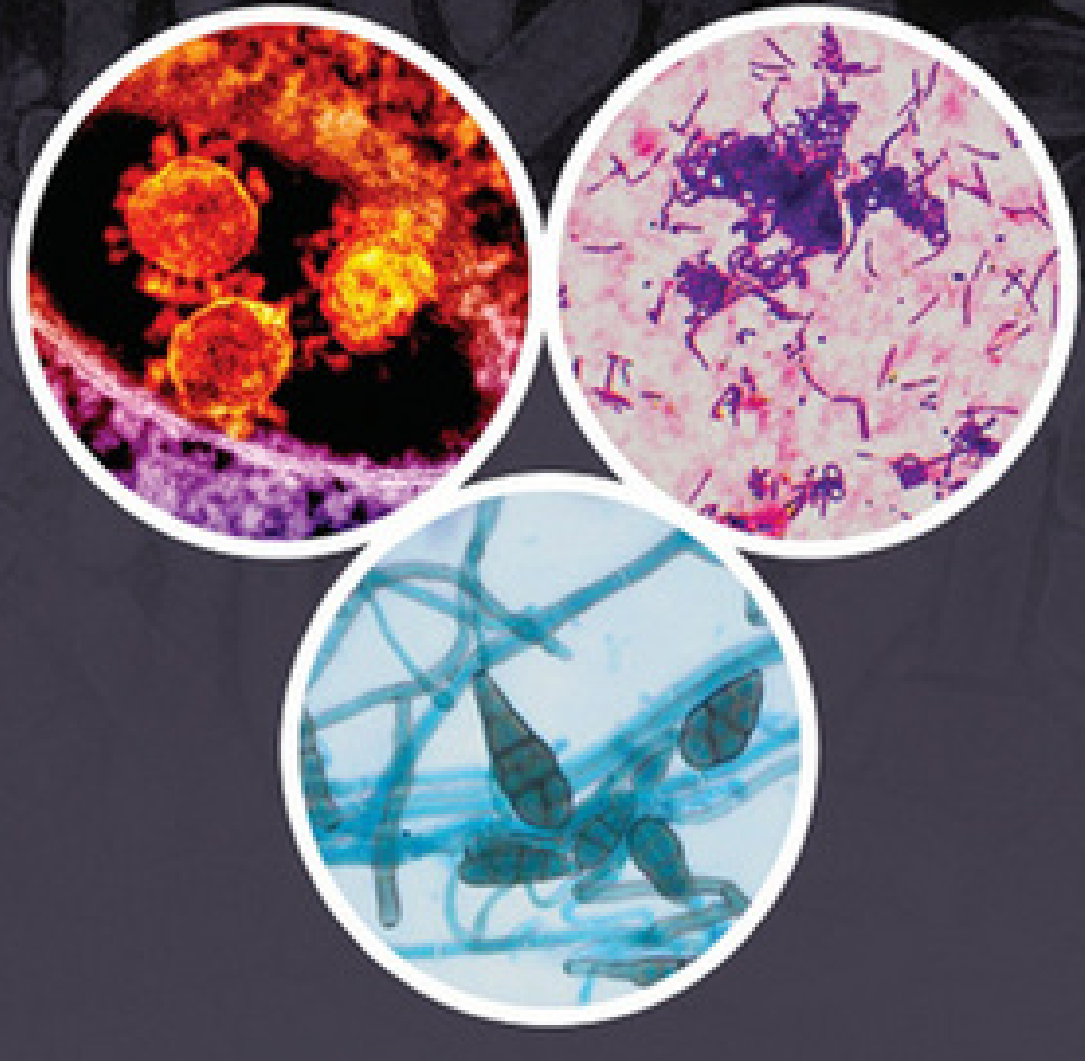

MEDICAL MICROBIOLOGY

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8TH EDITION

MEDICAL MICROBIOLOGY

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- *To all who use this textbook, that they may benefit from its use as much as we did in its preparation*

PREFACE

Our knowledge about microbiology and immunology is constantly growing, and by building a good foundation of understanding in the beginning, it will be much easier to understand the advances of the future.

Medical microbiology can be a bewildering field for the novice. We are faced with many questions when learning microbiology: How do I learn all the names? Which infectious agents cause which diseases? Why? When? Who is at risk? Is there a treatment? However, all these concerns can be reduced to one essential question: **What information do I need to know that will help me understand how to diagnose and treat an infected patient?**

Certainly, there are a number of theories about what a student needs to know and how to teach it, which supposedly validates the plethora of microbiology textbooks that have flooded the bookstores in recent years. Although we do not claim to have the one right approach to teaching medical microbiology (there is truly no one perfect approach to medical education), we have founded the revisions of this textbook on our experience gained through years of teaching medical students, residents, and infectious disease fellows, as well as on the work devoted to the seven previous editions. We have tried to present the basic concepts of medical microbiology clearly and succinctly in a manner that addresses different types of learners. The text is written in a straightforward manner with, it is hoped, uncomplicated explanations of difficult concepts. In this edition, we challenged ourselves to improve the learning experience even more. We are using the new technology on StudentConsult.com (e-version) to enhance access to the material. New to this edition, **chapter summaries** and learning aids are placed at the beginning of each of the microbe chapters, and on the e-version these are keyed to the appropriate sections in the chapter. In addition, many of the **figures** are enhanced to assist learning. **Details** are summarized in tabular format rather than in lengthy text, and there are colorful illustrations for the visual learner. **Clinical Cases** provide the relevance that puts reality into the basic science. **Important points** are emphasized in **boxes** to aid students, especially in their review, and the **study questions,** including Clinical Cases, address relevant aspects of each chapter. Each section (bacteria, viruses, fungi, parasites) begins with a chapter that summarizes microbial diseases, and this also provides **review material.**

Our understanding of microbiology and immunology is rapidly expanding, with new and exciting discoveries in all areas. We used our experience as authors and teachers to choose the most important information and explanations for inclusion in this textbook. Each chapter has been carefully updated and expanded to include new, medically relevant discoveries. In each of these chapters, we have attempted to present the material that we believe will help the student gain an interest in as well as a clear understanding of the significance of the individual microbes and their diseases.

With each edition of *Medical Microbiology* we refine and update our presentation. There are many changes to the eighth edition, both in the print and e-versions of the book. The book starts with a general introduction to microbiology and new chapters on the human microbiome and epidemiology of infectious diseases. The human microbiome (that is, the normal population of organisms that populate our bodies) can now be considered as another organ system with 10 times as many cells as human cells. This microbiota educates the immune response, helps digest our food, and protects us against more harmful microbes. Additional chapters in the introductory section introduce the techniques used by microbiologists and immunologists and are followed by chapters on the functional immune system. The immune cells and tissues are introduced, followed by an enhanced chapter on innate immunity and updated chapters on antigen-specific immunity, antimicrobial immunity, and vaccines. The sections on bacteria, viruses, fungi, and parasites have also been reorganized. Each section is introduced by the relevant basic science chapters and then the specific microbial disease summary chapter before proceeding into descriptions of the individual microbes, "the bug parade." Each chapter on the specific microbes begins with a summary (including trigger words), which is keyed to the appropriate part of the chapter in the e-version. As in previous editions, there are many summary boxes, tables, clinical photographs, and original clinical cases. **Clinical Cases** are included because we believe students will find them particularly interesting and instructive, and they are a very efficient way to present this complex subject. Each chapter in the "bug parade" is introduced by relevant questions to excite students and orient them as they explore the chapter. Finally, students are provided with access to the new Student Consult website, which provides links to additional reference materials, clinical photographs, animations (including new animations), and answers to the introductory and summary questions of each chapter. Many of the figures are presented in step-by-step manner to facilitate learning. A very important feature on the website is access to more than 200 **practice exam questions** that will help students assess their mastery of the subject matter and prepare for their course and licensure exams. In essence, this edition provides an understandable

text, details, questions, examples, and a review book all in one.

• To Our Future Colleagues: The Students

On first impression, success in medical microbiology would appear to depend on memorization. Microbiology may seem to consist of only innumerable facts, but there is also a logic to microbiology and immunology. Like a medical detective, the first step is to know your villain. Microbes establish a niche in our bodies; some are beneficial and help us to digest our food and educate our immune system, while others may cause disease. Their ability to cause disease, and the disease that may result, depend on how the microbe interacts with the host and the innate and immune protective responses that ensue.

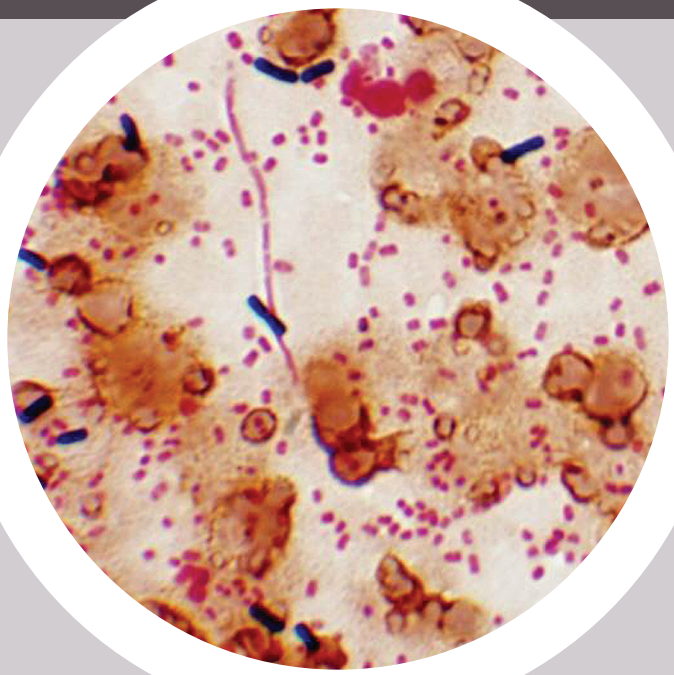
There are many ways to approach learning microbiology and immunology, but ultimately the more you interact with the material using multiple senses, the better you will build memory and learn. A **fun** and **effective** approach to learning is to **think like a physician and treat each microbe and its diseases as if it were an infection in your patient. Create a patient for each microbial infection, and compare and contrast the different patients.** Perform role-playing and ask the seven basic questions as you approach this material: Who? Where? When? Why? Which? What? and How? For example: Who is at risk for disease? Where does this organism cause infections (both body site and geographic area)? When is isolation of this organism important? Why is this organism able to cause disease? Which species and genera are medically important? What diagnostic tests should be performed? How is this infection managed? Each organism that is encountered can be systematically examined. Use the following acronym to create a clinical case and learn the essential information for each microbe: **DIVIRDEPT.** How does the microbial ***d***isease present in the patient and the differential diagnosis? How would you confirm the diagnosis and ***i***dentify the microbial cause of disease? What are the ***v***irulence properties of the organism that cause the disease? What are the helpful and harmful aspects of the ***i***nnate and ***i***mmune response to the infection? What are the specific conditions or mechanisms for ***r***eplicating the microbe? What are all the ***d***isease characteristics and consequences? What is the ***e***pidemiology of infection? How can you ***p***revent its disease? What is its ***t***reatment? Answering the DIVIRDEPT questions will require that you jump around in the chapter to find the information, but this will help you learn the material. For each of the microbes, learn three to five words or phrases that are associated with the microbe—words that will stimulate your memory (**trigger words,** provided in the new chapter summary) and organize the diverse facts into a logical picture. Develop **alternative associations.** For example, this textbook presents organisms in the systematic taxonomic structure (frequently called a "bug parade," but which the authors think is the easiest way to introduce the organisms). Take a given virulence property (e.g., toxin production) or type of disease (e.g., meningitis) and list the organisms that share this property. Pretend that an imaginary patient is infected with a specific agent and create the case history. Explain the diagnosis to your imaginary patient and also to your future professional colleagues. In other words, do not simply attempt to memorize page after page of facts; rather, use techniques that stimulate your mind and challenge your understanding of the facts presented throughout the text and **it will be more fun.** Use the summary chapter at the beginning of each organism section to **review** and help refine your "differential diagnosis" and classify organisms into logical "boxes." Get familiar with the textbook and its bonus materials and you will not only learn the material but also have a review book to work from in the future.

No textbook of this magnitude would be successful without the contributions of numerous individuals. We are grateful for the valuable professional help and support provided by the staff at Elsevier, particularly Jim Merritt, Katie DeFrancesco, and Rhoda Howell. We also want to thank the many students and professional colleagues who have offered their advice and constructive criticism throughout the development of this eighth edition of *Medical Microbiology*.

Patrick R. Murray, PhD; Ken S. Rosenthal, PhD; and Michael A. Pfaller, MD

SECTION

1



INTRODUCTION

CHAPTER

1

INTRODUCTION TO MEDICAL MICROBIOLOGY

Imagine the excitement felt by the Dutch biologist Anton van Leeuwenhoek in 1674 as he peered through his carefully ground microscopic lenses at a drop of water and discovered a world of millions of tiny "animalcules." Almost 100 years later, the Danish biologist Otto Müller extended van Leeuwenhoek's studies and organized bacteria into genera and species according to the classification methods of Carolus Linnaeus. This was the beginning of the taxonomic classification of microbes. In 1840, the German pathologist Friedrich Henle proposed criteria for proving that microorganisms were responsible for causing human disease (the "germ theory" of disease). Robert Koch and Louis Pasteur confirmed this theory in the 1870s and 1880s with a series of elegant experiments proving that microorganisms were responsible for causing anthrax, rabies, plague, cholera, and tuberculosis. Other brilliant scientists went on to prove that a diverse collection of microbes was responsible for causing human disease. The era of chemotherapy began in 1910, when the German chemist Paul Ehrlich discovered the first antibacterial agent, a compound effective against the spirochete that causes syphilis. This was followed by Alexander Fleming's discovery of penicillin in 1928, Gerhard Domagk's discovery of sulfanilamide in 1935, and Selman Waksman's discovery of streptomycin in 1943. In 1946, the American microbiologist John Enders was the first to cultivate viruses in cell cultures, leading the way to the large-scale production of virus cultures for vaccine development. Thousands of scientists have followed these pioneers, each building on the foundation established by his or her predecessors, and each adding an observation that expanded our understanding of microbes and their role in disease.

Our knowledge of microbiology is now undergoing a remarkable transformation founded in the rapid technologic advances in genome analysis. The Human Genome Project was a multinational program that concluded in 2005 with the comprehensive sequencing of the human genome. The techniques developed for this program have rapidly moved into the research and clinical laboratories, leading to microbial sequencing and revealing previously unappreciated insights about pathogenic properties of organisms, taxonomic relationships, and functional attributes of the endogenous microbial population. Clearly, we are at the early stages of novel approaches to diagnostics and therapeutics based on the monitoring and manipulations of this population (the microbiome).

The world that van Leeuwenhoek discovered was complex, consisting of protozoa and bacteria of all shapes and sizes. However, the complexity of medical microbiology we know today rivals the limits of the imagination. We now know that there are thousands of different types of microbes that live in, on, and around us—and hundreds that cause serious human diseases. To understand this information and organize it in a useful manner, it is important to understand some of the basic aspects of medical microbiology. To start, the microbes can be subdivided into the following four general groups: viruses, bacteria, fungi, and parasites, each having its own level of complexity.

• Viruses

Viruses are the smallest infectious particles, ranging in diameter from 18 to 600 nanometers (most viruses are < 200 nm and cannot be seen with a light microscope). Viruses typically contain either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) but not both; however, some viral-like particles do not contain any detectable nucleic acids (e.g., prions), whereas the recently discovered Mimivirus contains both RNA and DNA. The viral nucleic acids required for replication are enclosed in a protein shell with or without a lipid membrane coat. Viruses are true parasites, requiring host cells for replication. The cells they infect and the host response to the infection dictate the nature of the clinical manifestation. More than 2000 species of viruses have been described, with approximately 650 infecting humans and animals. Infection can lead either to rapid replication and destruction of the cell or to a long-term chronic relationship with possible integration of the viral genetic information into the host genome. The factors that determine which of these takes place are only partially understood. For example, infection with the human immunodeficiency virus, the etiologic agent of the acquired immunodeficiency syndrome (AIDS), can result in the latent infection of CD4 lymphocytes or the active replication and destruction of these immunologically important cells. Likewise, infection can spread to other susceptible cells, such as the microglial cells of the brain, resulting in the neurologic manifestations of AIDS. The virus determines the disease and can range from the common cold to gastroenteritis to fatal catastrophes such as rabies, Ebola, smallpox, or AIDS.

• Bacteria

Bacteria are relatively simple in structure. They are **prokaryotic** organisms—simple unicellular organisms with no nuclear membrane, mitochondria, Golgi bodies, or

endoplasmic reticulum—that reproduce by asexual division. The bacterial cell wall is complex, consisting of one of two basic forms: a gram-positive cell wall with a thick peptidoglycan layer, and a gram-negative cell wall with a thin peptidoglycan layer and an overlying outer membrane. Some bacteria lack this cell wall structure and compensate by surviving only inside host cells or in a hypertonic environment. The size (1 to 20 μm or larger), shape (spheres, rods, spirals), and spacial arrangement (single cells, chains, clusters) of the cells are used for the preliminary classification of bacteria, and the phenotypic and genotypic properties of the bacteria form the basis for the definitive classification. The human body is inhabited by thousands of different bacterial species—some living transiently, others in a permanent parasitic relationship. Likewise, the environment that surrounds us, including the air we breathe, water we drink, and food we eat, is populated with bacteria, many of which are relatively avirulent and some of which are capable of producing life-threatening disease. Disease can result from the toxic effects of bacterial products (e.g., toxins) or when bacteria invade normally sterile body tissues and fluids.

• Fungi

In contrast to bacteria, the cellular structure of fungi is more complex. These are **eukaryotic** organisms that contain a well-defined nucleus, mitochondria, Golgi bodies, and endoplasmic reticulum. Fungi can exist either in a unicellular form **(yeast)** that can replicate asexually or in a filamentous form **(mold)** that can replicate asexually and sexually. Most fungi exist as either yeasts or molds; however, some fungi can assume either morphology. These are known as **dimorphic** fungi and include such organisms as *Histoplasma, Blastomyces,* and *Coccidioides.*

• Parasites

Parasites are the most complex microbes. Although all parasites are classified as eukaryotic, some are unicellular and others are multicellular. They range in size from tiny protozoa as small as 4 to 5 μm in diameter (the size of some bacteria) to tapeworms that can measure up to 10 meters in length and arthropods (bugs). Indeed, considering the size of some of these parasites, it is hard to imagine how these organisms came to be classified as microbes. Their life cycles are equally complex, with some parasites establishing a permanent relationship with humans and others going through a series of developmental stages in a progression of animal hosts. One of the difficulties confronting students is not only an understanding of the spectrum of diseases caused by parasites but also an appreciation of the epidemiology of these infections, which is vital for developing a differential diagnosis and an approach to the control and prevention of parasitic infections.

• Immunology

It is difficult to discuss human microbiology without also discussing the innate and immune responses to the microbes. Our innate and immune responses evolved to protect us from infection. At the same time, the microbes that live in our bodies as normal flora or disease-causing organisms must be able to withstand or evade these host protections sufficiently long to be able to establish their niche within our bodies or spread to new hosts. The peripheral damage that occurs during the war between the host protections and microbial invaders contributes to or may be the cause of the symptoms of the disease. Ultimately, the innate and immune responses are the best prevention and cure for microbial disease.

• Microbial Disease

One of the most important reasons for studying microbes is to understand the diseases they cause and the ways to control them. Unfortunately, the relationship between many organisms and their diseases is not simple. Specifically, most organisms do not cause a single well-defined disease, although there are certainly ones that do (e.g., *Clostridium tetani* [tetanus], Ebola virus [Ebola], *Plasmodium* species [malaria]). Instead, it is more common for a particular organism to produce many manifestations of disease (e.g., *Staphylococcus aureus*—endocarditis, pneumonia, wound infections, food poisoning) or for many organisms to produce the same disease (e.g., meningitis caused by viruses, bacteria, fungi, and parasites). In addition, relatively few organisms can be classified as always pathogenic, although some do belong in this category (e.g., rabies virus, *Bacillus anthracis, Sporothrix schenckii, Plasmodium* species). Instead, most organisms are able to establish disease only under well-defined circumstances (e.g., introduction of an organism with a potential for causing disease into a normally sterile site such as the brain, lungs, and peritoneal cavity). Some diseases arise when a person is exposed to organisms from external sources. These are known as **exogenous infections,** and examples include diseases caused by influenza virus, *C. tetani, Neisseria gonorrhoeae, Coccidioides immitis,* and *Entamoeba histolytica*. Most human diseases, however, are produced by organisms in the person's own microbial flora that spread to normally sterile body sites where disease can ensue **(endogenous infections).**

The interaction between an organism and the human host is complex. The interaction can result in transient colonization, a long-term symbiotic relationship, or disease. The virulence of the organism, the site of exposure, and the host's ability to respond to the organism determine the outcome of this interaction. Thus the manifestations of disease can range from mild symptoms to organ failure and death. The role of microbial virulence and the host's immunologic response is discussed in depth in subsequent chapters.

The human body is remarkably adapted to controlling exposure to pathogenic microbes. Physical barriers prevent invasion by the microbe; innate responses recognize molecular patterns on the microbial components and activate local defenses and specific adapted immune responses that target the microbe for elimination. Unfortunately, the immune response is often too late or too slow. To improve the human body's ability to prevent infection, the immune system can be augmented either through the passive transfer of antibodies present in immune globulin preparations or through

active immunization with components of the microbes (vaccines). Infections can also be controlled with a variety of chemotherapeutic agents. Unfortunately, microbes can mutate and share genetic information, and those that cannot be recognized by the immune response because of **antigenic variation** or those that are resistant to antibiotics will be selected and will endure. Thus the battle for control between microbe and host continues, with neither side yet able to claim victory (although the microbes have demonstrated remarkable ingenuity). There clearly is no "magic bullet" that has eradicated infectious diseases.

• Diagnostic Microbiology

The clinical microbiology laboratory plays an important role in the diagnosis and control of infectious diseases. However, the ability of the laboratory to perform these functions is limited by the quality of the specimen collected from the patient, the means by which it is transported from the patient to the laboratory, and the techniques used to demonstrate the microbe in the sample. Because most diagnostic tests are based on the ability of the organism to grow, transport conditions must ensure the viability of the pathogen. In addition, the most sophisticated testing protocols are of little value if the collected specimen is not representative of the site of infection. This seems obvious, but many specimens sent to laboratories for analysis are contaminated during collection with the organisms that colonize mucosal surfaces. It is virtually impossible to interpret the testing results with contaminated specimens, because most infections are caused by endogenous organisms.

The laboratory is also able to determine the antimicrobial activity of selected chemotherapeutic agents, although the value of these tests is limited. The laboratory must test only organisms capable of producing disease and only medically relevant antimicrobials. To test all isolated organisms or an indiscriminate empirical selection of drugs can yield misleading results with potentially dangerous consequences. Not only can a patient be treated inappropriately with unnecessary antibiotics, but also the true pathogenic organism may not be recognized among the plethora of organisms isolated and tested. Finally, the in vitro determination of an organism's susceptibility to a variety of antibiotics is only one aspect of a complex picture. The virulence of the organism, site of infection, and patient's ability to respond to the infection influence the host-parasite interaction and must also be considered when planning treatment.

• Summary

It is important to realize that our knowledge of the microbial world is evolving continually. Just as the early microbiologists built their discoveries on the foundations established by their predecessors, we and future generations will continue to discover new microbes, new diseases, and new therapies. The following chapters are intended as a foundation of knowledge that can be used to build your understanding of microbes and their diseases.

CHAPTER

2

HUMAN MICROBIOME IN HEALTH AND DISEASE

Up until the time of birth, the human fetus lives in a remarkably protected and for the most part sterile environment; however, this rapidly changes as the infant is exposed to bacteria, archaea, fungi, and viruses from the mother, other close contacts, and the environment. Over the next few years, communities of organisms (**microbiota** or **normal flora** [Table 2-1]) form on the surfaces of the skin, nares, oral cavity, intestines, and genitourinary tract. The focus of this chapter is to gain an understanding of the role these communities play in the metabolic and immunologic functions of healthy individuals, factors regulating the composition of these communities, and how disruption of these communities can result in disease states.

• Human Microbiome Project

Our current knowledge of the **microbiome** is rooted in the successful completion of the Human Genome Project, a 13-year international effort initiated in 1990 that determined the sequences of the approximately 3 billion nucleotides that make up the 23,000 protein-encoding genes in human DNA. Much like efforts to send a man to the moon, the greatest legacy of this work was the development of technologies and informatic solutions that allow the generation and analysis of tremendous amounts of DNA and messenger RNA sequencing data.

The Human Microbiome Project was a 5-year multinational study to analyze the genetic composition **(microbiome)** of the microbial populations that live in and on healthy adults. To put the complexity of this program into perspective, it is estimated that bacterial cells outnumber human cells in the host by 10 : 1, and the bacterial population contributes at least 300-fold more protein genes.

The Human Microbiome Project was launched in 2007 with the collection of samples from the nose, mouth, skin, gut, and vagina from healthy adult volunteers. The microbes were identified by sequencing targeted regions of the 16S ribosomal RNA gene, and information about the gene content of the entire population was determined by sequencing the whole genome of a subset of specimens. These analyses showed that there is substantial variation in the species and gene composition for individuals and at different body sites. For example, bacteria colonizing the gut are different from those in the mouth, skin, and other body sites. The body site with the greatest taxonomic and genetic diversity was the intestine, and the vagina was the least complex. Microenvironments such as different regions of the mouth, gut, skin surface, and vagina also had their own unique microbiome (Figure 2-1).

• Core Microbiome

Most individuals share a **core microbiome,** arbitrarily defined as the species that are present at a specific site in 95% or more of individuals. The greatest numbers of shared species are present in the mouth, followed by the nose, intestine, and skin, and the fewest shared species are found in the vagina. Additionally, the small numbers of species that comprise the core microbiome are the most numerous, representing the majority of the total population, whereas the remaining portion of the population **(secondary microbiome)** consists of small numbers of many species that may not be widely shared by individuals. This would imply that the members of the core microbiome are critically important, providing essential functions that must be retained for normal metabolic and immunologic activities, and the functions provided by the secondary microbiome are also critically important but can be provided by a variety of organisms. In other words, although there is tremendous variation of species among individuals, there is less variation in the genetic composition at each site. The **taxonomic diversity** of a population is great, but the functional properties are highly conserved **(functional redundancy)** in microbiomes associated with health. This is not surprising if we consider that the microbiome is a community that exists in a symbiotic relationship with its host, providing needed metabolic functions, stimulating innate immunity, and preventing colonization with unwanted pathogens. Thus interpersonal variations of the microbiome can exist in healthy individuals as long as the needed functions are satisfied.

• Evolution of the Microbiome and Normal Flora

The **normal flora** of a particular site of the body consists of a unique community of core and secondary microbiota that evolved through a symbiotic relationship with the host and a competitive relationship with other species. The host provides a place to colonize, nutrients, and some protection from unwanted species (innate immune responses). The microbes provide needed metabolic functions, stimulate innate and regulatory immunity, and prevent colonization with unwanted pathogens (Figure 2-2). The ability to tolerate

Table 2-1 Glossary of Terms

Term	Definition
Microbiota	Community of microbes that live in and on an individual; can vary substantially between environmental sites and host niches in health and disease
Normal flora	Microbiota
Microbiome	Aggregate collection of microbial genomes in the microbiota
Core microbiome	Commonly shared microbial species among individuals at specific body sites; although typically represented by a limited number of species, these comprise the largest proportion of the microbial population
Secondary microbiome	Microbial species that contribute to the unique diversity of individuals at specific body sites; typically present in proportionately small numbers
Functional redundancy	Required functions (e.g., metabolism of nutrients, regulation of the immune response) that are provided by the diverse members of the microbiota
Taxonomic diversity	The diverse number of species that comprise the microbiota
Prebiotic	Food ingredient that supports the growth of one or more members of the microbiota
Probiotic	Live organism that when ingested is believed to provide benefit to the host

the amount of oxygen or lack thereof (redox state) and the pH and salt concentration, as well as to scavenge essential minerals and harvest and metabolize the available nutrients, determines the numbers and nature of the species that populate a site of the body. Anaerobic or facultative anaerobic bacteria colonize most of the sites of the body because of the lack of oxygen in sites such as the mouth, intestine, and genitourinary tract.

The composition of the microbiota is influenced by personal hygiene (e.g., use of soap, deodorants, mouthwash, skin peels, enemas, vaginal douches), diet, water source, medicines (especially antibiotics), and exposure to environmental toxins. Drinking well water versus chlorinated city water or a diet consisting of more or less fiber, sugar, or fats can select for different intestinal bacteria based on their ability to utilize the essential minerals (e.g., iron) and nutrients. Alteration of the environment with foods or medicines can also alter the microbiota (Figure 2-3). These changes can be acceptable if the core microbiome and critical functional properties of the microbiome are maintained but can result in disease if these functions are lost. Historically, the greatest concern with the use of broad-spectrum antibiotics was the selection of resistant bacteria; however, a greater concern should be the disruption of the microbiome and loss of essential functions.

Of the approximately 200 unique species of bacteria that colonize the gut, most are members of the Actinobacteria (e.g., *Bifidobacterium*), Bacteroidetes (e.g., *Bacteroides*), and Firmicutes (e.g., *Eubacterium, Ruminococcus, Faecalibacterium, Blautia*). Interestingly, the importance of many of these bacteria was not appreciated before gene sequencing was used to identify and quantitate the gut microbiota. Within the colon, some bacteria wage interspecies warfare to establish their niche with bacteriocins (e.g., colicins produced by *Escherichia coli*), other antibacterial proteins, and metabolites that deter other species from growing. These molecules also benefit the host by eliminating invading bacteria including *Salmonella, Shigella, Clostridium difficile, Bacillus cereus,* and other pathogens. The bacteria must also resist antimicrobial peptides and immunoglobulin (Ig)A produced by the host and released into the bowel.

Metabolism of nutrients plays a major role in the symbiotic relationship between the human host and microbe. Bacteria in the human gut are responsible for metabolizing complex carbohydrates (including cellulose) to provide small-chain fatty acids such as acetate, propionate, and butyrate that can be readily transported and used by the cells of our body. These acids also limit the growth of undesirable bacteria. Other bacteria graze on the carbohydrates, the mucins that line the epithelium, or the oils released in our sweat. Bacteroidetes and Firmicutes are more efficient than others at breaking down complex carbohydrates, including plant cell wall compounds (cellulose, pectin, xylan) as well as host-derived carbohydrates, including those attached to the mucins or chondroitin sulfates of the protective mucous layer of the intestine. Increases in the ratio of these bacteria in the gut microbiome can lead to a higher efficiency in storage of the metabolic byproducts. This can be a benefit for malnourished populations or patients with debilitating diseases such as cancer, or can lead to obesity in well-nourished populations.

• Role of the Microbiome in Disease

If the normal microbiome characterizes health, alterations in the microbiome can signify disease, a relationship we are only beginning to understand. In 1884 Robert Koch and Friedrich Loeffler defined the relationship between an organism and infection. The **Koch postulates** were based on the concept of one organism: one disease. Microbiome research has introduced a new concept—disease caused by a community of organisms rather than a single species of bacteria, and the influence extends beyond traditional "infectious" diseases to include immunologic and metabolic disorders such as inflammatory bowel disease, obesity, type 2 diabetes, and celiac disease. We are now at the forefront of a new era of defining infectious diseases.

Disruption of the normal microflora (commonly referred to as **dysbiosis**) can lead to disease by the elimination of needed organisms or allowing the growth of inappropriate bacteria. For example, following exposure to antibiotics and suppression of the intestinal normal flora, *C. difficile* is able to proliferate and express enterotoxins, leading to inflammation of the colon **(antibiotic-associated colitis).** Another disease of the colon, **ulcerative colitis,** is associated with an increased level of bacteria producing mucin-degrading sulfatases, leading to degradation of the protective mucosal lining of the intestinal wall and stimulation of inflammatory immune responses. Individuals with an intestinal microbiota that is more efficient at breaking down complex carbohydrates internalize rather than void these nutrients and are therefore susceptible to **obesity** and a predisposition to metabolic syndromes such as **type 2 diabetes.** Not all patients genetically predisposed to **celiac disease,** an

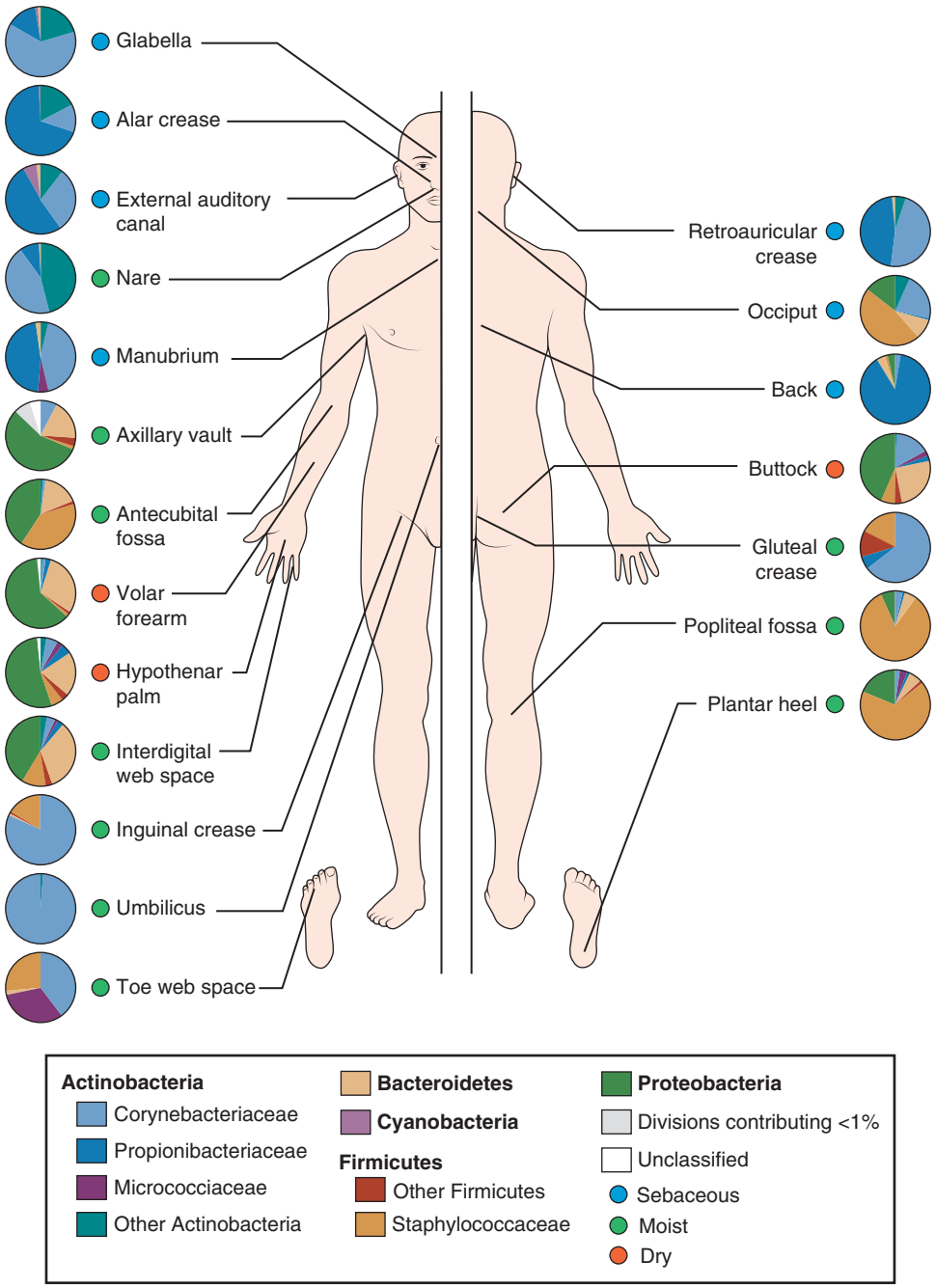


FIGURE 2-1 Topographical distribution of bacteria on skin sites. As at other body sites, the distribution of the skin microbiome is dependent on the microenvironment of the sampled site, such as sebaceous or oily *(blue circles),* moist *(green circles),* and dry, flat surfaces *(red circles).* (From Grice E, Segre J: The skin microbiome, *Nat Rev Microbiol* 9:244–253, 2011.)

immune-mediated enteropathology precipitated by exposure to gluten proteins, are symptomatic. The intestinal microbiota of most individuals is composed of bacteria capable of digesting glutens, which may be sufficient to protect these genetically predisposed individuals. In the absence of these bacteria, disease may occur. Shifts in the skin microbiome are associated with progression to **chronic wound infections** and episodic exacerbations of **atopic dermatitis.** Alteration in the vaginal microbiome from relatively few predominant organisms to a heterogeneous mixed population is associated with the progression to **vaginitis.**

• Diagnostics and Therapeutics

An understanding of the influence of dysbiosis on disease pathology can lead to both advanced diagnostic tests and paths for therapeutic intervention. Just as the presence of *Salmonella* or *Shigella* signifies disease, changes in the diversity and composition of the fecal microflora can also indicate susceptibility to or onset of disease. The most obvious example is *C. difficile* disease—clinical disease is preceded by a depletion of the normal flora owing to antibiotic use. Interestingly, patients with chronic relapsing *C. difficile* infections

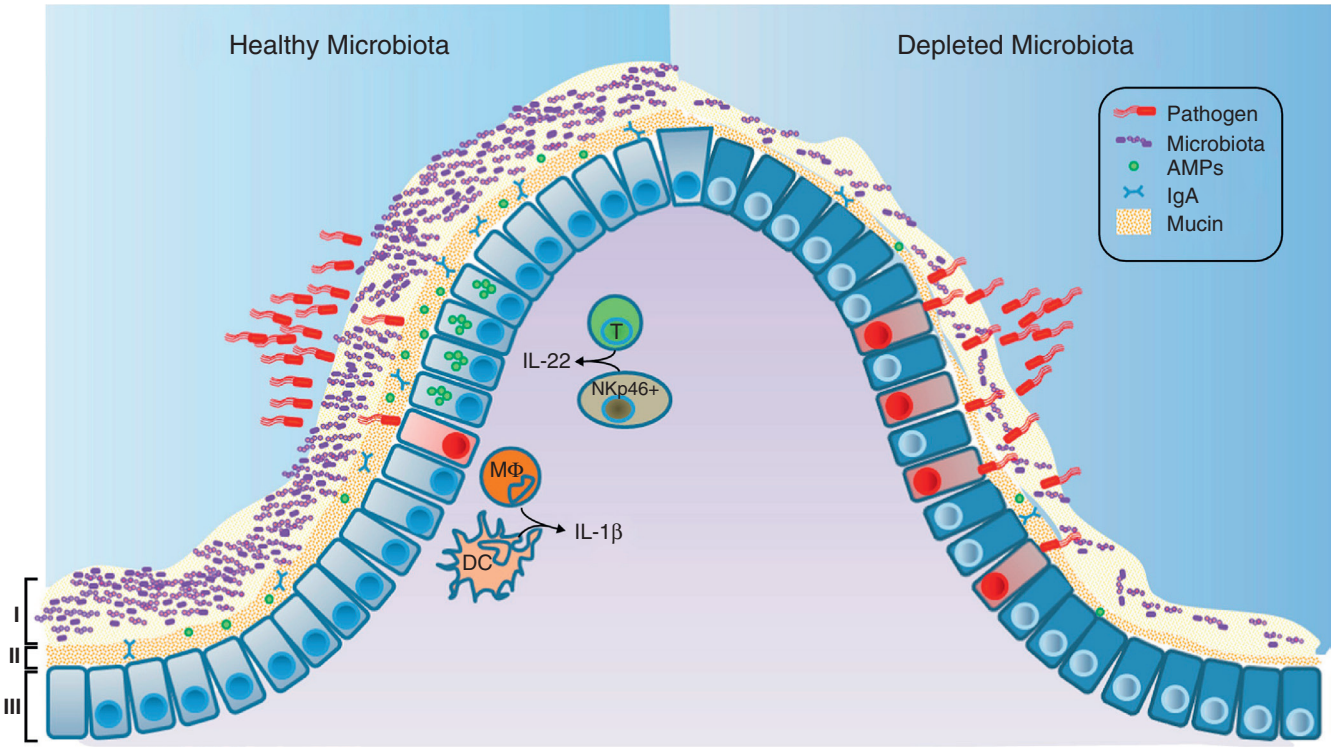


FIGURE 2-2 Intestinal microbiota protection against enteric infections. (I) Saturation of colonization sites and consumption of nutrients limit pathogen access to host tissues; (II) the microbiota prime innate immunity by stimulating mucin production, immunoglobulin *(Ig)*A, and antimicrobial peptides *(AMPs);* and (III) the microbiota stimulate interleukin *(IL)*-22 expression, which increases epithelial resistance, and IL-1β production, which promotes recruitment of inflammatory cells. (From Khosravi A, Mazmanian S: Disruption of the gut microbiome as a risk factor for microbial infections, *Curr Opin Microbiol* 16:221–227, 2013.)

are treated successfully by repopulating (some say **"repoopulating"**) the intestines with stool transplants from a healthy spouse or close relative, or with artificially created stool specimens consisting of a complex mixture of aerobic and anaerobic fecal organisms.

More subtle alterations in the gut microbiome may predict development of diseases such as **necrotizing enterocolitis (NEC),** inflammatory bowel disease, and a predilection for obesity. NEC is a devastating intestinal disease that afflicts preterm infants. Prospectively collected stool samples from infants younger than 29 weeks' gestational age who develop NEC demonstrate a distinct dysbiosis prior to the development of disease. Infants with early-onset disease have a dominance of Firmicutes (predominantly *Staphylococcus*), whereas infants with late-onset NEC have a dominance of Enterobacteriaceae.

The effects of microbiome alterations have also been described for the pathogenesis of inflammatory bowel disease and colorectal cancer. Proliferation of bacteria such as *Akkermansia muciniphila* that produce mucin-degrading sulfatases is responsible for degradation of the intestinal wall lining. Additionally, an increase in members of the anaerobic family Prevotellaceae leads to up-regulation of chemokine-mediated inflammation. Enterotoxigenic *Bacteroides fragilis* can also induce T helper cell–mediated inflammatory responses that are associated with colitis and are a precursor to colonic hyperplasia and colorectal tumors. Finally, *Methanobrevibacter smithii,* a minor member of the gut microbiome, enhances digestion of dietary glycans by *Bacteroides thetaiotaomicron* and other core intestinal bacteria, leading to accumulation of fat.

• Probiotics

Probiotics are mixtures of bacteria or yeast that upon ingestion colonize and proliferate, even temporarily, the intestine. Consumers of probiotics believe they act by rebalancing the microbiome and its functions, such as enhancing digestion of food and modulating the individual's innate and immune response. The most common reason people use over-the-counter probiotics is to promote and maintain regular bowel function and improve tolerance to lactose. Probiotics are commonly gram-positive bacteria (e.g., *Bifidobacterium, Lactobacillus*) and yeasts (e.g., *Saccharomyces*). Many of these microbes are found in ingestible capsules and as food supplements (e.g., yogurt, kefir). Probiotics have been used to treat *C. difficile*–associated diarrhea and inflammatory bowel disease, to provide protection from *Salmonella* and *Helicobacter pylori* disease, as therapy for pediatric atopic dermatitis and autoimmune diseases, and even for reduction in dental caries, although the value of probiotics for many of these conditions is unproven. Although probiotics are safe dietary supplements, not all probiotics are effective and for all people. The species, mixture of species, and dose and viability of the probiotic organisms within a probiotic formulation influence its potency, efficacy, and therapeutic potential. What is clear is that much like the use of complex artificial mixtures of

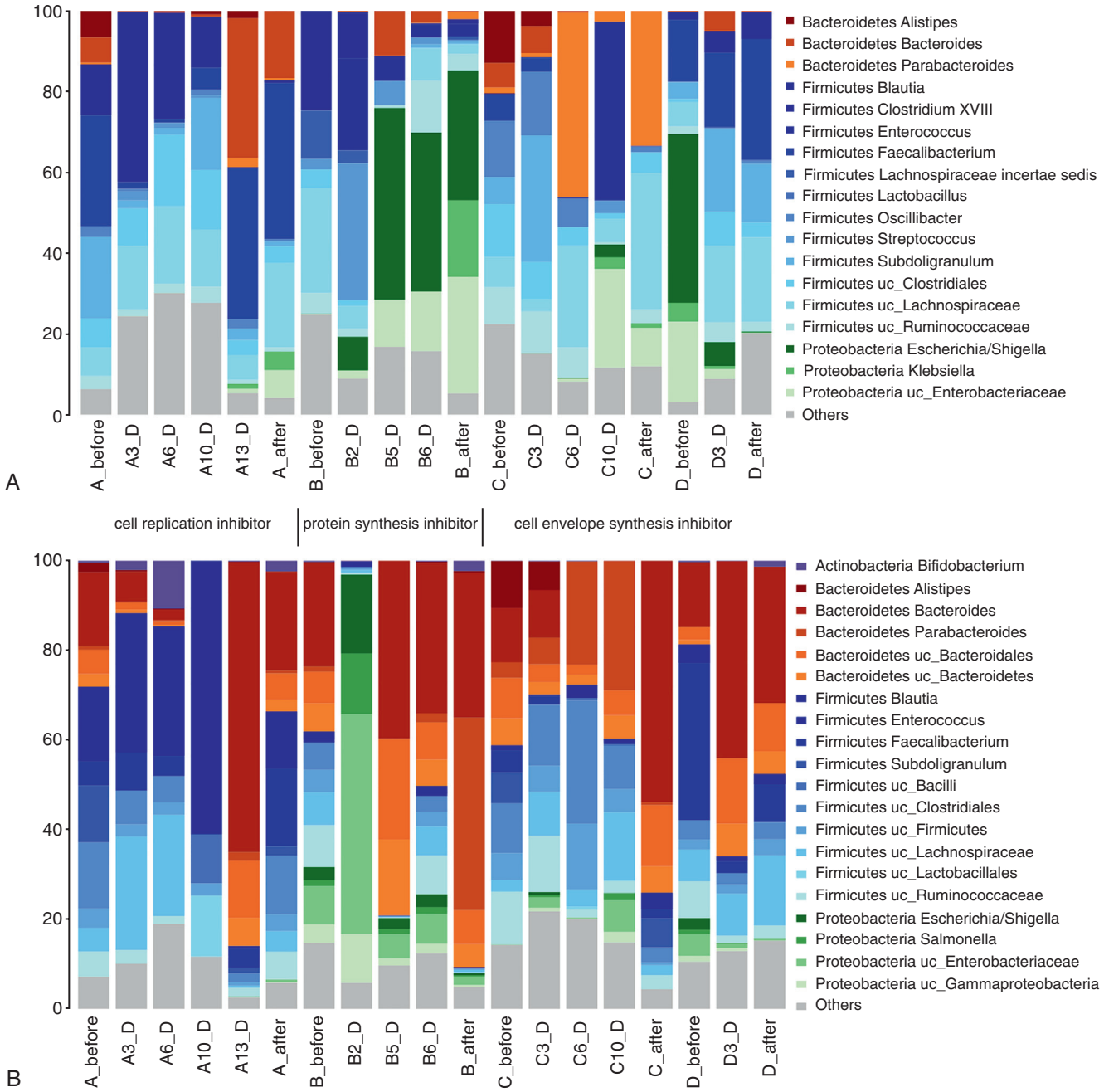


FIGURE 2-3 Effect of antibiotics on the gut microbiota. Fecal samples were collected from four patients treated with antibiotics: patient A, moxifloxacin; patient B, penicillin + clindamycin; patient C, cefazolin followed by ampicillin/sulbactam; patient D, amoxicillin. Fecal samples collected before, during (e.g., 3_D is day 3 of therapy), and after therapy were used to assess the total microbiota. Changes are noted both during therapy and after therapy is discontinued. **A,** Total microbiota (16S rRNA gene). **B,** Metabolically active microbiota (16S rRNA transcripts). (From Perez-Cobas AE, Artacho A, Knecht H, et al: Differential effects of antibiotic therapy on the structure and function of human gut microbiota, *PLoS One* 8:e80201, 2013.)

organisms to treat recurrent *C. difficile* disease, carefully designed "smart probiotics" will likely be an important adjunct to medical therapy in the future.

• Perspective

In the near future, with faster and cheaper DNA sequencing procedures, analysis of a person's microbiome may become a routine diagnostic test for predicting and treating a wide range of diseases. However, a number of questions remain to be resolved, including: can we predict disease in an individual by monitoring changes in the microbiome; which changes are most important—taxonomic or genetic function; can we prevent disease or treat disease by reestablishing a healthy microbiome; can this be done by prescribing specific replacement microbes (e.g., fecal transplant) or with a universal mixture (probiotic); can the use of metabolic

supplements **(prebiotics)** promote a healthy microbiota; and will use of antibiotics be replaced by use of "smart microbiome" therapies? Other questions include: what is the role of the host genome, environmental factors, and our hygienic practices in shaping the microbiome; and what will be the informatic requirements for guiding diagnostics or therapeutics? Regardless of the answers to these and other questions, it is certain that we are witnessing the beginning of a new era of microbiology that can radically change our approach to prediction, diagnosis, and treatment of disease.

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Questions

1. *What is the relationship between the human genome and microbiome genetic material?*
2. *Explain the concepts of taxonomic diversity and genetic diversity.*
3. *Explain the concept of the core microbiome.*
4. *Give three examples of alterations of the microbiome (dysbiosis) that are associated with specific diseases.*

Answers

1. The human genome consists of all the genes present in human chromosomes. These genes encode approximately 23,000 unique proteins. The microbiome genetic material includes all the genetic material present in the bacteria that live on and in us. This bacterial population contributes at least 100-fold more unique genes than the human genome.
2. Taxonomic diversity refers to the diverse population of bacterial species that compose the microbiome. The genetic diversity of this population refers to the number of unique protein-encoding genes in the microbiome. Whereas taxonomic diversity can be great (i.e., large number of different bacterial species in a community and highly variable from individual to individual), the genetic diversity is generally low in healthy individuals. This functional redundancy is necessary because the bacterial species perform a large number of critical functions to maintain health.
3. The core microbiome is the individual species of bacteria present in most individuals at a specific body site. The core microbiome typically consists of a small number of species but represents a large portion of the population. A large number of other bacterial species are less common in individuals and represent the minority population in the microbial community.
4. Diseases associated with microbiome dysbiosis include *Clostridium difficile* enterocolitis, inflammatory bowel disease, chronic wound infections, atopic dermatitis, vaginitis, and obesity.

CHAPTER

3

STERILIZATION, DISINFECTION, AND ANTISEPSIS

An important aspect of the control of infections is an understanding of the principles of sterilization, disinfection, and antisepsis (Box 3-1).

• Sterilization

Sterilization is the total destruction of all microbes, including the more resilient forms such as bacterial spores, mycobacteria, nonenveloped (non-lipid) viruses, and fungi. This can be accomplished using physical, gas vapor, or chemical sterilants (Table 3-1).

Saturated steam under pressure is a widely used, inexpensive, nontoxic, and reliable method of sterilization. Three parameters are critical: the time of exposure to steam, temperature, and amount of moisture. The most commonly used sterilization cycle is use of saturated steam heated at 121° C for 15 minutes. Maintaining the proper temperature is critical because a drop of 1.7° C increases the needed exposure time by 48%. If no moisture is present, then the temperature must reach 160° C. Dry heat sterilization requires prolonged exposure times and damages many instruments, so it is not currently recommended.

Ethylene oxide gas is used to sterilize temperature- or pressure-sensitive items. Treatment is generally for 4 hours, and sterilized items must be aerated for an additional 12 hours to eliminate the toxic gas before the items are used. Although ethylene oxide is highly efficient, strict regulations limit its use, because it is flammable, explosive, and carcinogenic to laboratory animals. For these reasons, ethylene oxide sterilization is avoided if acceptable alternatives are available.

Hydrogen peroxide vapors are effective sterilants because of the oxidizing nature of the gas. This sterilant is used for the sterilization of instruments. A variation is **plasma gas sterilization,** in which hydrogen peroxide is vaporized, and then reactive free radicals are produced with either microwave-frequency or radio-frequency energy. Because this is an efficient sterilizing method that does not produce toxic byproducts, plasma gas sterilization has replaced many of the applications for ethylene oxide. However, it cannot be used with materials that absorb hydrogen peroxide or react with it.

Two **chemical sterilants** have also been used: **peracetic acid** and **glutaraldehyde.** Peracetic acid, an oxidizing agent, has excellent activity, and the end products (i.e., acetic acid and oxygen) are nontoxic. In contrast, safety is a concern with glutaraldehyde, and care must be used when handling this chemical.

• Disinfection

Microbes are also destroyed by disinfection procedures, although more resilient organisms can survive. Unfortunately, the terms *disinfection* and *sterilization* are casually interchanged and can result in some confusion. This occurs because disinfection processes have been categorized as high level, intermediate level, and low level. High-level disinfection can generally approach sterilization in effectiveness, whereas spore forms can survive intermediate-level disinfection, and many microbes can remain viable when exposed to low-level disinfection.

Even the classification of disinfectants (Table 3-2) by their level of activity is misleading. The effectiveness of these procedures is influenced by the nature of the item to be disinfected, number and resilience of the contaminating organisms, amount of organic material present (which can inactivate the disinfectant), type and concentration of disinfectant, and duration and temperature of exposure.

High-level disinfectants are used for items involved with invasive procedures that cannot withstand sterilization procedures (e.g., certain types of endoscopes and surgical instruments with plastic or other components that cannot be autoclaved). Disinfection of these and other items is most effective if cleaning the surface to remove organic matter precedes treatment. Examples of high-level disinfectants include treatment with moist heat and use of liquids such as glutaraldehyde, hydrogen peroxide, peracetic acid, and chlorine compounds.

Intermediate-level disinfectants (i.e., alcohols, iodophor compounds, phenolic compounds) are used to clean surfaces or instruments where contamination with bacterial spores and other highly resilient organisms is unlikely. These have been referred to as semicritical instruments and devices and include flexible fiberoptic endoscopes, laryngoscopes, vaginal specula, anesthesia breathing circuits, and other items.

Low-level disinfectants (i.e., quaternary ammonium compounds) are used to treat noncritical instruments and devices, such as blood pressure cuffs, electrocardiogram electrodes, and stethoscopes. Although these items come into contact with patients, they do not penetrate through mucosal surfaces or into sterile tissues.

Box 3-1 Definitions

Antisepsis: Use of chemical agents on skin or other living tissue to inhibit or eliminate microbes; no sporicidal action is implied

Disinfection: Use of physical procedures or chemical agents to destroy most microbial forms; bacterial spores and other relatively resistant organisms (e.g., mycobacteria, viruses, fungi) may remain viable; disinfectants are subdivided into high-, intermediate-, and low-level agents

Germicide: Chemical agent capable of killing microbes; includes virucide, bactericide, sporicide, tuberculocide, and fungicide

High-level disinfectant: A germicide that kills all microbial pathogens except large numbers of bacterial spores

Intermediate-level disinfectant: A germicide that kills all microbial pathogens except bacterial endospores

Low-level disinfectant: A germicide that kills most vegetative bacteria and lipid-enveloped and medium-size viruses

Sterilization: Use of physical procedures or chemical agents to destroy all microbial forms, including bacterial spores

Table 3-1 Methods of Sterilization

Method	Concentration or Level
Physical Sterilants	
Steam under pressure	121° C or 132° C for various time intervals
Filtration	0.22- to 0.45-μm pore size; HEPA filters
Ultraviolet radiation	Variable exposure to 254-nm wavelength
Ionizing radiation	Variable exposure to microwave or gamma radiation
Gas Vapor Sterilants	
Ethylene oxide	450-1200 mg/L at 29° C to 65° C for 2-5 hr
Hydrogen peroxide vapor	30% at 55° C to 60° C
Plasma gas	Highly ionized hydrogen peroxide gas
Chemical Sterilants	
Peracetic acid	0.2%
Glutaraldehyde	2%

HEPA, High-efficiency particulate air.

Table 3-2 Methods of Disinfection

Method	Concentration (Level of Activity)
Heat	
Moist heat	75° C to 100° C for 30 min (high)
Liquid	
Glutaraldehyde	2%-3.2% (high)
Hydrogen peroxide	3%-25% (high)
Chlorine compounds	100-1000 ppm of free chlorine (high)
Alcohol (ethyl, isopropyl)	70%-95% (intermediate)
Phenolic compounds	0.4%-5.0% (intermediate/low)
Iodophor compounds	30-50 ppm of free iodine/L (intermediate)
Quaternary ammonium compounds	0.4%-1.6% (low)

The level of disinfectants used for environmental surfaces is determined by the relative risk these surfaces pose as a reservoir for pathogenic organisms. For example, a higher level of disinfectant should be used to clean the surface of instruments contaminated with blood than that used to clean surfaces that are "dirty," such as floors, sinks, and countertops. The exception to this rule is if a particular surface has been implicated in a nosocomial infection, such as a bathroom contaminated with *Clostridium difficile* (spore-forming anaerobic bacterium) or a sink contaminated with *Pseudomonas aeruginosa*. In these cases, a disinfectant with appropriate activity against the implicated pathogen should be selected.

• Antisepsis

Antiseptic agents (Table 3-3) are used to reduce the number of microbes on skin surfaces. These compounds are selected for their safety and efficacy. A summary of their germicidal properties is presented in Table 3-4. **Alcohols** have excellent activity against all groups of organisms except spores and are nontoxic, although they tend to dry the skin surface because they remove lipids. They also do not have residual activity and are inactivated by organic matter. Thus the surface of the skin should be cleaned before alcohol is applied. **Iodophors** are also excellent skin antiseptic agents, having a range of activity similar to that of alcohols. They are slightly more toxic to the skin than is alcohol, have limited residual activity, and are inactivated by organic matter. Iodophors and iodine preparations are frequently used with alcohols for disinfecting the skin surface. **Chlorhexidine** has broad antimicrobial activity, although it kills organisms at a much slower rate than alcohol. Its activity persists, although organic material and high pH levels decrease its effectiveness. The activity of **parachlorometaxylenol (PCMX)** is limited primarily to gram-positive bacteria. Because it is nontoxic and has residual activity, it has been used in hand washing products. **Triclosan** is active against bacteria but not against many other organisms. It is a common antiseptic agent in deodorant soaps and some toothpaste products.

• Mechanisms of Action

The following section briefly reviews the mechanisms by which the most common sterilants, disinfectants, and antiseptics work.

Table 3-3 Antiseptic Agents

Antiseptic Agent	Concentration
Alcohol (ethyl, isopropyl)	70%-90%
Iodophors	1-2 mg of free iodine/L; 1%-2% available iodine
Chlorhexidine	0.5%-4.0%
Parachlorometaxylenol	0.50%-3.75%
Triclosan	0.3%-2.0%

Table 3-4 Germicidal Properties of Disinfectants and Antiseptic Agents

Agents	Bacteria	Mycobacteria	Bacterial Spores	Fungi	Viruses
Disinfectants					
Alcohol	+	+	–	+	+/–
Hydrogen peroxide	+	+	+/–	+	+
Phenolics	+	+	–	+	+/–
Chlorine	+	+	+/–	+	+
Iodophors	+	+/–	–	+	+
Glutaraldehyde	+	+	+	+	+
Quaternary ammonium compounds	+/–	–	–	+/–	+/–
Antiseptic Agents					
Alcohol	+	+	–	+	+
Iodophors	+	+	–	+	+
Chlorhexidine	+	+	–	+	+
Parachlorometaxylenol	+/–	+/–	–	+	+/–
Triclosan	+	+/–	–	+/–	+

Moist Heat

Attempts to sterilize items using boiling water are inefficient because only a relatively low temperature (100°C) can be maintained. Indeed, spore formation by a bacterium is commonly demonstrated by boiling a solution of organisms and then subculturing the solution. Boiling vegetative organisms kills them, but the spores remain viable. In contrast, steam under pressure in an autoclave is a very effective form of sterilization; the higher temperature causes denaturation of microbial proteins. The rate of killing organisms during the autoclave process is rapid but is influenced by the temperature and duration of autoclaving, size of the autoclave, flow rate of the steam, density and size of the load, and placement of the load in the chamber. Care must be taken to avoid creating air pockets, which inhibit penetration of the steam into the load. In general, most autoclaves are operated at 121°C to 132°C for 15 minutes or longer. Including commercial preparations of *Bacillus stearothermophilus,* spores can help monitor the effectiveness of sterilization. An ampule of these spores is placed in the center of the load, removed at the end of the autoclave process, and incubated at 37°C. If the sterilization process is successful, the spores are killed and the organisms fail to grow.

Ethylene Oxide

Ethylene oxide is a colorless gas (soluble in water and common organic solvents) that is used to sterilize heat-sensitive items. The sterilization process is relatively slow and is influenced by the concentration of gas, relative humidity and moisture content of the item to be sterilized, exposure time, and temperature. The exposure time is reduced by 50% for each doubling of ethylene oxide concentration. Likewise, the activity of ethylene oxide approximately doubles with each temperature increase of 10°C. Sterilization with ethylene oxide is optimal in a relative humidity of approximately 30%, with decreased activity at higher or lower humidity. This is particularly problematic if the contaminated organisms are dried onto a surface or lyophilized. Ethylene oxide exerts its sporicidal activity through the alkylation of terminal hydroxyl, carboxyl, amino, and sulfhydryl groups. This process blocks the reactive groups required for many essential metabolic processes. Examples of other strong alkylating gases used as sterilants are formaldehyde and β-propiolactone. Because ethylene oxide can damage viable tissues, the gas must be dissipated before the item can be used. This aeration period is generally 16 hours or longer. The effectiveness of sterilization is monitored with the *Bacillus subtilis* spore test.

Aldehydes

As with ethylene oxide, aldehydes exert their effect through alkylation. The two best-known aldehydes are **formaldehyde** and **glutaraldehyde,** both of which can be used as sterilants or high-level disinfectants. Formaldehyde gas can be dissolved in water, creating a solution called formalin. Low concentrations of formalin are bacteriostatic (i.e., they inhibit but do not kill organisms), whereas higher concentrations (e.g., 20%) can kill all organisms. Combining formaldehyde with alcohol can enhance this microbicidal activity. Exposure of skin or mucous membranes to formaldehyde can be toxic, and vapors may be carcinogenic. For these reasons, formaldehyde is now rarely used in health care settings. Glutaraldehyde is less toxic for viable tissues, but it can still cause burns on the skin or mucous membranes. Glutaraldehyde is more active at alkaline pH levels ("activated" by sodium hydroxide) but is less stable. Glutaraldehyde is also inactivated by organic material, so items to be treated must first be cleaned.

Oxidizing Agents

Examples of oxidants include ozone, peracetic acid, and hydrogen peroxide, with the last used most commonly. **Hydrogen peroxide** effectively kills most bacteria at a concentration of 3% to 6% and kills all organisms, including

spores, at higher concentrations (10% to 25%). The active oxidant form is not hydrogen peroxide but rather the free hydroxyl radical formed by the decomposition of hydrogen peroxide. Hydrogen peroxide is used to disinfect plastic implants, contact lenses, and surgical prostheses.

Halogens

Halogens, such as compounds containing iodine or chlorine, are used extensively as disinfectants. **Iodine compounds** are the most effective halogens available for disinfection. Iodine is a highly reactive element that precipitates proteins and oxidizes essential enzymes. It is microbicidal against virtually all organisms, including spore-forming bacteria and mycobacteria. Neither the concentration nor the pH of the iodine solution affects the microbicidal activity, although the efficiency of iodine solutions is increased in acid solutions because more free iodine is liberated. Iodine acts more rapidly than do other halogen compounds or quaternary ammonium compounds. However, the activity of iodine can be reduced in the presence of some organic and inorganic compounds, including serum, feces, ascitic fluid, sputum, urine, sodium thiosulfate, and ammonia. Elemental iodine can be dissolved in aqueous potassium iodide or alcohol, or it can be complexed with a carrier. The latter compound is referred to as an *iodophor* (iodo, "iodine"; phor, "carrier"). Povidone iodine (iodine complexed with polyvinylpyrrolidone) is used most commonly and is relatively stable and nontoxic to tissues and metal surfaces, but it is expensive compared with other iodine solutions.

Chlorine compounds are also used extensively as disinfectants. Aqueous solutions of chlorine are rapidly bactericidal, although their mechanisms of action are not defined. Three forms of chlorine may be present in water: elemental chlorine (Cl_2), which is a very strong oxidizing agent; hypochlorous acid (HOCl); and hypochlorite ion (OCl_2). Chlorine also combines with ammonia and other nitrogenous compounds to form chloramines, or *N*-chloro compounds. Chlorine can exert its effect by the irreversible oxidation of sulfhydryl (SH) groups of essential enzymes. Hypochlorites are believed to interact with cytoplasmic components to form toxic *N*-chloro compounds, which interfere with cellular metabolism. The efficacy of chlorine is inversely proportional to the pH, with greater activity observed at acid pH levels. This is consistent with greater activity associated with hypochlorous acid rather than with hypochlorite ion concentration. The activity of chlorine compounds also increases with concentration (e.g., a twofold increase in concentration results in a 30% decrease in time required for killing) and temperature (e.g., a 50% to 65% reduction in killing time with a 10° C increase in temperature). Organic matter and alkaline detergents can reduce the effectiveness of chlorine compounds. These compounds demonstrate good germicidal activity, although spore-forming organisms are 10- to 1000-fold more resistant to chlorine than are vegetative bacteria.

Phenolic Compounds

Phenolic compounds (germicides) are rarely used as disinfectants. However, they are of historical interest because they were used as a comparative standard for assessing the activity of other germicidal compounds. The ratio of germicidal activity by a test compound to that by a specified concentration of phenol yielded the phenol coefficient. A value of 1 indicated equivalent activity, greater than 1 indicated activity less than phenol, and less than 1 indicated activity greater than phenol. These tests are limited because phenol is not sporicidal at room temperature (but is sporicidal at temperatures approaching 100° C), and it has poor activity against non–lipid-containing viruses. This is understandable because phenol is believed to act by disrupting lipid-containing membranes, resulting in leakage of cellular contents. Phenolic compounds are active against the normally resilient mycobacteria because the cell wall of these organisms has a very high concentration of lipids. Exposure of phenolics to alkaline compounds significantly reduces their activity, whereas halogenation of the phenolics enhances their activity. The introduction of aliphatic or aromatic groups into the nucleus of halogen phenols also increases their activity. Bisphenols are two phenol compounds linked together. The activity of these compounds can also be potentiated by halogenation. One example of a halogenated bisphenol is **hexachlorophene,** an antiseptic with activity against gram-positive bacteria.

Quaternary Ammonium Compounds

Quaternary ammonium compounds consist of four organic groups covalently linked to nitrogen. The germicidal activity of these cationic compounds is determined by the nature of the organic groups, with the greatest activity observed with compounds having 8- to 18-carbon-long groups. Examples of quaternary ammonium compounds include **benzalkonium chloride** and **cetylpyridinium chloride.** These compounds act by denaturing cell membranes to release the intracellular components. Quaternary ammonium compounds are bacteriostatic at low concentrations and bactericidal at high concentrations; however, organisms such as *Pseudomonas, Mycobacterium,* and the fungus *Trichophyton* are resistant to these compounds. Indeed, some *Pseudomonas* strains can grow in quaternary ammonium solutions. Many viruses and all bacterial spores are also resistant. Ionic detergents, organic matter, and dilution neutralize quaternary ammonium compounds.

Alcohols

The germicidal activity of alcohols increases with increasing chain length (maximum of five to eight carbons). The two most commonly used alcohols are **ethanol** and **isopropanol.** These alcohols are rapidly bactericidal against vegetative bacteria, mycobacteria, some fungi, and lipid-containing viruses. Unfortunately, alcohols have no activity against bacterial spores and have poor activity against some fungi and non–lipid-containing viruses. Activity is greater in the presence of water. Thus 70% alcohol is more active than 95% alcohol. Alcohol is a common disinfectant for skin surfaces and, when followed by treatment with an iodophor, is extremely effective for this purpose. Alcohols are also used to disinfect items such as thermometers.

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Questions

1. *Define the following terms and give three examples of each: sterilization, disinfection, and antisepsis.*
2. *Define the three levels of disinfection and give examples of each. When would each type of disinfectant be used?*
3. *What factors influence the effectiveness of sterilization with moist heat, dry heat, and ethylene oxide?*
4. *Give examples of each of the following disinfectants and their mode of action: iodine compounds, chlorine compounds, phenolic compounds, and quaternary ammonium compounds.*

Answers

1. There is not a uniform definition of *sterilization* or *disinfection*. In general, ***sterilization*** represents the total destruction of all microbes, including the more resilient forms such as bacterial spores, mycobacteria, nonenveloped viruses, and fungi. Examples of agents used for sterilization are ethylene oxide, hydrogen peroxide, peracetic acid, and glutaraldehyde. ***Disinfection*** results in the destruction of most organisms, although the more resilient microbes can survive some disinfection procedures. Examples of disinfectants include moist heat, hydrogen peroxide, and phenolic compounds. ***Antisepsis*** is used to reduce the number of microbes on skin surfaces. Examples of antiseptic agents include alcohols, iodophors, chlorhexidine, parachlorometaxylenol, and triclosan.
2. Disinfection is subdivided into high level, intermediate level, and low level. High-level disinfectants include moist heat, glutaraldehyde, hydrogen peroxide, peracetic acid, and chlorine compounds. Intermediate-level disinfectants include alcohols, iodophor compounds, and phenolic compounds. Low-level disinfectants include quaternary ammonium compounds. Although some agents are used for both sterilization and disinfection, the difference is the concentration of the agent and duration of treatment. The types of disinfectants used are determined by the nature of the material to be disinfected and how it will be used. If the material will be used for an invasive procedure but cannot withstand sterilization procedures (e.g., endoscopes, surgical instruments that cannot be autoclaved), then a high-level disinfectant would be used. Intermediate-level disinfectants are used to clean surfaces and instruments where contamination with highly resilient organisms is unlikely. Low-level disinfectants are used to clean noncritical instruments and devices (e.g., blood pressure cuffs, electrodes, stethoscopes).
3. The effectiveness of moist heat is greatest when applied under pressure. This allows the temperature to be elevated. Other factors that determine the effectiveness of moist heat are the duration of exposure and penetration of the steam into the contaminated material (determined by load size and flow rate of steam). Dry heat is effective if applied at a high temperature for a long duration. Ethylene oxide sterilization is a slow process that is influenced by the concentration of the gas, relative humidity, exposure time, and temperature. The effectiveness improves with a higher concentration of ethylene oxide, elevated temperatures, and a relative humidity of 30%.
4. Iodine compounds precipitate proteins and oxidize essential enzymes. Examples include tincture of iodine and povidone iodine (iodine complexed with polyvinylpyrrolidone). Chlorine compounds are strong oxidizing agents, although the precise mechanism of action is not well defined. Examples include elemental chlorine, hypochlorous acid, and hypochlorite ion. The most common commercial chlorine compound is bleach. Phenolic compounds act by disrupting lipid-containing membranes, resulting in a leakage of cellular contents. Examples include phenol (carbolic acid), *o*-phenylphenol, *o*-benzyl-*p*-chlorophenol, and *p*-tert-amyl-phenol. Quaternary ammonium compounds also denature cell membranes and include benzalkonium chloride and cetylpyridinium chloride.

SECTION

2

GENERAL PRINCIPLES OF LABORATORY DIAGNOSIS

CHAPTER

4

MICROSCOPY AND IN VITRO CULTURE

The foundation of microbiology was established in 1676 when Anton van Leeuwenhoek, using one of his early microscopes, observed bacteria in water. It was not until almost 200 years later that Pasteur was able to grow bacteria in the laboratory in a culture medium consisting of yeast extract, sugar, and ammonium salts. In 1881, Hesse used agar from his wife's kitchen to solidify the medium that then permitted the growth of macroscopic colonies of bacteria. Over the years, microbiologists have returned to the kitchen to create hundreds of culture media that are now routinely used in all clinical microbiology laboratories. Although tests that rapidly detect microbial antigens and nucleic acid–based molecular assays have replaced microscopy and culture methods for the detection of many organisms, the ability to observe microbes by microscopy and grow microbes in the laboratory remains an important procedure in clinical laboratories. For many diseases, these techniques remain the definitive methods to identify the cause of an infection. This chapter will provide an overview of the most commonly used techniques for microscopy and culture, and more specific details will be presented in the chapters devoted to laboratory diagnosis in the individual organism sections.

• Microscopy

In general, microscopy is used in microbiology for two basic purposes: the initial detection of microbes and the preliminary or definitive identification of microbes. The microscopic examination of clinical specimens is used to detect bacterial cells, fungal elements, parasites (eggs, larvae, or adult forms), and viral inclusions present in infected cells. Characteristic morphologic properties can be used for the preliminary identification of most bacteria and are used for the definitive identification of many fungi and parasites. The microscopic detection of organisms stained with antibodies labeled with fluorescent dyes or other markers has proved to be very useful for the specific identification of many organisms. Five general microscopic methods are used (Box 4-1).

Microscopic Methods

Brightfield (Light) Microscopy

The basic components of light microscopes consist of a light source used to illuminate the specimen positioned on a stage, a condenser used to focus the light on the specimen, and two lens systems **(objective lens** and **ocular lens)** used to magnify the image of the specimen. In brightfield microscopy the specimen is visualized by transillumination, with light passing up through the condenser to the specimen. The image is then magnified, first by the objective lens and then by the ocular lens. The total magnification of the image is the product of the magnifications of the objective and ocular lenses. Three different objective lenses are commonly used: low power (10-fold magnification), which can be used to scan a specimen; high dry (40-fold), which is used to look for large microbes such as parasites and filamentous fungi; and oil immersion (100-fold), which is used to observe bacteria, yeasts (single-cell stage of fungi), and the morphologic details of larger organisms and cells. Ocular lenses can further magnify the image (generally 10-fold to 15-fold).

The limitation of brightfield microscopy is the resolution of the image (i.e., the ability to distinguish that two objects are separate and not one). The **resolving power** of a microscope is determined by the wavelength of light used to illuminate the subject and the angle of light entering the objective lens (referred to as the **numerical aperture**). The resolving power is greatest when oil is placed between the objective lens (typically the 100× lens) and the specimen, because oil reduces the dispersion of light. The best brightfield microscopes have a resolving power of approximately 0.2 μm, which allows most bacteria, but not viruses, to be visualized. Although most bacteria and larger microorganisms can be seen with brightfield microscopy, the **refractive indices** of the organisms and background are similar. Thus organisms must be stained with a dye so they can be observed, or an alternative microscopic method must be used.

Darkfield Microscopy

The same objective and ocular lenses used in brightfield microscopes are used in darkfield microscopes; however, a special **condenser** is used that prevents transmitted light from directly illuminating the specimen. Only oblique scattered light reaches the specimen and passes into the lens systems, which causes the specimen to be brightly illuminated against a black background. The advantage of this method is that the resolving power of darkfield microscopy is significantly improved compared with that of brightfield microscopy (i.e., 0.02 μm versus 0.2 μm) and makes it possible to detect extremely thin bacteria such as *Treponema pallidum* (etiologic agent of syphilis) and *Leptospira* spp. (leptospirosis). The disadvantage of this method is that light passes around rather than through organisms, making it difficult to study their internal structure.

Box 4-1 Microscopic Methods

Brightfield (light) microscopy
Darkfield microscopy
Phase-contrast microscopy
Fluorescent microscopy
Electron microscopy

Phase-Contrast Microscopy

Phase-contrast microscopy enables the internal details of microbes to be examined. In this form of microscopy, as parallel beams of light are passed through objects of different densities, the wavelength of one beam moves out of "phase" relative to the other beam of light (i.e., the beam moving through the more dense material is retarded more than the other beam). Through the use of **annular rings** in the condenser and the objective lens, the differences in phase are amplified so that in-phase light appears brighter than out-of-phase light. This creates a three-dimensional image of the organism or specimen and permits more detailed analysis of the internal structures.

Fluorescent Microscopy

Some compounds called **fluorochromes** can absorb short-wavelength ultraviolet or ultrablue light and emit energy at a higher visible wavelength. Although some microorganisms show natural fluorescence **(autofluorescence),** fluorescent microscopy typically involves staining organisms with fluorescent dyes and then examining them with a specially designed fluorescent microscope. The microscope uses a high-pressure mercury, halogen, or xenon vapor lamp that emits a shorter wavelength of light than that emitted by traditional brightfield microscopes. A series of filters are used to block the heat generated from the lamp, eliminate infrared light, and select the appropriate wavelength for exciting the fluorochrome. The light emitted from the fluorochrome is then magnified through traditional objective and ocular lenses. Organisms and specimens stained with fluorochromes appear brightly illuminated against a dark background, although the colors vary depending on the fluorochrome selected. The contrast between the organism and background is great enough that the specimen can be screened rapidly under low magnification, and then the material is examined under higher magnification once fluorescence is detected.

Electron Microscopy

Unlike other forms of microscopy, **magnetic coils** (rather than lenses) are used in electron microscopes to direct a beam of electrons from a tungsten filament through a specimen and onto a screen. Because a much shorter wavelength of light is used, magnification and resolution are improved dramatically. Individual viral particles (as opposed to viral inclusion bodies) can be seen with electron microscopy. Samples are usually stained or coated with metal ions to create contrast. There are two types of electron microscopes: **transmission electron microscopes,** in which electrons such as light pass directly through the specimen, and **scanning electron microscopes,** in which electrons bounce off the surface of the specimen at an angle and a three-dimensional picture is produced. Today, electron microscopy is used more as a research tool than a diagnostic aid, with highly sensitive and specific nucleic acid amplification assays the primary diagnostic test in current use.

Examination Methods

Clinical specimens or suspensions of microorganisms can be placed on a glass slide and examined under the microscope (i.e., direct examination of a wet mount). Although large organisms (e.g., fungal elements, parasites) and cellular material can be seen using this method, analysis of the internal detail is often difficult. Phase-contrast microscopy can overcome some of these problems; alternatively, the specimen or organism can be stained by a variety of methods (Table 4-1).

Direct Examination

Direct examination methods are the simplest for preparing samples for microscopic examination. The sample can be suspended in water or saline **(wet mount),** mixed with alkali to dissolve background material **(potassium hydroxide [KOH] method),** or mixed with a combination of alkali and a contrasting dye (e.g., **lactophenol cotton blue, iodine**). The dyes nonspecifically stain the cellular material, increasing the contrast with the background, and permit examination of the detailed structures. A variation is the **India ink method,** in which the ink darkens the background rather than the cell. This method is used to detect capsules surrounding organisms, such as the yeast *Cryptococcus* (the dye is excluded by the capsule, creating a clear halo around the yeast cell) and encapsulated *Bacillus anthracis*.

Differential Stains

A variety of differential stains are used to stain specific organisms or components of cellular material. The **Gram stain** is the best known and most widely used stain and forms the basis for the phenotypic classification of bacteria. Yeasts can also be stained with this method (yeasts are gram-positive). The **iron hematoxylin** and **trichrome** stains are invaluable for identifying protozoan parasites, and the **Wright-Giemsa** stain is used to identify blood parasites and other selected organisms. Stains such as methenamine silver and toluidine blue O have largely been replaced by more sensitive or technically easier differential or fluorescent stains.

Acid-Fast Stains

At least three different acid-fast stains are used, each exploiting the fact that some organisms retain a primary stain even when exposed to strong decolorizing agents such as mixtures of acids and alcohols. The **Ziehl-Neelsen** is the oldest method used but requires heating the specimen during the staining procedure. Many laboratories have replaced this method with either the cold acid-fast stain **(Kinyoun method)** or the fluorochrome stain **(auramine-rhodamine method).** The fluorochrome method is the stain of choice because a large area of the specimen can be examined rapidly by simply searching for fluorescing organisms against a black background. Some organisms are "partially acid-fast," retaining the primary stain only when they are decolorized with a weakly acidic solution. This property is characteristic of only a few organisms (see Table 4-1), making it quite valuable for their preliminary identification.

Table 4-1 Microscopic Preparations and Stains Used in the Clinical Microbiology Laboratory

Staining Method	Principle and Applications
Direct Examination	
Wet mount	Unstained preparation is examined by brightfield, darkfield, or phase-contrast microscopy.
10% KOH	KOH is used to dissolve proteinaceous material and facilitate detection of fungal elements that are not affected by strong alkali solution. Dyes such as lactophenol cotton blue can be added to increase contrast between fungal elements and background.
India ink	Modification of KOH procedure in which ink is added as contrast material. Dye primarily used to detect *Cryptococcus* spp. in cerebrospinal fluid and other body fluids. Polysaccharide capsule of *Cryptococcus* spp. excludes ink, creating halo around yeast cell.
Lugol iodine	Iodine is added to wet preparations of parasitology specimens to enhance contrast of internal structures. This facilitates differentiation of amebae and host white blood cells.
Differential Stains	
Gram stain	Most commonly used stain in microbiology laboratory, forming basis for separating major groups of bacteria (e.g., gram-positive, gram-negative). After fixation of specimen to glass slide (by heating or alcohol treatment), specimen is exposed to crystal violet and then iodine is added to form complex with primary dye. During decolorization with alcohol or acetone, complex is retained in gram-positive bacteria but lost in gram-negative organisms; counterstain safranin is retained by gram-negative organisms (hence their red color). The degree to which organism retains stain is function of organism, culture conditions, and staining skills of the microscopist.
Iron hematoxylin stain	Used for detection and identification of fecal protozoa. Helminth eggs and larvae retain too much stain and are more easily identified with wet-mount preparation.
Methenamine silver	In general, performed in histology laboratories rather than in microbiology laboratories. Used primarily for stain detection of fungal elements in tissue, although other organisms (e.g., bacteria) can be detected. Silver staining requires skill because nonspecific staining can render slides unable to be interpreted.
Toluidine blue O stain	Used primarily for detection of *Pneumocystis* organisms in respiratory specimens. Cysts stain reddish-blue to dark purple on light blue background. Background staining is removed by sulfation reagent. Yeast cells stain and are difficult to distinguish from *Pneumocystis* cells. Trophozoites do not stain. Many laboratories have replaced this stain with specific fluorescent stains.
Trichrome stain	Alternative to iron hematoxylin for staining protozoa. Protozoa have bluish-green to purple cytoplasms with red or purplish-red nuclei and inclusion bodies; specimen background is green.
Wright-Giemsa stain	Used to detect blood parasites, viral and chlamydial inclusion bodies, and *Borrelia, Toxoplasma, Pneumocystis,* and *Rickettsia* spp. This is a polychromatic stain that contains a mixture of methylene blue, azure B, and eosin Y. Giemsa stain combines methylene blue and eosin. Eosin ions are negatively charged and stain basic components of cells orange to pink, whereas other dyes stain acidic cell structures various shades of blue to purple. Protozoan trophozoites have a red nucleus and grayish-blue cytoplasm; intracellular yeasts and inclusion bodies typically stain blue; rickettsiae, chlamydiae, and *Pneumocystis* spp. stain purple.
Acid-Fast Stains	
Ziehl-Neelsen stain	Used to stain mycobacteria and other acid-fast organisms. Organisms are stained with basic carbolfuchsin and resist decolorization with acid-alkali solutions. Background is counterstained with methylene blue. Organisms appear red against light blue background. Uptake of carbolfuchsin requires heating specimen (hot acid-fast stain).
Kinyoun stain	Cold acid-fast stain (does not require heating). Same principle as Ziehl-Neelsen stain.
Auramine-rhodamine	Same principle as other acid-fast stains, except that fluorescent dyes (auramine and rhodamine) are used for primary stain, and potassium permanganate (strong oxidizing agent) is the counterstain and inactivates unbound fluorochrome dyes. Organisms fluoresce yellowish-green against a black background.
Modified acid-fast stain	Weak decolorizing agent is used with any of three acid-fast stains listed. Whereas mycobacteria are strongly acid-fast, other organisms stain weaker (e.g., *Nocardia, Rhodococcus, Tsukamurella, Gordonia, Cryptosporidium, Isospora, Sarcocystis,* and *Cyclospora*). These organisms can be stained more efficiently by using a weak decolorizing agent. Organisms that retain this stain are referred to as partially acid-fast.
Fluorescent Stains	
Acridine orange stain	Used for detection of bacteria and fungi in clinical specimens. Dye intercalates into nucleic acid (native and denatured). At neutral pH, bacteria, fungi, and cellular material stain reddish-orange. At acid pH (4.0), bacteria and fungi remain reddish-orange, but background material stains greenish-yellow.
Auramine-rhodamine stain	Same as acid-fast stains.
Calcofluor white stain	Used to detect fungal elements and *Pneumocystis* spp. Stain binds to cellulose and chitin in cell walls; microscopist can mix dye with KOH. (Many laboratories have replaced traditional KOH stain with this stain.)

Table 4-1 Microscopic Preparations and Stains Used in the Clinical Microbiology Laboratory—cont'd

Staining Method	Principle and Applications
Direct fluorescent antibody stain	Antibodies (monoclonal or polyclonal) are complexed with fluorescent molecules. Specific binding to an organism is detected by presence of microbial fluorescence. Technique has proved useful for detecting or identifying many organisms (e.g., *Streptococcus pyogenes, Bordetella, Francisella, Legionella, Chlamydia, Pneumocystis, Cryptosporidium, Giardia*, influenza virus, herpes simplex virus). Sensitivity and specificity of test are determined by number of organisms present in test sample and quality of antibodies used in reagents.

KOH, Potassium hydroxide.

Fluorescent Stains

The auramine-rhodamine acid-fast stain is a specific example of a fluorescent stain. Numerous other fluorescent dyes have also been used to stain specimens. For example, the **acridine orange stain** can be used to stain bacteria and fungi, and **calcofluor white** stains the chitin in fungal cell walls. Although the acridine orange stain is rather limited in its applications, the calcofluor white stain has replaced the potassium hydroxide stains. Another procedure is the examination of specimens with specific antibodies labeled with fluorescent dyes **(fluorescent antibody stains).** The presence of fluorescing organisms is a rapid method for both detection and identification of the organism.

• In Vitro Culture

The success of culture methods is defined by the biology of the organism, the site of the infection, the patient's immune response to the infection, and the quality of the culture media. The bacterium *Legionella* is an important respiratory pathogen; however, it was never grown in culture until it was recognized that recovery of the organism required using media supplemented with iron and L-cysteine. *Campylobacter,* an important enteric pathogen, was not recovered in stool specimens until highly selective media were incubated at 42° C in a microaerophilic atmosphere. *Chlamydia,* an important bacterium responsible for sexually transmitted diseases, is an obligate intracellular pathogen that must be grown in living cells. *Staphylococcus aureus,* the cause of staphylococcal toxic shock syndrome, produces disease by release of a toxin into the circulatory system. Culture of blood will almost always be negative, but culture of the site where the organism is growing will detect the organism. In many infections (e.g., gastroenteritis, pharyngitis, urethritis), the organism responsible for the infection will be present among many other organisms that are part of the normal microbial population at the site of infection. Many media have been developed that suppress the normally present microbes and allow easier detection of clinically important organisms. The patient's innate and adaptive immunity may suppress the pathogen, so highly sensitive culture techniques are frequently required. Likewise, some infections are characterized by the presence of relatively few organisms. For example, most septic patients have less than one organism per milliliter of blood, so recovery of these organisms in a traditional blood culture requires inoculation of a large volume of blood into enrichment broths. Finally, the quality of the media must be carefully monitored to demonstrate it will perform as designed.

Relatively few laboratories prepare their own media today. Most media are produced by large commercial companies with expertise in media production. Although this has obvious advantages, it also means that media are not "freshly produced." Although this is generally not a problem, it can impact the recovery of some fastidious organisms (e.g., *Bordetella pertussis*). Thus laboratories that perform sophisticated testing frequently have the ability to make a limited amount of specialized media. Dehydrated formulations of most media are available, so this can be accomplished with minimal difficulty. Please refer to the references in the Bibliography for additional information about the preparation and quality control of media.

Types of Culture Media

Culture media can be subdivided into four general categories: (1) enriched nonselective media, (2) selective media, (3) differential media, and (4) specialized media (Table 4-2). Some examples of these media are summarized below.

Enriched Nonselective Media

These media are designed to support the growth of most organisms without fastidious growth requirements. The following are some of the more commonly used media:

Blood agar. Many types of blood agar media are used in clinical laboratories. The media contain two primary components—a basal medium (e.g., tryptic soy, brain heart infusion, *Brucella* base) and blood (e.g., sheep, horse, rabbit). Various other supplements can also be added to extend the range of organisms that can grow on the media.

Chocolate agar. This is a modified blood agar medium. When blood or hemoglobin is added to the heated basal media, it turns brown (hence the name). This medium supports the growth of most bacteria, including some that do not grow on blood agar (i.e., *Haemophilus,* some pathogenic *Neisseria* strains).

Mueller-Hinton agar. This is the recommended medium for routine antibiotic susceptibility testing of bacteria. It has a well-defined composition of beef and casein extracts, salts, divalent cations, and soluble starch that is necessary for reproducible test results.

Thioglycolate broth. This is one of a variety of enrichment broths used to recover low numbers of aerobic and anaerobic bacteria. Various formulations are used, but most include casein digest, glucose, yeast extract, cysteine, and sodium thioglycolate. Supplementation with hemin and vitamin K will enhance the recovery of anaerobic bacteria.

Table 4-2 Types of Culture Media

Type	Media (examples)	Purpose
Nonselective	Blood agar	Recovery of bacteria and fungi
	Chocolate agar	Recovery of bacteria including *Haemophilus* and *Neisseria gonorrhoeae*
	Mueller-Hinton agar	Bacterial susceptibility test medium
	Thioglycolate broth	Enrichment broth for anaerobic bacteria
	Sabouraud dextrose agar	Recovery of fungi
Selective, differential	MacConkey agar	Selective for gram-negative bacteria; differential for lactose-fermenting species
	Mannitol salt agar	Selective for staphylococci; differential for *Staphylococcus aureus*
	Xylose lysine deoxycholate agar	Selective, differential agar for *Salmonella* and *Shigella* in enteric cultures
	Lowenstein-Jensen medium	Selective for mycobacteria
	Middlebrook agar	Selective for mycobacteria
	CHROMagar	Selective, differential for selected bacteria and yeasts
	Inhibitory mold agar	Selective for molds
Specialized	Buffered charcoal yeast extract (BCYE) agar	Recovery of *Legionella* and *Nocardia*
	Cystine-tellurite agar	Recovery of *Corynebacterium diphtheriae*
	Lim broth	Recovery of *Streptococcus agalactiae*
	MacConkey sorbitol agar	Recovery of *Escherichia coli* O157
	Regan Lowe agar	Recovery of *Bordetella pertussis*
	Thiosulfate citrate bile salts sucrose (TCBS) agar	Recovery of *Vibrio* species

Sabouraud dextrose agar. This is an enriched medium consisting of digests of casein and animal tissue supplemented with glucose that is used for the isolation of fungi. A variety of formulations have been developed, but most mycologists use the formulation with a low concentration of glucose and neutral pH. By reducing the pH and adding antibiotics to inhibit bacteria, this medium can be made selective for fungi.

Selective Media and Differential Media

Selective media are designed for the recovery of specific organisms that may be present in a mixture of other organisms (e.g., an enteric pathogen in stool). The media are supplemented with inhibitors that suppress the growth of unwanted organisms. These media can be made differential by adding specific ingredients that allow identification of an organism in a mixture (e.g., addition of lactose and a pH indicator to detect lactose fermenting organisms). The following are some examples of selective and differential media:

MacConkey agar. This is a selective agar for gram-negative bacteria and differential for differentiation of lactose-fermenting and lactose-nonfermenting bacteria. The medium consists of digests of peptones, bile salts, lactose, neutral red, and crystal violet. The bile salts and crystal violet inhibit gram-positive bacteria. Bacteria that ferment lactose produce acid that precipitates the bile salts and causes a red color in the neutral red indicator.

Mannitol salt agar. This is a selective medium used for the isolation of staphylococci. The medium consists of digests of casein and animal tissue, beef extract, mannitol, salts, and phenol red. Staphylococci can grow in the presence of a high salt concentration, and *S. aureus* can ferment mannitol, producing yellow-colored colonies on this agar.

Xylose-lysine deoxycholate (XLD) agar. This is a selective agar used for detection of *Salmonella* and *Shigella* in enteric cultures. This is an example of a very clever approach to detecting important bacteria in a complex mixture of insignificant bacteria. The medium consists of yeast extract with xylose, lysine, lactose, sucrose, sodium deoxycholate, sodium thiosulfate, ferric ammonium citrate, and phenol red. Sodium deoxycholate inhibits the growth of the majority of nonpathogenic bacteria. Those that do grow typically ferment lactose, sucrose, or xylose, producing yellow colonies. *Shigella* does not ferment these carbohydrates, so the colonies appear red. *Salmonella* ferments xylose but also decarboxylates lysine, producing the alkaline diamine product cadaverine. This neutralizes the acid fermentation products, thus the colonies appear red. Because most *Salmonella* produce hydrogen sulfide from sodium thiosulfate, the colonies will turn black in the presence of ferric ammonium citrate, thus differentiating *Salmonella* from *Shigella*.

Lowenstein-Jensen (LJ) medium. This medium, used for the isolation of mycobacteria, contains glycerol, potato flour, salts, and coagulated whole eggs (to solidify the medium). Malachite green is added to inhibit gram-positive bacteria.

Middlebrook agar. This agar medium is also used for the isolation of mycobacteria. It contains nutrients required for the growth of mycobacteria (i.e., salts, vitamins, oleic acid, albumin, catalase, glycerol, glucose) and malachite

green for the inhibition of gram-positive bacteria. In contrast with LJ medium, it is solidified with agar.

CHROMagar. These selective differential agars are used for the isolation and identification of a variety of bacteria (e.g., *Staphylococcus aureus,* enteric bacteria) and yeasts. An example of design of these media is the one developed for *Candida* species. This medium has chloramphenicol to inhibit bacteria and a mixture of proprietary chromogenic substrates. The different species of *Candida* have enzymes that can use one or more of the substrates, releasing the color compound and producing colored colonies. Thus *Candida albicans* forms green colonies, *Candida tropicalis* forms purple colonies, and *Candida krusei* forms pink colonies.

Inhibitory mold agar. This medium is an enriched selective formulation used for the isolation of pathogenic fungi other than dermatophytes. Chloramphenicol is added to suppress the growth of contaminating bacteria.

Specialized Media

A large variety of specialized media have been created for the detection of specific organisms that may be fastidious or typically present in large mixtures of organisms. The more commonly used media are described in the specific organism chapters in this textbook.

Cell Culture

Some bacteria and all viruses are **strict intracellular microbes;** that is, they can only grow in living cells. In 1949, John Franklin Enders described a technique for cultivating mammalian cells for the isolation of poliovirus. This technique has been expanded for the growth of most strict intracellular organisms. The cell cultures can either be cells that grow and divide on a surface (i.e., **cell monolayer**) or grow suspended in broth. Some cell cultures are well established and can be maintained indefinitely. These cultures are commonly commercially available. Other cell cultures must be prepared immediately before they are infected with the bacteria or viruses and cannot be maintained in the laboratory for more than a few cycles of division **(primary cell cultures).** Entry into cells is frequently regulated by the presence of specific receptors, so the differential ability to infect specific cell lines can be used to predict the identity of the bacterium or virus. Additional information about the use of cell cultures is described in the following chapters.

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Questions

1. *Explain the principles underlying brightfield, darkfield, phase-contrast, fluorescent, and electron microscopy. Give one example in which each method would be used.*
2. *List examples of direct microscopic examinations, differential stains, acid-fast stains, and fluorescent stains.*
3. *Name three factors that affect the success of a culture.*
4. *Give three examples of enriched nonselective media.*
5. *Give three examples of selective differential media.*

Answers

1. In **brightfield microscopy,** visible light passes through a condenser, then through the object under observation, and finally through a series of lenses to magnify the image. This method is the most commonly used microscopic technique to examine specimens placed on glass slides. **Darkfield microscopy** uses the same series of lenses as brightfield microscopy; however, a special condenser is used to illuminate the subject material from an oblique angle. Thus the subject is brightly illuminated against a black background. This method is used to detect organisms that are too thin to be observed by brightfield microscopy (e.g., *Treponema,* the etiologic agent of syphilis). **Phase-contrast microscopy** illuminates objects with parallel beams of light that move out of phase relative to each other. This allows objects to appear as three-dimensional structures and is useful for observing internal structures. **Fluorescent microscopy** uses high-pressure mercury, halogen, or xenon vapor lamps that emit a short wavelength of light to illuminate the object. A series of filters block heat and infrared light and select a specific wavelength of light emitted by the object. This "fluorescence" is observed as a brightly illuminated object against a dark background. This technique is very useful for organisms with natural fluorescence (e.g., *Legionella*) and organisms stained with specific fluorescent dyes (e.g., *Mycobacterium*).
2. Methods of direct microscopic examination include suspending the sample in water (e.g., wet mount for fungi) or a contrasting dye (e.g., lactophenol cotton blue for fungi or iodine for parasites). Differential stains are used commonly to detect bacteria (e.g., Gram stain, acid-fast stain), parasites (e.g., iron hematoxylin and trichrome stains), and blood-borne pathogens (e.g., Giemsa stain for *Borrelia* and *Plasmodium*). A variety of acid-fast stain methods have been developed (e.g., Ziehl-Neelsen, Kinyoun, fluorochrome) that detect bacteria *(Mycobacterium, Nocardia, Rhodococcus)* and parasites *(Cryptosporidium, Cyclospora, Isospora)*. Common fluorescent stains have been used to detect fungi (calcofluor white stain) or acid-fast organisms (auramine-rhodamine stain).
3. Biology of the organism (Does the organism have special growth requirements or require supplementation of the medium with growth factors?); site of the infection (Is the submitted specimen from the area of infection?); patient's immune response to the infection (Is the organism inactivated or killed by the patient's immune response?); quality of the culture medium.
4. Blood agar, chocolate agar, thioglycolate broth.
5. MacConkey agar, mannitol salt agar, xylose lysine deoxycholate agar.

CHAPTER

5

MOLECULAR DIAGNOSIS

Like the evidence left at the scene of a crime, the DNA (deoxyribonucleic acid), RNA (ribonucleic acid), or proteins of an infectious agent in a clinical sample can be used to help identify the agent. In many cases, the agent can be detected and identified in this way even if it cannot be isolated or detected by immunologic means. New techniques and adaptations of older techniques are being developed for the analysis of infectious agents.

The advantages of molecular techniques are their sensitivity, specificity, and safety. From the standpoint of safety, these techniques do not require isolation of the infectious agent and can be performed on chemically fixed (inactivated) samples or extracts. Because of their sensitivity, very dilute samples of microbial DNA or RNA can be detected in a tissue even if the agent is not replicating or producing other evidence of infection. These techniques can distinguish related strains on the basis of differences in their genotype (i.e., mutants). This is especially useful for distinguishing antiviral drug–resistant strains, which may differ by a single nucleotide.

• Detection of Microbial Genetic Material

Electrophoretic Analysis of DNA and Restriction Fragment Length Polymorphism

The genome structure and genetic sequence are major distinguishing characteristics of the family, type, and strain of microorganism. Specific strains of microorganisms can be distinguished on the basis of their DNA or RNA or by the DNA fragments produced when the DNA is cleaved by specific restriction endonucleases **(restriction enzymes)** or selectively amplified (see later). Restriction enzymes recognize specific DNA sequences that have a palindromic structure; an example follows:

$$\begin{array}{l}\overrightarrow{\text{G}}\text{AATTC}\\ \text{CTTAA}\overleftarrow{\text{G}}\end{array}$$ EcoR 1 recognition sequence and cleavage

The DNA sites recognized by different restriction endonucleases differ in their sequence, length, and frequency of occurrence. As a result, different restriction endonucleases cleave the DNA of a sample in different places, yielding fragments of different lengths. The cleavage of different DNA samples with one restriction endonuclease can also yield fragments of many different lengths. The differences in the length of the DNA fragments among the different strains of a specific organism produced on cleavage with one or more restriction endonucleases is termed **restriction fragment length polymorphism (RFLP).**

DNA or RNA fragments of different sizes or structures can be distinguished by their electrophoretic mobility in an agarose or polyacrylamide gel. Different forms of the same DNA sequence and different lengths of DNA move through the mazelike structure of an agarose gel at different speeds, allowing their separation. The DNA can be visualized by staining with ethidium bromide. Smaller fragments (<20,000 base pairs), such as those from bacterial plasmids or viruses, can be separated and distinguished by normal electrophoretic methods. Larger fragments, such as those from whole bacteria, can be separated by using a special electrophoretic technique called *pulsed-field gel electrophoresis*.

RFLP is useful, for example, for distinguishing different strains of herpes simplex virus (HSV). Comparison of the restriction endonuclease cleavage patterns of DNA from different isolates can identify a pattern of virus transmission from one person to another or distinguish HSV-1 from HSV-2. RFLP has also been used to show the spread of a strain of *Streptococcus* causing necrotizing fasciitis from one patient to other patients, an emergency medical technician, and the emergency department and attending physicians (Figure 5-1). Often, comparison of the 16S ribosomal RNA is used to identify different bacteria.

Nucleic Acid Detection, Amplification, and Sequencing

DNA probes can be used like antibodies as sensitive and specific tools to detect, locate, and quantitate specific nucleic acid sequences in clinical specimens (Figure 5-2). Because of the specificity and sensitivity of DNA probe techniques, individual species or strains of an infectious agent can be detected even if they are not growing or replicating.

DNA probes are chemically synthesized or obtained by cloning specific genomic fragments or an entire viral genome into bacterial vectors (plasmids, cosmids). DNA copies of RNA viruses are made with the retrovirus reverse transcriptase and then cloned into these vectors. After chemical or heat treatments melt (separate) the DNA strands in the sample, the DNA probe is added and allowed to **hybridize** (bind) with the identical or nearly identical sequence in the sample. The **stringency** (the requirement for an exact sequence match) of the interaction can be varied so that related sequences can be detected or different strains (mutants) can be distinguished. The DNA probes are labeled with radioactive or chemically modified nucleotides (e.g.,

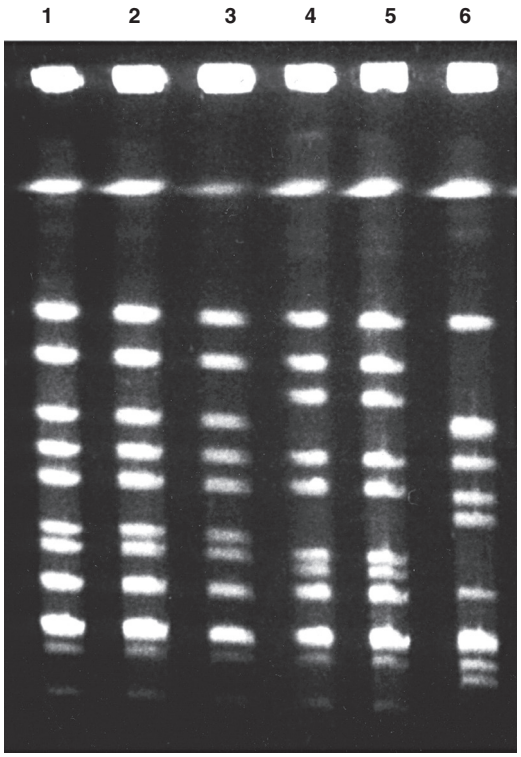

FIGURE 5-1 Restriction fragment length polymorphism distinction of DNA from bacterial strains separated by pulsed-field gel electrophoresis. Lanes 1 to 3 show Sma 1 restriction endonuclease-digested DNA from bacteria from two family members with necrotizing fasciitis and from their physician (pharyngitis). Lanes 4 to 6 are from unrelated *Streptococcus pyogenes* strains. (Courtesy Dr. Joe DiPersio, Akron, Ohio.)

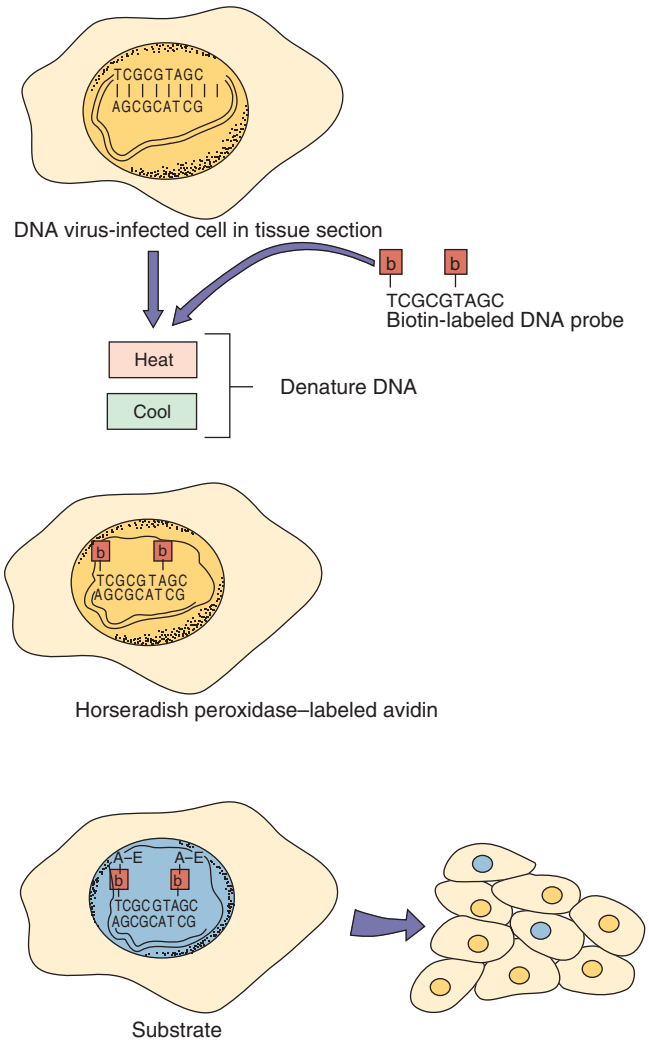

FIGURE 5-2 DNA probe analysis of virus-infected cells. Such cells can be localized in histologically prepared tissue sections using DNA probes consisting of as few as nine nucleotides or bacterial plasmids containing the viral genome. A tagged DNA probe is added to the sample. In this case, the DNA probe is labeled with biotin-modified thymidine, but radioactive agents can also be used. The sample is heated to denature the DNA and cooled to allow the probe to hybridize to the complementary sequence. Horseradish peroxidase–labeled avidin is added to bind to the biotin on the probe. The appropriate substrate is added to color the nuclei of virally infected cells. *A,* Adenine; *b,* biotin; *C,* cytosine; *G,* guanine; *T,* thymine.

biotinylated uridine) so that they can be detected and quantitated. The use of a biotin-labeled DNA probe allows the use of a fluorescent or enzyme-labeled avidin or streptavidin (proteins that bind tightly to biotin) molecule to detect viral nucleic acids in a cell in a way similar to how indirect immunofluorescence or an enzyme immunoassay localizes an antigen.

The DNA probes can detect specific genetic sequences in fixed permeabilized tissue biopsy specimens by **in situ hybridization.** When fluorescent detection is used, it is called **FISH: fluorescent in situ hybridization.** The localization of cytomegalovirus (CMV)-infected (Figure 5-3) or papillomavirus-infected cells by in situ hybridization is preferable to an immunologic means of doing so and is the only commercially available means of localizing papillomavirus. There are now many commercially available microbial probes and kits for detecting viruses, bacteria, and other microbes.

Specific nucleic acid sequences in extracts from a clinical sample can be detected by applying a small volume of the extract to a nitrocellulose filter **(dot blot)** and then probing the filter with labeled, specific viral DNA. Alternatively, the electrophoretically separated restriction endonuclease cleavage pattern can be transferred onto a nitrocellulose filter (**Southern blot**—DNA : DNA probe hybridization), and then the specific sequence can be identified by hybridization with a specific genetic probe and by its characteristic electrophoretic mobility. Electrophoretically separated RNA (**Northern blot**—RNA : DNA probe hybridization) blotted onto a nitrocellulose filter can be detected in a similar manner.

The **polymerase chain reaction (PCR)** amplifies single copies of viral DNA millions of times over and is one of the most useful genetic analysis techniques (Figure 5-4). In this technique, a sample is incubated with two short DNA oligomers, termed **primers,** that are complementary to the ends of a known genetic sequence within the total DNA, a heat-stable DNA polymerase (Taq or other polymerase obtained from thermophilic bacteria), nucleotides, and buffers. The oligomers hybridize to the appropriate sequence of DNA and