Chapter 16 – Microbiology Basics

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ABSTRACT

Microbiology is the study of organisms too small to be seen by the naked eye. Clinical microbiology encompasses the study of pathogens such as bacteria, viruses, fungi, and parasites, which cause disease or infection in humans. Issues of concern in clinical microbiology include the nature and epidemiology of etiological agents, how they interact with the immune system, rapid diagnosis, and providing information on treatment, such as antimicrobial susceptibility.

Fundamental knowledge of microorganisms, their identification, significance, and basic laboratory techniques provide the infection preventionist with an understanding of pathogenic organisms. This chapter includes an overview of microorganisms, clinical laboratory methods that are used to evaluate the presence and/or significance of microorganisms, and methods to analyze the relatedness of microorganisms for epidemiological purposes.

Readers who desire more information regarding the technical laboratory aspects of specific organism identification are encouraged to consult the supplemental resources provided at the end of the chapter. Spending a few days in the microbiology laboratory under the direction of a certified laboratory professional is also an excellent method of developing practical knowledge.

KEY CONCEPTS

- The clinical microbiology laboratory is an important partner in the practice of infection prevention.
- The clinical microbiology laboratory can provide information on microorganisms determined to be of clinical significance.
- Proper specimen collection and transport are integral to the recovery of valid microbiological information.
- The presence of microorganisms in a clinical specimen does not always indicate the presence of infection.
- A variety of methods can be used to identify bacteria, fungi, and viruses.
- Antimicrobial susceptibility testing is commonly used to assist in the selection of appropriate antimicrobial therapy. Monitoring resistance patterns is an important function of the clinical microbiology laboratory.
- The clinical microbiology laboratory can be useful during outbreak investigations and situations requiring environmental sampling.

BACKGROUND

The field of microbiology includes the study of bacteria, fungi (molds and yeasts), protozoa, viruses, and algae. The infection preventionist is likely to encounter most of these microorganism types in the course of their practice with the exception of algae. Most microorganisms are single cells and exhibit characteristics common to all biological systems: reproduction, metabolism, growth, irritability, adaptability, mutation, and organization. Microorganisms are traditionally placed in their own kingdom (Protista), because they share characteristics that might cause them to be classified in both the plant and animal kingdoms.

Microorganism Classification

Microorganisms are grouped into three categories based on cell organization and function: prokaryotes, eukaryotes, and Archaebacteria. Archaebacteria are microorganisms that grow under extreme environmental conditions. Because they are not encountered in clinical microbiology, they are not discussed further in this chapter. Prokaryotes are probably the smallest living organisms, ranging in size from 0.15 μm (mycoplasmas) to about 2.0 μm (many of the bacteria). Prokaryotic cells are different from eukaryotic cells in that they have no distinct membrane around their nuclear deoxyribonucleic acid (DNA), they replicate by binary fission, have plasmids (small pieces of DNA found in the cytoplasm), and typically have a cell wall. The characteristics of prokaryotic cells apply to bacteria and cyanobacteria (formerly known as blue-green algae), as well as rickettsiae, chlamydiae, and mycoplasmas.

Eukaryotic cells are generally larger and more complex than prokaryotic cells. They contain a distinct nucleus and organelles (compartments for localizing metabolic function), replicate by mitosis and meiosis, and, with the exception of fungi, typically do not have a cell wall. Fungi have cell walls made of chitin and other polysaccharides. Eukaryotes include microorganisms such as fungi, protozoa, and simple algae.

Viruses and prions are considered neither prokaryotes nor eukaryotes because they lack the characteristics of living things, except the ability to replicate, which they accomplish only in living cells.

Classification Schemes

The subgrouping of microorganisms is based ideally on evolutionary lines of descent. Traditionally these have been determined on the basis of morphological or biochemical characteristics. With the advent of DNA and ribonucleic acid (RNA) testing, schemes have been revised based on the degree of genetic similarity between different species. This has resulted in the reclassification or renaming of many organisms. Using the example of Escherichia coli, the groupings (from most to least inclusive) are as follows:

1. Kingdom: Prokaryotae (includes all bacteria)
2. Division: Protophyta
3. Class: Schizomycetes

4. Order: Eubacteriales
5. Family: Enterobacteriaceae
6. Genus: *Escherichia*
7. Species: *coli*

Genus and species are of primary importance in designating a microorganism. The correct format for naming an organism is genus (capitalized, italicized, or underlined), species (lowercase, italicized, or underlined): *Escherichia coli* (abbreviation, *E. coli*).

For some organisms, further identification to the subspecies level can be determined based on serotypes. Serological testing of the organism is performed to identify the presence or absence of known cell surface antigens such as the somatic antigen designated with the letter O, the flagella antigen designated with the letter H, or the capsular antigen designated with the letter K, etc. In the *E. coli* example, the organism could be further tested to identify or rule out the presence of *E. coli* O157:H7, a Shiga-toxin–producing strain of the bacterium that causes bloody diarrhea and occasionally kidney failure in infected patients.

Bacteria

Cell Structure

Internal Structures

Bacteria are very small, relatively simple, single-celled organisms. They contain a single long circular molecule of double-stranded DNA. This "bacterial chromosome" is not surrounded by a nuclear envelope and is attached to the plasma membrane. In addition to the bacterial chromosome, bacteria often contain small circular, double-stranded DNA molecules called plasmids. Although plasmids are not necessary for cell survival, they may carry genes for activities such as antibiotic resistance, production of toxins, and synthesis of enzymes. Plasmids can be transferred from one bacterium to another and genes may move from plasmid to chromosome. These genes are called transposable genetic elements or transposons. Bacteria also contain ribosomes that function as the site of protein synthesis. The plasma membrane encloses the cytoplasm of the cell and provides selective permeability for nutrients to enter.

When essential nutrients are depleted, certain Gram-positive bacteria (e.g., *Clostridium* and *Bacillus*), form "resting" cells called endospores or spores. These spores contain condensed nuclear material and protein and can survive extreme heat, lack of water, and exposure to toxic chemicals. When growth conditions permit, the cell germinates into a dividing bacterium also known as a vegetative state. An example of this is seen with *Clostridium difficile*–associated diarrhea. When the patient has active diarrheal disease, the feces contains both the vegetative and spore forms of the bacteria. Once shed from the body, the vegetative bacteria form spores in order to survive the inhospitable temperatures and detergents/disinfectants used for cleaning. When the spore encounters appropriate living conditions, it germinates back into the vegetative state and begins to reproduce.

<u>External Structures</u>

The cell wall of bacteria is a complex, semirigid structure responsible for the shape of the cell. It surrounds the underlying, fragile plasma membrane and protects it and the interior of the cell from the environment. The cell wall is made up of a macromolecular network called peptidoglycan. In most Gram-positive bacteria, the cell wall consists of many layers of peptidoglycan, forming a thick rigid structure. By contrast, Gram-negative cell walls contain only one (or very few) layers of peptidoglycan. Gram-negative cells possess an outer membrane that is composed of lipoproteins, lipopolysaccharides, and phospholipids. This outer membrane helps some organisms evade phagocytosis, provides a barrier to certain antibiotics, and confers properties of virulence (endotoxins). Glycocalyx is a general term used for substances that surround cells with a chemical composition that can vary widely. If this substance is organized and firmly attached to the cell wall, it is described as a capsule. Capsules may contribute to bacterial virulence and may offer protection from phagocytosis. If the glycocalyx is unorganized and only loosely attached to the cell wall, it is described as a slime layer.

Some bacteria have flagella which are long filamentous appendages that can propel the cell. Many Gram-negative bacteria possess hair-like appendages that are used for attachment rather than for motility. These are divided into two types, fimbriae and pili. Fimbriae enable a bacterial cell to adhere to surfaces (including other cells), whereas pili join bacterial cells in preparation for the transfer of DNA from one cell to another.

Size, Shape, and Arrangement of Bacterial Cells

There are many sizes and shapes among bacteria. Most range from 0.2 to 2.0 μm in diameter and 2 to 8 μm in length. They have a few basic shapes: spherical coccus (plural, cocci), rod-shaped bacillus (plural, bacilli), and spiral. Cocci are usually round but can sometimes be irregularly shaped. Cocci that remain in pairs after dividing are called diplococci; those that remain attached in a chain are called streptococci; and those that remain attached in clusters or broad sheets are called staphylococci. Most bacilli appear as single rods and are fairly uniform in shape. However, some bacilli are oval and look so much like cocci they are called coccobacilli. Spiral bacteria have one or more twists. Bacteria that look like curved rods are called vibrios; others that look like corkscrews and have fairly rigid structures are called spirilla; and those that are helical and flexible are called spirochetes (Fig. 16-1).
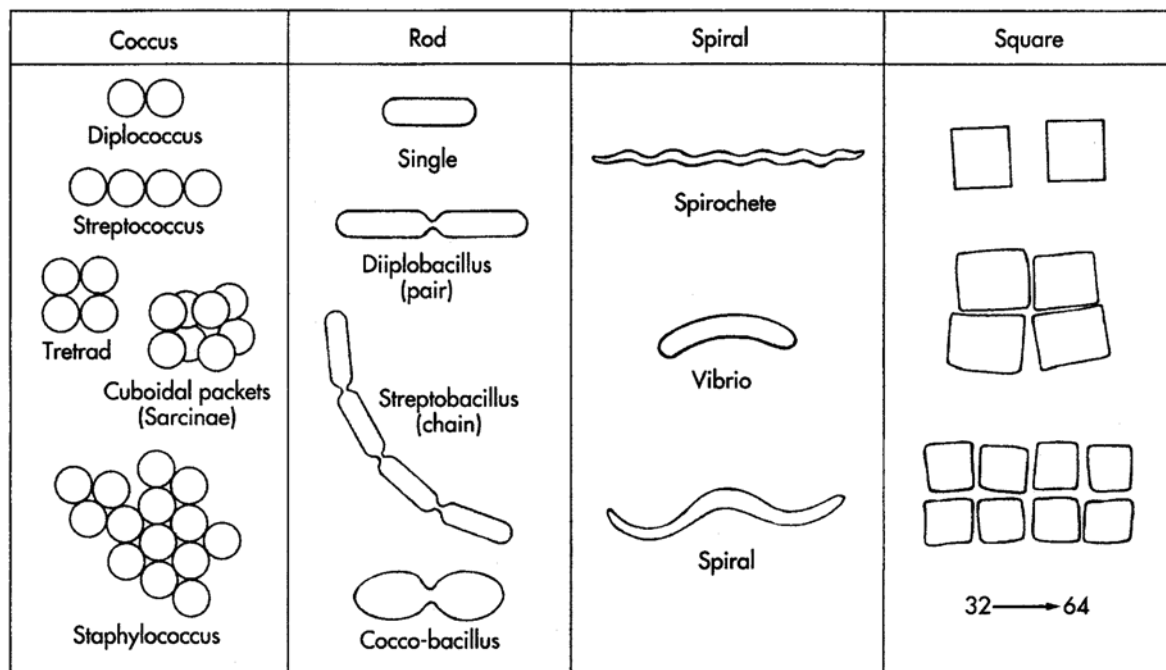
Figure 16-1. Characteristic bacterial cell shapes and arrangements.

Bacterial Replication

Bacteria normally reproduce by binary fission, with one cell dividing into two cells. The rate of replication can vary from slow (*Myobacterium tuberculosis* may replicate every 12 to 24 hours) to rapid (*E. coli* may divide every 15 minutes). The first step in division is cell elongation and replication of chromosomal DNA. The cell wall and cell membrane begin to grow inward from all sides. Eventually the in-growing cell walls meet, forming a cross-wall (septation) and two individual cells are formed. These "daughter cells" are essentially identical to the parent cell.

However, it is possible for bacteria to change through either genetic recombination or mutation. Genetic recombination refers to the exchange of genes between two DNA molecules to form a new combination of genes on a chromosome. Exchange of genes happens in one of three ways.

1. Transformation occurs when genes are transferred from one bacterium to another as "naked" DNA in the environment. The transferring DNA either replaces existing chromosomal DNA or adds to the plasmid DNA pool.

2. Conjugation occurs when all or part of a plasmid is transferred from a donor to a recipient cell. The cells must be in direct contact and transfer occurs via the sex pilus. Conjugation can occur between widely separated species, leading to the rapid dissemination of genetic information (e.g., antibiotic resistance genes).

3. Transduction occurs when bacterial DNA is transferred from a donor cell to a recipient cell inside a virus that infects bacteria, called a bacteriophage, or phage. Mutation usually results

from random mistakes in DNA replication. In bacteria, the spontaneous mutation rate is thought to be about one in a billion reproductions. Many mutations are never expressed and do not significantly impact the bacteria. However, if the mutation offers some type of advantage, such as antibiotic resistance, the mutants will survive when an antibiotic is applied.

Submicroscopic Bacteria

Mycoplasma

Mycoplasmas are extremely small pleomorphic bacteria (0.2–0.8 μm), below the resolving power of a light microscope. They lack cell walls and are surrounded only by an outer plasma membrane. Because they lack a rigid cell wall, they are resistant to cell wall–active antibiotics (e.g., penicillins and cephalosporins). Mycoplasmas can be grown on artificial media that provide them with sterols (exogenous cholesterol) and other special nutritional or physical requirements. Because colonies are extremely small, cell culture methods are often used. Although there are approximately 70 species of mycoplasmas, not all are associated with human disease. Organisms associated with human infection include *Mycoplasma pneumoniae* (atypical pneumonia or walking pneumonia), Ureaplasma urealyticum (urogenital tract infections), and *M. hominis* (urogenital infections) (also see Chapters 22, Pneumonia; and 99, Sexually Transmitted Diseases).

Chlamydiae

Chlamydiae are obligate intracellular parasitic bacteria. They are Gram-negative coccoid organisms that range in size from 0.2 to 1.5 μm. Chlamydiae display a growth cycle that takes place in host cells. The bacteria invade the cells and differentiate into dense bodies called reticulated bodies. The reticulated bodies reproduce and eventually form new chlamydiae in the host cell called elementary bodies. These elementary bodies lyse the host cell and begin a new infection cycle. Organisms associated with human infection include *Chlamydia trachomatis* (male and female genital tract infection, causes lymphogranuloma venereum), *C. pneumoniae* (pneumonia, pharyngitis, risk factor for Guillain-Barré syndrome), and *C. psittaci* (causes psittacosis or parrot fever) (also see Chapters 22, Pneumonia; and 99, Sexually Transmitted Diseases).

Rickettsiae

Rickettsiae are obligate intracellular parasitic bacteria. They are Gram-negative rod-shaped bacteria or coccobacilli (0.8–2.0 μm long) that divide by binary fission. Rickettsiae infect humans as well as arthropods, such as ticks, mites, and lice. Infection is transmitted to humans through the bite of an infected insect (tick, mouse mite, body louse, and rat flea). Rickettsiae have not been grown in cell-free media; however, molecular diagnostics can detect rickettsiae in tissues. Diagnosis is usually achieved using antibody testing (Weil-Felix agglutination test, latex agglutination test), microimmunofluorescence test on serum or immunofluorescent staining of tissue biopsy specimens. Organisms causing human infection include *Rickettsia rickettsii* (Rocky Mountain Spotted Fever), *R. prowazekii* (epidemic typhus), *R. typhi*(endemic [murine] typhus), *Ehrlichia canis* (Ehrlichiosis), and *Coxiella brunette* (Q fever).

Fungi

Fungi are eukaryotic organisms that derive nutrients from organic materials. They have a cell wall that contains chitin and lack photosynthetic capability. Some fungi are well-adapted human pathogens (e.g.,*Candida albicans*). Most, however, are accidental pathogens that humans acquire through contact with decaying organic matter or airborne spores in the environment. Typically fungi are divided based on the appearance of the organism into two separate groups: yeasts and molds (also see Chapter 90, Fungi).

Yeasts

Yeasts are single-celled, microscopic, round-to-oval organisms ranging in size from 2 to 60 μm. In culture, yeasts usually form a smooth, creamy colony without aerial hyphae. They have a single nucleus with a nuclear membrane and contain organelles. They typically reproduce by a process of budding. In this process a parent cell forms a "bud" on its outer surface. As the bud elongates the parent cell's nucleus divides and one nucleus migrates into the bud. Eventually the cell wall closes between the parent cell and the bud, and the bud breaks free. Some yeasts reproduce through the process of fission, similar to bacterial reproduction. Commonly pathogenic yeasts include *Candida* spp. (mucositis, vaginitis, dermatitis, systemic dissemination) and *Cryptococcus neoformans* (meningitis, pneumonia in compromised hosts).

Molds

Molds consist of long, branching filaments of cells called hyphae. A tangled mass of hyphae visible to the naked eye is a mycelium. Arial mycelium gives mold a fuzzy or woolly appearance in culture. The hyphae's physical characteristics, such as shape (e.g., antler, racquet, or spiral), pigmentation, and the presence of rootlike structures called rhizoids are used to differentiate and identify molds. Some molds reproduce asexually by fragmentation of their hyphae. Additionally, molds may reproduce sexually and asexually by the formation of spores. Asexual spores are formed by the hyphae of one organism. When these spores germinate they become organisms identical to their parent cell. Sexual spores result from fusion of nuclei from two opposite mating strains of the same species of fungus. Organisms that grow from sexual spores have characteristics of both parental strains. Common opportunistic pathogenic molds include *Aspergillus* spp. (necrotizing pneumonia) and agents of mucormycosis (e.g., *Rhizopus* and *Mucorspp*.).

Dimorphic Fungi

Some fungi exhibit dimorphism and can grow as either a mold or yeast form. The mold-like forms produce vegetative and aerial hyphae; the yeast-like forms reproduce by budding. Typically, dimorphism in fungi is temperature dependent: at 37°C the fungus is yeast-like, and at 25°C it is mold-like. Common pathogenic dimorphic fungi include *Histoplasma capsulatum* (acute pulmonary histoplasmosis, disseminated infection), *Blastomyces dermatitidis* (chronic skin infections, pulmonary lesions), and *Coccidioides immitis* (respiratory tract, meningeal infection). *Pneumocystis carinii*, previously classified as a parasite, was recently reclassified as a fungus based on DNA characteristics. *P. carinii* (now named *Pneumocystis jiroveci)* is a major

cause of pneumonia in acquired immunodeficiency syndrome (AIDS) and other immunosuppressive conditions.

Viruses

Viruses are obligate intracellular parasites that need living cells to grow and reproduce. Viruses are ultramicroscopic particles containing nucleic acid (either RNA or DNA) surrounded by protein, and in some cases, other components such as a membrane-like envelope. Originally, viruses were classified by their type of host and/or the type of diseases caused (e.g., human poliovirus). With the advent of genetic testing, viruses are now classified in families and genera based on genome type, the number of strands in the genome (double-stranded [ds] or single-stranded [ss]), morphology, and the presence or absence of an envelope. Using the example of Varicella-zoster virus, the taxonomy is as follows:

1. Genome: dsDNA
2. Envelope: Yes
3. Family: Herpesviridae
4. Subfamily: Alphaherpesvirinae
5. Genus: Varicellovirus
6. Member: Human herpesvirus 3, Synonym: Varicella-zoster virus

Outside the host cell, the virus particle is known as a virion. The virion is metabolically inert and does not grow or multiply. All viruses replicate in a similar fashion, which occurs in five steps:

1. *Attachment*. The virion attaches to a complementary receptor site on the host cell. Certain types of viruses "seek out" or target specific types of host cells. For example, Epstein-Barr virus seeks out receptor sites on B lymphocytes.

2. *Penetration*. The virion enters the host cell through a process called endocytosis, an active cellular process by which nutrients and other molecules are brought into a cell.

3 *Replication*. Viral DNA or RNA directs the host cell to begin synthesis of viral components. Viral replication uses host cell ribosomes, energy sources, and amino acids to produce these components.

4. *Maturation*. The viral components essentially assemble into a viral particle spontaneously; daughter virions are formed.

5. *Release*. The host cell lyses or the virus buds through the cell wall and the new virions are released. Some viruses lie dormant in the host cell for months or years; after this latent period, new virions form and cause damage to host cells.

Parasites

Human parasites vary greatly in size and complexity. They may be single-celled microscopic protozoa or complex worms over 10 feet in length. Protozoa are unicellular, free-living eukaryotic organisms. Most protozoan parasites exist in two different forms: the pleomorphic trophozoite stage (feeds, and produces effects in the host) and the cyst stage (most responsible for transmission). Other types of parasites include flukes, tapeworms, roundworms, and ectoparasites such as lice and scabies. Please refer to Chapter 92, Parasites, for more specific information.

CLINICAL MICROBIOLOGY

The presence and identification of organisms in a clinical specimen may be indicative of infection. The primary goals of clinical microbiology are to identify the presence of pathogenic organisms in tissues, body fluids, excretions, or secretions and to classify those pathogens to species level based on morphological and biochemical properties. Additional goals are to predict response to antimicrobial therapy and assist in epidemiological investigations.

Microscopy

Because microorganisms are invisible to the naked eye, the essential tool in microbiology is the microscope. The microscope allows direct examination of clinical and culture materials. Further, it yields information on the presence, relative size and shape, and preliminary identification of microorganisms.

Light Microscope

The most commonly used microscope in the clinical laboratory is the light microscope. It is a compound microscope because it contains two types of lenses that function to magnify an object. The lens closest to the eye is called the ocular, whereas the lens closest to the object is called the objective. Most microscopes of this type have four objective lenses: the scanning lens (43 or 4 times magnification); the low-power lens (10X); the high-power lens (40X); and the oil immersion lens (100X). With the ocular lens that magnifies 10X, the total magnification will be 40X for the scanning lens and 1000X for the oil immersion lens. Depending on the type of light source used to illuminate the slide (specimen), the compound microscope can be used in several ways.

Bright-field microscopy uses bright light sources (usually incandescent) and is the most common type of microscopy used in the clinical lab. This is the method regularly used to examine specimens that have been stained using the Gram stain method.

Dark-field microscopy is utilized to examine fresh material and permits observation of motile organisms that are not able to be stained by common methods (e.g., treponemes, *Borrelia* spp.). This type of microscopy uses a special condenser that scatters light and causes it to reflect off the

specimen at an angle. Bright objects appear against a dark background and can offer better resolution than bright-field microscopy.

Phase-contrast microscopy is helpful in direct observation of unstained material. It uses a special condenser that throws light "out of phase" and causes it to pass through objects at different speeds. This type of microscopy takes advantage of the different densities of cellular elements and makes them appear to stand out from their backgrounds. Phase-contrast microscopy is typically used for examination of living cells, particularly tissue cultures for viral isolation and identification.

The fluorescent microscope employs an ultraviolet (UV) light source. This type of microscopy depends on the ability of naturally fluorescent substances or dyes to absorb energy in non-visible UV and short visible wavelengths, become excited, and emit energy in longer visible wavelengths. This method is very popular because of ease of interpretation of stained materials and the speed at which materials can be reviewed. Coupled with specific antibodies (e.g., direct or indirect fluorescent antibody [DFA or IFA] tests), rapid diagnoses of specific organisms can be made.

A fairly recent development in light microscopy is known as confocal microscopy. Specimens are stained with fluorochromes so they will emit or return light. A laser is used to illuminate the specimen. Most confocal microscopes are used in conjunction with a computer to construct three-dimensional images.

Electron Microscope

The energy source in the electron microscope is a beam of electrons produced by an electron-emitting tungsten filament. Because the beam has an extremely short wavelength, it strikes most objects in its path and increases the resolution of the microscope significantly. Viruses and some large molecules can be seen with this type of instrument. Special gold or palladium stains are used to prepare the specimens before viewing. Images are typically viewed on a computer monitor. Transmission electron microscopy (TEM) uses a finely focused beam, whereas scanning electron microscopy (SEM) sends the beam through an electromagnetic lens that allows for three-dimensional views. Because of the significant cost of electron microscopes, they are rarely used in clinical laboratories but are useful as research tools.

Specimen Preparation

In order to observe clinical or culture materials through a microscope, the specimen must be prepared for observation. In some cases the specimen may be placed onto a slide for direct observation. Some fungi and parasites are large and distinct enough to be examined directly (e.g., protozoa, Cryptococcus in India ink). However, most observations are made with stained preparations.

Direct examination or direct wet mount of clinical specimens should be performed as soon as possible after collection; consequently, it is often performed in the clinic or ambulatory care

setting. Because some materials are very thick, they require dilution with sterile saline; however, this practice increases the risk of aerosolization and should be performed in a biosafety cabinet. Types of specimens examined by direct wet mount include sputum, drainage from lesions, body fluid aspirates, stool, vaginal discharge, and urine sediment. Examples of pathogens identified by direct wet mount include the motile trophozoites of *Giardia lamblia* in stool, *Trichomonas vaginalis* in vaginal discharge or urine sediment, or *Entamoeba histolytica* from a liver abscess aspirate.

Before microorganisms can be stained they must be fixed (attached) to a microscope slide. A thin film of material is spread over the surface of a slide. This "smear" is then fixed with either heat or chemicals. Fixing the smear not only ensures that the organisms are attached to the slide, but also kills the organisms, making the slide safe to handle.

Staining Methods

Staining is one of the most useful tools available in the microbiology laboratory. It rapidly provides the clinician with confirmation that the specimen is representative of the patient's condition (e.g., deep cough quality sputa vs. oral secretions or saliva), identifies the cellular elements within the specimen as well as inflammatory debris expected in the presence of an infection (e.g., white blood cells in cerebrospinal fluid), and can rapidly assist in presumptive identification of specific infectious agents (e.g., acid-fast stain for the detection of mycobacteria). Staining simply means coloring the microorganisms with a dye that emphasizes certain structures. Stains are usually acidic (negatively charged) or basic (positively charged) salts. Basic dyes react with nuclear cell components; acidic dyes react with cytoplasm and granules. Simple staining uses only one dye and may be used to demonstrate the shape, size, and arrangement of organisms or the presence of spores. Differential staining uses two or more dyes to demonstrate shape and biochemical color reaction. Differential stains react differently with various microorganisms and thus can be used to distinguish among them. They are used to divide nearly all bacteria into major groups. The most commonly used differential stains are the Gram stain and the acid-fast stain. Figure 16-2 provides stains mostly commonly used in the clinical microbiology laboratory setting.

Gram Stain

The Gram stain was developed in 1884 by the Danish bacteriologist Hans Christian Gram. In this procedure, a heat-fixed smear is covered with a basic purple dye, usually crystal violet. After a short time, the purple dye is washed off and the slide is covered with iodine, a mordant that sets and intensifies the crystal violet stain. When the iodine is washed off, all of the bacterial cells present are dark violet or purple. The slide is then rinsed with alcohol or an alcohol-acetone solution. This "decolorizing" solution causes some of the cells to lose their purple color, whereas others maintain their color. At this point some of the cells have no color and so a counterstain of safranin, a basic red dye, is applied to the smear. The smear is rinsed with water and allowed to dry prior to microscopic examination.

Differences in cell-wall structure affect the retention or loss of the combination of crystal violet/iodine complex. Gram-positive bacteria have a thick peptidoglycan cell wall that does not

allow the crystal violet/iodine complex to be removed during the alcohol wash. Under the microscope, Gram-positive organisms appear dark violet, purple, or blue. Gram-negative bacteria contain a lipopolysaccharide layer as part of their cell wall. The alcohol wash disrupts this layer and the crystal violet/iodine complex is rinsed out of the cell wall. As a result, Gram-negative cells are colorless until counterstained with safranin. Under the microscope, Gram-negative organisms appear pink or red. Some organisms absorb an increased amount of the stains and are said to have a strong avidity, whereas others are weakly stained and present a pale appearance (low avidity). Gram-negative enteric pathogens have a strong avidity to the safranin stain and are bright red. Pseudomonads are less avid and only uptake a moderate amount of safranin. Anaerobic bacilli and other thin-walled Gram-negative organisms (e.g., Borrelia, Legionella) stain weakly and appear pale red or pink. In addition to bacteria, many fungi and some protozoa and helminths stain with the Gram stain process. *Chlamydia, Rickettsia, Mycobacterium*, and *Nocardia* organisms stain poorly and may require special staining techniques for identification.
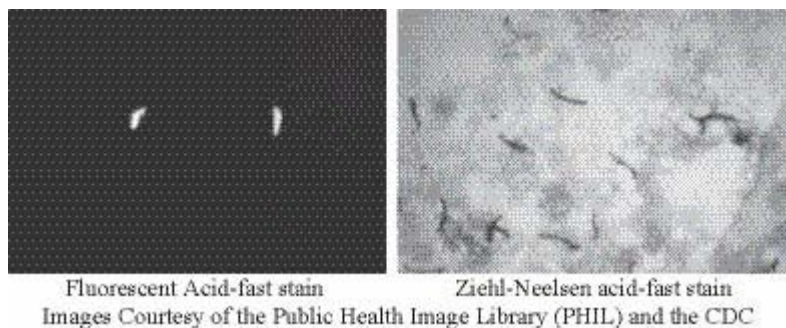

Fluorescent Acid-fast stain   Ziehl-Neelsen acid-fast stain
Images Courtesy of the Public Health Image Library (PHIL) and the CDC

Figure 16-2. **A**. Fluorescent acid-fast stain. **B.** Ziehl-Neelsen acid-fast stain. Images Courtesy of the Public Health Image Library and the CDC.

The Gram stain is an important tool for the clinical microbiologist. Gram stain reaction, along with cell shape/arrangement, can be used to determine the type of media that should be used for culture, the appropriate identification procedures that should be done, and the types of antimicrobial testing that should be initiated. For these reasons, it is imperative that the microbiologist maintain a high degree of skill as well as a comprehensive quality assurance program. As stated earlier, Gram staining of a specimen with interpretation is important to clinicians. It may help to determine the quality of a specimen, initial direction for therapy (empiric), or the need for isolation precautions (e.g., Gram-negative diplococci in cerebrospinal fluid, suggesting meningococci).

Acid-Fast Stain

Cells of certain bacteria and parasites contain long-chain fatty acids (mycolic acids) that make them impervious to crystal violet and other basic dyes. Heat or detergents can be used to force dye into this type of cell. Once this occurs, the cell cannot be decolorized by acid-alcohol, hence the term acid-fast. Acid-fast stains are very useful in identifying *Mycobacterium* spp., an acid-fast bacillus (AFB), as well as Nocardia (stains blue) and *Actinomyces* organisms.

Generally, one of two types of procedures is used for acid-fast stains: a fluorescent or a nonfluorescent stain. Both the Ziehl-Neelsen and Kinyoun nonfluorescent staining procedures use the red dye carbolfuchsin as the primary stain and methylene blue as the counterstain; however, the Ziehl-Neelsen process uses heat with the carbolfuchsin, whereas the Kinyoun process is a cold stain. Acid-fast organisms will retain the carbolfuchsin and will appear red under the microscope (where the AFB nickname "red-snapper" comes from) (Table 16-1). The auramine-rhodamine method uses a fluorescent stain as the primary stain and acid-fast organisms exhibit bright yellow-orange fluorescence under ultraviolet light (see Fig. 16-2). As the number of organisms shed by the infected patient can vary greatly, the overall sensitivity of the acid-fast smear varies from 20% to 80%. In an effort to standardize reporting, the U.S. Department of Health and Human Services has published recommendations for the reporting and interpretation of acid-fast smears (Table 16-2). It should be noted that the auramine-rhodamine fluorochrome stains are more sensitive than the carbolfuchsin stains.

Table 16-1. Stains Commonly Used in the Clinical Microbiology Laboratory[5]

Stain	Application
Gram stain	Most bacterial species; bacteria can be grouped based on their Gram stain reactions; Routinely used as the primary microscopic examination
Acid-fast stain Ziehl-Neelsen nonfluorescent	Direct smear for the detection of mycobacteria; Identification of acid-fast organisms
Kinyoun nonfluorescent	Direct smear for the detection of mycobacteria, cryptosporidia and Cyclospora parasites in stool
Fluorochromes (fluorescent stains) Acridine Orange	Detection of cell wall–deficient bacteria such as mycoplasmas
Auramine-rhodamine	Detection of mycobacteria as well as some sporozoan parasites
Calcofluor white	Direct smear for the differentiation of fungi from background materials; bronchoalveolar fungi and some parasitic cysts
Immunofluorescent	Diagnostic antibody or DNA probe-mediated stains directed specifically at an organism
Modified Toluidine Blue O Stain	Detect *Pneumocystis carinii* in respiratory tract material as well as other parasites and fungi
Trichrome stain	Differentiates the internal structures of cysts, trophozoites or other forms of parasites; useful for examination of stool specimens
Wright-Giemsa	Detect parasitic protozoan nuclei in blood (e.g., *Plasmodium* species, *Babesia, Trypanosoma cruzi*)

Table 16-2. Acid-fast Smear Interpretation[6]

Carbolfuchsin Stain # AFB seen (1000×)	Fluorochrome Stain #AFB seen (450×)	Quantitative Report
0	0	No AFB seen
1–2/300 fields	1–2/70 fields	Doubtful AFB seen; resubmit a new specimen
1–9/100 fields	1–2/70 fields	1+
1–9/10 fields	2–18/50 fields	2+
1–9/field	4–36/field	3+
>9/field	>36/field	4+

Calcofluor White Stain

Calcofluor white is used for rapid screening of specimens for fungal elements and Pneumocystis cysts. This colorless dye binds to the cellulose and chitin in the cell walls of fungi and fluoresces when exposed to ultraviolet light. Yeast cells, pseudohyphae, and hyphae display a bright apple-green or blue-white fluorescence.

Miscellaneous Stains

Other staining procedures can be useful in identifying the presence of specific microorganisms. Immunofluorescent staining combines an antibody directed/probe mediated to a specific organism with a dye that converts ultraviolet light into visible light. If the antibody binds with the organism, the organism fluoresces under the microscope. Immunofluorescent stains may be used to detect *Chlamydia, Legionella pneumophila, Bordetella pertussis, herpes simplex* virus, varicella-zoster virus, cytomegalovirus, adenovirus, and respiratory viruses from clinical specimens. The trichrome stain is useful in the identification of many fecal parasites. It is used to enhance the structures of protozoa, cysts, and some ova.

Microbial Growth and Identification

Once a specimen is received in the microbiology laboratory, it is assessed for potential microbial pathogens. In most cases, the specimen is placed into or onto special media to cultivate the growth of microbes. Once the microbe grows, further test methods are used to classify/identify the organism.

Bacteria

Like most living organisms, bacteria require the proper type of nutrition, appropriate temperature, and correct atmospheric conditions. In order to cultivate the growth of bacteria,

clinical specimens are inoculated (plated) onto media that provides the nutrients necessary for growth. The choice of media depends on the site being cultured (e.g., throat, blood, urine), the growth requirements of common or suspected pathogens, and the likelihood of normal flora or commensal bacteria being present. Commensal bacteria live in a relationship in which one organism derives food or other benefits from another organism without hurting or helping it (e.g., normal flora in the mouth). Most growth media are in agar form (a gelatin-like substance in a Petri dish). There are several categories of growth media including: (1) nutrient agar, a general-purpose growth medium that supports the growth of a wide variety of bacteria (e.g., trypticase soy agar with 5% sheep blood); (2) enrichment medium which contains special nutrients necessary for the growth of hard-to-grow (fastidious) bacteria (e.g., chocolate agar for the growth of *Neisseria meningitidis*); (3) selective media that contain chemicals or antibiotics designed to inhibit normal commensals, whereas allowing organisms of interest to grow (e.g., bismuth sulfate agar for the isolation of *Salmonella* spp.); and (4) differential media that promotes the differentiation of specific organisms while inhibiting others (e.g., acetate agar to differentiate *E. coli* from *Shigella*).

Once the specimen is plated, it is systematically spread out on the media surface to assist in separation and quantification of organisms that may be present. The medium is then incubated for at least 24 hours in a warm, moist environment. Most cultures are incubated at human body temperature (35°C). However, some are incubated at room temperature, whereas others are incubated at 42°C. Most specimens are incubated for a minimum of 48 hours.

Atmospheric conditions (i.e., the presence or absence of oxygen) are also considered during the incubation process. Organisms that have an absolute requirement for air (oxygen gas) and do not grow in the absence of oxygen are called aerobic organisms. Bacteria that grow only in complete or nearly complete absence of ambient atmospheric oxygen and are inhibited or killed by oxygen are known as obligate anaerobes. Facultative anaerobes are organisms that can use oxygen if it is present but can grow without it. Microaerophilic organisms require oxygen in concentrations of 2% to 10%, and, in addition, they may also require an increased carbon dioxide concentration.

Once bacterial growth is present, further identification can take place. The microbiologist observes the growth for colony morphology—each viable bacterium showing growth is counted as a colony-forming unit, or growth may be otherwise quantified for relative amount, depending on laboratory procedure. The colonies may consist of commensal bacteria that must be identified and differentiated from potentially pathogenic organisms. In addition to colonial growth characteristics, classification of bacteria is based on Gram stain characteristics (Gram-positive vs. -negative), morphological features (cocci vs. bacilli), and oxygen utilization. Gram-positive cocci can undergo several types of basic tests to further classify the specific organism. For example, a catalase test can be used to differentiate streptococci (negative) from staphylococci (positive) and a coagulase test can be used to differentiate *S. aureus* (positive) from other staphylococci (negative). For species level identification of Gram-positive organisms, a battery of biochemical tests may need to be performed. These tests may be conducted manually (e.g., individual test tubes) or in an automated instrument (e.g., Vitek® or Microscan®).

Gram-negative bacilli are generally grouped by their ability to ferment lactose as a nutrient. Lactose fermenting Gram-negative bacilli include the family Enterobacteriaceae, whereas non-

lactose fermenting Gram-negative bacilli include *Pseudomonas* spp. as well as *Proteus* spp. As with Gram-positive organisms, species level identification of Gram-negative organisms requires a battery of biochemical tests. These tests may be conducted manually (e.g., individual test tubes or test strip API 20E®) or in an automated instrument (e.g., Vitek® or Microscan®).

Fungi

Depending on the type of organism, some fungi can be identified directly from a clinical specimen. For example, a skin scraping may be directly examined for the presence of fungal hyphae (may require special staining techniques). Additionally, some yeasts, especially *Candida* spp., grow on routine blood agar and require no special culture techniques. Yeasts such as *Candida* can be identified to the species level through a series of tests, including germ-tube tests (positive, *C. albicans*) and sugar assimilation (done manually or in an automated instrument).

In some cases, cultures need to be done to identify and classify potential fungal pathogens. A selective media such as Sabouraud (suppresses the growth of bacteria) is generally used for culturing fungus. The specimens are usually incubated at room temperature (25°C–30°C) for several weeks. Identification of fungal isolates is based on the appearance of the colony and on microscopic examination.

In addition to culture, other methods exist to identify the presence of certain fungi. Direct antigen detection methods (e.g., latex agglutination) may be used to identify *C. neoformans*. Serologic test methods can be used to identify coccidiomycosis, histoplasmosis, and *Aspergillus* spp.

Mycobacteria

Mycobacteria require special culture techniques to be isolated. In general, specimens undergo procedures to kill commensal bacteria that may be present (so that the mycobacteria can be isolated). Once the specimen has been processed, it is planted on special media and incubated for 4 to 6 weeks. Once the organism grows, further testing must be conducted to identify the species (also see Chapter 91, Mycobacteria).

Because conventional culture techniques may take weeks to recover mycobacteria, more rapid techniques have been developed. The BACTEC method utilizes a radiometric culture technique. This technique is used not only to isolate mycobacteria from clinical specimens, but also to differentiate *M. tuberculosis* complex from other mycobacteria and for antimicrobial susceptibility testing. Polymerase chain reaction (PCR) methods have also been developed. In this method, genetic chromosomal parts can be detected by DNA probes. DNA probes are usually used after the mycobacteria have been isolated from the culture.

Mycoplasma

Because of their fastidious growth requirements, *Mycoplasma* culture is rarely attempted in the clinical laboratory. *M. hominis* may be grown from wounds using special media and from blood

using radiometric techniques (e.g., BACTEC®). In general, serological tests are used to diagnose Mycoplasma infection.

Chlamydiae

Because of their parasitic nature, Chlamydiae growth requires tissue culture and is not attempted in most laboratories. Direct-detection methods enjoy the greatest popularity for diagnosis. It is now possible to detect the antigen by direct fluorescent antibody slide staining and enzyme-linked immunosorbent assay (ELISA) technique. Invasive infection does produce an immunogenic response. Therefore, *Chlamydia* antibodies can be measured using complement fixation, microimmunofluorescence, and ELISA techniques.

Rickettsiae and Other Tick-borne Microbes

Because of their parasitic nature and host requirements, Rickettsiae are rarely cultured (never in clinical labs). Early diagnosis is usually made on clinical grounds. Currently, serological studies are the most sensitive and specific tests for detection of specific infections (i.e., Lyme disease). ELISA is the best diagnostic test and determines specific levels of antibodies (immunoglobulins M and G, [IgM and IgG, respectively]).

Viruses

There are three major methods to diagnose viral infections: direct detection in the clinical specimen, specific antibody assay to detect viral antibodies in the serum, and viral culture. Direct detection methods include: (1) electron microscopy, which is used primarily by reference laboratories; (2) ELISA for viruses such as respiratory syncytial virus (RSV), hepatitis B surface antibody, and rotavirus; (3) latex agglutination for viruses such as rotavirus and RSV; (4) DNA probes for viruses such as cytomegalovirus (CMV); (5) polymerase chain reaction for DNA detection for viruses such as HIV types 1 and 2; (6) optical immunoassay (OIA), an antibody antigen-based test that produces a reflection change for detection of influenza viruses A and B from respiratory specimens; and (7) light microscopy of cell scrapings from infected sites can detect Cowdry type A inclusion bodies from herpes simplex virus and varicella zoster virus, Papanicolaou (Pap) smears for the effect of human papillomavirus on squamous cells, and Negri bodies for the diagnosis of rabies.

Viral infections cause an immunogenic response; therefore, antibody detection methods can be useful in the diagnosis of infection. Simple antibody tests can determine the presence or absence of immunoglobulin (Ig). This can be used to determine if a patient has ever been infected with a specific virus (e.g., varicella, adenovirus). Complex antibody detection systems use a battery of viral antigens and often distinguish IgM (early) from IgG (late) antibodies.

Specific antibody detection has some inherent issues that can limit effective use of this methodology, including:

• Measurement of the patient's response to the virus and not actual detection of the virus

• Antibody production varies based on the patient's immune system

• Antibody level does not necessarily correlate with acuteness or activity level of the disease state

• Testing is most often retrospective as diagnosis by paired sera requires both acute and convalescent samples

• There is possibility of cross-reaction with nonspecific antibodies

• There is the possibility of passive transfer of antibodies transplacentally or after transfusion

Advantages of serological testing methodology include diagnosis of viral infection from nonculturable organisms (e.g., hepatitis viruses), confirmation of immune status for diseases such as rubella, measles, Varicella, and hepatitis B, and usefulness in epidemiological or prevalence studies.

Virus culture traditionally requires specialized media containing antibacterial and antifungal agents in prepared plastic or glass tubes and flasks. Clinical specimens are cultured on an array of different mammalian cell culture lines, depending on the agent suspected clinically. Growth is viewed microscopically and is identified through changes in the host cells rather than as discrete viral "colonies." Although tissue cultures are still considered to be the gold standard, many viral infections are diagnosed through other rapid methods. Advantages of viral culture include sensitivity, detection of many types of viruses, adaptability to viral variation, and options for susceptibility testing.

Parasites

Microscopy is the cornerstone of most parasite diagnosis. Direct or concentrated examination of stool, urine, vaginal secretions, or duodenal aspirates may yield protozoans or eggs of helminths. Specific identification is based on characteristic morphological appearance. Direct antigen detection methods have been developed for giardiasis and are widely used. Serological test methods may be performed when direct examination of tissue is difficult or unrevealing. These tests are usually conducted by reference laboratories and may be useful in diagnosing parasitic infections such as amebiasis, schistosomiasis, cysticercosis, echinococcosis, and malaria (also see Chapter 92, Parasites).

Antimicrobial Susceptibility Testing

Antimicrobial therapy seeks to suppress (bacteriostatic) or kill (bactericidal) microorganisms by exploiting biochemical reactions unique to the pathogenic microbe. Ideally, this should be accomplished using the simplest agent with minimal toxicity to the patient. Originally, the purpose of susceptibility testing, sometimes referred to as sensitivity testing, was to determine whether the organism isolated was able to resist the effect of the therapeutic agent chosen for treatment. However, as processes have evolved, there are antimicrobial susceptibility testing (AST) methods available that directly measure the activity of one or more antimicrobial agents

against a bacterial isolate, directly detect the presence of a specific resistance mechanism in a bacterial isolate, and measure complex antimicrobial-organism interactions. The type and extent of the AST conducted depends on the organism isolated, the source of the culture (body site), available antimicrobial agents, and typical susceptibility patterns. Several methods may be used for susceptibility testing.

Disk Diffusion (Kirby-Bauer Method)

Once a potential pathogen has been isolated by culture, a standardized suspension of bacteria is spread in a lawn fashion onto Mueller-Hinton agar. Paper disks impregnated with a standard amount of an antibiotic are placed onto the agar surface and the agar plate is incubated overnight. During incubation, the antibiotic diffuses out into the agar, resulting in decreasing concentrations of the agent as it moves further from the disk. Organism growth is either inhibited by the concentration of the antibiotic in the agar or not. That area in which the concentration of the antibiotic prohibits the growth of the organism is called the zone of inhibition (Fig. 16-3).

Measurements of the zone size have been standardized for each antibiotic/pathogen interaction and are used to interpret the effectiveness of that antibiotic or class of antibiotics with that specific isolate. Once the zone of inhibition has been measured in millimeters, it is compared with the Clinical Laboratory Standards Institute's (CLSI) guidelines for interpretation.1 AST by disk diffusion is reported in one of three ways: (1) susceptible, indicating that the antimicrobial agent may be effective against the identified pathogen; (2) intermediate, indicating that the antimicrobial agent may be less effective than an antimicrobial agent with a susceptible result, that the organism may become resistant, or an antimicrobial agent may potentially be used if a high drug concentration is administered; or (3) resistant, a result indicating that the antimicrobial agent should not be used for therapy.

Figure 16-3. Disk diffusion antimicrobial susceptibility test. **A**. The light halo surrounding the disk is the zone of inhibition. A ruler is used to measure the zone size. **B**. A Mueller Hinton plate with multiple antibiotic disks. Note the small zone size around some disks. (Images courtesy of the PHIL and the CDC.)

Broth Dilution Antimicrobial Susceptibility Testing

Broth dilution test methods are used to determine the least amount of antibiotic necessary to inhibit growth of the organism or the minimal inhibitory concentration (MIC). This method uses replicate inoculation of a standardized suspension of bacteria in broth into a series of micro-wells containing antibiotics in descending concentration expressed in micrograms per milliliter

(µg/mL). After a period of incubation, the wells are examined for bacterial growth (seen as turbidity). The first well in the series that shows no bacterial growth contains the minimum inhibitory concentration of the antibiotic that is effective against the organism being tested. Broth dilution test results are reported in µg/mL. The reading is compared with set breakpoints determined by CLSI for interpretation as sensitive, intermediate, or resistant.

Broth dilution AST can be conducted and read manually; however, most laboratories use some type of automated or computer-assisted instrumentation. In an automated system, the instrument actually reads the test results; in a computer-assisted system a technologist visually assesses bacterial growth and records the results. Both systems generate interpretive criteria and cumulative susceptibility profiles. Although most instruments require overnight incubation of the test trays, some of the newer systems read growth photometrically and can generate results in as little as 3 to 10 hours (e.g., Vitek®, WalkAway®).

E-Test

The E-test was developed to combine the ease and flexibility of disk diffusion with the ability to quantify resistance provided by broth dilution. In this method, a standardized suspension of bacteria is spread in a lawn fashion onto an agar plate. One or more nonporous plastic strips, impregnated with a serial dilution of a selected antimicrobial agent and marked with a MIC reading scale, are applied to the agar. The plate is then incubated overnight. After incubation, the plate is examined and the number at the point at which the border of the growth inhibition intersects the E-strip (at the meniscus) represents the MIC (Fig. 16-4). The same MIC interpretive criteria are used for E-tests and broth dilutions. E-tests are often used when the level of resistance can be clinically important (e.g., penicillin or cephalosporins against *Streptococcus pneumoniae*) with results reported in µg/mL. This test method is limited to certain antibiotics and is typically used for penicillin, ampicillin, and vancomycin.

Special Test Methods

β-Lactamase

Depending on the organism, additional tests may be used to evaluate the potential effectiveness of antimicrobial agents. Organisms such as *Haemophilus influenzae, Neisseria gonorrhoeae, Staphylococcus* spp., and *Pseudomonas* spp. should be tested for β-lactamase production. β-Lactamase is an enzyme present in some bacteria that can induce antibiotic resistance in three ways (Table 16-3): (1) Alter the antibiotic by destroying the β-lactam ring of the antibiotic agent resulting in the antibiotic being unable to bind to proteins thereby interfering with cell wall synthesis; (2) alter the target of the antibiotic, specifically the penicillin binding proteins (PBP) in the organism's cell wall, resulting in decreased binding of the antibiotic to the organism; (3) change the number or character of the organism's porin channels (decreased uptake) to affect the route by which beta-lactam antibiotics cross the outer membrane to reach the PBP of Gram-negative bacteria. The presence of the enzyme is sufficient to determine resistance, but not all strains of an organism produce β-lactamase.

Disk Approximation Test

In recent years, inducible clindamycin resistance has been observed in some *Staphylococcus* spp. These isolates test resistant to erythromycin and susceptible to clindamycin using routine AST methods. The presence of the "erm" gene in some strains of *Staphylococcus* induces production of the enzyme methylase, which allows clindamycin resistance to be expressed. In order to test for this inducible clindamycin resistance, a simple disk approximation test, commonly referred to as the "D test," can be performed. An erythromycin disk is placed 15 to 26 mm (edge to edge) from a clindamycin disk in a standard disk diffusion test. After incubation, a flattening of the zone (Fig. 16-5) in the area between the disks where both drugs have diffused indicates that the organism has inducible clindamycin resistance.
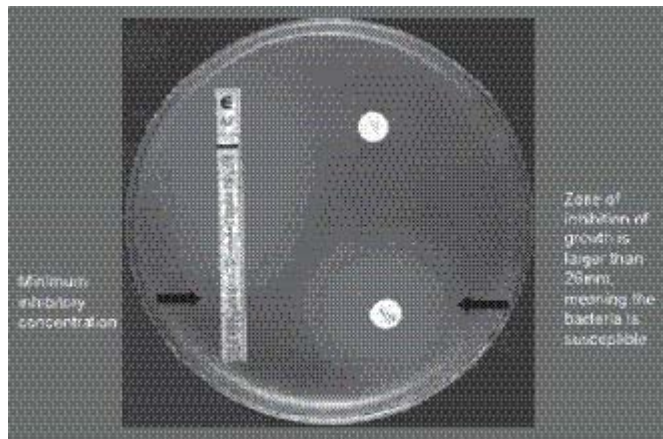

Image courtesy of PHIL and the CDC

Figure 16-4. E-test and disk diffusion. (Image courtesy of PHIL and the CDC.)

Reporting clindamycin as susceptible for *Staphylococcus* spp. that test erythromycin resistant and clindamycin susceptible without checking for inducible clindamycin resistance may result in inappropriate clindamycin therapy. The test described is acceptable for all *Staphylococcus* spp., including oxacillin susceptible or oxacillin-resistant *S. aureus* or coagulase-negative staphylococci.

Table 16-3. Examples of Organisms with β-lactamase Resistance

Resistant Mechanism	Organism	Antibiotic Effected
Enzyme alters antibiotic	*Staphylococcus* spp.	Penicillins
	Pseudomonas aeruginosa	Penicillins, cephalosporins, and aztreonam
Altered target	*Staphylococcus* spp.	Methicillin and other beta-lactam agents
	Streptococcus pneumoniae	Penicillin and cephalosporin
Decreased uptake	*Pseudomonas aeruginosa*	Imipenem

Miscellaneous Testing

Synergy testing, which is used to determine the inhibitory ability of combinations of antibiotics, is often used with *Enterococcus* spp. And *Staphylococcus* spp. The Hodge test is used to detect the presence of extended spectrum β-lactamase (ESBL) resistance in Gram-negative organisms (e.g., *Klebsiella pneumoniae, E. coli, P. aeruginosa*). This disk diffusion-based test has been modified to detect other enzyme related (e.g., metallo-β-lactamase, carbapenemase, and Amp C β-lactamase) antimicrobial resistance producing strains of Gram-negative organisms. Minimal bactericidal concentration (MBC) test methods are used to determine minimal concentration of antibiotic necessary to kill (not merely inhibit growth of) an organism. Although MBC testing is infrequently performed, it may be helpful in certain clinical situations. Susceptibility testing is not commonly conducted for anaerobic organisms. If testing is required, MIC agar-based or broth dilution techniques may be used. Likewise, susceptibility testing for viruses and fungi is generally performed in reference laboratories and often is not standardized. Some laboratories can provide CLSI approved MIC testing for *Candida* spp. for amphotericin B, fluconazole, and other antifungal agents.[2]

Specimen Collection and Transport

Specimen collection and transport to the laboratory is an essential part of the culture process. Improperly selected, collected, or transported specimens can generate misleading data that may result in inappropriate patient management. Consequently, improper specimen collection and transport is a reason for specimen rejection. In general, all specimens should be collected aseptically and placed in a sterile container. In some cases, specimens may be placed directly into culture media (e.g., blood cultures, genital cultures). Special handling techniques may be necessary for some specimens, such as those for anaerobic culture. Prompt delivery to the laboratory is essential to prevent the death of pathogenic organisms and/or the overgrowth of commensal organisms. If transport is delayed, some specimens may be refrigerated (e.g., urine, stool, sputum), whereas others should be maintained at room temperature (e.g., genital, eye, spinal fluid).
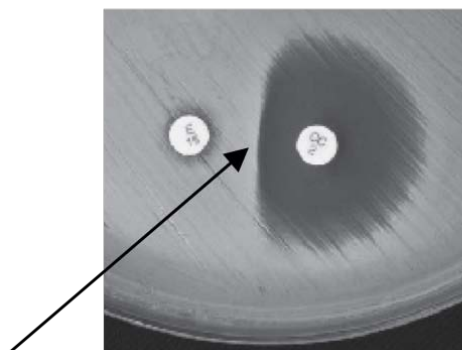
Figure 16-5. D test for inducible clindamycin resistance. The flattened side of the zone of inhibition gives the zone a D shape. (Image courtesy of the PHIL and CDC.)

Specific procedures for specimen collection and transport are institution dependent; however, there are some general guidelines to facilitate collection of the optimum specimen for examination (Fig. 16-4). Please refer to your institution's laboratory manual for specific procedures and protocols.

Table 16-4. General Guidelines for Collection of Optimum Specimens[5]

1. Collect the material from the site in which the etiologic agent will most likely be found.

2. Collect the specimen at the optimum time (e.g., early morning sputum for acid-fast bacillus, AFB).

3. Obtain cultures prior to administration of antibiotics whenever possible.

4. Collect adequate volume of material. Inadequate amounts of specimen may yield false-negative results.

5. Collect specimen in a manner that minimizes or eliminates contamination from indigenous flora as possible to ensure that the sample will be representative of the infected site.

6. Use appropriate collection devices, transport media, and sterile, leakproof containers.

7. Use sterile equipment and aseptic technique to collect specimen to prevent introduction of microorganisms during invasive procedures.

8. Clearly label the specimen including specific information regarding site of collection (e.g., blood obtained via blue lumen of right subclavian central catheter) and complete the ordering process.

9. Identify the specimen source and/or specific site correctly so that proper processing methods and culture media will be selected by the laboratory personnel.

10. If the specimen is collected through intact skin, cleanse the skin first with 70% alcohol, an iodine solution (e.g., povidone-iodine), or chlorhexidine/alcohol combination. If iodine is used, remove excess iodine after the specimen has been collected.

11. Provide clear instructions to patients if they are collecting their own specimen (e.g., clean catch urine or stool) in order to obtain the best quality specimen and allay their fears.

12. Deliver the specimen promptly to the laboratory. Delay in transport may compromise the specimen.

13. As with all patient contact episodes, consistent attention must be given to hand hygiene and use of appropriate personal protective equipment.

14. Use appropriate safety devices to minimize risk of accidental needlestick, cut, or puncture. It is advisable to make sure the user is knowledgeable about how the safety device works prior to its use.

MICROBIAL PATHOGENESIS

Normal Flora and Colonization

Microorganisms are ubiquitous in nature and are naturally present in and on humans; the term used for those found on healthy surfaces is normal flora. Typical normal flora organisms vary by body site (Table 16-5). The term colonization generally denotes the presence of a microorganism in the absence of symptoms or deep tissue invasion. Normal flora may be described as colonization in most cases (e.g., *E. coli* colonization of stool), whereas potentially pathogenic organisms may exist as colonizers. Colonizing organisms (e.g., *N. gonorrhoeae* colonization of pharynx, *Salmonella* spp. colonization of stool, MRSA colonization of the nares, yeast in genital tract) may facilitate transmission to others or may lead to disease in the colonized individual during a disruptive situation (e.g., normal flora out-of-balance from antimicrobial treatment, invasive device, or wound).

Infection

The term *infection* refers to a condition in a host resulting from the presence and invasion of microorganisms. Infection implies either recovery of an organism from a normally sterile body site or the production of an inflammatory response to a microorganism. An asymptomatic infection occurs when viable organisms are present in a body site without causing any obvious symptoms (e.g., latent tuberculosis, chronic hepatitis B, latent syphilis). The immune status of the host plays a large role in determining the likely pathogenic potential of a microorganism. Infections with organisms that cause disease primarily in immunodeficient hosts are called opportunistic infections (e.g., *C. neoformans* meningitis in patients with deficient cell-mediated immunity, *Legionella pneumophila* pneumonia in patients with chronic lung disease or transplant recipients). Although almost any organism can cause an infection if introduced into a normally sterile body site, certain organisms are commonly associated with specific types of infections as shown in Table 16-6.

Sources of Microorganisms

Microorganisms can come from a variety of sources. Exogenous organisms are those that come from outside of the person or host. Exogenous sources include other humans (e.g., *Herpes virus, M. tuberculosis*), foodstuffs (e.g., *Salmonella* spp.), contaminated water sources (e.g., *Giardia, Enterovirus*), insects (e.g., Lyme disease, malaria), animals (e.g., *Brucellosis, Pasteurella* spp.), and airborne sources (e.g., *Histoplasma, Legionella* spp.). Endogenous organisms are derived from the host's own microbial flora (e.g., *S. aureus* carried on skin, E. coli carried on the perineum). For some organisms, the distinction is not clear-cut; some pathogens may be acquired first as colonizers, only to cause disease later (e.g., *Pneumococcus* spp. acquired from another host first causes pharyngeal colonization and then subsequent pneumonia).

Table 16–5. Bacteria Commonly Found on Healthy Human Body Sites

Body Site	Common / Prominent Bacteria	Irregular Bacteria
Conjunctiva	Staphylococci, Corynebacteria, anaerobic Gram (−) cocci	*S. viridans, S. pneumoniae*, Neisseriae, Haemophilus, Enterobacteriaceae
Genitourinary Tract-External Genitalia	Staphylococci, *S. viridans*, Enterococci, Corynebacteria, Enterobacteriaceae, Bacteroides, Fusobacteria, anaerobic Gram (+) cocci	Propionibacteria, anaerobic Gram (−) cocci
Genitourinary Tract-Anterior Urethra	Staphylococci, Enterococci, Neisseriae, Corynebacteria, Bacteroides, Fusobacteria, anaerobic Gram (−) cocci	*S. viridans*, Enterobacteriaceae, Clostridia, Lactobacilli, anaerobic Gram (+) cocci
Genitourinary Tract-Vagina	Staphylococci, *S. viridans*, Enterococci, Neisseriae, Corynebacteria, Lactobacilli, Bifidobacteria, Bacteroides, anaerobic Gram (+) cocci	Clostridia, Fusobacteria
Mouth	Staphylococci, *S. viridans*, Enterococci, *S. pneumoniae*, Neisseriae, Corynebacteria, Haemophilus, Enterobacteriaceae, Actinomyces, Lactobacilli, Bifidobacteria, Fusobacteria, anaerobic Gram (+) cocci, anaerobic Gram (−) cocci	Group A Streptococci, Clostridia, Propionibacteria
Lower Intestine	*S. viridans*, Enterococci, Corynebacteria, Enterobacteriaceae, Clostridia, Lactobacilli, Bifidobacteria, Fusobacteria, anaerobic Gram (+) cocci	Staphylococci, Propionibacteria, Actinomyces
Upper Respiratory Tract	Staphylococci, *S. viridans, S. pneumoniae*, Corynebacteria, Haemophilus, Propionibacteria, Actinomyces, Bacteroides, Fusobacteria, anaerobic Gram (+) cocci, anaerobic Gram (−) cocci	Group A Streptococci, Group D Streptococci, Neisseriae, Enterobacteriaceae
Skin	Staphylococci, Corynebacteria, Propionibacteria, anaerobic Gram (+) cocci	*S. viridans*

Table 16–6. Infections and Common Organisms

Infection / Site	Common Organisms	Less Common Organisms
Bronchitis	*S. pneumoniae, H. influenzae*, respiratory viruses	*B. pertussis*, RSV
Device-Related	Coagulase-negative staphylococci, *Corynebacteria sp.*	Gram (−) bacilli, *Candida sp.*
Empyema	*S. aureus*, Streptococci, anaerobes	*S. pyogenes, H. influenzae*
Endocarditis	*S. viridans, S. aureus*, Enterococci	*Haemophilus sp., S. epidermidis, Candida sp.*
Gastroenteritis	*Salmonella sp., Shigella sp., Campylobacter sp., E. coli 0157:H7*, viruses	*Giardia sp., Yersinia sp., Vibrio sp.*
Meningitis	*H. influenzae, N. meningitides, S. pneumoniae*	*L. monocytogenes, C. neoformans, M. tuberculosis, Pseudomonas sp., E. coli*
Pelvic Inflammatory Disease	*C. trachomatis, N. gonorrhoeae, Bacteroides sp.*, Enterobacteriaceae	
Peritonitis	*Bacteroides sp.*, anaerobic cocci, Enterococci, Enterobacteriaceae	*S. aureus, Candida sp.*
Pharyngitis	*S. pyogenes*, respiratory viruses	*C. albicans, N. gonorrhoeae, C. diphtheriae*
Pneumonia (Community)	*S. pneumoniae, H. influenzae, M. pneumoniae, C. pneumoniae, M. tuberculosis*	*S. aureus*, Gram (−) bacilli, anaerobes, *L. pneumophila, P. carinii*
Pneumonia (Healthcare-assoc)	*Pseudomonas sp., S. aureus*, Enterobacteriaceae	*Legionella sp., S. pneumoniae*
Osteomyelitis	*S. aureus*	*Salmonella sp., Pseudomonas sp., S. agalactiae*
Septic Arthritis	*S. aureus, N. gonorrhoeae*	*S. pneumoniae, S. pyogenes*
Septicemia	*S. aureus, S. pneumoniae, E. coli, Klebsiella sp., Salmonella sp.*	*Clostridium sp., Candida sp., Listeria sp.*
Sinusitis	*S. pneumoniae, H. influenzae, S. pyogenes, S. aureus*	Gram (−) bacilli
Skin	*S. aureus, S. pyogenes, Candida sp.*, dermatophytes	Gram (−) bacilli, *Clostridium sp.*
Urinary Tract	*E. coli*, Enterococci, *Candida sp., Klebsiella sp., Proteus sp.*	*Pseudomonas sp., S. saprophyticus*

ENVIRONMENTAL TESTING

Microbiological environmental testing is not generally recommended. Environmental culturing can be costly and may require special laboratory procedures. Additionally, in most cases no standards for comparison exist. Because of the lack of standards, environmental testing may generate inconclusive data that could result in the implementation of unnecessary procedures or treatment. Rationale for special environmental monitoring should be carefully planned and limited to epidemiological investigations. In limited situations, "routine" environmental sampling may be indicated.

Routine Environmental Testing

Routine microbiologic sampling for quality assurance purposes should be limited to: (1) biologic monitoring of sterilization processes; (2) monthly cultures and endotoxin testing of water and dialysate in hemodialysis units; and (3) short-term evaluation of the impact of infection prevention measures or changes in infection prevention protocols.[3]

Biological monitoring of sterilization procedures is designed to provide a maximal challenge to the sterilizer to ensure that other items in the load are sterile without physically opening and culturing a number of items in the load. Standardized preparations of bacterial spores (biological indicators) are commercially available as self-contained indicator systems. Different bacterial spores and incubation temperatures are used to test different types of sterilizing procedures. (See Chapters 21, Cleaning, Disinfection, and Sterilization; and 55, Central Services, for more details.)

Dialysate and water in hemodialysis units are tested to satisfy local regulations and/or national standards for water quality. These samples are typically tested monthly using standardized protocols and guidelines (see Chapter 48, Dialysis, for more details). Short-term environmental sampling can be conducted to evaluate the effectiveness of infection prevention protocols. Examples of this type of testing are evaluating new cleaning procedures and/or products and for education of employees and staff members or water culturing after Legionella abatement.

Special Environmental Testing

Environmental testing may be indicated when epidemiological investigation suggests that a source or reservoir of microorganisms may exist. Testing may involve personnel, medical devices, air, water, food, and/or surfaces. The type of sampling depends on the causative organism, type of infection, and potential sources/reservoirs. Quantitative test methods (determines the amount of an organism present) should be used rather than qualitative methods (determines only if an organism is present).

A variety of methods can be used for solid surface test samples. Swab-rinse sampling uses a template to swab a standardized area. Sponge-rinse and wipe-rinse methods use sterile sponges or wipes rubbed over a large area. Rinse-sampling involves direct immersion of an item if it can

be totally exposed to a rinse solution. Impression plating is a method in which the culture media is placed directly onto the surface being tested.

Liquid or water testing is generally more difficult to conduct than solid surface testing. There are typically fewer organisms present in liquids and they may be harder to culture. One quantitative test method is the agar spread plate, in which a known quantity of the fluid is spread on solid culture media. A second quantitative method is the membrane filter method, in which a standard volume of fluid is passed through a membrane filter and placed on a pad containing media. If tests are being conducted to detect *Legionella* spp., special culture media must be used because of the growth requirements of the organism. For more information about environmental testing for *Legionella* spp., see Chapter 77, *Legionella pneumophila*.

Fungal spores are ubiquitous in the environment and generally cause no harm to normal hosts; therefore, air sampling without reason is not recommended (refer to Chapters 15, The Immunocompromised Host; 104, Heating, Ventilation, and Air Conditioning; and 105, Water Systems Issues and Prevention of Waterborne Infectious Diseases in Healthcare Facilities). There are no recommendations regarding routine microbiological air sampling before, during, or after construction or renovations (refer to Chapter 106, Construction and Renovation). The United States Pharmacopeia (USP) standards addressing air quality and systematic testing in pharmacies or compounding areas are being phased into place.[4] USP is an official public standards–setting authority for all prescription and over-the-counter medicines and other healthcare products manufactured or sold in the United States (refer to Chapter 61, Pharmacy Services).

THE ROLE OF THE LABORATORY IN OUTBREAK INVESTIGATION

The clinical laboratory plays a pivotal role in both endemic and epidemic epidemiology, but the awareness is heightened during the investigation of an outbreak. The laboratory can assist in the identification of an outbreak by confirming organism identities, recognizing organism clusters, and detecting unusual organisms and/or antimicrobial susceptibility patterns. Additionally, they can retrieve and review archival data to determine background rates of organism isolation and help determine if an outbreak situation actually exists. Finally, the laboratory can save organism isolates from suspected cases and assist in testing to determine if the organisms are the same or related.

Determining Organism Relatedness

Historically, a variety of methods have been utilized to identify pathogenic microorganisms and evaluate their potential epidemiological interrelationships. In general, these methods have primarily relied on phenotypic (i.e., observable) characteristics. However, as test methods have advanced, more emphasis has been placed on genotypic methods (i.e., molecular or chromosomal) to determine if organisms are related.

Phenotypic Methods

Biotyping characterizes organisms based on the patterns of metabolic activities such as biochemical reactions, colony morphology, or nutritional and environmental requirements. Although biotyping can be used with any organism, it has limited ability to distinguish epidemiologically related organisms from unrelated organisms.

Antimicrobial susceptibility testing has also been used to determine if organisms are related. Although this type of testing is routinely performed in the clinical laboratory, it is relatively nonspecific and has limited usefulness in determining true relatedness.

Serotyping is based on the immunological (i.e., antisera) detection of specific antigenic determinants on the surface of bacterial cells. Serotyping is most commonly used with *Salmonella* spp., *Shigella* spp., and *Pneumococci*. It is not as discriminating as genotypic analysis and requires maintenance of large stocks of typing antisera.

Bacteriophage typing is only useful with bacterial species that are susceptible to infection and lysis by viruses (bacteriophages). The potential interrelationship between different bacterial isolates (i.e., the bacteriophage type) is assessed on the basis of bacteriophage lytic patterns. This methodology was most widely used with *S. aureus*, but is no longer recommended as an epidemiological typing method by the CDC.

Electrophoresis may be used to compare the rate of movement of metabolic enzymes or proteins from different isolates. This method assumes that minor differences in enzyme genes or proteins are reflected in their movement across the test gel. When the test gel is examined, related organisms have the same pattern of movement. Although this test method may be used with some bacterial pathogens, it is labor intensive and time consuming.

Genotypic Methods

Plasmid analysis compares bacterial isolates based on the presence of self-replicating extrachromosomal genetic elements (plasmids). Because plasmids often encode antibiotic resistance, clinical bacterial isolates frequently carry several different plasmid types. The bacterial cells are enzymatically lysed to release the plasmid DNA, which can then be analyzed by conventional agarose gel electrophoresis. This type of analysis may be used with common bacterial pathogens that frequently carry plasmids, however, many organisms are "nontypeable," as they do not carry plasmids.

Restriction endonuclease enzymes can subdivide both plasmids and chromosomal DNA into smaller fragments. Using agarose gel electrophoresis, the smaller pieces of genetic material are separated into different-sized restriction fragments, which are compared to assess genetic relatedness. Unfortunately, because chromosomal patterns are composed of hundreds to thousands of restriction fragments, interpretation of results may be difficult.

Southern blot analysis of restriction fragment length polymorphisms (RFLPs) is a method in which chromosomal DNA is extracted from clinical isolates and digested with restriction enzymes. Using gel electrophoresis, the restriction fragments are separated and transferred to a synthetic membrane. The fragments are then labeled with a homologous piece of DNA that acts as a probe to find complimentary base pairs. Variations in the number and sizes of fragments detected are called RFLPs. This testing method may be used with any organism that has available defined probes, and the use of probes derived from genes for ribosomal RNA is termed *ribotyping*. One advantage of RFLP testing is that some probes may allow simultaneous assessment of epidemiological interrelationships and definition of other clinically relevant characteristics (e.g., mechanisms of antibiotic resistance or the presence of specific antibiotic resistance genes). Unfortunately, this method is time consuming, labor intensive, and technically difficult.

Pulsed-field gel electrophoresis (PFGE) begins with the lysis of organisms and digestion of their chromosomal DNA with restriction enzymes. The fragments are separated into a pattern of discrete bands by switching the direction of the electrical current. This pattern serves as a "bar code" of the bacterial chromosome which can be used to assess the relatedness of different clinical isolates. This test method may be used with any organism from which chromosomal DNA can be properly isolated; it has been used with a wide variety of bacterial pathogens to assess epidemiological interrelationships. PFGE is probably the most widely used method for "molecular epidemiology" and is generally considered to be the gold standard for most clinically important organisms.

Amplification techniques, such as polymerase chain reaction (PCR), are widely used in epidemiological investigation of healthcare-acquired pathogens. In PCR, target DNA is extracted from the study organism. Short DNA molecules (primers), which specifically attach to each end of the target sequence, are added to the PCR reaction mixture along with a thermostable DNA polymerase and other reagents essential for DNA synthesis. In the PCR instrument (thermocycler), the target DNA is heated to denature it to single strands. After cooling, the primers attach to each end of the specific target sequence if it is present. DNA polymerase allows duplication (amplification) of the target DNA, resulting in two double-stranded molecules from each original sequence. After many cycles, millions of copies of the target sequence are produced that may be identified by a variety of means (e.g., electrophoresis, reaction with a specific probe, etc.). A number of other amplification methods are variations of PCR procedures and testing methods are continually being developed. As new techniques are developed they must be assessed to determine their usefulness in epidemiological investigations.

BIOSAFETY IN MICROBIOLOGICAL AND BIOMEDICAL LABORATORIES

In 1969, the Centers for Disease Control's booklet, *Classification of Etiologic Agents on the Basis of Hazard* detailed the code of practice for biosafety. Although the intent of the document was originally advisory, some regulatory agencies have made adherence mandatory. The principles of biosafety are based on containment and risk assessment.[2]

Containment refers to those employee or microbiological practices; selection, provision and use of safety equipment; and facility safeguards that protect laboratory workers, the environment, patients, and visitors from exposure to infectious microorganisms collected, processed, stored, and disposed of in the facility. In order to prevent laboratory-associated infections, a risk assessment is required to identify appropriate microbiological practices, safety equipment, and facility safeguards. Refer to Chapter 60, Laboratory Safety, for more information.

FUTURE TRENDS

Clinical microbiology plays an important role in the practice of infection prevention as well as epidemiology. The many advances in molecular microbiology and virology are providing new avenues of microorganism detection and identification. Ongoing identification of the mechanisms causing antimicrobial resistance will require the adaptation or modification of current tests used as well as development of new testing methodologies. Research into the complex microbiology of biofilms is needed to support efforts to eliminate healthcare-associated infections. The lean managed laboratories of the future will need to address the issue of a shrinking trained microbiology workforce. In an effort to offer clinicians rapid accurate results, research is being directed toward developing new molecular methods and or making existing molecular test methods more widely available.

INTERNATIONAL PERSPECTIVE

The epidemiology and incidence of specific microorganisms can vary widely depending on the environment, commensal flora, and transmission risks. Organisms that may be common in one geographic area may be uncommon in another. However, given the ease of international travel, few microorganisms are "confined" to specific countries or areas. Laboratory techniques are similar in most parts of the world and testing is fairly standard. Additionally, international agencies, such as the Centers for Disease Control and Prevention and the World Health Organization, can assist with laboratory analysis of unusual or epidemiologically important pathogens. Clinical microbiology laboratories must work closely with clinicians if unusual pathogens are suspected.

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