the herd.

Other ways to transmit infectious agents are through inapparent cases or silent infections (or **nonclinical**, in contrast to **clinical disease**, in which clinical signs are apparent), which designate the situations in which a host does not develop clinical signs despite being infected and infectious (i.e. is able to infect other hosts with the agent). In the case of *M. bovis* mastitis infection, inapparent cases may occur (**subclinical** mastitis); that is, dairy cattle may be infected and shedding the agent, but showing none of the clinical signs that are characteristic of clinical mastitis, which makes disease control difficult. Diagnosis is often achieved by serological or culture methods. Besides subclinical disease, there are other types of nonclinical disease that include **preclinical disease**, designating disease with clinical signs yet to appear, **persistent** or **chronic disease**, referring to disease that persists for years or for life, and **latent disease**, in which the genetic material of the infectious agent remains present in the host, but no infection or clinical signs occur.

Lastly, carriers and reservoirs can also transmit infectious agents to susceptible individuals. **Carrier** animals are non-diseased animals that may nevertheless be a source of infection to other animals or the environment, as they are infected (transiently or persistently) and potentially shedding the agent, although they are not clinically ill. The carrier status may be short or chronic. **Reservoirs** are animals, persons, arthropods, plants, or substances in which the infectious agent finds appropriate conditions for survival and multiplication, allowing transmission to other susceptible hosts. Both vectors and intermediate hosts may be considered as reservoir animals. Reservoir animals may also serve as amplifying hosts when the agent multiplies in

that animal. For instance, pigs are the reservoir and amplifying hosts for the Japanese encephalitis virus, being a focus of infection when they receive an infectious bite from a competent mosquito species (Figure 72.1).

Describing Patterns of Disease

Some diseases are very rare in a population (e.g. rabies in the United States) to the point that the occurrence of one case may warrant an epidemiologic investigation, whereas others are normally present and often expected with only unusual deviations necessitating further investigation. As such, patterns of disease in populations can be defined in terms of their *level of occurrence,* distribution over *time* and *space*. **Endemic** diseases (e.g. Johne's disease in dairy cattle in the United States) are those that are usually present throughout the year in a given geographic area (i.e. constant) or that reoccur frequently in that same area (i.e. frequent). Endemic diseases tend to follow predictable patterns that represent a long-term balance between agent and host. **Epidemic** diseases are those that spread at a particular time in a given region and season and whose occurrence exceeds what would be expected. They occur in clusters in time, and their temporal distribution can be represented in an epidemic curve. Japanese encephalitis, for instance, follows these two different epidemiological patterns; whereas the disease is endemic in the Southern, tropical regions of Southeast Asia, the pattern of transmission is epidemic in the Northern regions, where the climate is temperate. The difference between the two patterns of transmission is due to temperature and climatic conditions that allow for the presence of mosquitoes, which are an important determinant of disease transmission and spread. A **pandemic** is an epidemic occurring over several countries or continents and affecting a large number of people. Examples include the influenza pandemic of 1918 and the COVID-19 pandemic of 2019.

A **Sporadic** pattern refers to a disease that occurs in an irregular and infrequent fashion (e.g. rabies, which occurs sporadically in dogs in countries where the disease has not been eradicated). A disease **outbreak** is defined as the occurrence of disease cases beyond the number that would be normally expected within a geographical area or season; it refers to an epidemic in a more limited geographic area. An outbreak may occur in a restricted region or in an extended area covering many countries, lasting for just a few days to years. Outbreaks can be described as common source (point source, continuous, and intermittent) when a group of individuals are exposed to an infectious agent from the same common source; *point source* refers to animals (or persons) being exposed over a brief period where disease among individuals occurs over one incubation period (e.g. foodborne disease outbreaks in humans). The range of exposure widens in a *continuous* outbreak, whereas in an *intermittent* common-source outbreak, the pattern reflects the intermittent exposure of individuals. Conversely, *propagated* outbreaks are spread from an animal (or person) to the other, rather than originating from the same common source. Propagated outbreaks usually last for longer periods than point-source outbreaks, and may lead to multiple peaks of infection, with the occurrence of secondary and tertiary cases (e.g. measles), occurring over more than one incubation period. Transmission can occur through several of the pathways described, including direct (e.g. syphilis, person to person), through vehicles (e.g. hepatitis B, needles), or vectors (e.g. yellow fever, mosquitoes).

Over *time*, diseases may also present patterns that are **seasonal**, **cyclical**, or **secular**. *Seasonal* patterns follow weather (climatic) trends and are usually present in vector-borne diseases, as vector (e.g. mosquitoes) breeding is usually weather dependent. Hence, seasonal trends of disease translate into fluctuations of disease frequency within a year. Similarly, a *cyclical* pattern follows the same fluctuations, but these can occur over a period of several years (e.g. influenza). *Secular* patterns are those in which there are gradual changes in disease frequency over longer periods of time (years). They can be associated with the reintroduction of a pathogen, mutation of an agent, or with chronic diseases (e.g. diabetes).

Disease patterns can also be described *spatially*. The geographical distribution of infectious diseases can reveal the occurrence of diseases globally, giving important information regarding burden of disease, biosurveillance and outbreak likelihood, and the emergence and evolution of infectious diseases. The geographical mapping of disease distribution can improve preparedness in bordering regions where the disease has not yet been reported.

Distribution patterns and disease severity may vary according to host characteristics, which include age, sex, breed (or race), biological characteristics (e.g. immune or physiological status), and other acquired aspects (e.g. marital status, economic status, occupation, or drug use). Age is one of the most important host characteristics when defining disease patterns. Patterns can be **linear**, when disease risk increases with age (e.g. cancer), **U-shaped**, when there is a higher risk of disease in younger and older people (e.g. influenza); **curvilinear**, when the risk is higher in adult age, rather than in young or older people (or animals) (e.g. sexually [venereal] transmitted diseases); and **multinodal**, when disease risk is higher in different age groups.

Understanding Infection Dynamics

To understand the dynamics of infection and to successfully assess the effects of interventions that may be implemented to halt transmission implemented, it is essential to determine transmission probabilities, as well as their variability within a population. The probability that a susceptible host becomes infected with an infectious agent after contacting an infective source is defined as the **transmission probability**.

The estimation of transmission probabilities depends on the characteristics of the infectious source (i.e. person/host, vector, or fomite), the infectious agent, the host, and the type of contact that is established among them. The type of contact needed for infection to occur is determined by the mode of transmission of each infectious agent, as well as its strain and infectiousness, and the susceptibility of the host.

To estimate transmission probabilities, one can (i) identify infectious individuals and the proportion of successful contacts with susceptible hosts (that result in transmission), that is, the **secondary attack rate (**SAR**)**; or (ii) identify susceptible hosts, the proportion of contacts with infectious individuals, and the resulting outcome, that is, the **binomial model**.

Contact patterns can be defined by density, as occurs with influenza cases, or by choice, which is the case with sexually transmitted diseases. Random mixing is a type of contact pattern in which each individual, person, or animal, has the same chance of contacting another individual, and thus be exposed to the infectious agent. However, groups tend to mix differently within populations. These can be described by a mixing matrix in which entries represent the rate of contacts of individuals within and between groups.

Another important parameter in the transmission of infectious diseases is the **basic reproductive number (R_0)**, which refers to the ease (or speed) with which an infectious agent spreads within a population. The R_0 value is considered a threshold parameter that helps predict the spread of an infection, providing a summary of several aspects of an infectious disease. Table 72.2 includes approximate basic reproductive numbers for well-known infectious diseases.

This parameter can be useful in understanding an outbreak, as well as the transmission dynamics of a certain infectious agent, and in assessing the strategies required to control an epidemic (vaccination efforts or other public health risk reduction plans). It can be further defined as the average expected number of new infectious hosts (i.e. secondary cases) per infectious host during the period of infectiousness in a susceptible population at the beginning of an epidemic (assuming that all contacts are susceptible ones and not including noninfectious secondary cases):

$$R_0 = \text{number of contacts per unit time}\left(c\right)* \text{transmission probability per contact}\left(p\right)* \text{duration of infectiousness}\left(d\right)$$

For instance, an $R_0 = 5$ for a specific disease means that an infectious case will produce five new infectious cases in that population. Basic reproductive numbers can help us elucidate the magnitude and risk of occurrence of an emerging infectious disease, as an $R_0 > 1$ indicates that an epidemic can occur. If $R_0 < 1$, it is expected that infection will cease. Disease transmission is a stochastic process; as such, there is always a chance component dictating transmission, where the size and timing of epidemics are hard to predict. Furthermore, the heterogeneity of contact rates, transmission probabilities, and duration of infectiousness lead to different R_0 values in certain clusters of individuals, within a population and in different populations.

In an epidemic, estimation of R_0 can be implemented during the early stages (during the exponential phase) or at its peak. For endemic diseases, in which a continued supply of susceptible individuals is needed (usually through birth or loss of immunity), the incidence rate of new infections in the population and the proportion of the population that is susceptible are needed to estimate R_0. For diseases with a stable prevalence, the prevalence of infection is also needed.

Estimates are specific to the infectious agent and host populations at a given time. For diseases transmitted indirectly, such as vector-borne diseases, R_0 values are calculated considering the components of transmission of the two host populations involved. Values of R_0 for vector-borne diseases are higher during mosquito seasons and lower off-season. Whenever contact rates peak, R_0 values will also be the highest.

Table 72.2 Approximate[a] basic reproductive numbers (R_0) for well-known infectious diseases.

Infection	Transmission route	R_0
Diphtheria	Saliva	6–7
Ebola	Body fluids	1.5–2.5
Influenza	Airborne droplet	2–3
Measles	Airborne	5.4–18 (3.7–203.3)[b]
Mumps	Airborne droplet	4–7
Polio	Fecal–oral	5–7
Smallpox	Airborne droplet	5–7

[a] These values are approximate as they do not necessarily reflect the range and diversity between populations.
[b] *Source:* Based on Guerra et al. (2017).

In real-life scenarios, where not all contacts are susceptible, as some people or animals may already be immune, one can estimate the effective reproductive number (R), in which the expected number of new cases is lower than R_0:

$$R = R_0 * x$$

where x is the proportion of contacts with susceptible individuals.

The value of R decreases as herd immunity increases. **Herd immunity**, **indirect protection**, or the **herd effect** occurs when the risk of infection in a population is reduced because a proportion of individuals within that population is immune to that specific disease. The presence or proximity of immune individuals, usually due to vaccination, protects the rest of the individuals that are susceptible, as the vaccine protects not only against infection, but also against infectiousness (e.g. by reducing nasal or ocular discharge of the infectious agent). The critical percentage, or the estimated vaccine coverage, that is needed to prevent the spread of infection, assuming the vaccine is 100% effective, can be computed as $1–1/R_0$. Other formulas exist to account for partially protective vaccines or vaccines that reduce the duration of the infectious period or the infectiousness of infected individuals.

This phenomenon is important when considering control and prevention measures against infectious diseases, as it allows the selective vaccination of certain groups within a population, thus reducing costs while maintaining targeted levels of protection. The selected individuals are those that play a major role in transmission and whose vaccination will indirectly help in slowing transmission. The magnitude of the indirect effect depends on the infectiousness of the infectious agent, the type of immunity that the vaccine induces, the overall immune status of the individuals within that population, and the transmission and contact patterns of the specific infectious agent in the population.

Modeling Infectious Disease Transmission

Modeling is helpful in depicting the likely progression of an epidemic, evaluating potential control strategies, and forecasting future outbreaks. Combined with experimental data, models can be used to predict disease transmission; combined with observational data, they help explain disease dynamics and can be used to calculate fixed parameters such as the recovery rate, or dynamic parameters, such as R_0 as it changes during an outbreak. When developing epidemiological models to predict transmission probabilities of infectious diseases, one should take into consideration the intrinsic assumptions that are embedded within the classical frameworks. By modifying and adapting these assumptions to the specific epidemiological scenarios, more accurate predictions can be obtained.

Transmission parameters can be estimated using *statistical*, *mathematical,* and *empirical*-based models. Models of disease transmission can be **deterministic**, when a given set of parameters produces a single outcome, or **stochastic**, when chance is incorporated in the model, as transmission events have a probabilistic nature and a probability distribution of possible outcomes is produced, rather than a single outcome. Stochasticity in the models helps define the certainty around the outcome.

Within *statistical* methods, spatial models, regression methods, and time-series analysis can assist surveillance efforts by estimating or predicting cases. Caution must be exercised when considering some of the ad hoc assumptions regarding the dependence between the dynamics of disease and the independent variables determining its transmission. The statistical properties based on the chosen distributions may not be readily applicable to emerging disease conditions. Although still constrained under some of their statistical properties, in time-series analysis and neural networks, assumptions of autocorrelation are relaxed, allowing incorporation of seasonal changes and forecasting of disease evolution, which can be useful when modeling certain infectious diseases.

To simplify *mathematical* modeling of infectious diseases, the population can be divided into compartments – hence their designation as *compartmental models* – assuming that individuals in the same compartment share the same infection status. Individuals can only be located in a single compartment at a given time, and they transition between compartments at specific rates. In particular, the **Susceptible-Infectious-Recovered (**SIR**)** model (Diagram 72.1) represents the simplest modeling framework that can be adapted to describe the transmission dynamics of a multitude of infectious diseases. The SIR model assumes that susceptible individuals (S) become infected and infectious at rate β (infection rate, R_0) and that infected individuals (I) recover (R) and become immune at rate γ (recovery rate, 1/duration of infection), so that $S+I+R$ equals the total population.

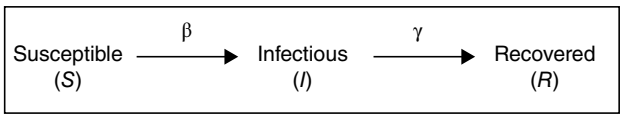


Diagram 72.1 Schematics of the Susceptible-Infectious-Recovered (SIR) epidemiological model of disease transmission.

This model is helpful in defining important parameters that are needed for modeling infectious diseases, such as contact rates (c), probability of transmission (p), incidence (i.e. rate of new infections in the population) (i), duration of the infectious period (d), R_0, and R.

Rate β represents the chance of individuals becoming infected within a defined period of time, which contrasts with incidence (i), a concept in epidemiology that stands for the rate at which new infections occur in an entire population. Thus, whereas the incidence of a certain disease may be low (as the proportion of susceptible individuals decreases during the course of an epidemic), β remains the same, as the chance of individuals becoming infected does not change.

SIR epidemiological models can be used to determine vaccine effectiveness. The reduction of virus transmission in susceptible calves during foot-and-mouth disease (FMD) epidemics in Europe has been assessed by estimating the R_0 after vaccination. In this example, infected/infectious calves (I) were those presenting clinical signs of FMD or testing positive in laboratory tests, S represented the total number of susceptible calves, and R the total number of recovered calves at the end of the experiment (not considering that calves could be carriers of FMD virus).

An extension of the SIR model that considers more complex interactions occurring between the infectious agent and host is the **Susceptible-Exposed-Infectious-Recovered** (SEIR) model. This model does not assume that an infected individual becomes immediately infectious, instead considering the possibility of a latent period. Moreover, after the infectious period, individuals may become immune, die, or be removed from the population at a certain rate (γ) (Figure 72.2).

The SEIR model has been applied to influenza outbreaks, which have known latent periods of one to four days and infectious periods lasting for four or five days more. By including a latent period in the model, rather than using the simpler SIR model, the results provide more accurate estimates of R_0 values (contrasting with the underestimation that results from ignoring the latent period in the calculations). Further improvements of the model use alternative assumptions in which the model is fitted to more realistic distributions of transmission probabilities (p) and also the incorporation of gamma-distributed latent and infectious periods (rather than exponentially distributed). These SEIR models better incorporate uncertainty, resulting in more accurate quantitative predictions to be used in public health management and control strategies.

By solving a set of **differential equations**, one can obtain estimates for different parameters that are key in disease transmission dynamics. These equations can be solved using software packages and fit to the data. An example of a dynamic model that studied livestock-to-human transmission of brucellosis in Mongolia applied a deterministic model using stochastic parameters (mostly using triangular and uniform probability distributions) fit to a specified period of disease transmission (with and without vaccination interventions in livestock). Adapting the model to the available data from that specific time period (1991–1999), the results were able to expose the trends in the different compartments that were considered: (i) sheep-to-sheep susceptible, (ii) sheep-to-sheep seropositive; (iii) sheep-to-sheep vaccinated; (iv) cattle-to-cattle susceptible; (v) cattle-to-cattle seropositive; (vi) cattle-to-cattle vaccinated; (vii) total Mongolian human population; (viii) new cases of brucellosis in humans annually; and (ix) recovered brucellosis patients.

One of the simplest stochastic epidemic models, the **Reed–Frost model,** is a "chain binomial" or iterative model in which the transmission probability relating the number of new cases to the total number of contacts is estimated in discrete time units. This model was developed in the 1920s by Lowell Reed and Wade Frost to accurately describe how diseases spread through populations. This model assumes that exposures to two or more infectious individuals in the same time period are independent events. In contrast, the **Greenwood model** considers the exposures to two or more infectious individuals as a single exposure. Although both models are used in epidemiology for understanding disease transmission, they have not been used to estimate the p parameter (probability of transmission). The Reed–Frost model has been applied to evaluate the within-herd transmission of tuberculosis in farmed fallow deer herds in Sweden by fitting the model to the observed herd incidence and thus estimate the number of effective contacts made by an animal during a specified (discrete) time period.

Network models help exploring individual-based and population-level models, while considering the impact of spatial structures on transmission. These models consider the heterogeneity in disease transmission (due to differences in susceptibility to infection, level of infectiousness, or contact rates) and the fact that networks are dynamic structures. Hierarchical networks can be used to model the spread of vector-borne diseases, such as Rift Valley fever, using either empirical data or stochastic parameters that include the most appropriate probability distributions to parameterize the model. This type of modeling approach helps identify at-risk areas and understand potential patterns of infection spread within populations and geographically, which helps planning and implementation of control and mitigation strategies during an outbreak.

Agent-based models can simulate complex epidemic systems as they consider details such as demographics, mobility of the population, transportation infrastructure of the simulated area, evolution of the disease within a host, and transmission between hosts. An agent-based simulation evaluated potential containment interventions to prevent the spread of the H5N1 influenza A pandemic in Southeast Asia. Data on mobility of the population, location of households, schools, and workplaces were considered among different interventions.

In recent years, **machine learning** techniques have been employed to detect early indicators of disease through the use of Web search and social platforms. Using Google Web queries, researchers can detect early cases of disease (e.g. malaria and influenza).

The research question at hand and the available data define the type and structure of the models to be fit. Models can be useful tools to estimate frequency and distribution of diseases, predict trends, understand disease dynamics, or predict the relative impacts of intervention strategies. To inform stakeholders and policymakers, more complex models incorporating risk mitigation strategies and social and economic considerations may be required. It is important to note, however, that models are an approximate and simplified description of reality and hence cannot capture all the intricacies of the complex processes involved in the dynamics of infectious diseases.

To enhance communication and decision-making, it is crucial to understand the lexicon of infectious disease epidemiology, enabling better acceptance and appreciation of disease outcomes, especially relevant in the midst of disease outbreaks.

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ICH *see* infectious canine hepatitis
Ics *see* intercellular spread
IEM *see* immune electron microscopy
IFA *see* indirect immunofluorescence assay
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