**Table of Normal Values**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
<th>Newborn</th>
<th>Age 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>4,000–12,000/µl</td>
<td>[4–12 × 10⁹/liter]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2,000–7,500/µl</td>
<td>[2–7.5 × 10⁹/liter]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>40–400/µl</td>
<td>[0.04–0.40 × 10⁹/liter]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>150,000–400,000/µl</td>
<td>[150–400 × 10⁹/liter]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pO₂</td>
<td>85–100 mmHg</td>
<td>[11.3–13.3 kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD₄ count</td>
<td>430–1,185/µl (adults)</td>
<td>[Same]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>13.4–17.4 g/dl</td>
<td>12.3–15.7 g/dl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Haemoglobin]</td>
<td>[Same]</td>
<td>[Same]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td>40–54%</td>
<td>38–47%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Haematocrit]</td>
<td>[0.4–0.54 liter/liter]</td>
<td>[0.38–0.47 liter/liter]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>0–20 mm/h</td>
<td>0–30 mm/h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ESR is usually calculated by age: male (ESR = 0.5 × age); female (ESR = 0.5 × (age + 10)); alternatively, the American values given here usually apply.]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>10–53 U/liter</td>
<td>7–30 U/liter</td>
</tr>
<tr>
<td>AST</td>
<td>11–40 U/liter</td>
<td>9–26 U/liter</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.8–1.5 mg/dl</td>
<td>0.6–1.2 mg/dl</td>
</tr>
<tr>
<td>[Creatinine (male and female) = 70–150 µmol/liter]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine kinase</td>
<td>61–200 U/liter</td>
<td>30–125 U/liter</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.5–5.0 g/dl</td>
<td>[35–50 g/liter]</td>
</tr>
<tr>
<td>Serum glucose (fasting)</td>
<td>65–110 mg/dl</td>
<td>&lt;3.6–6.1 mmol/liter</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>39–117 U/liter</td>
<td>[Same]</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>0–1.2 mg/dl</td>
<td>[0–20 µmol/liter]</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>108–215 U/liter</td>
<td>[Same]</td>
</tr>
<tr>
<td>CSF glucose</td>
<td>50–75 mg/dl</td>
<td>[2.8–4.2 mmol/liter, or 2/3 blood glucose]</td>
</tr>
<tr>
<td>CSF protein</td>
<td>15–45 mg/dl</td>
<td>[0.15–0.45 g/liter]</td>
</tr>
<tr>
<td>CSF total nucleated cells</td>
<td>0–3/µl</td>
<td>[Same]</td>
</tr>
<tr>
<td>Body temperature</td>
<td>37°C</td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>60–100/min; higher for infants and children</td>
<td></td>
</tr>
<tr>
<td>Respiratory rate</td>
<td>9–18/min; higher for infants and children</td>
<td></td>
</tr>
<tr>
<td>Blood pressure</td>
<td>90–150/50–90; lower for infants and children</td>
<td></td>
</tr>
</tbody>
</table>

*Values in brackets indicate European equivalents. If no value is given, the American value is used.*
Cases in Medical Microbiology and Infectious Diseases

THIRD EDITION

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ASM Press, Washington, D.C.
To our children
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We would like to thank Charles Upchurch for updating the excellent glossary originally compiled by Susan Gibbs and Paul Walden. We thank Mary Ellen Mangum and Melissa Jones for collecting clinical materials that were used for the many new photographs in this edition. We thank Bob Bagnell for his excellent photographic work. We are grateful to Joan Barenfanger for the Ehrlichia photos; Thomas Bouldin for the Creutzfeldt-Jakob figure; Lynn Garcia for the Trichomonas figure; Jerome O. Klein for the measles rash photo; Frederick T. Koster for the hantavirus case; Krishnan Parayth for the photos of the coccidiodomycosis patient; Thomas Treadwell for the meningococcemia and dengue cases and patient photos; and Alison Holmes and Fiona Cooke for their contributions toward making the Table of Normal Values relevant to health care professionals who work with units that are not commonly in use in the United States.

We would like to thank Mary McKenney for her excellent copyediting. We would particularly like to thank Ellie Tupper, ASM Press, for overseeing this project with diligence and encouragement. Any shortcomings in this text are solely the responsibilities of the authors.

Finally, we would like to remember our colleague and friend Roy S. Hopfer, who passed away much too young in spring of 2001. Roy’s photographs have graced all three editions of this text, including photos used on the covers of the first two editions. Roy had a remarkable fund of medical mycology knowledge. He taught us a great deal, and what we learned from him is reflected in these pages. He is greatly missed.
INTRODUCTION
TO THE THIRD EDITION

Since the publication of the second edition of this text, two events in the United States clearly have spotlighted infectious diseases. In the fall of 1999, an outbreak of encephalitis in New York City due to West Nile virus, newly introduced to the United States, brought the problem of emerging infectious disease into the public discourse. In the fall of 2001, bioterrorism in the form of “anthrax” letters sent through the U.S. mail resulted in five deaths. The specter of bioterrorism has resulted in a renewed interest, not only in the medical community but also in the general public, in the epidemiology, detection, pathogenesis, treatment, and prevention of infectious diseases, especially in those that have the potential to sicken and kill thousands to millions. The worldwide spread of HIV and AIDS as well as the ever-increasing problem of antimicrobial drug resistance continue to be major health problems that will not be solved anytime soon. In this setting, we have prepared the third edition of this text.

The goal of this text continues to be to challenge students to develop a working knowledge of the variety of microorganisms that cause infections in humans. Initially, this text was geared exclusively to medical students for use during their basic science curriculum. We have learned from many students that they found this text to be valuable in preparation for both Parts 1 and 2 Examinations of the National Board of Medical Examiners. We also see students using this text during their clinical infectious disease rotations. Although medical students continue to be our main target audience, we recognize that this text is used by a variety of different learners, including advanced undergraduate students, other professional students, physicians in training at both the resident and fellow stage, and faculty members at all levels from high schools to community colleges to colleges and universities and medical schools. We hope all readers of this new edition find it useful.

To try to make this book of greater value to all of our readers, we have included an extensive introductory chapter called “A Primer on the Laboratory Diagnosis of Infectious Disease.” The goal of this primer is to explain in simple and concise language the strengths and weaknesses of different approaches that the clinical laboratory uses in assisting the physician in making the diagnosis of infectious diseases. All of the approaches described in this introductory section will later be encountered in the cases. It is our hope that this section will assist readers as they work through the cases that follow in understanding the strategies used in establishing an infectious disease diagnosis.
The cases are presented as “unknowns” and represent actual case histories of patients we have encountered during our professional duties at two university teaching hospitals. Each case is accompanied by several questions to test knowledge in four broad areas:

- the organism’s characteristics and laboratory diagnosis
- pathogenesis and clinical characteristics
- epidemiology
- prevention, and in some cases, drug treatment and resistance

Whenever possible, we have tried to incorporate an exciting new area of study, cellular microbiology, into the case discussions. Cellular microbiology is the study of the manner in which microbes interact with their target host cell. As information gained from work within the Human Genome Project is translated into the area of microbial pathogenesis, the understanding of the interaction of the microbial invader with the target host cell will lead to exciting new targets for antimicrobial therapy. This is becoming of greater importance as resistance to conventional antimicrobial therapies becomes more widespread among an ever-increasing number of human pathogens.

There are 15 new cases in the third edition. As with the second edition, we have tried to find cases that represent contemporary trends in infectious diseases. This has led us to delete some cases. We have deemphasized opportunistic infections in HIV patients because the use of highly active antiretroviral therapy fortunately has resulted in a significant reduction in these types of complications. We have tried to increase the number of cases in transplant recipients, because the number of these patients is increasing, and opportunistic infections remain a major problem in this immunosuppressed population. We try to balance these tertiary care problems with typical cases of community-acquired infections that will be more frequently encountered by the majority of physicians. Because this text relies on actual case studies seen in our two institutions, we are happy to say that no cases due to bioterrorism are presented here, although organisms that are possible bioterrorism agents are represented and their potential for use is highlighted.

The book continues to be richly illustrated to familiarize students with the appearance of specific microorganisms in appropriate clinical settings. This is done in response to the decision by many medical schools to limit or eliminate hands-on laboratory experiences. Although photographs are an imperfect substitute for direct observation, we believe that students should develop an appreciation for the importance of microscopy and direct observation in infectious disease diagnosis.
This text has three secondary goals. One is to help students learn how to develop a differential diagnosis. The second is to help students begin to understand the language of medicine. An extensive glossary, originally compiled for the first edition by T. Paul Walden, M.D., and greatly expanded in the second edition by Susan Gibbs, M.D., has been updated for this edition by a current medical student, Charles Upchurch. The purpose of the glossary is to explain the medical terminology so the cases are accessible to readers, especially those who are not physicians, clinical scientists, or physicians-in-training.

The third goal of this text is to present interesting and challenging cases that make the study of medical microbiology and infectious diseases enjoyable. We hope that we have succeeded in this goal and, as always, are open to feedback from interested readers on how we can make future editions of the textbook reflect the fascination that we have for microbiology and infectious diseases.
TO THE STUDENT

This text was written for you. It is an attempt to help you better understand the clinical importance of the basic science concepts you learn either in your medical microbiology or infectious disease course or through your independent study. You may also find that this text is useful in reviewing for Part I of the National Board of Medical Examiners exam. It should be a good reference during your Infectious Disease rotations.

Below is a sample case, followed by a discussion of how you should approach a case to determine its likely etiology.

SAMPLE CASE
A 6-year-old child presented with a 24-hour history of fever, vomiting, and complaining of a sore throat. On physical examination, she had a temperature of 38.5°C, her tonsillar region appeared inflamed and was covered by an exudate, and she had several enlarged cervical lymph nodes. A throat culture plated on sheep blood agar grew many beta-hemolytic colonies. These colonies were small with a comparatively wide zone of hemolysis.

What is the likely etiologic agent of her infection?

The first thing that should be done is to determine what type of infection this child has. She tells you that she has a sore throat. On physical examination, she has sign of an inflamed pharynx with exudate, which is consistent with her symptoms. (Do you know what an exudate is? If not, it’s time to consult the glossary in the back of this text.) She also has enlarged regional lymph nodes, which support your diagnosis of pharyngitis (sore throat).

What is the etiology of her infection? The obvious response is that she has a “strep throat,” but in reality there are many agents which can cause a clinical syndrome indistinguishable from that produced by group A streptococci, the etiologic agent of “strep throat.” For example, sore throats are much more frequently caused by viruses than streptococci. Other bacteria can cause pharyngitis as well, including Mycoplasma spp., various Corynebacterium spp., Arcanobacterium sp., and Neisseria gonorrhoeae. All of these organisms would be in the differential diagnosis, along with other perhaps more obscure causes of pharyngitis.

However, further laboratory information narrows the differential diagnosis considerably; small colonies that are surrounded by large zones of hemolysis are consistent with beta-hemolytic streptococci, specifically group A streptococci. On the basis of presenting signs and symptoms and the laboratory data, this child most likely has group A streptococcal pharyngitis.
Specific aids have been added to the book to assist you in solving the cases.

1. For this edition, a new section called “A Primer on the Laboratory Diagnosis of Infectious Diseases” has been added. The purpose of this section is to explain the application and effectiveness of different diagnostic approaches used in the clinical microbiology laboratory. We recommend that you read this primer before beginning your study of the cases.

2. At the beginning of each book section is a brief introduction and a list of organisms. Only organisms on this list should be considered when solving the cases in that section. These lists have been organized on the basis of important characteristics of the organisms.

3. A table of normal values is available inside the front cover of this book. If you are unsure whether a specific laboratory or vital sign finding is abnormal, consult this table.

4. A glossary of medical terms which are frequently used in the cases is available at the end of the text. If you do not understand a specific medical term, consult the glossary. If the term is not there, you will have to consult a medical dictionary or other medical texts.

5. Figures demonstrating microscopic organism morphology are presented in many of the cases, as are key radiographic, laboratory, clinical, or pathologic findings. They provide important clues in helping you determine the etiology of the patient’s infection.

A FINAL THOUGHT

The temptation for many will be to read the case and its accompanying questions and then go directly to reading the answers. You will derive more benefit from this text by working through the questions and subsequently reading the case discussion.

Have fun and good luck!
A PRIMER ON THE LABORATORY DIAGNOSIS OF INFECTIOUS DISEASES

The accurate diagnosis of infectious diseases often but not always requires the use of diagnostic tests to establish their cause. The utilization of diagnostic tests in the managed care environment is carefully monitored and is frequently driven by standardized approaches to care called “clinical pathways.” These pathways include using a pre-defined set of diagnostic tests for patients who present with signs and symptoms characteristic of certain clinical conditions, such as community-acquired pneumonia. Currently, the Infectious Disease Society of America has published over 30 different “practice guidelines” dealing with various infectious diseases, including HIV (human immunodeficiency virus), tuberculosis, group A streptococcal pharyngitis, diarrheal disease, and pneumonia, from which clinical pathways can be derived. Clinical pathways and practice guidelines fall under the concept of “evidence-based medicine.” “Evidence-based medicine” relies on review and interpretation of data in the medical literature as a basis for clinical decision-making.

In some patients, such as an otherwise healthy child with a rash typical of varicella (chicken pox), the etiology of the child’s infection can be established with a high degree of certainty by physical examination alone. The use of diagnostic testing in this setting would be viewed as wasteful of the health care dollar. On the other hand, a 4-year-old who presents with enlarged cervical lymph nodes and a sore throat should have a diagnostic test to determine whether he or she has pharyngitis due to group A streptococci. The reason why such testing is necessary is that certain viral syndromes are indistinguishable clinically from group A streptococcal pharyngitis. Since group A streptococcal pharyngitis should be treated with an antibiotic to prevent poststreptococcal sequelae, and viral infections do not respond to antibiotics, determining the cause of the infection in this particular case is central to appropriate patient management. Far too often, antibiotics are given without diagnostic testing in a child with a sore throat. As a result, many children with viral pharyngitis are given antibiotics. This inappropriate use of antibiotics increases antibiotic selective pressure. This can result in greater antimicrobial resistance among organisms in the resident microflora of the throat, such as *Streptococcus pneumoniae*. In addition, patients may develop antibiotic-associated complications, such as mild to severe allergic reactions or gastrointestinal distress including diarrhea. One of the goals of the third edition of this text is to help you think in a cost-effective way about how best to use laboratory resources. As an introduction to this edition, we will present a general overview of the various laboratory approaches that are used in the diagnosis and management of infectious diseases.
ACCURACY IN LABORATORY TESTING

The clinical microbiology laboratory must balance the requirements of timeliness with those of accuracy.

As an example, consider the identification of a gram-negative rod from a clinical specimen. If the organism is identified with the use of a commercially available identification system, an identification and an assessment of the probability of that identification will be made on the basis of biochemical test results and a comparison of these results with a database. So, if the result states that the organism is *Enterobacter cloacae* with 92% probability, the laboratory may very well report this identification. Assuming that the 92% probability figure generated by the commercial system is on target (many commercial systems do a worse job with anaerobic bacteria), this means that there is a probability of 8%, or about 1 time in 12, that this identification will be incorrect.

Certainly, it would be possible for the laboratory to perform additional testing to be more certain of the identification. The problem is that by doing so there would be a delay, perhaps a clinically significant one, in the reporting of the results of the culture. In some cases such a delay is unavoidable (e.g., when the result of the identification in the commercial system is below an arbitrary acceptable probability and manual methods must be used) or clinically essential (e.g., when a specific identification is required and the isolate must be sent to a reference laboratory for identification; an example is *Brucella* spp., which require prolonged therapy and are potential agents of bioterrorism).

Similarly, the methods most commonly used in the clinical laboratory for susceptibility testing are imperfect. The worst errors, from the clinical point of view, are those in which the laboratory reports an organism as susceptible to a particular antibiotic to which, in fact, it is resistant. In some cases additional tests are employed to minimize the risk of this occurring. For example, in addition to standard testing using either an automated or a manual method, recommended susceptibility testing of *Staphylococcus aureus* includes the use of Mueller-Hinton agar, in which the antibiotic oxacillin is present at a known concentration. Even if the results of the standard susceptibility testing indicate susceptibility to oxacillin, if there is growth of the *S. aureus* isolate on the oxacillin-containing Mueller-Hinton plate, the organism is reported as resistant to oxacillin (as well as to all other beta-lactams).

Unfortunately, very few such checks exist to correct erroneous bacterial susceptibility assays. In addition to the oxacillin-containing plate, there are plates containing medium with vancomycin. These are used in the laboratory to screen for the possibility of vancomycin resistance in enterococci and the rarely seen increase in the minimum inhibitory concentration (MIC) in *S. aureus*. In general, there is a delay in the
ability of automated susceptibility methods to reliably identify newly described mechanisms of antibiotic resistance. As a result, manual methods are often required. The performance of automated susceptibility testing methods varies, and certain combinations of organism and antibiotic have an unacceptably high error rate. In such cases, backup methods, such as disk diffusion or MIC testing, should be employed. Laboratories with a significant number of susceptibility tests to perform commonly use automated susceptibility methods because of the labor-intensive nature of manual testing and the speed with which automated systems are able to report results—often in a few hours as compared with overnight incubation, as is the case with manual methods.

Diagnostic tests vary in their sensitivity and specificity. As an example, consider a hypothetical STD (sexually transmitted disease) clinic in which the rapid plasma reagin (RPR) test, a screening test for syphilis, is being evaluated in 1,000 patients with genital ulcer disease who are suspected of having primary syphilis:

<table>
<thead>
<tr>
<th>PRIMARY SYPHILIS</th>
<th>RPR TEST RESULT</th>
<th>PRESENT</th>
<th>ABSENT</th>
<th>Positive predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSITIVE</td>
<td></td>
<td>420</td>
<td>60</td>
<td>420/(420 + 60) = 0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive predictive value = 88%</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td></td>
<td>220</td>
<td>300</td>
<td>300/(300 + 220) = 0.58</td>
</tr>
</tbody>
</table>

On the basis of these data, the sensitivity of this test (the true-positive rate, calculated as true-positive results divided by the number of patients with disease) in primary syphilis is 66%. The specificity (1 minus the false-positive rate) is 83%. Note that in this high-prevalence population (the prevalence here is the total number of cases in which primary syphilis is present—640 divided by the total number of individuals, 1,000—and is thus 0.64 or 64%), the predictive value of a positive test is fairly good, at 88%. The positive predictive value of an assay varies with the prevalence of the disease in the population. This is a key point. An example of this in our syphilis serology example in a low-prevalence population will serve to illustrate the point.

The same RPR serologic assay is being used in a hypothetical population of octogenarian nuns, none of whom are or have been sexually active in at least 6 decades,
and all of whom have been confined to their abbey since before the Second World War.

<table>
<thead>
<tr>
<th>SYPHILIS</th>
<th>PRESENT</th>
<th>ABSENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPR TEST RESULT</td>
<td>1</td>
<td>169</td>
</tr>
<tr>
<td>POSITIVE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>0</td>
<td>830</td>
</tr>
</tbody>
</table>

Positive predictive value = 1/170 = 0.006
Positive predictive value = 0.6%

Negative predictive value = 830/830 = 1.00
Negative predictive value = 100%

Specificity = 830/999 = 0.83
Specificity = 83%

In this patient population, there is only one true case of syphilis, presumably acquired many years previously. The specificity of the test in this patient population is essentially the same as it is in the individuals attending the STD clinic (in reality, it is likely to be different in different populations and also in different stages of syphilis). Since there is one case of syphilis, and 169 of the positive RPR results are false-positive test results, the positive predictive value in this patient population is only 0.6%. Clearly, this is a patient population in which the decision to test for syphilis using the RPR assay is not cost-effective.

In making a decision to order a specific test, the physician should know what he or she will do with the test results—essentially, how the results will alter the care of the patient. In a patient who the physician is certain does not have a specific disease, if the test for that disease has an appreciable rate of false-positive results, a positive test result is likely to be false positive and should not alter clinical care. Conversely, if the physician is certain that a patient has a disease, there is no good reason to order a test with a low sensitivity, as a negative result will likely be false negative. Tests are best used when there is uncertainty and when the results will alter the posttest probability and, therefore, the management of the patient.

**SPECIMEN SELECTION, COLLECTION, AND TRANSPORT**

Each laboratory test has three stages.

1. **The preanalytical stage:** The caregiver selects the test to be done, determines the type of specimen to be collected for analysis, ensures that it is properly labeled with the patient’s name, and facilitates rapid and proper transport of this specimen to the laboratory.
2. **The analytical stage:** The specimen is analyzed by the laboratory for the presence of specific microbial pathogens. The remaining sections of this chapter describe various analytic approaches to the detection of pathogens.

3. **The postanalytical stage:** The caregiver uses the laboratory results to determine what therapies, if any, to use in the care of the patient.

The preanalytical stage is the most important stage in laboratory testing! If the wrong test is ordered, if the wrong specimen is collected, if the specimen is labeled with the wrong patient’s name, or if the correct specimen is collected but is improperly transported, the microbe causing the patient’s illness may be not detected in the analytical stage. As a result, at the postanalytical stage, the caregiver may not have the appropriate information to make the correct therapeutic decision. The maxim frequently used in laboratory medicine is “garbage in, garbage out.”

Specimen selection is important. A patient with a fever, chills, and malaise may have an infection in any one of several organ systems. If a patient has a urinary tract infection and if urine is not selected for culture, the etiology and source of the infection will be missed. Careful history taking and physical examination play an important role in selecting the correct specimen.

Continuing with the example of a patient with a fever due to a urinary tract infection, the next phase in the diagnosis of infection is the collection of a urine specimen. Because the urethra has a resident microflora, urine specimens typically are not sterile. A properly collected urine specimen is one in which the external genitalia are cleansed and midstream urine is collected. Collection of midstream urine is important because the initial portion of the stream washes out much of the urethral flora. Even with careful attention to detail, clean-catch urine can be contaminated with urethral flora, rendering the specimen uninterpretable at the postanalytical stage.

An important concept when considering the transport of clinical specimens for culture is to recognize that they contain living organisms whose viability is influenced by transport conditions. These organisms may be killed by changes in temperature, drying of the specimen, exposure to oxygen, lack of vital nutrients, or changes in specimen pH. Transport conditions that support the viability of any clinically significant organisms present in the specimen should be established. It should also be noted that the longer the transport takes, the less likely it is that viability will be maintained. Rapid transport of specimens is important for maximal accuracy at the analytical stage.

If the correct test is selected, the proper specimen is collected and transported, but the specimen is labeled with the wrong name, the test findings might be harmful to two different patients. The patient from whom the specimen came might not receive the proper therapy, while a second patient whose name was mistakenly used to label the specimen might receive a potentially harmful therapy.
DIRECT EXAMINATION

Macroscopic

Once a specimen is received in the clinical laboratory, the first step in the determination of the cause of an infection is to examine it. Frequently, infected urine, joint, or cerebrospinal fluid specimens will be “cloudy” because of the presence of microorganisms and white blood cells, suggesting that an infectious process is occurring. Occasionally, the organism can be seen by simply looking for it in a clinical specimen or by looking for it on the patient. Certain worms or parts of worms can be seen in the feces of patients with ascariasis or tapeworm infections. Careful examination of an individual’s scalp or pubic area may reveal lice, while examination of the anal region may result in the detection of pinworms. Ticks can act as vectors for several infectious agents, including Rocky Mountain spotted fever, Lyme disease, and ehrlichiosis. When found engorged on the skin, physicians may remove and submit these ticks to the laboratory to determine their identity. This is done because certain ticks (deer ticks) act as a vector for certain infectious agents (Borrelia burgdorferi, the organism that causes Lyme disease). Knowing the vector may help the physician determine the patient’s diagnosis.

Microscopic

Because most infectious agents are visible only when viewed with the aid of a light microscope, microscopic examination is central to the laboratory diagnosis of infectious diseases. Microscopic examination does not have the overall sensitivity and specificity of culture or the newer molecular diagnostic techniques. However, microscopic examination is very rapid, is usually relatively inexpensive (especially when compared with molecular techniques), is available around the clock in at least some formats in most institutions, and in many clinical settings but by no means all is highly accurate when done by highly skilled laboratorians. The organisms can be detected either unstained or by using a wide variety of stains, some of which are described below. Microbes have characteristic shapes that are important in their identification. Morphology can be very simple, with most clinically important bacteria generally appearing as either cocci (Fig. 1a) or rods (Fig. 1b). The rods can be very long or so short that they can be confused with cocci (coccobacilli); they can be fat or thin, have pointed ends, or be curved. The arrangement of cocci can be very helpful in determining their identity. These organisms can be arranged in clusters (staphylococci), pairs or diplococci (S. pneumoniae), or chains (various streptococcal and enterococcal species).

Fungi are typically divided into two groups based on morphology. One is a yeast (Fig. 2), which is a unicellular organism, and the other is a mold which is a multicellular organism with complex ribbon-like structures called hyphae (Fig. 3). Organisms that are referred to as parasites may be unicellular—the protozoans (Fig. 4)—or highly
complex—the nematodes (Fig. 5) and cestodes. Parasites are typically identified on the basis of morphology alone.

Because of their small size, viruses cannot be visualized by light microscopy. Alternative approaches described below are needed to detect these microbes in clinical specimens.

**Wet mounts**

This technique is extremely simple to perform. As the name implies, the clinical specimen is usually mixed with a small volume of saline, covered with a glass coverslip, and examined microscopically. It is most commonly utilized to examine discharges from
the female genital tract for the presence of yeasts or the parasite *Trichomonas vaginalis*. Wet mounts are also used to make the diagnosis of oral thrush, which is caused by the yeast *Candida albicans*. Using a special microscopic technique—dark-field microscopy—scrapings from genital ulcers and certain skin lesions can be examined for the spirochete *Treponema pallidum*, the organism that causes syphilis. This technique is not particularly sensitive but is highly specific in the hands of an experienced microscopist. It is typically done in STD clinics where large numbers of specimens are available, enabling the microscopist to maintain his or her skill in detecting this organism.

The wet mount can be modified by replacing a drop of saline with a drop of a 10% KOH solution to a clinical specimen. This technique is used to detect fungi primarily in sputum or related respiratory tract specimens, skin scrapings, and tissues. The purpose of the KOH solution is to “clear” the background by “dissolving” tissue and bacteria, making it easier to visualize the fungi.

Another modification of the wet mount is to mix a drop of 5% Lugol’s iodine solution with feces. This stains any protozoans or eggs of various worms that may be present in the stool, making them easier to see and identify.

**Gram stain**

The most frequently utilized stain in the microbiology laboratory is the Gram stain. This stain differentiates bacteria into two groups. One is referred to as gram positive because of its ability to retain crystal violet stain, while the other is referred to as gram negative because it is unable to retain this stain (see Fig. 1). These organisms can be further subdivided based on their morphological characteristics.

The structure of the bacterial cell envelope determines an organism’s Gram stain characteristics. Gram-positive organisms have an inner phospholipid bilayer membrane surrounded by a cell wall composed of a relatively thick layer of the polymer peptidoglycan. Gram-negative organisms also have an inner phospholipid bilayer membrane surrounded by a peptidoglycan-containing cell wall. However, in the gram-negative organisms, the peptidoglycan layer is much thinner. The cell wall in gram-negative organisms is surrounded by an outer membrane composed of a phospholipid bilayer. Embedded within this bilayer are proteins and the lipid A portion of a complex molecule called lipopolysaccharide. Lipopolysaccharide is also referred to as **endotoxin** because it can cause a variety of toxic effects in humans.

Because of their size or cell envelope composition, certain clinically important bacteria cannot be seen on Gram stain. These include all species of the genera *Mycobacterium*, *Mycoplasma*, *Rickettsia*, *Coxiella*, *Ehrlichia*, *Chlamydia*, and *Treponema*. Yeasts typically stain as gram-positive organisms, while the hyphae of molds may inconsistently take up stain but generally will be gram positive.
Gram stains can be performed quickly, but attention to detail is important to get an accurate Gram reaction. One clue to proper staining is to examine the background of the stain. The presence of significant amounts of purple (gram-positive) in the epithelial cells, red or white blood cells, or proteinaceous material, all of which should stain gram negative, suggests that the stain is under-decolorized and that the Gram reaction of the bacteria may not be accurate. This type of staining characteristic is frequently seen in “thick” smears. The detection of over-decolorization is much more difficult and is dependent on the observation skills of the individual examining the slide.

**Staining of acid-fast organisms**

*Mycobacterium* spp., unlike other bacteria, are surrounded by a thick mycolic acid coat. This complex lipid coat makes the cell wall of these bacteria refractory to staining by the dyes used in the Gram stain. As a result, bacteria within this genus usually cannot be visualized or, infrequently, may have a beaded appearance on Gram stain. Certain stains, such as carbol fuchsin or auramine-rhodamine, can form a complex with the mycolic acid. This stain is not washed out of the cell wall by acid-alcohol or weak acid solution, thus the term “acid-fast” bacterium.

Auramine and rhodamine are nonspecific fluorochromes. Fluorochromes are stains that “fluoresce” when excited by light of a specific wavelength. Bacteria that retain these dyes during the acid-fast staining procedure can be visualized with a fluorescent microscope (Fig. 6). In clinical laboratories with access to a fluorescent microscope, the auramine-rhodamine stain is the method of choice because the organisms can be visualized at a lower magnification. By screening at lower magnification, larger areas of the microscope slide can be examined more quickly, making this method more sensitive and easier to perform than acid-fast stains using carbol fuchsin.

Several other organisms are acid-fast, although they typically are not alcohol-fast. As a result, they are stained using a modified acid-fast decolorizing step whereby a weak acid solution is substituted for an alcohol-acid one. This technique is frequently used to distinguish two genera of gram-positive, branching rods from each other. *Nocardia* species are acid-fast when the modified acid-fast staining procedure is used, while *Actinomyces* species are not. *Rhodococcus equi* is a coccobacillus that may also be positive by modified acid-fast stain when first isolated. The modified acid-fast stain has also been effective in the detection of two gastrointestinal protozoan parasites, *Cryptosporidium* and *Cyclospora*. It should be noted that *Cyclospora* stains
inconsistently, with some organisms giving a beaded appearance while others do not retain the stain at all.

**Trichrome stain**

The trichrome stain is used to visualize protozoans in fecal specimens. This stain is particularly effective at staining internal structures, the examination of which is important in determining the identity of certain protozoans, such as *Entamoeba histolytica*. Modification of the trichrome stain is used in the detection and identification of microsporidia.

**Direct fluorescent-antibody stains**

The development of monoclonal antibodies has enhanced both the sensitivity and the specificity of staining techniques that use antibodies to detect microbes in clinical specimens. The most widely used staining technique that incorporates the use of antibodies is the direct fluorescent-antibody (DFA) stain. In this technique, a highly specific antibody is coupled to a fluorochrome, typically fluorescein, which emits an “apple-green” fluorescence. The antibody binds specifically either to antigens on the surface of the microbes or to viral antigens expressed by virally infected cells which can be visualized under the fluorescent microscope (Fig. 7). This technique is rapid, usually requiring 1 to 2 hours. In the hands of a skilled operator, the test is highly specific, although it frequently has a sensitivity of only 60 to 70% when compared with bacterial culture. Because of its rapidity, the test has been used to detect some relatively slow-growing or difficult-to-grow bacteria, such as *Bordetella pertussis* and *Legionella pneumophila*. For respiratory and herpes viruses, the sensitivity of this technique approaches 90% of the sensitivity of culture. The development of molecular amplification techniques, particularly real-time polymerase chain reaction (PCR) assays, for the detection of these bacterial and viral agents may render this application of DFA testing obsolete.

DFA staining is frequently used for the detection of microbes that cannot be cultured. DFA is the method of choice for detection of the nonculturable fungus *Pneumocystis carinii*, a common cause of pneumonia in people with AIDS. DFA is much more sensitive than other commonly used staining techniques, such as silver, Giemsa, or toluidine blue O staining. Likewise, for the gastrointestinal protozoans *Giardia lamblia* and *Cryptosporidium parvum*, DFA staining has been found to be much more sensitive than examination of wet mounts or the use of trichrome (for *Giardia*) or modified acid-fast stain (for *Cryptosporidium*).
A novel application of the DFA technique is its use in detecting bloodstream infections with the cytomegalovirus (CMV). This virus is an important cause of infections in immunocompromised patients, especially in those who have received solid-organ or bone marrow transplant. In this technique, CMV antigens are detected on the surface of peripheral white blood cells. The number of infected cells per 100,000 white blood cells is determined. This technique, along with quantitative PCR, is currently believed to be the most accurate for diagnosing disseminated CMV infections and predicting those individuals who need to be receiving antiviral therapy.

**Infectious disease diagnosis from peripheral blood smears and tissue sections**

Not all staining used in the diagnosis of infectious disease is done in the microbiology laboratory. The hematologist and the anatomical pathologist can play important roles in the diagnosis of certain infectious diseases.

The peripheral blood smear is the method of choice for detection of one of the most important infectious diseases in the world, malaria, which is caused by protozoans within the genus *Plasmodium*. The various developmental stages of these parasites are detected in red blood cells. Other, less frequently encountered parasites seen in a peripheral blood smear include *Babesia* species, trypanosomes, and the microfilariae.

Bacterial and fungal pathogens may be seen in peripheral smears on occasion. The most likely of these is *Histoplasma capsulatum*, which is seen as small, intracellular yeasts in peripheral white blood cells. *Ehrlichia* spp. can produce a characteristic lesion, the morulae, which can be seen in peripheral white blood cells. There are two forms of the disease, one in which mononuclear cells are infected, and the other in which granulocytic cells are infected.

Examination of tissue by the anatomical pathologist is an important technique for detecting certain infectious agents. Tissue cysts due to toxoplasmosis can be detected in brain biopsy material from patients with encephalitis. The diagnosis of Creutzfeldt-Jakob disease is based on the finding of typical lesions on brain biopsy. The finding of hyphal elements in lung tissue is an important tool in the diagnosis of invasive aspergillosis and pulmonary zygomycosis. The observation of ribbon-like elements in a sinus biopsy is pathognomonic for the diagnosis of rhinocerebral zygomycosis, a potentially fatal disease most frequently seen in diabetic patients.

**Antigen detection**

Visual examination of a clinical specimen is not the only means by which an infectious agent can be directly detected. A variety of tests have been developed that, like DFA, are dependent on the availability of highly specific antibodies to detect antigens of specific bacteria, fungi, viruses, and parasites. The most widely used antigen detection tests are various formats of the enzyme immunoassay (EIA) or the latex agglutination
assay. These tests take anywhere from 10 minutes to 2 hours. The test most widely used is a 10- to 15-minute EIA for the detection of group A streptococci. The sensitivity of these various formats has been reported to be 80 to 90%, with a specificity usually greater than 95%. In the United States, there are over 50 different test formats marketed for the detection of this organism. The test is done in a wide variety of laboratories, clinics, and physicians’ offices. Antigen detection tests are widely used in the United States to detect a variety of infectious agents, including *Cryptococcus neoformans*, *Clostridium difficile* toxin, respiratory syncytial virus (RSV), rotavirus, influenza virus, and *Giardia* and *Cryptosporidium* spp.

**MOLECULAR DIAGNOSTICS**

In addition to standard methods of culturing and identifying pathogenic microorganisms, there are now a number of molecular methods available that are able to detect the presence of the specific nucleic acid of these organisms. These methods are used in demonstrating the presence of the organism in patient specimens as well as in determining the identification of an isolated organism. In some cases, these methods are able to determine the quantity of the nucleic acid.

As an example, bacteria of a particular species will have a chromosomal nucleic acid sequence significantly different from that of another bacterial species. On the other hand, the nucleic acid sequence within a given species has regions that are highly conserved. For example, the base sequence of the *Mycobacterium tuberculosis* 16S ribosomal RNA gene differs significantly from the base sequence in the *Mycobacterium avium* complex 16S ribosomal RNA gene, yet the sequence of bases in this region among members of the *M. tuberculosis* complex is highly conserved. These properties form the basis for the use of genetic probes to identify bacteria to the species level. There are a number of commercially available genetic probes that can detect specific sequences in bacteria, mycobacteria, fungi, and viruses.

Nucleic acid hybridization is a method by which there is the in vitro association of two complementary nucleic acid strands to form a hybrid strand. The hybrid can be a DNA-RNA hybrid, a DNA-DNA hybrid, or, less commonly, an RNA-RNA hybrid. To do this, one denatures the two strands of a DNA molecule by heating to a temperature above which the complementary base pairs that hold the two DNA strands together are disrupted and the helix rapidly dissociates into two single strands. A second nucleic acid is introduced which will bind to regions that are complementary to its nucleic acid sequence. The stringency, or specificity, of the reaction can be varied by reaction conditions such as the temperature.

In addition to the direct demonstration of a nucleic acid sequence by hybridization, amplification assays (the process of making additional copies of the specific sequence of interest) are of increasing importance in clinical microbiology. The most
commonly used amplification assay is PCR (Fig. 8). PCR uses a DNA polymerase that is stable at high temperatures that would denature and inactivate most enzymes. This thermostable DNA polymerase most often is isolated from the bacterium *Thermus aquaticus*. Its stability at high temperature enables the enzyme to be used without the need for replacement after the high-temperature conditions of the DNA denaturation step that occurs during each cycle of PCR:

1. The target DNA sequence is heated to a high temperature that causes the double-stranded DNA to denature into single strands.

2. An annealing step follows, at a lower temperature than the denaturization step above, during which sets of primers, with sequences designed specifically for the PCR target sequences, bind to these target sequences.

3. Last is an extension step, during which the DNA polymerase completes the target sequence between the two primers.

Assuming 100% efficiency, the above three steps generate two copies of the target sequence. Multiple cycles (such as 30) in a thermal cycler result in a tremendous amplification of the number of sequences, so that the sequence is readily detectable using any of a variety of methods—colorimetric, chemiluminescent, or gel electrophoretic.
When the specific target nucleic acid is RNA rather than DNA, a complementary DNA (cDNA) sequence is made with the enzyme reverse transcriptase (RT) before PCR amplification in a procedure known as RT-PCR. Examples of pathogens for which RT-PCR is used include the RNA-containing viruses HIV-1 and hepatitis C virus (HCV).

An additional feature of PCR is that the amplified nucleic acid products can be directly sequenced. These sequences can be compared with sequences found in publicly accessible databases. This allows, for example, the identification of a bacterial organism to the level of species on the basis of a sequence of hundreds of bases in the 16S ribosomal RNA gene or, if the sequence is less closely related to sequences within the database, to the level of genus. In some cases, the organism may be an entirely new one. This method of PCR and sequencing of the product for the purposes of bacterial identification remains a research tool in clinical microbiology, but the rapidity with which it can be performed and the high quality of the databases makes it likely that this method will be increasingly used.

After the invention of PCR, a number of other amplification assays were developed, some of which have entered the clinical microbiology laboratory. These include ligase chain reaction (LCR), in which DNA ligase is used (as is a thermal cycler) to produce ligation products, and transcription-mediated amplification (TMA), which does not require a thermal cycler and relies on the formation of cDNA from a target single-stranded RNA sequence, the destruction of the RNA in the RNA:DNA hybrid by RNase H, and the formation of double-stranded cDNA (which can serve as transcription templates for T7 RNA polymerase). A similar procedure occurs during the nucleic acid sequence-based amplification (NASBA) assay. Strand-displacement amplification (SDA) does not require a thermal cycler and has two phases in its cycle: a target generation phase during which a double-stranded DNA sequence is heat denatured, resulting in two single-stranded DNA copies, and an exponential amplification phase in which a specific primer binds to each strand at the cDNA sequence. DNA polymerase extends the primer, forming a double-stranded DNA segment that contains a specific restriction endonuclease recognition site, to which a restriction enzyme binds, cleaving one strand of the double-stranded sequence and forming a nick, followed by extension and displacement of new DNA strands by DNA polymerase.

All of these assays—PCR, LCR, TMA, and SDA—have one thing in common: they amplify the target nucleic acid sequence, making many, many copies of the sequence. As you might imagine, there is the possibility that small quantities of the billions of amplified target nucleic acid sequences can contaminate a sample that will then undergo amplification testing, resulting in false-positive results. Steps are taken to minimize contamination, including physical separation of specimen preparation.
and amplification areas, positive displacement pipettes, and both enzymatic (in PCR) and nonenzymatic methods to destroy the amplified products.

An alternative method of demonstrating the presence of a specific nucleic acid sequence that does not require the amplification of the target is by amplification of the signal. One commonly used method that does so is the branched DNA assay (b-DNA) (Fig. 9), which is used particularly in quantitative assays, such as HIV and HCV viral load determinations. In this assay, specific oligonucleotides hybridize to the sequence of interest and capture it onto a solid surface. In addition, a set of synthetic enzyme-conjugated branched oligonucleotides hybridize to the target sequence. When an appropriate substrate is added, light emission is measured and compared with a standard curve. This permits quantitation of the target sequence. As there is no amplified sequence to be concerned about, the risk of contamination is dramatically reduced.

There are several commercially available molecular diagnostic assays for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. These include direct hybridization assays as well as tests that amplify the nucleic acid in specimens of cervical and urethral swabs and urine. The amplified assays are being used with increasing frequency both because of the convenience of using urine rather than a sample that requires more invasive specimen collection and because of the increased sensitivity of amplified as compared with

![Diagram of bDNA-based signal amplification](image)

*Figure 9  bDNA-based signal amplification. Target nucleic acid is released by disruption and is captured onto a solid surface via multiple contiguous capture probes. Contiguous extended probes hybridize with adjacent target sequences and contain additional sequences homologous to the branched amplification multimer. Enzyme-labeled oligonucleotides bind to the bDNA by homologous base pairing, and the enzyme-probe complex is measured by detection of chemiluminescence. All hybridization reactions occur simultaneously. (Reprinted from *Manual of Clinical Microbiology*, 7th ed., ©1999 ASM Press, with permission.)*
nonamplified assays. Since *N. gonorrhoeae* is a fastidious organism that may not survive specimen transport, this test is of particular benefit in settings in which there may be a delay in the transport of the specimen to the laboratory; i.e., the viability of the organisms is not required to detect the presence of its nucleic acid. Similarly, the previous “gold standard” for the detection of *C. trachomatis*—tissue culture—was labor-intensive, required the use of living cell lines, and required rapid specimen transport on wet ice to ensure the viability of the organisms in the specimen. In many laboratories, *C. trachomatis* tissue culture has been replaced by amplification technologies, which have been shown to be more sensitive. As you might imagine, since these assays do not require the presence of living organisms, patients who have been treated with appropriate antibiotics may continue to have a positive assay for some time because of the presence of dead, and therefore noninfectious, organisms that contain the target nucleic acid.

Quantitative assays are now available for several different pathogens. These include tests that determine the level of HIV RNA in patients with HIV infection and are now recognized as one component of the standard clinical management of these patients. With the availability of highly active antiretroviral therapy (HAART) but the potential for antiviral drug resistance, it is important to be able to closely monitor the plasma level of HIV RNA, also known as the viral load. A clinical response to antiretroviral therapy can be demonstrated by a decrease in the viral load. Similarly, an increase in the viral load may indicate either the development of viral resistance to one or more of the antiviral agents being used to treat the patient or merely patient non-compliance with therapy. Modification of therapy may be made on the basis of a rising HIV viral load and the results of HIV genotyping studies.

HIV genotyping is a test that determines the specific nucleic acid sequence present in the virus infecting a patient. There are a number of ways that this test can be performed, and direct sequencing of amplified cDNA (using RT-PCR) is one example. These results are routinely compared with a database that contains nucleic acid sequences from viral strains that are known to be both sensitive and resistant to specific antiretroviral medications. This comparison permits the clinician to note what, if any, mutations are present in the virus infecting the patient and to predict with some reasonable degree of probability whether the viral isolate is resistant to antiretroviral medications, including those being taken by the patient. These data can help the physician make a rational choice of an antiretroviral regimen in a patient whose therapy is failing. One difficulty with this test is that patients are often infected with a mixture of different HIV viral populations, both because of the high frequency of mutation that occurs with HIV and because of the selection of resistant subpopulations while the patient receives antiretroviral therapy. As a result, there may be resistant subpopulations that are below the level of detection of the standard HIV
genotyping assay and that could become clinically relevant under the selective pressure of continued antiretroviral therapy.

Detection of HCV RNA using PCR can be used both diagnostically and for following the effectiveness of therapy. The PCR product generated during the HCV RNA assay can be used for genotyping. Genotype is determined by a hybridization assay in which specific genes associated with specific genotypes are detected. Genotype 1 is more refractory to therapy than genotypes 2 and 3. Therefore, therapy is much more prolonged (48 versus 24 weeks) for genotype 1 than for 2 and 3.

CULTURE

Detection of bacterial and fungal pathogens by culture
Culture on artificial medium is the most commonly used technique for detecting bacteria and fungi in clinical specimens. Although not as rapid as direct examination, it is more sensitive and much more specific. For the majority of human pathogens, culture requires only 1 to 2 days of incubation. For particularly slow-growing organisms, such as *M. tuberculosis* and some fungi, the incubation period may last for weeks. By growing the organism, it is available for further phenotypic and genotypic analysis, such as antimicrobial susceptibility testing, serotyping, virulence factor detection, and molecular epidemiology studies.

Environmental and nutritional aspect of bacterial and fungal culture
Certain basic strategies are used to recover bacterial and fungal pathogens. These strategies are dependent upon the phenotypic characteristics of the organisms to be isolated and the presence of competing microflora in a patient’s clinical specimen. Most human pathogens grow best at 37°C, human body temperature. Most bacterial and fungal cultures are performed, at least initially, at this temperature. Certain skin pathogens, such as dermatophytes and some *Mycobacterium* spp., grow better at 30°C. When seeking these organisms, cultures may be done at this lower temperature. A few clinically significant microorganisms will grow at low temperatures (4°C), while others prefer higher temperatures (42°C). These incubation temperatures may be used when attempting to recover a specific organism from specimens with a resident microflora, such as feces, since few organisms other than the target organism can grow at these temperature extremes.

Another important characteristic of human bacterial and fungal pathogens is the impact of the presence of oxygen on the growth of these organisms. Microbes can be divided into three major groups based on their ability to grow in the presence of oxygen. Organisms that can only grow in the presence of oxygen are called aerobes. Fungi and many bacteria are aerobic organisms. Organisms that can only grow in the absence of oxygen are called anaerobes. The majority of bacteria that make up the
resident microflora of the gastrointestinal and female genital tracts are anaerobic organisms. Some bacteria can grow either in the presence or in the absence of oxygen. These organisms are called **facultative** organisms. A subgroup of facultative organisms is called **microaerophiles**. These organisms grow best in atmosphere with reduced levels of oxygen. *Campylobacter* spp. and *Helicobacter* spp. are examples of microaerophiles.

Besides temperature and oxygen, nutrients are an important third factor in the growth of microbes. Many bacteria have very simple growth requirements. They require an energy and carbon source, such as glucose; a nitrogen source, which may be ammonium salts or amino acids; and trace amounts of salts and minerals, especially iron. Some human pathogens have much more complex growth requirements, needing certain vitamins or less well defined nutrients such as animal serum. Organisms with highly complex growth requirements are often referred to as being **fastidious**. A fastidious bacterium which is frequently encountered clinically is *Haemophilus influenzae*. This bacterium requires both hemin, an iron-containing molecule, and NAD for growth.

**Media**

The selection of media to be used in isolation of pathogens from clinical specimens is dependent on several factors. First, the nutritional requirements of the specific pathogen must be met. For example, fastidious organisms require a medium which is enriched with specific nutrients, such as animal blood, serum, or other growth factors. If the clinical specimen is obtained from a site that has a resident microflora, certain strategies will be necessary to isolate a specific pathogen from the accompanying resident microflora. Often in this setting, a special type of medium called selective medium is used to recover these pathogens. This medium selects for the growth of a specific group of organisms. This is done by adding substances, such as dyes, antibiotics, or bile salts, that inhibit the growth of one group of organisms while permitting the growth of another. For example, MacConkey agar is a selective medium that contains bile salts and the dye crystal violet. These two substances are inhibitory for gram-positive organisms as well as some gram-negative ones. A wide variety of gram-negative rods grow on this medium. Some selective media are also differential. MacConkey agar is an example of a selective and differential medium. The gram-negative rods that grow on this agar can be differentiated from one another on the basis of the organism’s ability to ferment the carbohydrate lactose. Organisms that ferment lactose are called lactose positive, and organisms that are unable to ferment lactose are called lactose negative (Fig. 10). When selecting media for culturing clinical specimens from sites with a resident microflora, typically both enriched and selective media are used. If gram-negative rods are a component of this
microflora, then a selective-differential medium might be used as well.

Certain organisms will not grow on media commonly used to culture clinical specimens, because the media may not be enriched enough or may contain inhibitory substances. When these organisms are sought, the laboratory must be notified so that special isolation medium can be used. Two important respiratory tract pathogens, *B. pertussis* and *L. pneumophila*, are examples of organisms that do not grow on standard laboratory media and require special media for their isolation.

**Organism identification and susceptibility testing**

Once organisms are isolated, they may be identified, and in some cases susceptibility testing needs to be performed. Bacteria and fungi grow as colonies on agar plates. The appearance of these colonies is often useful in determining the identity of the organism. Colonies may appear flat or raised, smooth or rough, may pit the agar, or may hemolyze red blood cells in blood-containing agar. Molds, for example, have very characteristic “fuzzy” growth on agar. Colonies of organisms such as *S. aureus* may be pigmented or may secrete a diffusible pigment, as seen with *Pseudomonas aeruginosa*. Skilled microbiologists often have a very good idea of the identification of a microorganism based solely on its colonial appearance.

In specimens that come from an area of the body with a resident microflora, it is important to separate the colonies of organisms that may represent the resident microflora from the colonies of organisms that may be pathogens. Much of the time, this can be done on the basis of colonial appearance. However, some potential pathogens, such as *S. pneumoniae*, a common cause of bacterial pneumonia, cannot be readily differentiated from viridans group streptococci, a member of the resident oropharyngeal flora. In patients with suspected bacterial pneumonia, a sputum specimen may be obtained. Sputum consists of secretions coughed up from the lower airways that are expectorated through the oropharynx and submitted for culture. Because they pass through the oropharynx, sputum specimens almost always contain viridans group streptococci. The appearance of colonies produced by viridans group streptococci is very similar to that produced by *S. pneumoniae*. To determine whether or not these colonies are *S. pneumoniae*, one must do tests based on the phenotypic characteristics of the organism; these are referred to as biochemical tests. The biochemical test which is done most often to distinguish between these two organisms is the disc diffusion test, in which the organism’s susceptibility to the compound
optochin is examined. *S. pneumoniae* (Fig. 11) is susceptible to optochin, while the viridans group streptococci are not. On the basis of this easily performed test, the identity of *S. pneumoniae* can be determined from a sputum specimen.

Bacteria are typically identified on the basis of colonial morphology, Gram stain reaction, the primary isolation media on which the organism is growing, and biochemical and serologic tests of various degrees of complexity. Figures 12 and 13 are flow charts that give fairly simple means of distinguishing commonly encountered human pathogens. Yeasts are identified in much the same way that bacteria are, while molds are generally identified on the basis of the arrangement of microscopic reproductive structures called conidia. It is important to accurately identify bacteria and fungi because certain organisms (e.g., *B. pertussis*) are the cause of certain clinical syndromes (in this case, whooping cough). Other bacteria (e.g., *Staphylococcus epidermidis*) may represent contamination with resident microflora in a clinical specimen (e.g., a wound culture). The accurate identification of a bacterium or fungus may help determine what role a particular microbe may be having in the patient’s disease process.

Antimicrobial susceptibility typically is performed on rapidly growing bacteria if the organism is believed to play a role in the patient’s illness and if the profile of antimicrobial agents to which the organism is susceptible is not predictable. Let’s take three clinical scenarios to explain this concept.

A patient with a “strep throat” has group A streptococci recovered from his throat. Although the organism is clearly playing a role in the illness of this patient, antimicrobial susceptibility testing is not warranted. This organism is uniformly susceptible to first-line therapy—penicillin—and is susceptible more than 98% of the time to second-line therapy—the macrolide antibiotics such as erythromycin—although recent reports suggest that erythromycin resistance is becoming more frequent in this organism.

A patient presents with a leg abscess from which *S. aureus* is recovered. Susceptibility testing is indicated because some strains are resistant to the first-line drugs used to treat this infection—semisynthetic penicillins, including oxacillin and dicloxacillin—and the second-line drug, clindamycin. In this situation, the patient may be started on empiric antimicrobial therapy until the susceptibility of the organism is known. If the organism is resistant to the agent used for empiric therapy, then the patient should be treated with an alternative antimicrobial agent to which the organism is susceptible.
Important Groups of Gram-Positive Pathogenic Bacteria

**Cocci**
- Cocci in clusters
  - Aerobic catalase (+)
    - Staphylococcus sp.
  - Alpha-hemolytic
  - Beta-hemolytic
  - Gamma-hemolytic (non)
  - Streptococcus pneumoniae
  - Streptococcus mutans
  - Streptococcus anginosus
- Diplococci/cocci in chains
  - Aerobic catalase (+)
  - Beta-hemolytic
  - Streptococcus pyogenes (GAS)
  - Streptococcus agalactiae (GBS)
  - Enterococcus faecalis
  - Enterococcus faecium
- Anaerobic catalase (-)
  - Peptostreptococcus sp.

**Rods**
- Aerobic
  - Listeria monocytogenes
  - Corynebacterium diphtheriae
  - Bacillus anthracis
  - Nocardia sp.
- Anaerobic
  - Clostridium tetani
  - Clostridium botulinum
  - Clostridium difficile
  - Clostridium perfringens
  - Clostridium septicum
  - Actinomyces sp.
  - Propionobacterium sp.

**Organisms for Which the Gram Stain Is Not Used**
- Chlamydia trachomatis
- Chlamydia psittaci
- Chlamydia pneumoniae
- Treponema pallidum
- Borrelia burgdorferi
- Rickettsia rickettsii
- Ehrlichia chafeensis
- Mycobacterium tuberculosis
- Mycobacterium sp.
- Mycoplasma pneumoniae
Figure 13

Important Groups of Gram-Negative Pathogenic Bacteria

**Gram-negative rods**
- MacConkey (−)
  - Oxygen tolerant
    - *Haemophilus influenzae*
    - *Bordetella pertussis*
    - *Legionella pneumophila*
    - *Brucella sp.*
    - *Francisella tularensis*
    - *Pasteurella multocida*
    - *Campylobacter jejuni*
    - *Helicobacter pylori*
    - *Vibrio cholerae*
  - Strictly anaerobic
    - *Bacteroides fragilis*
    - *Fusobacterium sp.*
    - *Prevotella sp.*
    - *Porphyromonas sp.*

**Gram-negative cocci**
- MacConkey (+)
  - Lactose (+)
    - *Escherichia coli*
    - *Klebsiella pneumoniae*
    - *Enterobacter sp.*
  - Lactose (−)
    - *Salmonella sp.*
    - *Shigella sp.*
    - *Proteus sp.*
    - *Providencia sp.*
    - *Serratia sp.*
    - *Yersinia sp.*
- Glucose (+)
  - *Pseudomonas aeruginosa*
  - *Acinetobacter calcoaceticus*
  - *Stenotrophomonas maltophilia*
  - *Burkholderia cepacia*
- Glucose (−)
  - *Neisseria gonorrhoeae*
  - *Neisseria meningitidis*
  - *Moraxella catarrhalis*
The third scenario is more subtle. A patient comes to the hospital with a high fever. He has two sets of blood cultures drawn in the emergency room. Two days later, \textit{S. epidermidis} is recovered from one of these blood culture sets. As with \textit{S. aureus}, this organism may show resistance to a variety of antimicrobial agents that are used to treat infected patients. However, no susceptibility testing is done by the laboratory, and this practice is acceptable to the clinician caring for the patient. Why? \textit{S. epidermidis} is a component of skin microflora and may have contaminated the culture. If the laboratory had performed the susceptibility testing without considering that this isolate was a potential contaminant, they would be validating that the isolate was clinically significant. In this setting, the laboratory should only do susceptibility testing if instructed to by the caregiver, who is in a better position to know if this organism is clinically important.

There are several approaches to antibacterial susceptibility testing. All the approaches are highly standardized to ensure that the susceptibility results will be consistent from laboratory to laboratory. Screening of selected organisms for resistance to specific antimicrobial agents is one strategy that is frequently used, especially with the emergence of resistance in three organisms: \textit{S. aureus} to oxacillin, \textit{S. pneumoniae} to penicillin, and \textit{Enterococcus faecium} and \textit{Enterococcus faecalis} to vancomycin. Other strategies are to determine susceptibility to a preselected battery of antimicrobial agents using automated or manual systems that determine the MIC of antibiotics to the organism being tested or by using the disk diffusion susceptibility testing technique.

A novel approach to susceptibility testing is to perform MIC determinations using the E-test. The E-test is a plastic strip that contains a gradient of a specific antimicrobial agent. This strip is applied to a lawn of bacteria on an agar plate. Where the zone of inhibition intersects with the strip is the MIC value of that antibiotic for the organism tested. This test has many applications but is used most frequently for determining penicillin MIC values for \textit{S. pneumoniae} isolates that show resistance to penicillin in the screening test previously described (Fig. 14).
Susceptibility testing is not routinely performed on fungal isolates. Because of their slow growth, special susceptibility testing techniques are used for the mycobacteria.

**Tissue culture for Chlamydia and viruses**

Both *Chlamydia*, a bacterium, and the viruses are obligate intracellular parasites. As such, they do not grow on artificial media, as fungi and other bacteria do. Rather, they can only grow by parasitizing living animal cells (including human cells) that are maintained by continuous tissue culture. Animals such as mice or chicken eggs can be inoculated in an attempt to isolate certain viruses, but this approach is rarely done. Tissue culture for *Chlamydia* may still be attempted, especially in situations where the detection of *C. trachomatis* is at issue in a legal proceeding, such as a case of sexual abuse of a child. However, molecular detection has become the standard method for diagnosis of *C. trachomatis* infection.

Tissue culture is still an important technique for the detection of viruses. Herpes simplex virus can be isolated from skin and genital tract lesions, often within the first 24 hours of incubation. Another herpesvirus, varicella-zoster virus, the etiologic agent of chicken pox and zoster, can also be isolated from skin lesions, but it typically requires 3 to 7 days to grow. The enteroviruses are the major etiologic agents of aseptic meningitis and can be isolated from cerebrospinal fluid.

A modification of the tissue culture technique is done to detect cytomegalovirus and several respiratory viruses in clinical specimens. In this method, the specimen is centrifuged onto tissue culture cells that are growing on a round glass coverslip inside a vial referred to as a shell vial. The cells are incubated for a brief period of time (24 to 72 hours) and then stained with fluorescent antibodies to detect the virus. This technique is much more rapid and sensitive than conventional tissue culture.

**SEROLOGY**

It is not always possible to isolate a microorganism by culture, visualize it microscopically, or detect it by antigenic or molecular detection techniques. In those situations, an alternative approach is to determine if the patient has mounted an immune response against a specific agent as evidence that he or she has been infected with that agent. The immune response is generally measured by detecting antibodies in the serum of patients. Thus the name serology.

Serology has both advantages and disadvantages. (i) Specimens for testing are readily available; (ii) antibodies are relatively stable molecules, so transport is not a major concern as it is with culture; and (iii) tests have been designed that can detect most known agents, such as HIV and HCV, which are difficult to detect by other means. Depending upon the target antigen against which the immune response is measured, the test can show both high sensitivity and high specificity. Compared with
other techniques, these tests are relatively inexpensive and easy to perform, in part because they have been automated. As a result, they can be used to screen large numbers of specimens for selected infectious agents. For example, this approach is used to screen blood products used for transfusions to ensure that the transfused patient does not receive blood contaminated with hepatitis B and C viruses, HIV, or *T. pallidum*, the agent of syphilis.

Serologic tests also have several disadvantages and should be interpreted with some caution. To have a positive test, the patient must have mounted an immune response. Serum obtained from an acutely ill patient may have been taken during the window period in an infection before the patient had time to mount an immune response. Therefore, to get the most accurate result, acute and convalescent specimens should be obtained. The convalescent specimen should show a significant increase (or, in some cases, decrease) from the antibody level of an acute specimen. This is usually a fourfold change in the titer. Because the convalescent specimen should be obtained a minimum of 2 weeks after the acute specimen, serologic diagnosis is often retrospective. Because obtaining a convalescent specimen is often difficult logistically, the only value that may be available is that from the acute specimen. Patients may have relatively high antibody levels because of previous infection with the test organism and, as a result, may have a false-positive result. Antigenic cross-reactions between the test organism and other antigens may also lead to false-positive results. Some immunocompromised patients are unable to mount a response and may never have a positive serologic test.

Serologic tests can be done in combination using a screening test followed by a confirmatory test. This approach is used most commonly in the diagnosis of syphilis, HIV infection, HCV infection, and Lyme disease. The screening test should be highly sensitive so that all true-positive results will be detected. This test may not be highly specific, meaning that some results may be false positives. It should also be easily performed so that large numbers of specimens can be tested fairly inexpensively. The confirmatory test needs to be highly specific so that the correct diagnosis can be applied to the patient who screens positive for the infectious agent. It tends to be much more expensive and technically complex than the screening test. Western blotting or an equivalent technique is used in the confirmatory tests for Lyme disease, HIV infection, and HCV infection. In this technique, a patient is considered to be positive for the agent only if the patient has antibodies to multiple specific antigens.
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