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**Medical Laboratory
Journeyman-Microbiology**

Volume 1. Bacteriology Introduction



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MICROBIOLOGY has undergone many significant changes within the second half of the twentieth century, during which time tremendous scientific progress was achieved. Such progressive changes are continuous and require the updating and revising of this course. Thus, in this and subsequent volumes, we introduce the latest applied literature in the field for the bacteriology student and the technologist with the ultimate goal of meeting the professional training of both.

This volume enables students to review fundamentals of diagnostic bacteriology. The opening unit briefly traces the historical development of current bacteriological procedures, bacterial taxonomy and cell anatomy, and safety principles. Unit 2 reviews specimen collection and transport. Unit 3 describes media selection and background information regarding media. Unit 4 explains how knowledge of bacterial morphology and physiology permits the technician to process, isolate, and cultivate pathogenic microbes in the laboratory. Last, Unit 5 discloses the basis for antimicrobial sensitivity testing and drug assays. In Volume 2, *Bacteriology* you will enhance your knowledge of familiar bacteria and procedures. In Volume 3, *Other Medically Important Organisms*, you will learn about some of the infrequently encountered organisms causing disease, and some emerging pathogens. Volume 4, *Parasitology* will provide an extensive overview of important human parasites.

A glossary of terms, abbreviations and acronyms, is included at the end of this volume.

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1993	Outline of a typical test card used by the Vitek Auto Microbic System	Figure 22–8	Figure 4–4

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NOTE:

In this volume, the subject matter is divided into self-contained units. A unit menu begins each unit, identifying the lesson headings and numbers. After reading the unit menu page and unit introduction, study the section, answer the self-test questions, and compare your answers with those given at the end of the unit. Then do the unit review exercises.

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Unit 1. General Information

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BACTERIOLOGY, as it is known today, is a science that demands an understanding of the basic morphology and physiology of bacteria. You must have a keen sense of perception to be able to observe bacterial growth, make intelligent interpretations of this growth, and record your observations. As a laboratory technician working in bacteriology, you must be a *detective* ready to analyze clues gathered from your efforts in the laboratory to the identity of a particular disease-associated microorganism. This analysis is routine today, but hasn't always been.

Investigators of long ago were not aware of anything smaller than what was visible to the unaided eye. Belief in the supernatural, fear of reprisal, and restricted practices did not stimulate research. The invention of the microscope was an important stimulus to learn about the tiny world of animalcules (microscopic animals). Through a slow and gradual process, knowledge about bacteria was sought and diseases associated with them were documented. The world began to realize that small microscopic matter was important in health and disease as this process continued.

By being aware of some of the historical high points in the development of bacteriology as a science, you'll better appreciate the obstacles that were overcome. Possibly, you'll be stimulated to add to the progress in this science. Before going into a discussion of present-day laboratory techniques, let's look at some of the events that helped shape the development of bacteriology.

1–1. Brief History of Microbiology

Hippocrates (460–377 B.C.), known as the Father of Medicine, was not a bacteriologist, but his observations and writings gave a foundation to the field of bacteriology, even though bacteria, as such, were unknown. He attributed diseases to changes and disorders in the *vital fluids* of the body. He stressed the use of boiled water for irrigating wounds and called attention to the importance of clean hands and fingernails of the *surgeon*. He coined such terms as *acute*, *chronic*, *endemic*, and *epidemic*—which are still with us today, although they may be used in a slightly different context.

001. Major contributions to bacteriology

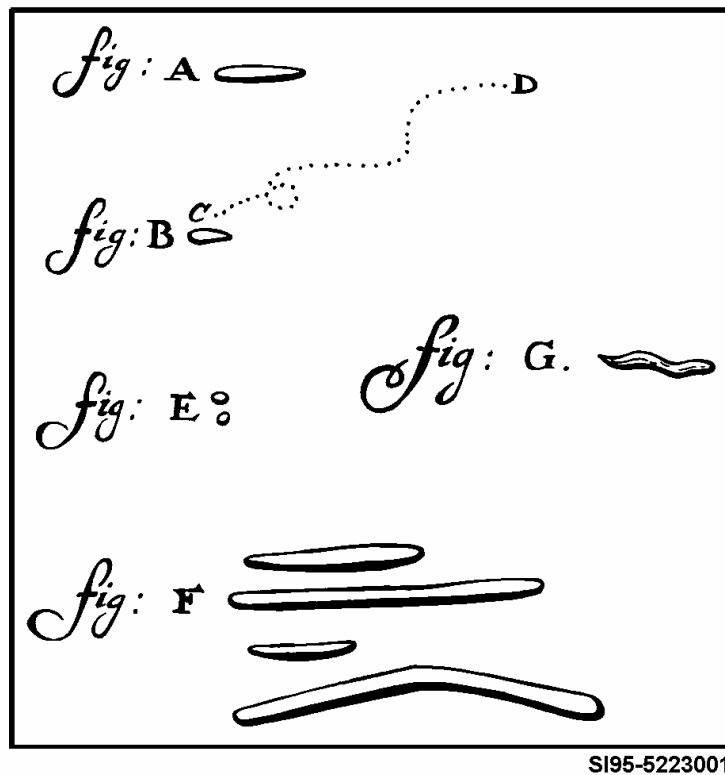
Let's briefly discuss some of the significant, major contributions in the discovery of bacteria.

Concepts of contagion

As the Dark Ages (A.D. 476-1350) replaced the progressiveness of the Romans and Greeks with mysticism and fear; witches were boiled in water. Filth, pestilence, and plagues covered Europe. This situation prevailed until the 18th century. Changes were made, but their acceptance was slow. The discovery of infectious agents was long preceded by the concept of contagious disease. Contagious disease was initiated by contact with a diseased person or with contaminated objects. It was during the Renaissance (1453-1600) that bacteriology had its real beginning. In 1546, Girolamo Fracastoro, Father of the Germ Theory of Disease, wrote, "Contagion is an infection that passes from one thing to another." He recognized there were three basic sources of contagious material: (1) by contact, (2) by fomites, and (3) from a distance. This typified the level of scientific knowledge at the beginning of the 17th century, and, from this level, science began to emerge as a systematic method of investigation. Fracastoro gets credit for giving the venereal disease "syphilis" its name, which is still with us today. Fracastoro published a poem that recounted the legend of the shepherd Syphilus, who had been afflicted with the disease.

The first microscopic observations

Development of the microscope was probably the most significant occurrence in the field of bacteriology. The first simple lens was made by Roger Bacon. This invention was followed by the compound lens of Zacharias Janssen in Holland. The world of microbes became visible to all who were interested. The first notable observation of microbes was recorded by Anton van Leeuwenhoek in 1675. With his crude microscope, composed of a biconvex lens, Leeuwenhoek discovered bacteria in water and body fluids, as well as the yeast in beer. He saw different shapes and observed that certain bacteria could move from one place to another under its own power. His drawings of these organisms, shown in figure 1-1, formed a foundation of the modern-day classification of bacteria. Invention of the microscope provided a means to study the causes of fermentation and disease. Naturally, knowledgeable men of the day wanted to learn more about the identity and functions of microbes. However, they were handicapped in their efforts because Leeuwenhoek



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Figure 1-1. Leeuwenhoek's drawing of bacteria.

jealously guarded the secret of his microscope until his death. After Leeuwenhoek's death, with the aid of this new instrument and with the information in the more than 200 letters Leeuwenhoek wrote to the Royal Society of London about his experiments and observations, the science of microbiology had begun.

Contributions of important bacteriologists

Belief in the theory that living organisms could originate from non-living matter made it easy to explain the presence of biological beings, but it hampered research for scientific truths. In fact, the theory of spontaneous generation was so commonly accepted that one could purchase a recipe book containing a formula for producing mice by simply placing a pair of old trousers, an old shoe, and a few crumbs of food in a quiet, dark corner. In a few days or so, mice would indeed be found in the corner or at least evidence that they had been there. Fortunately, there were individuals who would not accept such examples as truths.

Francesco Redi

Francesco Redi (1626–1697) wanted to disprove once and for all the theory that putrefying meat gave rise, spontaneously, to living worms or maggots. His simple and classic experiment consisted of three jars into which he placed pieces of fresh meat. He left one jar open, thus exposing the meat to air and flies. Another he covered with gauze; while still another he sealed with a tight-fitting lid. He noticed flies visited the jars exposed to the air, and, indeed, observed that white worms began forming on the meat in the jar left uncovered. White worms were also found on the gauze that covered one of the jars. No worms were found in the tightly sealed jar although putrefaction was evident. Redi's experiment started the beginning of the end for the theory of spontaneous generation. This experiment was scientific proof for the day. The worms or maggots were simply fly larvae that eventually turned into flies. Of course, advocates of spontaneous generation tried to disprove this evidence, but their efforts were to no avail.

Lazzaro Spallanzani

Lazzaro Spallanzani (1729–1799), interested in the work of Redi and others, approached the problem of spontaneous generation in another way. He boiled beef broth for an hour and sealed the flasks in which they were boiled. He noticed that neither putrefaction nor other apparent changes occurred in the broth even after long periods. Spontaneous generation advocates decried this experiment, claiming the heat had destroyed the air. Other scientific minds later applied Spallanzani's principle to preserve foodstuffs, resulting in benefits still enjoyed today.

Schroeder and von Dusch

To further disprove the theory of spontaneous generation, two scientists, Schroeder and von Dusch, followed up the experiments of boiled broths. However, instead of sealing containers, they closed them with gauze plugs, allowing air to enter the vessels. Eventually, some decomposition took place in the broths naturally. This led Schroeder to wonder if the broth changes were caused by organized microscopic germs or some unknown chemical substance. The decomposition was later attributed to oxygen. The cotton plugs had kept bacteria, flies, and other particles out of the broth, and are still used to great advantage in modern-day bacteriology.

Edward Jenner

The 18th century saw many innovations and scholarly contributions to the science of microbiology. Particularly important were the discoveries of Edward Jenner (1749–1823) regarding immune principles. His classical experiments in developing and administering vaccines are known worldwide.

Joseph Lister

The 19th century saw the use of antiseptics in surgery. The use of antiseptics in surgery was advocated by Lord Joseph Lister (1827–1912). He proposed the theory that “infection was due to passage of minute bodies capable of self-multiplication from infector to infected.” He soaked ligatures in disinfectants, and even went to the extreme of performing operations under a spray of phenol, in an effort to destroy and/or prevent these minute bodies from infecting the patient. Because of his many contributions, the title, “Father of Aseptic Surgery,” was bestowed on him. One of his least-known credits, although debatable, is of being the first person to obtain a pure culture of a microorganism.

Louis Pasteur

Probably the greatest blow to the theory of spontaneous generation was dealt by Louis Pasteur (1822–1895). Pasteur’s simple experiment, using flasks with long, swan-neck tubular openings, revealed that contamination of nutrient material only took place when the material had access to air laden with living microorganisms. As the air passed through the tubular neck, the lack of air currents prevented the organisms from rising up the tube and gaining access to the nutrient material within the flask. Consequently, the flasks remained uncontaminated. Louis Pasteur is well known for his work on fermentation, rabies, and anthrax. Pasteurization of many foodstuffs is a result of his experiments and observations. A major contributor to the “Golden Age of Bacteriology,” Pasteur is recognized as the Father of Bacteriology. In disproving the theory of spontaneous generation, he developed basic microbiology procedures.

Robert Koch

Knowledge of bacteria is important, and bacteriological techniques are of prime concern to clinical bacteriologists. The work of Robert Koch (1843–1910) provided a firm foundation for modern-day techniques. He discovered and developed the use of culture media, isolated pure cultures of microorganisms, and introduced the rapid-air drying of thin bacterial films for staining. In laboratory research and development of bacteriological techniques, he established valid criteria for determining the cause of infectious disease. These criteria are known as “Koch’s Postulates” and form the basis for the identification of many bacteria. His classic work with tuberculosis stands as a tribute to these problems. These postulates, briefly stated, are:

1. A specific organism must always be associated with a specific disease.
2. The organism must be isolated in a pure culture.
3. The organism must cause the specific disease in a susceptible animal.
4. The organism must, in turn, be isolated in a pure culture from the diseased animal.

Improved bacteriological techniques make it unnecessary to go through each step of Koch’s postulates in laboratory diagnosis of most disease agents. There are many organisms that do not produce disease in experimental animals. Furthermore, certain organisms in pure culture require close association with another organism in order to produce infection. These postulates have made a science out of microbiology and are required for successful investigation of certain diseases. Through careful observation and recording of experimental

results, you can make valid conclusions in the bacteriology laboratory. It requires practice, perseverance, and astuteness to become a successful clinical bacteriologist on whom the physician and patient rely for determining the cause of an infectious disease. You, as a medical laboratory technician, must be constantly alert to new discoveries and concepts that improve medical bacteriological techniques. The words of Pasteur (1854) admirably illustrate this point: "Without theory, practice is only routine; governed by the force of habit. Only theory can breed and develop the spirit of invention." Perhaps, in this course, your spirit of invention will be stimulated.

002. The health professions and microbiology

Although nursing in some form has existed from the start of human life, the beginning of professional nursing is usually credited to Florence Nightingale (1820–1910). She began her work during the Crimean war (1854–1856), when microbiology was in its infancy. Subsequent discoveries concerning the relationships of microorganisms to disease necessitated the development of nursing procedures far more complex and refined than those used by the "Angel of the Crimea." In her time, little was known about the transfer of infections from person to person. The development of aseptic surgery after Lister's discoveries (about 1865) opened the whole field of operating room technique to the surgeon and the professional nurse.

Advancements made possible by microbiology

The earlier discovery of preventive measures, such as administration of smallpox vaccine (Edward Jenner, 1796) and later, of diphtheria and tetanus toxins and antitoxins or immune sera (von Behring, Frankel, and Kitasato, 1890), to say nothing of polio and measles vaccines (Enders, Weller, and Robins, 1949; Salk, 1954; Sabin and others, 1954–1967) and gamma globulins for the prevention of measles, rabies, pertussis, and some other diseases, are but a few of the major advancements brought about by microbiology. All of these made necessary the development of new concepts, education, and training in the preparation and administration of these substances. The discovery of diagnostic tests in the field of microbiology necessitated more advanced microbiologic training of medical technologists, doctors, nurses, and other health professionals in methods of collecting specimens and reporting specific laboratory findings so therapy could be started early. The discovery of specific chemicals (chemotherapeutic agents, such as sulfonamides) and antibiotic substances (such as penicillin, streptomycin, the tetracyclines, and chloramphenicol) have altered medical care and presented new problems and new opportunities for the health care team. Discoveries of disease transmission led to the development of effective methods to prevent their spread. For example, the transmission of some diseases through the bites of insects and arachnids was demonstrated in 1893 by Theobald Smith, in connection with Texas fever, a disease of cattle caused by infectious ticks. In 1895, Sir Ronald Ross (1857–1932), an army physician in India, demonstrated the transmission of malarial protozoa by mosquitoes. The parasites had, however, been seen in human red blood cells as early as 1881 by Laveran, a French army surgeon in Algeria. In 1900, the transmission of the yellow fever virus by a particular species of mosquito (*Aedes aegypti*) was demonstrated in Cuba by Drs. Walter Reed (1851–1902), James Carroll (1854–1907), Aristides Agramonte (1868–1931), and Jesse Lazear (1866–1900) of the United States Army. The discoveries of the modes of transmission of malaria and yellow fever are among the most brilliant in medicine and have been epoch-making in their results.

Advancement of microbiology

Modern surgical procedures would be impossible without disinfection and sterilization. Today's dairy, frozen and packaged food, and canning industries are dependent on

microbiology. Sanitation of water supplies and reclamation of polluted waters (previously called sewage disposal) are possible only because of knowledge gained from microbiology in the past century. The human life span has been extended partly because of what is now known about prevention of deadly transmissible diseases. In many ways, every health profession is dependent on the knowledge, understanding, and utilization of information from microbiology.

Microbiology professional

Thanks to the efforts of many scientists, the incidence of certain microbial diseases has decreased. Only constant vigilance on the part of all members of the health professions, however, continues this downward trend in preventable diseases. The registered medical microbiologist, the practicing physician, and the professional nurse need to apply knowledge from microbiology to the everyday practice of their professions. Although certain techniques and skills could be learned by memorization, you as a true professional understand the scientific facts and principles underlying these techniques. The qualified professional also knows how these techniques should be modified for certain kinds of patients—for example, those who have had cardiac surgery; what techniques must be used in the operating room, delivery room, and nursery, and how these can be safely modified in emergency conditions, like war or other disasters; and why patients, families, and the general public should be informed about available immunizing agents, such as polio vaccines.

Increased life expectancy

Microbiology, the study of microscopically small living organisms, has progressed in the last century from a relatively simple science to one producing great advances in preventive, diagnostic, and therapeutic medicine. It has improved food preservation, sanitation, and increased the human life expectancy by approximately 50 percent. We are at the threshold of even greater advances involving recombinant deoxyribonucleic acid (DNA), transferring genetic material of mammalian origin into selected bacteria, which then produce specific chemical products for medical or industrial uses.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

001. Major contributions to bacteriology

1. What concept preceded the discovery of infectious agents?
2. During what period of history did bacteriology probably have its real beginning?
3. Who is considered to be the father of the germ theory of disease?
4. What three sources of contagious material were identified by Girolamo Fracastoro?

5. What was probably the most significant occurrence in the development of the field of bacteriology?
6. Who is credited with making the first simple lens?
7. Who is credited with the first notable observation of microbes?
8. Match each of the early bacteriologists in column B with the statement in column A. Items in column B may be used once, more than once, or not at all.

Column A

- ____ (1) His experiment started the beginning of the end for the theory of spontaneous generation.
- ____ (2) He wanted to disprove once and for all the theory that putrefying meat gave rise, spontaneously, to living worms or maggots.
- ____ (3) He boiled beef broth for an hour, sealed the flasks, and observed no formation of microbes.
- ____ (4) Other scientific minds later applied his principle to preserve foodstuffs.
- ____ (5) Instead of sealing, the experiment involved containers closed with gauze plugs.
- ____ (6) His classical experiments in developing and administering vaccines are known worldwide.
- ____ (7) One of the investigators who wondered if the changes in the plugged broth container were caused by organized microscopic germs or some unknown chemical substance.
- ____ (8) He proposed the theory that infection was due to passage of minute bodies capable of self-multiplication from infector to infected.
- ____ (9) He is recognized as the Father of Bacteriology.
- ____ (10) Although questionable, he is said to have been the first person to obtain a pure culture of a microorganism.
- ____ (11) He discovered and developed the use of culture media.
- ____ (12) In laboratory research and development of bacteriological techniques, he established valid criteria for determining the cause of infectious disease.

Column B

- a. Lazzaro Spallanzani.
- b. Louis Pasteur.
- c. Francesco Redi.
- d. Robert Koch.
- e. Edward Jenner.
- f. Joseph Lister.
- g. Schroeder.
- h. Franz Schulze.
- i. John Tyndall.
- j. von Dusch.

002. The health professions and microbiology

1. Match each item in column B with the statements it relates to in column A. Items in column B may be used once, more than once, or not at all.

Column A

- ____ (1) Angel of the Crimea.
- ____ (2) Administration of smallpox vaccine.
- ____ (3) Chemotherapeutic agent.
- ____ (4) Texas fever.
- ____ (5) Transmission of the malarial parasite by mosquitoes.
- ____ (6) Transmission of yellow fever.
- ____ (7) Transferring genetic material of mammalian origin into selected bacteria.
- ____ (8) Vaccination against diphtheria.
- ____ (9) Malarial parasite observed in human red blood cells.

Column B

- a. Laveran.
- b. Microbiology.
- c. von Behring.
- d. Recombinant DNA.
- e. *Aedes aegypti*.
- f. Ross.
- g. Smith.
- h. Sulfonamides.
- i. Jenner.
- j. Nightingale.

1-2. Bacterial Taxonomy

One of the new areas in your studies is the classification of bacteria. Taxonomy is the science of classification. As a science, it is dynamic and subject to change based on available data. New data often necessitates changes in taxonomy. These changes frequently result in changes in the existing classification, in nomenclature, in criteria for identification, and in the recognition of new species. In this section, let's discuss bacterial taxonomy, classification of bacteria, and anatomy of a bacterial cell.

003. How bacteria are classified

As you learn about the varieties of bacteria, you'll obviously need to differentiate them from each other, and have some accepted method by which they can be classified and named. This process is known as taxonomy (*taxis* = orderly arrangement; *nomos* = law).

Bacterial taxonomy

Taxonomy is divided into three interrelated but separate areas:

1. Classification — A systematic arrangement of organisms into classes or groups (taxonomic groups) based on similarities.
2. Nomenclature — A set or classified system of names used in any science.
3. Identification — The process of deciding where new organisms belong.

The taxonomy process is not easy. Apart from the difficulty involved in agreeing on terms, some groups of bacteria may differ very little from each other, and some workers do not regard these particular differences as valid. Furthermore, a more difficult problem is in the definition of a bacterial species. In higher life forms, a species may be defined as a group of creatures able to reproduce with one another, but not with other species. This definition cannot be applied to bacteria. With higher life forms, some evidence of an evolutionary relationship and development may exist according to which classification of the different species may be made, using the evolutionary pattern as a guide. Again, this type of approach is not valid for bacteria since their evolutionary history is not evident and there are no fossil remains. Despite all of the difficulties and disagreements, the need for classification and naming remains. These are needed because microbiologists need to have some form of common nomenclature so they can communicate with one another and with their clinical colleagues.

Bacterial classification

There is no official classification of bacteria, but bacterial nomenclature is governed by internationally agreed rules and decisions. Generally accepted classification of bacteria is in *Bergey's Manual of Determinative Bacteriology*, which publicizes agreed on definitions. The International Committee for Systematic Bacteriology with taxonomic subcommittees exists to make sure the definitions in the manual are current.

Phenetic classification

Phenetic classification is based on easily recognizable characteristics and similarities that are weighed in importance. That is, certain features are stressed to indicate groups that are related to one another (for example, motility versus nonmotility, oxidative versus fermentative metabolism of carbohydrates, and so forth).

Key method

The basis of the *key* method of identification, to be precise, is the demonstration of identity of the unknown organism with a known organism already in its taxonomic place. The *key* method of identification is often shown in a diagrammatic form as a series of dichotomies (a series of pairs distinguished by opposite qualities), as shown in the following table looking at biochemical classification.

	Lactose	Indole	Glucose	Urease	Motility
<i>E. coli</i>	+	+	AG	-	+
<i>Enterobacter aerogenes</i>	+	-	AG	-	+
<i>P. vulgaris</i>	-	+	AG	+	+
<i>Salmonella</i>	-	-	AG	-	+
<i>S. typhi</i>	-	-	A	-	+
<i>Sh. dysenteriae</i>	-	-	A	-	-

Identifying pattern

Generally, in medical bacteriology, especially under the urgency of clinical pressures, a battery of tests for different characteristics is done simultaneously and not by the slower dichotomous routes indicated earlier. To accommodate this approach, diagnostic tables are drawn, using identical information, and these give the bacteriologist the identifying pattern of the results.

Numerical classification

Numerical classification is concerned with phenetic relationships. Relatedness among a large number of organisms is estimated by a numerical analysis in which 100 to 300 biochemical characteristics are compared for each strain.

Similarity index

A similarity index is calculated for each pair of organisms, using the formula:

$$S = NS / (NS + ND)$$

where

S is the similarity index.

NS is the number of characteristics common to both organisms.

ND is the number of characteristics that are not shared.

Related pairs

The higher the value of S, the more related the pair of strains. Examination of a large number of biochemical parameters reduces the bias inherent in less extensive classification schemes that are based on the presence or absence of only a few key enzymes.

Serologic classification

Serologic classifications are commonly used to distinguish differences among species within a given genus. These tests employ antibodies as very sensitive and specific probes for the presence of various chemicals and antigenic conformations present on the bacterial surface. The use of monoclonal antibodies greatly increased the specificity for many such antigenic probes.

Chemotaxonomy

In the past 20 years, chemical and physical techniques to interpret the chemical composition of whole bacterial cells produced information aiding in the classification and identification of bacteria. This information was found so useful in classifying bacteria, that the word “chemotaxonomy,” used to describe the classification of bacteria by their chemical composition, is now used in the literature. Cell wall composition, lipid composition, isoprenoid quinones, cytochrome composition, amino acid sequences of the various proteins, protein profiles, enzyme characterization, and fermentation product profiles comprise chemotaxonomy.

Genetic classifications

Like all other life forms (with the exception of some viruses, that have ribonucleic acid (RNA) as their genetic material), bacteria have DNA as the repository of their inheritance and as the coordinator of their cellular activity. Through careful study and comparison of an unknown bacteria’s genetic material with the genetic material of a known strain of bacteria, classification of the unknown bacteria is accomplished.

DNA homology

In recent years, the phylogenetic relationships occurring in bacteria, even among those not sharing many biochemical properties, has been estimated by determining the degree of similarity existing between their nucleic acid sequences. In the most direct test for DNA homology, the DNA from one organism is made radioactive and mixed with an excess of small DNA fragments obtained from the test organism. The degree of DNA homology between the two organisms is revealed by the rate and extent to which the test DNA forms stable double-stranded duplexes with that from the reference organism.

Ribosomal RNA

All bacteria possess ribosomal RNAs (rRNA) whose structures and functions have been strongly conserved; that is, the sequences have changed slowly since the time any two organisms diverged from a common progenitor. Thus, the degree of identity of the rRNA sequences can be related to the time the evolutionary tracks of two organisms separated. The relatedness of rRNAs (primarily 5S or 16S) can be easily estimated from the number of identical oligonucleotide spots seen following enzymatic digestion and separation of an rRNA. Rapid techniques for the determination of DNA sequence make feasible comparisons of the entire sequence for certain rRNAs. In this way, organisms are compared for the number of base changes that have occurred in this portion of the genome, providing a more meaningful estimator of the genetic relatedness of two organisms.

DNA sequences

Certainly, determination of the DNA sequence is not the type of technique that finds immediate application in the clinical laboratory. However, using cloning techniques, it is possible to obtain portions of a genome that are specific for a given genus, species, or subspecies. Such cloned DNA fragments could provide the basis for simple and rapid tests for the presence of that type of organism in a clinical specimen. The ease, specificity, and sensitivity of hybridization techniques to detect homologous sequences promise to make this type of approach practical even for routine use. Continual advances allow for the development of methods to use the DNA from a drug-resistant determinant to test for the presence of that determinant in a clinical sample, helping to identify an organism and determine its drug-resistance properties with immediacy instead of waiting for culture results.

Classification advantages

The four advantages of genetic classification are:

1. A more consolidating concept of bacterial species is possible.
2. Less frequent or radical changes occur.
3. More reliable identification schemes can be prepared.
4. Useful information on how bacteria evolved and their arrangement according to their ancestral relationships can be obtained.

More bacterial classification

For a more in-depth study of bacterial classification, see *Bergey's Manual of Systematic Bacteriology* and *Bergey's Manual of Determinative Bacteriology*.

004. The evolution of bacterial taxonomy

Because bacteriology is a progressive and advancing science, it is continually subject to revision. Such revision is often reflected in nomenclature since this is the expression of the result of advanced research and techniques.

An example of a change

A recent example of changes in nomenclature reflecting advances in knowledge is occurring in the genera *Proteus* and *Providencia*. Until recently, it was accepted that there were four medically important species of *Proteus*—*P. mirabilis*, *P. vulgaris*, *P. rettgeri*, and *P. morganii*, and two species of *Providencia*—*Prov. alcalifaciens* and *Prov. stuartii*. However, *P. morganii* by DNA homology is closer to *E. coli* and *Salmonella* and less close to other *Proteus* species. Hence, it was decided to form a new genus and species—*Morganella morganii*. With *Proteus rettgeri*, it became apparent that, by DNA homology, some varieties are merely urea positive *Providencia stuartii*, and the urease gene may be plasmid borne. Other strains of *P. rettgeri* appear both genetically and biochemically like *Providencia* and are now known as *Providencia rettgeri*. The easy division of *Proteus* from *Providencia*, by the presence or absence of urease, respectively, has crumbled. Thus, in place of the four protei and two providencias, there are, tentatively at least, two protei, three providencias, and a new genus—*Morganella*. Other similar changes are occurring in other genera.

Classification in general use

As mentioned earlier, the most common classification for bacteria is that given in *Bergey's Manual of Systematic Bacteriology*, and is kept current by the International Committee for Systematic Bacteriology. In the classification adopted by Bergey's manual, there is a progressive division of the plant kingdom through numerous subdivisions to individual species. Each sublevel includes two or more at that level. The naming of the groups as the

classification proceeds is shown in the following table. Following the name of the group are the endings of the words used to describe that group. Thus *eubacteriales* is an order, whereas *streptococceae* denotes a tribe.

CLASSIFICATION SYSTEM
Kingdom
↓↓
Divisions (2 or more)
↓↓
Classes
↓↓
Orders (-ales)
↓↓
Suborders (-inae)
↓↓
Families (-aceae)
↓↓
Subfamilies (-oideae)
↓↓
Tribes (-eae)
↓↓
Subtribes (-inae)
↓↓
Genera
↓↓
Species

Style and convention

It is usual to use a capital letter for a word indicating a genus or for any group above a genus. A genus is usually printed in italics, and the specific word relating to the species (known as the specific epithet) also is italicized. The first letter of this specific epithet is not capitalized, although it may be derived from a proper noun; for example, *Staphylococcus albus*, *Salmonella london*, and *Haemophilus ducreyi* (after Augusto Ducrey).

Generic name

When the generic name is used as an adjective, the capital letter is dropped, as are the italics—streptococcal, staphylococcal, and so forth. The first letters of names of groups above that of genus are capitalized, but apart from that, there are no specific conventions.

Common name

Common names for bacteria are used in conversation and by long tradition for some organisms. It is easier to say “pneumococci” than *Streptococcus pneumoniae* and “gonococci” than *Neisseria gonorrhoeae*. Some terms are used as euphemisms to avoid impact to anxious ears or eyes—“acid-fast bacilli” for *Mycobacterium tuberculosis*, KLB (Klebs-Löffler bacillus) for *Corynebacterium diphtheriae*, “gram-negative intracellular diplococci” for *N. gonorrhoeae*, and so forth. In the microbiology laboratory, you’ll be more concerned with the genera and species of bacteria for identification and antimicrobial therapy.

005. The basic anatomy of a bacterial cell

Knowledge of the bacterial cell structure is of value in understanding bacteriogenic disease and identification of bacterial species.

The cell

Refer to figure 1–2 when reading this material. The fundamental unit of all living creatures is the cell. Cells are classified into two major groups—eukaryotes (Greek for “true nucleus”) and prokaryotes (Greek for “primitive nucleus”). One of the primary differences between the two groups is the nuclear membrane. Eukaryotes contain a nucleus with a classic membrane; algae, animals, fungi, plants, and protozoa belong to this group. Prokaryotes contain a nucleus without a nuclear membrane; bacteria and blue-green algae belong to this group.

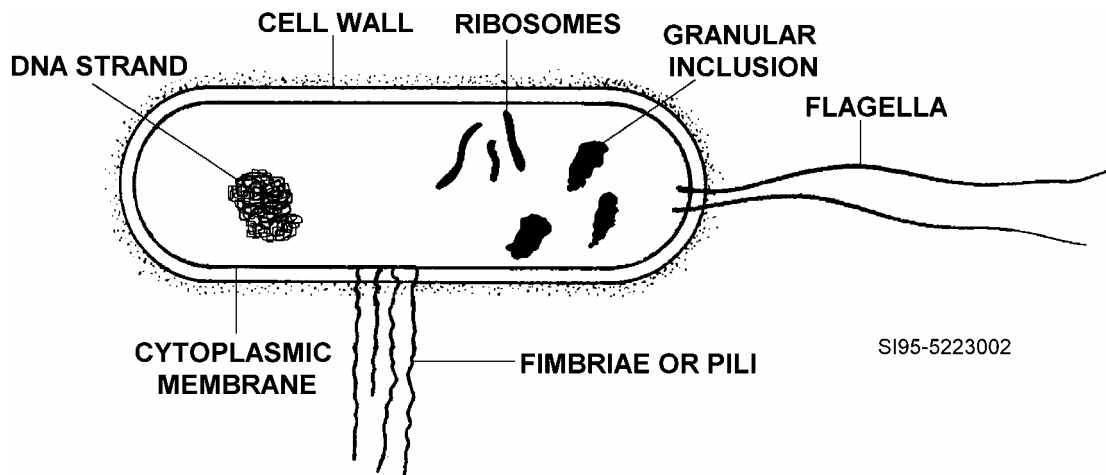


Figure 1–2. Schematic representation of a bacterial cell.

Nuclear region

Within the cell wall and cytoplasmic membrane, the cell itself has a nuclear region, also called nucleoid, nuclear body, or chromatin body. The nuclear region has no nuclear membrane and lacks a definitive shape. It contains the genetic material DNA and a small amount of protein. The DNA present in bacteria is a single, closed strand of DNA that aids in its binary fission process for reproduction. The DNA is looped and coiled extensively; for example, in *Escherichia coli* (a gram-negative rod measuring 2–6 μm long), the DNA strand is reported to be approximately 1,400 μm in length.

Cytoplasmic matrix or Cytoplasm

Within the cell is the cytoplasmic matrix (formerly the protoplasm-substance of a living cell), exclusive of the nucleus, that contains ribosomes, endospores, and various inclusions that store reserve material for cell survival. The cytoplasmic membrane and everything within the cell is called the protoplast. The cytoplasmic matrix is a major portion of the protoplast.

Ribosomes

Within the cell are numerous ribosomes (composed of RNA and protein) that are the sites of protein synthesis. They are present in all cells and are essential for conveying the genetic code of the nucleus into instructions in the manufacture of cellular components. Their

presence is of little significance morphologically, but some antimicrobials bind to bacterial ribosomes, disturbing transcription and interfering with peptide chain formation and protein synthesis, thus causing death of the bacteria.

Endospores

Endospores are formed by species of *Clostridium* and *Bacillus*. It would be logical to consider endosporulation as a response to environmental adversity, but they are produced by cells growing under optimal conditions just after the maximal growth rate. The spore includes the chromosomal material surrounded by several layers of a wall. Within the wall is dipicolinic acid, which is found only in relation to spores and is not in vegetative cells. The resistant properties of a spore extend to stains that are allowed to enter if they are avid dyes and heated. The shape of spores and their position in the cell can be of diagnostic assistance; for example, spores of *Clostridium tetani* are terminal, and their diameter is larger than the parent cell, so they exhibit a characteristic drumstick appearance. The heat resistance of spores, especially of the *Bacillus* species, is of paramount significance in operating room sterilization procedures.

Inclusion bodies

Cytoplasmic inclusion bodies are seen in some bacteria and can be organic or inorganic. Organic inclusion bodies usually contain glycogen or poly- β -hydroxybutyrate (PHB), and are carbon storage reservoirs providing material for energy and biosynthesis. There are two major types of inorganic inclusion bodies—volutin granules and sulfur granules. Volutin granules store phosphate that is a component of cell constituents, such as nucleic acids. Sulfur granules temporarily store sulfur in which hydrogen sulfide is used as a photosynthetic electron donor.

Cell wall

Bacterial cell walls are one of the most distinguishing features, having a rigidity and strength that protect the cellular content and provide shape. The nonselective permeable cell wall also plays a role in division and biosynthesis. The gram-positive cell wall consists of 3 layers—(1) capsule or slime layer, (2) a large peptidoglycan (murein or mucopeptide) layer, (3) and cytoplasmic membrane. This cell wall is usually devoid of lipids and proteins, and contains teichoic acids that are common surface antigens. The cell wall of gram-negative organisms consists of a periplasmic space and 4 layers—(1) capsule or slime layer, (2) outer membrane (lipid bilayer), (3) a small peptidoglycan layer, and (4) a cytoplasmic membrane. This wall is more structurally and chemically complex, and contains enzymes, proteins, lipoproteins, and lipopolysaccharides (LPS or endotoxin). The difference between gram-positive and gram-negative organisms resides in the cell wall. If the cell wall of a gram-positive organism is removed after staining but before washing, it can be decolorized with acetone or alcohol. It is still unclear why gram-positive walls block the dye-extraction step, although the chemical composition of the gram-positive and gram-negative cell walls is fairly well known.

Cytoplasmic membrane

The cytoplasmic membrane, sometimes referred to as a cellular membrane, is present in both the eukaryotic and prokaryotic cells. The membrane is arranged in two layers composed of phospholipids and proteins. This lipid bilayer is a barrier against leakage of cellular substances and protects the cell from entry of unwanted material. It also facilitates the transport of substances across the cell wall and it carries out various metabolic reactions. The cytoplasmic membranes' most important role is aerobic respiration, due to the absence of cytoplasmic structures, (i.e., mitochondria) in the bacterial cell.

Capsule or slime layer

Surrounding the cell wall is a large amount of extracellular polymer (substance of high molecular weight) or material. When this polymer is well-defined and closely associated to the cell, it is called a capsule. When the polymer is loose, nonuniform in thickness, and extends outward from the cell, it is referred to as a slime layer. In most organisms, the primary function of the capsule or slime layer is to protect against phagocytosis. They also protect the cell from environmental hazards and play an important role in adherence of bacteria to surfaces. The organism *Streptococcus mutans*, in the presence of sucrose, forms an extracellular polysaccharide glucan that adheres to the surface of teeth. This glucan is the basic component of dental plaque, in which a heterologous group of bacteria are able to live in continual contact with the dental enamel, eventually producing dental caries (incidentally the world's most prevalent disease). The glycan binding to dental enamel is an example of a method by which bacteria are able to adhere to vulnerable cells or structures.

Flagella

Emerging from some cells are flagella, which are anchored in the cytoplasmic membrane by a complex structure of a hook and basal body. They are relatively long and actively motile, helically coiled, and composed of protein. They vary in arrangement and number, depending upon species, and provide motion for some bacteria.

Fimbriae and Pili

Bacterial fimbriae are so named because of their resemblance to fringes or small hairs. They also arise from the cytoplasmic membrane, project from the bacterial surface, and are protein in nature. Pili, which is now used for gram-negative bacterial species involved in conjugal pairs, are of considerable importance in the "sexual mating" of bacteria. Pili allow the transfer of DNA between bacteria through a hollow pilus "tube." The act itself is not sexual, but rather dependent on the presence of a plasmid—an extrachromosomal piece of DNA usually coding for antibiotic resistance. The primary function of fimbriae is in the adherence of the bacteria to other bacteria, to hard or soft surfaces, and to mammalian cells. This is a major step in the initiation of disease of the respiratory, gastrointestinal, and genitourinary tracts. Specific adherence by bacteria to specific cells also explains the pattern of bacterial disease; for example, why *Salmonella typhi* is a cause of gastrointestinal disease and why *Streptococcus pneumoniae* most often causes primary disease in the upper respiratory tract.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

003. How bacteria are classified

1. What are the definitions of classification, nomenclature, and identification?
2. What is the basis of the phenetic classification of bacteria?
3. How has medical bacteriology adapted the identification process to meet the urgency of clinical pressures?

4. The use of what type of antibodies has greatly increased the specificity of the serological classification of bacteria?
5. Describe the most direct test for DNA homology.
6. How is the relatedness of rRNAs easily estimated?

004. The evolution of bacterial taxonomy

1. Where can the most common nomenclature for bacteria be found?
2. What organization is responsible for keeping Bergey's manual current?
3. What suffix denotes a tribe in Bergey's manual?
4. What is the rule governing capitalization of an epithet?

005. The basic anatomy of a bacterial cell

1. Of what value is the knowledge of the basic anatomy of a bacterial cell to you as a bacteriology technician?
2. Bacterial cells are in what major group of classified cells?
3. What is the primary difference between eukaryote and prokaryote cells?
4. What components can be found within the cytoplasm?

5. What are the 3 layers of the gram-positive cell wall?
6. What are the 4 layers of the gram-negative cell wall?
7. In most organisms, what is the primary function of the capsule or slime layer?

1–3. The Microbiology Laboratory Environment

In the context of this lesson, the term “microbiology laboratory environment” refers to the overall physical and functional design of a workplace dedicated to the specific task of diagnostic clinical microbiology. Thus, it is clearly distinct from other types of work environments by virtue of the nature of the work performed. In no other section of the clinical laboratory is the environment more complex and more important to the function of the laboratory than in clinical microbiology. The environment must be conducive to productive work, and the health and safety of the laboratory workers.

006. Biohazard safety principles

Laboratory safety is an important and complex subject. This lesson is a general review of a good laboratory safety program with emphasis on biohazard safety. The most important element of laboratory safety is achieved primarily through the education and training of laboratory personnel about safety procedures and equipment. Fire extinguishers are useless if the people who need them don’t know where they are and how to use them. It also involves educating personnel on the proper handling of infectious specimens and warning others, either verbally or in writing, on hazards in the laboratory. This may include a written notice or “sign” to alert housekeeping or visitors of the biohazards in the area.

Fire safety

Basic fire safety equipment includes the telephone and fire alarm system; fire retarding doors; Class A, B, and C fire extinguishers; fire hoses; and fire blankets. However, the most important elements of a good fire safety system are knowledgeable and trained laboratory personnel. This is reinforced by periodic fire drills and continuing training sessions. Fire prevention is an important element as well.

Chemical safety

Basic chemical safety equipment includes: acid and alkali spill stations where material is stored and used to neutralize and contain those types of chemical spills; protective clothing, monitoring devices, and absorbent material that can be used for containing and decontaminating radioactive spills; and drench showers and eye washes for removing and diluting toxic or caustic chemicals.

Biohazard safety

The basic strategy of a biohazard safety program is to prevent exposure to infectious disease agents. The Centers for Disease Control (CDC) Standard Precautions cover exposure to blood and body fluids for protection against biohazards. As microbiology personnel, you’ll come in contact with blood in the form of blood cultures and body fluid secretions and excretions that

contain visible blood. You must also be cautious of the samples that do not contain visible blood. These specimens should also be treated as potentially infectious for blood-borne pathogens. The CDCs' Standard Precautions may not mention bacteria, fungi, or parasites directly, but using these guidelines in the microbiology laboratory can protect you against infectious agents. Using personal protective equipment (PPE) as outlined in the CDCs' Standard Precautions, whether or not for blood-borne pathogens or in a microbiology setting, can prevent infection. An excellent reference for biosafety is *Biosafety in Microbiological and Biomedical Laboratories*, a booklet by the Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH), in cooperation with United States Department of Health and Human Services.

Biohazard safety program

Fundamentals of a biohazard safety program include procedures controlling access to the laboratory, the use of PPE, and instructions for correct specimen handling. Specimens should be clearly labeled as to source. A biohazard safety program also involves periodic assessment of exposure by skin testing, chest roentgenogram or serum antibody titers, and, if warranted, a vaccination or prophylactic treatment program. The Force Protection Element (formerly Public Health) at your medical treatment facility tracks these periodic assessments.

Training

Another important facet of a biohazard safety program is ensuring all personnel are properly trained on laboratory techniques. Reducing or eliminating procedures that generate aerosols primarily prevents exposure to infectious agents by inhalation. Over the past few decades, published data indicates aerosols of infectious agents are the largest source of potential laboratory-acquired infections. Frequent hand washing and the absolute elimination of mouth pipetting are advised to avoid ingestion. The ban on smoking, applying cosmetics, contact lenses, and eating or storing food in the laboratory is vital. The careful handling and disposal of needles and broken glassware avoids direct inoculation. Covering small cuts and abrasions helps prevent the spread of infectious agents. Other fundamentals of a biohazard safety program include the proper disposal of infectious waste into covered and well-marked receptacles; the availability of sinks and a good-quality antiseptic soap; and readily available disinfectants including phenolic, glutaraldehyde, and hypochlorite solutions. Proper wear of all required PPE is a must.

Air handling

The air handling system in a clinical laboratory is also a critical factor. The air handling system ensures the air flow proceeds from areas of low risk to areas of high risk, and replenishment with outside air reducing the biohazard burden on the auxiliary air handling systems. The Infection Control Manager, along with the Civil Engineers, is responsible for the air handling system within the hospital.

Biological safety cabinets

Using biological safety cabinets or hoods to protect laboratory personnel from aerosol exposure of infectious agents is on the rise. In these cabinets, the infectious agents are usually sterilized by passing through a high-efficiency particulate air (HEPA) filter, although heat and ultraviolet light can also be used. There are three classes of biosafety cabinets. Class I and II are "partial containment" and Class III is "total containment," which means it is totally enclosed with negative pressure. A Class I cabinet provides protection to the technician, but does not provide sterility to the specimen. In the Class I cabinet room, air is pulled through the cabinet over the work surface, specimen and/or material, then through the HEPA filter, sterilized, and exhausted back into the room. In Class II cabinets, the room air is sterilized as

it flows over the specimen, then pulled through the HEPA filter and exhausted. Class II biosafety cabinets are the most common cabinets found in hospital laboratories. Room air is filter sterilized through HEPA filters coming in and going out of Class III cabinets. The cabinet is completely enclosed with rubber gloves connected and sealed to the cabinet. The gloves are then used for handling highly infectious specimens or material.

Biosafety levels

In conjunction with good laboratory safety procedures, PPE, and biosafety cabinets, the assignment of biosafety levels to infectious agents helps inform and protect laboratory personnel. In the hospital laboratory, we do not routinely assign or think about biosafety levels, yet we may deal with known or identified infectious agents (i.e., blood cultures drawn from an Acquired Immune Deficiency Syndrome (AIDS) patient). Recommendations for biosafety levels for specific agents are made on the basis of the potential hazard of the agent and of the laboratory function or activity. An overview of these levels is presented in the following table:

Biosafety levels	Definition	Procedures	Equipment
1	Minimal hazard risk	Open-bench procedures, lab access uncontrolled, basic facility.	Standard laboratory safety procedures and equipment used.
2	Low to moderate hazard risk	Level 1 plus limited access to lab, warning signs as needed, decontamination of work area and infectious waste, basic facility.	Level 1 plus use of Class I or II safety cabinet if risk of infectious aerosols, wear of lab coats, and gloves.
3	Moderate risk to potentially lethal or serious agents	Level 2 plus controlled access to lab, special procedures for handling infectious waste, containment facility.	Level 2 plus use of Class I and II safety cabinet is mandatory, special laboratory clothing and PPE used.
4	High risk to lethal or exotic agents	Level 3 plus secured access to lab, showers upon exit from containment area, decontamination of all materials and waste, maximum containment facility.	Class III cabinets used, removal of clothing and full-body, positive-pressure, air-supplied suits used for all procedures.

In the laboratory environment, it is impossible to know if a specimen contains an infectious disease agent. For this reason, standard precautions were developed and you must learn how to protect yourself and others.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

006. Biohazard safety principles

1. How is good laboratory safety primarily achieved?
2. What is the basic strategy of a biohazard safety program?
3. What is the largest source of potential laboratory acquired infections?

4. What are the three classes of biosafety cabinets? Briefly describe each.
5. Match each item in column B with the statements it relates to in column A. by placing the letter(s) of the column B item beside the number of the column A item that most nearly describes it. Each element in column B may be used once, more than once, or not at all.

<i>Column A</i>	<i>Column B</i>
___ (1) Low to moderate hazard risk.	a. Biosafety Level 1.
___ (2) Class III cabinets used.	b. Biosafety Level 2.
___ (3) Special procedures for handling of infectious waste.	c. Biosafety Level 3.
___ (4) Open bench procedures.	d. Biosafety Level 4.
___ (5) Minimal hazard risk.	
___ (6) Wear of lab coats and gloves.	
___ (7) Mandatory use of class I or II safety cabinet.	
___ (8) High risk to lethal or exotic agents.	
___ (9) Use class I or II safety cabinet if risk of infectious aerosols.	
___ (10) Moderate risk to potentially lethal or serious agents.	

007. Sterilization, disinfection, and antisepsis

Although the definitions of sterilization, disinfection, and antisepsis are generally accepted, it is common to see all three terms misused.

Definitions

The precise definitions of the three terms, and the basic knowledge of how to achieve and monitor each state, are extremely important if long-known principles of laboratory asepsis are to be effectively applied. Death has occurred despite warnings of disinfecting and cleaning the work area immediately when finished. Well-known physicians H. T. Ricketts, S. J. M. von Prowazek, and E. Weil died while studying rickettsial diseases.

Sterilization

Sterilization is the complete destruction of all living organisms by physical or chemical means. This means freeing an article from viruses, mycobacteria, bacteria and their spores, and fungi and their spores, both pathogenic and nonpathogenic. Sterility then is an **absolute** state; thus, an article is either sterile or contaminated, and must not be described as being “relatively sterile.” Sterilization is required for culture media, suspending fluids, reagents, containers, and equipment used in microbiology. It is also required for medical and surgical instruments, and materials used in procedures that involve penetration into the blood, tissues, and other parts of the body that are normally sterile. Some examples include any equipment used in surgical procedures, intravenous infusions, hypodermic injections, and diagnostic aspirations.

Disinfection

Disinfection means the freeing of an article from some or all of its contamination with live pathogenic bacteria that might cause infection during use. Disinfection is destruction of microbes by chemical means. There are three levels of disinfection:

1. High-level that can generally approach sterilization.
2. Intermediate-level in which spores, mycobacteria, and nonlipid virus can survive.

3. Low-level in which many microbes can remain viable.

The term is relative, and disinfection may be described as being partially or highly effective according to the proportion of the pathogenic organisms killed or removed. Generally, microorganisms, but not heat-resistant spores, are destroyed. Synonyms for disinfectant are germicide and bactericide, both of which are used quite extensively. Under circumstances in which sterility is unnecessary or sterilizing procedures are impractical, disinfection rather than sterilization is attempted. There is still some merit in obtaining a partial or complete removal of nonsporing pathogens. For example, it is impractical to apply sterilizing procedures to laboratory furniture, counter tops, equipment, eating utensils, washbasins, bedclothes, and other fomites that might spread infection in a hospital or laboratory. Since vegetative pathogens present on these articles are capable of causing infection, it is useful to disinfect the articles by procedures lethal only to vegetative organisms. It is also impractical to apply sterilizing procedures to the skin.

Antisepsis

An antiseptic is a substance that can inhibit the growth of microorganisms without actually killing them. Antiseptics are generally used to inhibit organisms that typically contact the body from the environment. Although the terms “bacteriostasis” and “antibiotic” have the same definition as antiseptic, they have a different significance. The suffix of bacteriostasis, “*stasis*,” means “to stand still.” Bacteriostatic agents do not cause the immediate death of microorganisms, but, instead, prevent multiplication. The microorganisms eventually die without a significant increase in their number. Good examples of bacteriostatic agents are low temperature, desiccation (of some organisms), and antibiotics. An antibiotic, literally translated, means “antigrowth substance” or “growth inhibitor.” Its effects are similar to bacteriostasis and, in fact, some act as a bacteriostatic agent.

Physical agents of sterilization

There are many physical means of killing or inhibiting the growth of microorganisms. Methods in common use include:

- Moist heat.
- Steam under pressure.
- Dry heat.
- Incineration.
- Radiation.
- Mechanical filtration.

We'll discuss each separately and give you a working knowledge of the principles on which they work.

Moist heat

Moist heat (boiling water and steam) coagulates the protein within the bacterial cell. This occurs in two stages: (1) the water reacts with the protein (denaturation), and (2) the altered protein separates out as particles (flocculation). Coagulation of the protein is directly related to time and temperature. Different genera of microorganisms vary in their susceptibility to heat.

Boiling water

Using boiling water for disinfecting purposes is a simple procedure. You can use it almost any place where water and a container to boil the water is available. Boiling is a good method

for disinfecting; however, it should never be depended upon for complete sterilization, especially at elevations above sea level. Ordinarily, a 5-minute boiling procedure in New York City, that is at sea level, would be sufficient to sterilize water. However, it would take a much longer time to sterilize water at Tahoe, Nevada, where the altitude is 6,225 feet. If boiling is used for sterilization, as might be the case with outpatients for sterilizing their own containers, sufficient time must be allowed to compensate for the difference in altitude and the change in the boiling point. Remember, spores may remain alive after hours of boiling. Use boiling as a means of sterilization only under carefully controlled conditions.

Free-flowing (live) steam

To get free-flowing steam, use a covered container to hold steam without building up pressure. Remember, free steam, as with boiling water, does not reach a temperature above 100°C or 212°F. In higher elevations, the temperature would be even less. A British scientist, Tyndall, noticed that after boiling and standing for a period of 24 hours at room temperature, the dormant and heat-resistant spores in a solution would germinate and grow. Reboiling would then kill these forms. Thus, free steam is sometimes used to accomplish *fractional sterilization* or *tyndallization*. Tyndall devised a process of sterilization by steaming for a few minutes at 100°C on three or four successive occasions, separated by 24-hour intervals at room temperature. The intervals permit the dormant, resistant spores to become active, vulnerable vegetative cells readily destroyed by 100°C. This process renders an infusion sterile, whereas one single continuous boiling for 1 hour may not, since many spores can remain in their dormant and resistant state during this time. An advantage is it requires no special apparatus. A disadvantage is it is time-consuming and in some fluids, such as water, spores may not grow out promptly. Also, if the material is freely exposed to air, anaerobic spores will not germinate and may survive the process. If not freely exposed to air, aerobic spores will not grow.

Steam under pressure

If you are familiar with the operation of a home pressure cooker, then you should understand the principle of an *autoclave*. The autoclave, as shown in figure 1-3, kills microorganisms, including heat-resistant spores, with moist heat at temperatures above that of boiling water. Water boils when the vapor pressure is equal to the pressure of the surrounding atmosphere. Hence, if the pressure is increased in a sealed vessel, the boiling point rises above 100°C. In a closed system, the exact temperature at which water boils depends on the pressure employed. Since it is the temperature and moisture, not the pressure, that are the effective lethal agents, the autoclave is equipped with a thermometer, as well as a pressure indicator. The exposure time should be based on the temperature reading rather than the pressure reading, because air trapped in the chamber may cause the pressure to build up before the desired temperature is reached. The maximum temperature corresponding to any given pressure can only be obtained when the interior of the vessel is saturated with steam. Only culture media, solutions, or material that are not destroyed or altered by excessive heat should be subjected to autoclave sterilization. An autoclave temperature of 121°C at 15 pounds pressure per square inch (psi) for 15 minutes or longer is required for the sterilization of most of the materials routinely used in the bacteriology laboratory. Keep in mind that the “set time” of the autoclave rarely equals the “contact time” (the time the steam contacts all portions of the load). Larger loads must be set for a longer time. Figure 1-3 illustrates the parts of the autoclave and the flow of steam through the autoclave.

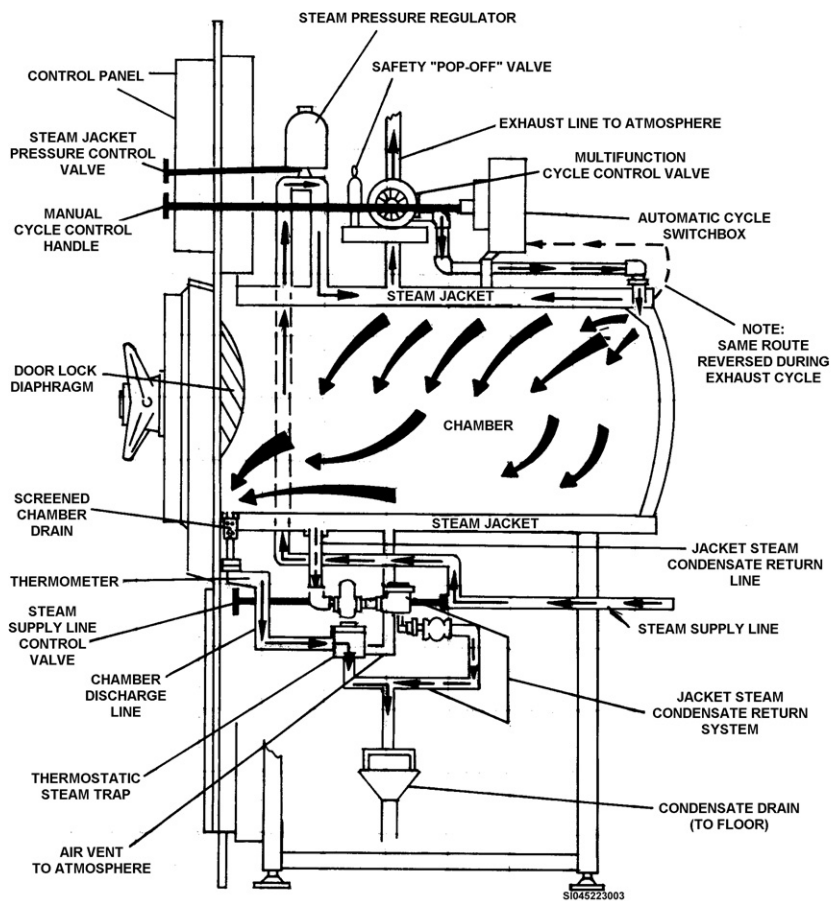


Figure 1-3. Parts of an autoclave.

Process of autoclaving

In order to achieve the desired results through autoclave sterilization, you must observe the following principles:

- The autoclave should never be overloaded.
- Tubes should be packed loosely in baskets or racks, and never placed in containers capable of trapping air.
- Large quantities of media in single containers should be preheated to avoid undue lag time. If possible, distribute media to several smaller containers to effect complete heating and sterilization. The pressure must be allowed to subside slowly after heating is completed or the superheated fluids in open (cotton-plugged) vessels boil over. Tightly sealed vessels may explode.
- All air must be exhausted from the autoclave and the temperature reached before timing the sterilization cycle.
- Media should never be over-autoclaved or reautoclaved.
- Flasks and tubes should never be filled to more than two-thirds capacity to avoid boil-over (one-half capacity is better).
- All media should be removed from the autoclave as soon as possible after sterilizing.

Most effective

Heat sterilization is usually the most effective with moist heat being the most commonly used.

Dry heat

This method is used when direct contact of saturated steam to all surfaces of a material is impractical. Sterilization by dry heat requires higher temperatures and a longer period of heating than sterilization with steam. Its use is limited primarily to the sterilization of glassware and such material as oils, jellies, and powders that are impervious to steam. The lethal action results from the heat conveyed from the material with which the organisms are in contact and not from the hot air that surrounds them. As a result, the importance of uniform heating of the whole of the material to be sterilized is stressed in this method. The hot air oven is the most widely used to provide dry heat. A general rule of thumb is sterilizing times of 2 hours at 180°C are adequate even for spore-forming organisms.

When loading the dry-heat sterilizer, observe the following rules:

- Do not load the chamber to capacity.
- Do not pack containers close together—allow room for air circulation.
- Do not stack articles against the walls.
- All items to be sterilized by the dry-heat method must be thoroughly cleansed before exposure. Otherwise, heat fixes the grease, dirt, and other material to the equipment.

Incineration

Incinerating or burning is a method to assure complete destruction of contaminated materials. You should use common sense when deciding what may be burned, and take caution to ensure complete burning of the discarded material. Always observe local practice when using the incinerator.

Radiation

This method of sterilization came about after prolonged investigation. There is no doubt that ionizing radiation kills microorganisms. Gamma radiation is used for sterilizing some food products and certain medical products, but its use in the laboratory is not practical. It must be applied directly to the surface being sterilized. It cannot penetrate glass, and exposure must be prolonged. Eyes must be protected from the X-rays. It can be used to sterilize rooms in a hospital, such as the operating room. It is quite effective in killing microorganisms on the floor, walls, and instrument tables, and in the air.

Biological materials

The treatment of certain biological materials, such as vaccines and plasma, to kill any contaminating viruses is becoming common practice. In every case, it is the radiation and energy absorbed by the bacterium that bring about its destruction, with resultant damage to the DNA, and not the energy applied.

Ultraviolet radiation

On the other hand, ultraviolet (UV) radiation, when used as a disinfectant, is practical for inactivating viruses, bacteria, and fungi in the laboratory under certain conditions. The use of UV radiation is limited by its low penetrating power. Microorganisms inside dust or soil particles are protected. It cannot be a substitute for chemicals in surface decontamination. UV light is used primarily in biosafety cabinets and laboratory rooms to reduce the levels of viable airborne microorganisms.

WARNING! UV light can cause burns and cornea damage; it must only be used in the absence of all personnel.

Mechanical (filtration)

Filtration is a mechanical means of sterilization. The sterilizing effect of filters is achieved mostly by absorption of the microorganisms to the surface of the filter. It removes rather than destroys microorganisms from the material to be sterilized. Certain types of heat-labile compounds, including some types of media, cannot be heat sterilized. In the past, filters of sintered glass, asbestos pads, and diatomaceous earth were used. Today, membrane filters are most commonly used. Membrane filters of cellulose ester or polycarbonate are made with various pore sizes, ranging from 8 μm to 0.025 μm . A pore size of 0.22 μm is small enough to remove bacteria; however, viruses and some mycoplasma can pass through this pore size.

Needless to say, not all items that need to be sterilized or disinfected can be boiled, autoclaved, irradiated, or filtered. Surgical instruments, patient beds, and other furniture, including laboratory benches, are examples. For these items, chemical disinfectants must be used.

Evaluating the effectiveness of sterilization techniques

Quality control in the laboratory demands that some means be available to evaluate the effectiveness of sterilization techniques performed. This quality assurance function should be especially relevant to you since failure to assure sterility of various materials used in the microbiology laboratory and other areas of the hospital may negate all your efforts to provide valid laboratory data.

Asepsis testing

Asepsis is briefly defined as the absence of septic matter, or freedom from infection. As a bacteriology technician, you'll test the effectiveness of hospital aseptic techniques and practice aseptic techniques in much of your routine daily work. This material deals primarily with the laboratory's responsibility toward preventing the spread of nosocomial (hospital-acquired) infections and ensuring the effectiveness of sterilization techniques. There are many behind-the-scene activities in a hospital. One of these is the prevention of the spread of disease within the hospital. There are many different facets to this particular activity, and you'll be required to lend support to this extremely important program. The Hospital Infection Control Committee, with the approval of the commander, makes recommendations to the individual working sections for the prevention and control of infections. The following paragraphs inform you about aseptic techniques and nosocomial infections.

Sterility testing

This refers to those procedures that test the effectiveness of the various methods of sterilization—specifically, the autoclave and room disinfection. You can use the autoclave to sterilize medical equipment. To ensure sterility of this equipment, it must be tested at regular intervals. You can do this testing in three ways:

1. Testing the equipment itself by culturing.
2. Using heat-sensitive dye indicator strips.
3. Using spore strips.

First, you must test the equipment by opening the packs and taking cultures. In this method, you open the packs containing the equipment. Swab the various articles using a sterile, cotton-tipped applicator, and then place the swab into a suitable culture medium and incubate it. Check the broth for growth. A disadvantage of this method is you can only assume that, if

no growth occurs from a particular article, the specific article was sterilized by the autoclave. However, it is possible the article was sterilized when being cleansed. The other articles in the pack may not be sterile. This method is a reasonably reliable substitute when heat-sensitive dyes or spore strips are not available. If the piece of equipment is small, you can place the entire article in a suitable culture medium to check for sterility.

When you are operating the autoclave, the use of a dye-indicator strip is a quick means to test the equipment for proper operation. The indicator is a thermolabile dye on paper strips or wrappers, or on tape. The dye turns a specific color if the contents have been sterilized at the correct temperature for a sufficient length of time. You need only to observe these color changes to ensure sterility.

You can make a more direct, but more time-consuming check on sterilization by the use of spore strips. A spore-impregnated strip of filter paper enclosed in a sterile envelope is placed in the center of the package to be sterilized. For autoclave testing, the spores of *Bacillus stearothermophilus* are most often used, and for dry heat and ethylene oxide, the spores of *Bacillus subtilis (globigii)* are preferred. After sterilization, remove the strip from its envelope (using sterile forceps), place it in a suitable culturing medium, and incubate. If growth occurs within 7 days, sterilization has not been complete. The absence of growth indicates sterilization has been complete. To assure yourself that sterility is the reason for the spores not germinating and growing, you must also use a control strip. This control strip is not autoclaved, and should grow organisms when cultured. Sealed ampules containing culture media and spores of *Bacillus stearothermophilus* are also available on the market. After sterilization, the ampules are incubated at 55°C. As *Bacillus stearothermophilus* is a strict thermophile, lack of sterility is indicated by growth of the organism in the control ampule. As *Bacillus stearothermophilus* is one of the most heat-resistant organisms known, its use offers a good indication of sterility.

Basic principles of chemical disinfection and factors that influence effectiveness

There are three components in the bacterial cell that are susceptible to the action of chemical agents. These are surface layer, nuclear material, and enzyme systems. Chemical agents perform their disinfecting and sterilizing action on these sites by coagulation of proteins, by specific chemical combinations, and by nonspecific chemical combinations. Action by chemical agents may be by one or more of the above methods. Let's briefly discuss each of these methods.

Coagulation of proteins by surfactants

The coagulation of proteins is similar to that of cooking an egg. The protein (albumin or white) of the egg coagulates and turns white. Any agent that can induce coagulation is lethal to the living cell. Surfactants are chemical agents that coat the surface of the organism and/or the surface of certain enzyme systems to prevent the microorganism from absorbing or utilizing nutritional substances. This causes the organism to die. Certain chemical agents injure or destroy the cell wall, causing immediate lysis of the cell or changes in the cell membrane permeability. Polymixin, an antibiotic, works in this manner.

Specific chemical combinations

In low concentrations, these agents enter the cell of the microorganism and stop or interfere with enzyme groups. This leads to bacteriostasis or death, depending upon the type of cell and chemical agent. The sulfonamide drugs and many antibiotics are effective in this manner.

Nonspecific chemical combinations

These chemicals combine indiscriminately with the protein and other compounds within a bacterial cell. These substances are nonspecific in their action and combine readily with feces, blood, body tissue, and mucus, as well as the protein of the microorganisms. Chlorine, iodine, formaldehyde, and phenol are examples of the chemicals used in this method.

Basic principles of chemical disinfection

Certain basic principles form the basis for all procedures involved in chemical disinfection:

- The type and resistance of microorganisms determine the effectiveness of a chemical agent.
- Disinfectants vary in their level of effectiveness according to the makeup of the chemical agent and the manner in which it is used.
- The solution must be of sufficient strength to be lethal to the microorganism for which it is intended.
- The entire surface of the item to be disinfected must be exposed to the disinfectant.
- The exposure period must be accurately timed, as time is important for bactericidal effect.
- The disinfectant should be economical and safe for patients and personnel.

Factors determining effectiveness of chemical agents

There are many factors that determine the effectiveness of the particular agents in producing disinfection. Because the process of chemical disinfection implies an interaction between the agent used and the microorganisms involved, some aspects of both basic components of each limits the effectiveness of chemical disinfection. The following factors must be considered when selecting the chemical agent and procedures to use for disinfecting specific items.

Cleanliness of item or surface

The first step in chemical disinfection must be cleaning. The active ingredient of certain agents is inactivated by body proteins, by levels of pH, or by residual soaps. All items are cleaned and dried before they are chemically disinfected. Contaminated items require special preparation in order to control the massive microorganism population. If the chemical disinfectant is diluted with a large amount of organic material, or if residual cleaning liquid is present on the object to be disinfected, the agent's concentration and effectiveness is decreased. A clean surface not only decreases the number of microorganisms and prevents dilution of the disinfecting agent, but prevents coagulation of an outer coating or protein that, in turn, prevents penetration of some chemicals to the item's surface.

Type of microorganisms

You must consider the type of microorganisms to be investigated. Most chemical disinfectants control or destroy vegetative bacteria. However, few chemical agents control the resistant forms. The order of resistance is, starting with the most resistant, bacterial spores, mycobacteria, nonlipid or small viruses, fungi, vegetative bacteria, and lipid or medium-size viruses, which are the easiest to destroy.

Number of microorganisms

The number of microorganisms in a colony also determines the effectiveness of chemical disinfection for two reasons:

1. Because of the mass effect of number (the more microorganisms present, the longer it takes the disinfectant to destroy them).
2. Because within any given colony of large numbers of microorganisms, there are individual microorganisms with varying susceptibility to a given chemical agent. Large colonies of microorganisms are more likely to contain greater numbers of resistant organisms.

The decrease in the number of microorganisms caused by cleaning further points to the need for cleansing before beginning the chemical disinfectant process.

Type of object or material

Because all chemical disinfection is based on a chemical reaction, an adequate exposure of the entire surface of the item to the chemical agent is required. The best way to do this is with liquid agents and articles that are disassembled. Tubing and catheters require special attention to ensure both the inner and outer surfaces are in complete contact with the chemical agent. To do this, submerge the tube or catheter in the disinfectant solution and then force the solution through the lumen using a syringe.

Agent strength or concentration

Each chemical agent has a definite concentration and pH that the manufacturer suggests is required for a specific effect. The alcohol solutions undergo evaporation that results in decreased concentration. Chemical compatibility is occasionally important, because the concentration of some germicides is decreased by incompatibility with acidity, alkalinity, or oxidation reduction properties. The presence of hard water interferes with the effect of certain germicides. It is important to thoroughly dry all items or surfaces to be disinfected because the presence of additional moisture and/or humidity dilutes the concentration of the disinfectant, reducing its germicidal action.

Time requirements

All mechanisms of a chemical disinfecting agent action require time to be effective; therefore, all agents are more effective with increasing exposure to the item being disinfected. The time required for effective disinfection also depends on the microorganism to be controlled, the number of microorganisms involved, and the ability to expose all parts of the item to the agent. Time is not only important for bactericidal effect, but also to keep corrosive agents from damaging rubber or plastic goods.

Temperature

The temperature of a disinfectant solution influences its effectiveness. *Normally*, the higher the temperature, the faster a germicide reacts (and destroys microbes) because the heat of the solution speeds up the chemical reactions that occur when the disinfectant contacts microbial cells.

Use of containers

First, sterilize the container and then place gauze on the bottom of the container to protect articles being chemically disinfected. Always keep the container covered to prevent the solution from evaporating and dust particles from settling on the solution surface. Only plastic containers should be used to disinfect instruments because many chemical disinfectants corrode metal surfaces.

Chemical disinfectants

Now that you know the basic principles of disinfection, and are aware of the factors that influence the effectiveness of chemical disinfection, it is time you learned how disinfectants are classified, the properties of an ideal disinfectant agent, and some of the major characteristics of disinfectants you'll be working with.

Classification of disinfectants

There are numerous disinfectant agents on the market today, but only certain ones are suitable for use in a hospital environment. All chemical disinfectants must be registered with the Environmental Protection Agency (EPA) before they are approved for sale. To be approved for hospital use by the EPA, a chemical disinfectant must be proven effective against three highly-resistant bacteria: *Staphylococcus aureus*, *Salmonella choleraesuis* (bacteria linked to enteric fever), and *Pseudomonas aeruginosa*. The product label must identify exactly what type of microorganisms the agent is supposed to destroy. Agents that destroy all forms of vegetative bacteria are called disinfectants, germicides, or bactericides. Stronger agents, designed to penetrate the waxy coating of the tubercle bacilli and destroy it, are known as tuberculocidal agents. Those that kill fungi are fungicides; viruses, virucides; and spores, sporicides. Most chemical disinfectants can kill vegetative bacteria and tubercle bacilli, but not all can kill spores or certain viruses (like the hepatitis virus).

This varying ability to kill different types of microorganisms, coupled with the intended use of the agent, leads to different classification systems of the agents. The EPA uses a system that classifies chemical germicides as sporicides, general disinfectants, hospital disinfectants, sanitizers, and others. A second, more widely accepted classification system for disinfectants involves breaking them into three levels or categories according to their germicidal action (what general types of microorganisms they kill). The three categories of this classification are:

1. High-level disinfectant — can be sporicidal as well as bacteriocidal and virucidal, if contact time is sufficient.
2. Intermediate-level disinfectant — *is not* sporicidal, but kills the more resistant bacteria and viruses.
3. Low-level disinfectant — is not sporicidal and kills only less resistant bacteria and viruses.

Because no chemical agent currently available can be used in all situations, you must be familiar with some of the properties that an ideal disinfectant should possess so you can make intelligent decisions regarding disinfectant selections.

Properties of an ideal disinfectant

To be considered an “ideal” disinfectant, a chemical agent should meet the following criteria:

- Must be effective against a variety (broad spectrum) of microorganisms. The agent must kill or irreversibly inactivate all vegetative bacteria, fungi, and viruses.
- Demonstrate a rapid germicidal action.
- Must be effective in the presence of organic or inorganic foreign matter.
- Is not affected by physical and chemical changes. This means the ideal agent should be equally effective under all acid-alkaline conditions and temperature ranges, should mix well with and remain active in hard water, and not be deactivated by detergents.
- Is not absorbed by gauze or fabrics.
- Will not be accumulated in the environment (agent must be biodegradable after use).

- Is not toxic to humans, animals, or the environment.
- Will not chemically or physically alter surfaces to which it is applied (nondamaging).
- Have a long storage life without being physically or chemically altered.
- Have residual germicidal effects. The agent should be able to inhibit the growth of microorganisms after treated surfaces have dried.
- Easy to mix (dilute) and use.
- Effective in low concentration so dilution would have minimal effect on germicidal action.
- Is equally effective in single-use dilution on all types of surfaces.
- Is virtually odorless.
- Is economical to use.

Obviously, it's difficult (if not impossible) for a manufacturer to develop a chemical agent that meets all the criteria we just outlined. Therefore, hospital personnel must decide on the combination of properties needed, based on the type and population of microorganisms to be killed and the intended use of the disinfectant. Next, let's discuss some of the more common chemical disinfectants used in a hospital and their characteristics.

Characteristics of commonly used disinfectants

Although there are many chemical agents used as hospital disinfectants, the seven types that follow are the most common.

Alcohols

The alcohols, ethyl and isopropyl, are useful as antiseptics in concentrations of 70 to 90 percent. They rapidly kill vegetative bacteria and the tubercle bacillus, but not bacterial spores. As a result, the alcohols are also classified as intermediate-level disinfectants. Alcohols are relatively nontoxic, easy to use, and inexpensive. Because they evaporate quickly and leave no residue, alcohols are commonly used to damp-dust environmental surfaces. Alcohols are irritating to open wounds and skin lesions, and prolonged use dries the skin. They are highly flammable, inactivated by the presence of organic material, and, after long-term exposure, harden and swell plastic tubing. Alcohol should not be used on lensed instruments because it dissolves the cement holding the lens in place. To be an effective disinfectant, alcohol must be in contact with a surface for 10–15 minutes. Because of the prolonged exposure time needed, and the fact that untreated alcohols are corrosive to metal surfaces, their use in the laboratory is limited. Isopropyl alcohol in 70 percent concentration is the most commonly used agent in this group.

Iodine and iodophors

Iodine is used primarily as a skin antiseptic because, in the proper concentration, it is bactericidal, virucidal, fungicidal, and tuberculocidal, yet relatively nontoxic to tissue. However, you need to be aware that some patients do have allergies to iodine and its compounds, and their reactions may be severe. A suitable iodine substitute must be used for these patients. Iodine is easy to use, economical, and remains stable for a long time. One of the major drawbacks of iodine is it stains porous surfaces, such as linens and certain plastics. To reduce the staining properties and enhance the ability to disinfect skin, iodine is often combined with detergents. These compounds are called iodophors. Wescodyne, Betadine, and Povidone are some examples of iodophors. Iodophors with 100 parts per million (ppm) of available iodine are effective in destroying vegetative bacteria, and, in concentrations above 450 ppm, effective against the tubercle bacillus and viruses. Although frequently used as a

skin antiseptic, iodophors can cause chemical burns if allowed to remain in contact with the skin for prolonged periods. Also, many people are allergic to iodine or iodine-containing compounds and can have severe allergic reactions from even slight exposure to the chemical. Iodophors can be used to disinfect instruments at higher concentrations, but 0.2 percent sodium nitrite must be added to the solution to prevent corrosion of metal.

Chlorine

Sodium hypochlorite, more commonly known as household bleach, is an effective low- to intermediate-level disinfectant in concentrations from 1 to 5 percent. It is mainly used as a sanitizing agent for environmental surfaces, particularly in food preparation areas. It may also be used for spot cleaning of floors and furniture, and is the recommended agent for disinfecting basic cardiac life support practice mannequins. Chlorine, a universal disinfectant, is bactericidal, fungicidal, virucidal, tuberculocidal, and even sporicidal (in high concentrations). It acts rapidly, leaves no residue, is easy to use, and is inexpensive. However, chlorine compounds usually have an objectionable odor; are very toxic to skin, eyes, respiratory tract and mucus membranes; and corrosive to some metals and plastics.

Phenolics

Phenolics are derived from phenol (phenolic or carbolic acid) that is obtained from coal tar. Pure phenol is very toxic and suspected to be potentially carcinogenic (studies are ongoing), and rarely used as a disinfectant; however, many of the most effective detergent-germicides used today are phenol-containing compounds. In proper concentrations, phenolics are considered low- to intermediate-level disinfectants capable of killing vegetative bacteria, fungi, tubercle bacilli, and some viruses, but not spores. Phenolics are easy to use, economical, stable, and noncorrosive to environmental surfaces, and remain active after mild heating or prolonged drying. Because of the residue left by the agent, a dry surface that was previously treated with a phenolic compound becomes bactericidal when moistened. Because of this residual germicidal action, phenolic detergent-germicides are widely used in the laboratory and are the agents of choice when dealing with fecal contamination. Phenolic compounds are very toxic and should not be used on porous materials or in food preparation areas. They are also highly corrosive and, if used as an instrument disinfectant, must be mixed with 0.5 percent sodium bicarbonate to prevent metal corrosion. Prolonged skin contact with phenol compounds can lead to depigmentation (loss of color) of the skin, so protective gloves should always be worn when handling these agents.

Quaternary ammonium compounds ("quats")

Some of the synthetic cationic detergents that have quaternary ammonium compounds have some germicidal activity. The quats in the proper concentrations are effective in destroying vegetative bacteria, but the length of exposure time required limits their usefulness. They are ineffective against the tubercle bacillus, spores, and viruses. The quats are bland in nature. The quats are not good antiseptics as they are inactivated by soaps and detergents. Fabrics absorb the quats from a solution and rapidly dilute the concentration.

Formaldehyde

This agent can be used in solution or gas form. It is bactericidal, virucidal, and tuberculocidal, and can even be sporicidal if the exposure time is a minimum of 12 hours. Formalin is the solution of formaldehyde and water commonly used as a tissue preservative. Formalin is normally used at room temperature and loses its effectiveness below 5°C. The solution is irritating to the skin and the fumes are irritating to the eyes and mucus membranes. Formaldehyde vapor is used as a space disinfectant for sterilizing refrigerators, biosafety cabinets, rooms, and even buildings. The use of this agent must be carefully controlled. The

fumes are highly toxic to humans and prolonged inhalation should be avoided. Prolonged exposure to formaldehyde has been linked to occupational nasal cancer. Proper protective attire, rubber gloves, aprons, and eye/face protective devices should always be worn when handling this agent. Formaldehyde solutions are corrosive and are not recommended for instrument disinfection.

Glutaraldehyde

An aqueous, 2-percent solution of glutaraldehyde is often used for high-level disinfection of heavily contaminated lensed instruments. Glutaraldehyde is a broad-spectrum chemical disinfectant that kills vegetative bacteria, viruses, fungi, and tubercle bacilli within 10 minutes, and is sporicidal in 10 hours. This agent remains effective in the presence of organic material, is effective in hard water, is easy to use, contains a rust inhibitor, and acts very rapidly. Because it is an effective sporicide, activated glutaraldehyde in a 2-percent solution can also be used for cold sterilization of items that cannot be steam or ethylene-oxide sterilized. Cidex and sporicidin are the two most common types of glutaraldehyde solutions used today. Cidex can kill spores (sterilize) on instruments in 10 hours, and sporicidin in 6 to 7 hours. Typical glutaraldehyde solutions have a limited shelf life after they are prepared. Usually, this is for a period of 14 days after the solution is activated, but some products are available with an extended shelf life of up to 28 days. Despite all of its advantages, activated glutaraldehyde poses some problems. The agent is toxic and can irritate or burn eyes, respiratory tract, and skin. Heated solutions release noxious fumes potentially toxic to humans. Most glutaraldehyde solutions are alkaline, but special acidic solutions exist that are used mainly in automatic decontamination machines or ultrasonic equipment. Acidic glutaraldehyde solutions have an extended shelf life of 28 days after mixing.

You can see there are a wide variety of chemical agents that can be used in the laboratory and throughout the hospital for disinfection purposes. The main things you need to remember when handling these agents are always read and follow the manufacturer's instructions on the container label, and always follow all local policies and procedures regarding safe use of chemical disinfectants.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

007. Sterilization, disinfection, and antisepsis

1. What is sterilization?
2. Can an article be relatively sterile?
3. What is disinfection?
4. What are the three levels of disinfection?

5. What is antiseptic?
6. The process of destroying bacteria by moist heat, such as boiling and steam, is accomplished in two stages by what principles?
7. Coagulation of the protein (bacteria) is directly related to what two factors?
8. What two factors affect the effectiveness of boiling as a means of sterilization, and to what other key factor are they both related?
9. What is the principle of fractional sterilization or tyndallization with free-flowing steam?
10. What are four disadvantages of free-flowing steam?
11. How does the autoclave destroy microorganisms?
12. What are the two effective lethal agents in the process of sterilization by the autoclave?
13. What time, temperature, and pressure are routinely used to sterilize bacteriology laboratory materials?
14. What could happen to the super heated fluids in open (cotton-plugged) vessels if the autoclave pressure is not allowed to subside slowly?
15. If the autoclave pressure is not allowed to subside slowly, what could happen to tightly sealed vessels?
16. To what capacity should flasks and tubes be filled?

17. How soon after sterilizing should all media be removed from the autoclave?
18. What does asepsis mean?
19. Who coordinates activities within USAF hospitals for the prevention and control of hospital-borne infections?
20. What are three main techniques used for testing the sterility of the autoclave?
21. What is a disadvantage of testing equipment by swabbing various articles?
22. In using spore strips for sterility testing, where is the strip initially placed?
23. What spores are most often used for autoclave testing?
24. What spores are preferred for dry heat and the ethylene oxide test for sterilization?
25. What is done with the spore strip after sterilization?
26. What does the presence of growth within 7 days indicate?
27. Since the control strip is not autoclaved, what occurs when the strip is cultured?
28. Chemical sterilization is achieved by attacking what three sites of a bacterium?
29. How do surfactant chemical agents affect the cell?

30. How is the cell affected by nonspecific chemical combinations?
31. What are two factors related to microorganisms that determine the effectiveness of a disinfectant?
32. What causes disinfectants to vary in their level of effectiveness?
33. What is the first step in the disinfection process? Why is this necessary?
34. What type of bacteria will most disinfectants easily destroy?
35. How does the number of microorganisms affect disinfection?
36. Why is it important to thoroughly dry all surfaces or items before they are disinfected?
37. How does time affect disinfection?
38. How does increasing the temperature of a disinfectant solution normally increase the rate of microbial destruction?
39. What type of containers should be used as disinfectant containers? Why?
40. What three highly-resistant bacteria must a chemical agent be proven effective against in order to be approved for hospital use by the EPA?
41. What are the five categories used by the EPA to classify chemical disinfectants?
42. What are high-, intermediate-, and low-level disinfectants?

43. Why should alcohols *not* be used to disinfect lensed instruments?
44. What is one of the major drawbacks of using iodine as a disinfectant?
45. What is an iodophor?
46. What chemical disinfectant is recommended for cleaning basic cardiac life support practice mannequins and is commonly used to sanitize food preparation areas?
47. What type of chemical agents leave a residue on surfaces that again becomes bactericidal if the surface is moistened?
48. Why are the quaternary ammonium compounds considered poor disinfectants?
49. What chemical disinfectant is commonly used as a tissue preservative?
50. What chemical agent is primarily used as a high-level instrument disinfectant, but can achieve sterilization in 10 hours?

Answers to Self-Test Questions

001

1. Contagious disease.
2. The Renaissance, 1453–1600.
3. Girolamo Fracastoro.
4. (1) By contact.
(2) By fomites.
(3) From a distance.
5. The development of the microscope.
6. Robert Bacon.
7. Anton van Leeuwenhoek.
8. (1) c.
(2) c.
(3) a.

- (4) a.
- (5) g.
- (6) e.
- (7) g.
- (8) f.
- (9) b.
- (10) f.
- (11) d.
- (12) d.

002

- 1. (1) j.
- (2) i.
- (3) h.
- (4) g.
- (5) f.
- (6) e.
- (7) d.
- (8) c.
- (9) a.

003

- 1. Classification is a systematic arrangement of organisms into classes or groups (taxonomic groups) based on similarities. Nomenclature is a set or classified system of names used in any science. Identification is the process of deciding where new organisms belong.
- 2. On easily recognizable characteristics that are weighed in importance.
- 3. A battery of tests for different characteristics is done simultaneously.
- 4. Monoclonal.
- 5. Radioactive DNA from one organism is mixed with an excess of small DNA fragments obtained from the test organism. The degree of DNA homology between the two organisms is revealed by the rate and extent to which the test DNA forms stable double-stranded duplexes with that from the reference organism.
- 6. From the number of identical oligonucleotide spots seen following enzymatic digestion and separation of an rRNA.

004

- 1. *Bergey's Manual of Systematic Bacteriology*.
- 2. International Committee for Systematic Bacteriology.
- 3. "-eae."
- 4. They are not capitalized, even if they are proper nouns.

005

- 1. In understanding bacterial disease and in the identification of bacterial species.
- 2. Prokaryotes.
- 3. Eukaryotes contain a nucleus with a classic membrane and prokaryotes contain a nucleus without a nuclear membrane.
- 4. Ribosomes, endospores, and various inclusions that store reserve material for cell survival.
- 5. (1) Capsule or slime layer.

- (2) A large peptidoglycan (murein or mucopeptide) layer.
- (3) Cytoplasmic membrane.
- 6. (1) Capsule or slime layer.
- (2) Outer membrane (lipid bilayer).
- (3) A small peptidoglycan layer.
- (4) A cytoplasmic membrane.
- 7. To protect against phagocytosis.

006

- 1. Through the education and training of laboratory personnel about safety procedures and equipment.
- 2. Prevent exposure to infectious disease agents.
- 3. Aerosols of infectious agents.
- 4. Class I, II, and III. Class I and II are “partial containment” and Class III is “total containment” that means it is totally enclosed with negative pressure.
- 5. (1) b.
- (2) d.
- (3) c and d.
- (4) a.
- (5) a.
- (6) b, c, and d.
- (7) c.
- (8) d.
- (9) b.
- (10) c.

007

- 1. Complete destruction of all living organisms by physical or chemical means.
- 2. No.
- 3. Freeing of an article of some or all of its contamination with live pathogenic bacteria that might cause infection during use.
- 4. High, intermediate, and low.
- 5. An antiseptic is a substance that can inhibit the growth of microorganisms without actually killing them.
- 6. Water reacts with the protein, causing denaturation; and the altered protein separates out as particles.
- 7. Time and temperature.
- 8. Time and temperature, both of which are related to altitude (pressure).
- 9. A process whereby alternate heating and incubation destroy vegetative forms, and allow spores to germinate to vegetative stages and then be destroyed by successive heating.
- 10. (1) It is time consuming.
- (2) In some fluids, such as water, spores may not grow out promptly.
- (3) If the material is freely exposed to air, anaerobic spores may not germinate.
- (4) If the material is not freely exposed to air, aerobic spores will not grow out.
- 11. With moist heat at temperatures above that of boiling water.
- 12. Temperature and moisture.

13. 15 minutes, 121°C, and 15 psi.
14. They boil over.
15. They may explode.
16. Never more than two-thirds capacity; one-half capacity is better.
17. As soon as possible.
18. The absence of septic matter, or freedom from infection.
19. Hospital Infection Control Committee.
20. (1) Testing the equipment itself by culturing.
(2) Using heat-sensitive dye indicator strips.
(3) Using spore strips.
21. You can only assume that, if no growth occurs that an article was sterilized by the autoclave.
22. In the center of the package to be sterilized.
23. *Bacillus stearothermophilus*.
24. *Bacillus subtilis (globigii)*.
25. It is removed from its envelope using sterile forceps, placed in a suitable culture medium, and incubated.
26. Sterilization is incomplete.
27. It should grow organisms when cultured.
28. (1) Surface layer.
(2) Nuclear material.
(3) Enzyme systems.
29. Surfactants coat the cell wall of the bacteria and prevent it from absorbing or utilizing nutritional materials.
30. These chemicals combine indiscriminately with the protein and other compounds within a bacterial cell. They are nonspecific in their action and combine readily with feces, blood, body tissue, and mucus, as well as the protein of the microorganisms.
31. The type and resistance of microorganisms.
32. The make-up of the chemical agent and how it is used.
33. Cleaning; a clean surface decreases the number of microorganisms, prevents dilution of the disinfectant, and prevents coagulation of an outer protein coating that would, in turn, interfere with the disinfectant's ability to contact the surface of the item.
34. Vegetative.
35. The more microorganisms present, the longer it takes the disinfectant to destroy them. Larger colonies of microorganisms are more likely to contain resistant organisms.
36. Because any moisture left on an item dilutes the concentration of the disinfectant, reducing its germicidal action.
37. Chemical agents are more effective with increased exposure to the item being disinfected.
38. The heat of the solution speeds up the chemical reactions that occur when the disinfectant contacts microbial cells.
39. Only plastic containers because many disinfectants corrode metal.
40. (1) *Staphylococcus aureus*.
(2) *Salmonella choleraesuis*.
(3) *Pseudomonas aeruginosa*.
41. (1) Sporicides.
(2) General disinfectants.
(3) Hospital disinfectants.

- (4) Sanitizers.
- (5) Others.
- 42. (1) High Level: sporicidal, bacteriocidal, and virucidal, if contact time is sufficient.
(2) Intermediate Level: not sporicidal, but kills the more resistant bacteria and viruses.
(3) Low Level: not sporicidal, and kills only less resistant bacteria and viruses.
- 43. The alcohol dissolves the cement that holds the lenses in place.
- 44. It stains porous surfaces, such as linens and certain plastics.
- 45. Iodine combined with a detergent.
- 46. Sodium hypochlorite (common household bleach) solutions.
- 47. Phenolics.
- 48. They are inactivated by soaps and detergents, and do not kill the tubercle bacillus, spores, or viruses.
- 49. Formalin.
- 50. Activated glutaraldehyde, 2 percent.

Do the unit review exercises before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter. When you have completed all unit review exercises, transfer your answers to ECI (AFIADL) Form 34, Field Scoring Answer Sheet.

Do not return your answer sheet to AFIADL.

- 1. (001) The discovery of infectious agents was long preceded by the concept of
 - a. spontaneous generation.
 - b. contagious disease.
 - c. self-multiplication.
 - d. microfermentation.
- 2. (001) The first notable observation of microbes was made by
 - a. Roger Bacon.
 - b. Zacharias Janssen.
 - c. Girolamo Fracastoro.
 - d. Anton van Leeuwenhoek.
- 3. (001) The scientist known for his discoveries regarding immune principle, and developing and administering vaccines is
 - a. Joseph Lister.
 - b. Edward Jenner.
 - c. Francesco Redi.
 - d. Lazzaro Spallanzani.
- 4. (001) The father of bacteriology is
 - a. von Dusch.
 - b. Schroeder.
 - c. Pasteur.
 - d. Ross.

5. (002) Human life expectancy in this century has been increased through the application of discoveries in microbiology by approximately
 - a. 10 percent.
 - b. 20 percent.
 - c. 50 percent.
 - d. 70 percent.
6. (003) A set or classified system of names used in any science is
 - a. classification.
 - b. identification.
 - c. consolidating.
 - d. nomenclature.
7. (003) The phenetic classification of bacteria is based on
 - a. antigenic conformations present on the bacterial cell surface.
 - b. the structure and function of ribosomal ribonucleic acid (rRNA).
 - c. easily recognizable characteristics that are weighed in importance.
 - d. the degree of similarity existing between bacterial nucleic acid sequences.
8. (003) What antibodies have greatly increased the specificity of the serological classification of bacteria?
 - a. Fluorescein tagged.
 - b. Monoclonal.
 - c. High titer.
 - d. Activated.
9. (004) What suffix denotes a family in Bergey's Manual of Systematic Bacteriology?
 - a. "ales."
 - b. "ineae."
 - c. "aceae."
 - d. "oideae."
10. (005) The *primary* differences between the eukaryote and prokaryote group of cells is the
 - a. cell wall.
 - b. cytoplasm.
 - c. fimbriae and pili.
 - d. nuclear membrane.
11. (005) What component, within the bacterial cell cytoplasm, is composed of ribonucleic acid (RNA) and protein?
 - a. Nucleus.
 - b. Granules.
 - c. Ribosomes.
 - d. Endospores.
12. (006) The *basic* strategy of a biohazard safety program is
 - a. enforcing laboratory safety guidelines.
 - b. preventing exposure to infectious agents.
 - c. containing and decontaminating radioactive spills.
 - d. limiting exposure to infectious agents by restricting access to the laboratory.

13. (006) Over the past few decades, published data indicates the *largest* source of potential laboratory-acquired infections
 - a. is mouth pipetting.
 - b. are aerosols of infectious agents.
 - c. is uncontrolled access to the laboratory.
 - d. is eating or storing food in the laboratory.
14. (006) A biosafety cabinet considered a *total containment* cabinet is Class
 - a. I.
 - b. II.
 - c. III.
 - d. IV.
15. (007) Sterility is
 - a. a relative state.
 - b. an absolute state.
 - c. a temporary condition.
 - d. a permanent condition.
16. (007) The *required* temperature, pressure in pounds per square inch (psi), and time requirements for the sterilization of most of the materials routinely used in the bacteriology laboratory is
 - a. 115°C at 15 psi for 10 minutes.
 - b. 115°C at 15 psi for 15 minutes.
 - c. 121°C at 10 psi for 10 minutes.
 - d. 121°C at 15 psi for 15 minutes.
17. (007) The sulfonamide drugs are specific chemical combinations that are effective chemical sterilization agents because they
 - a. enter the cell and inhibit enzyme activity.
 - b. are lethal to living cells due to protein coagulation.
 - c. combine indiscriminately with compounds within a bacterial cell.
 - d. prevent the microorganism from receiving sufficient nutritional substances.
18. (007) The order of resistance, starting with the *most resistant* is
 - a. lipid or medium-size viruses, vegetative bacteria, fungi, nonlipid or small viruses, mycobacteria, and bacterial spores.
 - b. fungi, vegetative bacteria, nonlipid or small viruses, bacterial spores, mycobacteria, and lipid or medium-size viruses.
 - c. vegetative bacteria, lipid or medium-size viruses, fungi, mycobacteria, nonlipid or small viruses, and bacterial spores.
 - d. bacterial spores, mycobacteria, nonlipid or small viruses, fungi, vegetative bacteria, and lipid or medium-size viruses.
19. (007) When disinfecting tubes and catheters, what action is taken to make sure both inner and outer surfaces are completely contacted by the chemical agent?
 - a. Water is blown out of the lumen of the tubes and catheters.
 - b. Gauze is inserted into the lumens to “wick” the disinfectant inside.
 - c. The lumens are scrubbed with a hard-bristle brush to remove caked-on soil.
 - d. The tube and catheter are submerged in the disinfectant and a syringe is used to force the agent through the lumens.

20. (007) In order to kill spores, immerse an item in 2-percent aqueous glutaraldehyde for a *minimum* of
- a. 10 minutes.
 - b. 30 minutes.
 - c. 1 hour.
 - d. 10 hours.

Please read the unit menu for unit 2 and continue ➔

Student Notes

Unit 2. Selection, Collection, and Transport of Specimens

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THE microbiology laboratory plays a critical role in successful patient care, but the value of laboratory reports depends on the very first steps in specimen handling—selecting, collecting, and transporting specimens. In the final analysis, a clinical microbiology laboratory can be of little value to the physician and thus offer only minimal service to patient care if specimens are improperly collected and submitted for the isolation and identification of microorganisms. The laboratory technician must also be aware that misleading or insignificant information on collection procedures given to a physician can be as harmful as incorrect results.

2–1. Specimen Considerations and Selection

The specimen chosen for submission to a laboratory is, for the most part, determined by the health care provider at the time a patient is seen, and it is subsequently collected outside of the laboratory. Ideally, the person determining the specimen to be collected and the collection procedure should be thoroughly familiar with the proper collection procedures listed in the Laboratory Guide. A major step in maintaining standards for proper collection and transport of specimens is the establishment of an operating instruction (OI). The Laboratory Guide also provides information concerning what does and does not constitute an acceptable specimen.

008. General considerations concerning specimen collection

Generally, a report from the microbiology laboratory can indicate only what has been found by microscopic and cultural examination. An etiologic diagnosis is thus confirmed or denied. Failure to isolate the causative organism, however, is not necessarily the fault of inadequate technical methods; it is frequently the result of faulty collecting or transporting techniques. In a busy hospital, the collection of specimens is too often relegated to persons who do not understand the requirements and consequences of such procedures. The laboratory staff may also deserve criticism, on occasion, for neglecting to provide adequate supplies or proper instructions that could result in poorly collected samples. The following paragraphs discuss general considerations regarding the collection of material for culture. Specific instructions for the handling of a variety of specimens are given in subsequent lessons. Please keep in mind these are general considerations and instructions. You should always check with your laboratory's OIs for the procedures used at your medical treatment facility (MTF).

General considerations

As you can see, the *most* important step in the recovery of microorganisms responsible for infectious disease is proper specimen collection, but there are other considerations. Whenever possible, specimens are obtained before antimicrobial agents are administered. Often, a purulent cerebrospinal fluid (CSF) reveals no bacterial pathogens on smear or culture when an antibiotic has been given within the previous 24 hours. A patient with salmonellosis

may have a negative stool culture if the specimen was collected while he or she received suppressive antibacterial therapy, only to reveal a positive culture several days after therapy is terminated. If the culture is taken after initiation of antibacterial therapy, the laboratory is informed so specific counteractive measures, such as adding penicillinase or merely diluting the specimen, can be carried out.

Special procedures

It is an accepted rule that material is collected where the suspected organism is most likely to be found, with as little external contamination as possible. The skin and all mucosal surfaces are populated with normal flora (also known as indigenous flora or commensal organisms) and often acquire a transient flora or even become colonized for extended periods with potential pathogens from the hospital environment. The latter is particularly true of individuals who are quite ill, especially if they are receiving antimicrobial therapy (resistant organisms colonize as normal flora is suppressed). Accordingly, special procedures are employed to help distinguish between organisms involved in an infectious process and those representing normal flora or “abnormal” colonizers that are not actually causing infection. The four major approaches utilized to resolve this problem are:

1. Collect specimens using aseptic techniques. For example, first cleanse the area with a germicide using enough friction for mechanical cleansing. Start centrally and go out in ever enlarging circles. Repeat this several times, using a new swab each time. Alcohol (70 percent) is satisfactory for skin, but a full 2 minutes of wet contact time is needed. Iodine (2 percent) and povidone-iodine work more quickly (1 min.) and are effective against spore-forming organisms. Collection of normally sterile body fluids (such as joint, pleural, or CSF) by percutaneous needle aspiration should always be preceded by thorough skin decontamination as described here.
2. Bypass areas of normal flora entirely (i.e., percutaneous transtracheal aspiration rather than coughed sputum).
3. Culture only for a specific pathogen (i.e., group A streptococci in the throat).
4. Quantitative culture results as a means of determining the likelihood of organisms involved in infection (i.e., quantitative urine culture). Less formal quantitation are also satisfactory and should be used routinely; this can involve grading on a scale from 4+ to 1+, or, simply, heavy growth and four colonies.

Stage of disease factor

Another factor contributing to the successful isolation of the causative agent is the stage of the disease at which the specimen is collected for culture. Enteric pathogens are present in much greater numbers during the acute, or diarrheal, stage of intestinal infections, and are more likely to be isolated at that time. Viruses responsible for causing meningoencephalitis are isolated from CSF with greater frequency when the fluid is obtained soon after the onset of the disease, rather than when the symptoms of acute illness subside.

Patient participation

There are occasions when patients must participate actively in the collection of a specimen, such as a sputum sample. They should be given full instructions and cooperation should be encouraged by the staff. Another specimen for which proper collection procedures are essential for reliable culture results is the clean-catch, midstream urine specimen. These specimens, usually collected by the patient without the assistance of a trained medical care worker, often comprise a large portion of the specimens received by a clinical microbiology laboratory. You may be asked to help prepare guidelines for proper specimen collection. The use of a printed card with the procedure clearly described can help ensure patient compliance.

Separate cards are given to males and females. Careful patient education is key to improving the quality of such urine specimens received by the laboratory.

Specimen quality and quantity

Specimens should be of a sufficient quantity to permit complete examination and placed in sterile, leak-proof containers that avoid hazard to the patient, nurse, or ward clerk. Serious dangers to laboratory personnel, as well as to all others involved, are the soiled outer surface of a sputum container, a leaking stool sample, and possible contact with blood-containing exudates. The hazard of spreading disease by inadequately trained personnel is frequently overlooked. Its control requires continued education and constant vigilance by those in responsible and supervisory positions.

Specimen delivery

Provisions must be made for the prompt delivery of specimens to the laboratory, if the results are to be valid. It is difficult, for example, to isolate *Shigella* from a fecal specimen that remained on the hospital ward too long, permitting overgrowth by normal flora, and an increasing death rate of the *Shigellae*. In some instances, it may be necessary to take culture media and other equipment to the patient's bedside to ensure prompt inoculation of the specimen.

Specimen care

Although most pathogenic microorganisms are not greatly affected by small changes in temperature, they are generally susceptible to drying out, particularly on cotton swabs. However, some bacteria, such as the meningococcus in CSF, are quite sensitive to low temperatures and require immediate culturing. Clinical material that is likely to contain abundant microbial flora is, in most instances, held at 4°C in a refrigerator for several hours before culturing if it cannot be processed right away. Take measures to ensure the specimen is processed before the end of the shift upon which it arrived. This is particularly true with such specimens as urine, feces, and material on swabs taken from a variety of sources, with the exception of wound cultures, that may contain oxygen-sensitive anaerobes. For this reason, wound cultures should be inoculated promptly. Not only does refrigeration preserve the viability of most pathogens, it also minimizes overgrowth of normal flora, increased numbers of which could make the isolation of a significant microbe more difficult. However, the sooner an organism leaving the sheltered environment of its host is transferred to an appropriate artificial culture medium, the better the chances of its survival and subsequent multiplication.

Anaerobic specimen collection considerations

The prominence given to anaerobes in the current medical literature is a reflection of the increased knowledge of these organisms as a consequence of a number of revolutionary laboratory developments during the past several decades. Anaerobic infections have always plagued man. Infections, such as brain abscess, aspiration pneumonia, necrotizing pneumonia, endocarditis, cellulitis, abscesses of the liver or subphrenic space, appendicitis, tubo-ovarian abscess, and septic thrombophlebitis are a few clinical syndromes that have existed for a long time and are now known to be caused by anaerobic, as well as aerobic, organisms. On the other hand, the anaerobic nature of the etiologic agents of gas gangrene and actinomycosis has been recognized for some time. More recently, a new clinical syndrome, pseudomembranous enterocolitis, has been described.

Basic clinical features

Regardless of the actual etiologic agent, there are some basic clinical features of these infections of importance to the clinician. One of the key indicators to anaerobic infection is its proximity to a mucosal surface, particularly following some surgical procedure where sterile tissue has been exposed to organisms that constitute the normal flora of the body. Another key feature is the presence of any degree of necrosis with or without pus or foul smell. The infection-promoting effect of immunosuppressive drugs and certain broad spectrum antibiotics, such as aminoglycosides, are also important considerations.

Proper collection

Proper collection (i.e., taking care to avoid inclusion of normal flora) cannot be overemphasized because normal flora anaerobes are often present in such large numbers that even minimal contamination of a specimen with normal flora can give very misleading results and cause much wasted effort.

Coughed sputum

Coughed sputum is unsuitable because it becomes contaminated with normal flora on its passage through the mouth and pharynx. For the same reason, bronchoscopic specimens or those obtained by nasotracheal tube suctioning also should not be cultured anaerobically. The sampling tube always contacts normal flora on its downward path. The efficacy of a double lumen plugged catheter in preventing such contamination is questioned by some, but it is possible to obtain reliable results by careful attention to detail in carrying out these procedures and by doing quantitative cultures. Adequate pleuropulmonary specimens for anaerobic culture can be obtained by transtracheal aspiration, thoracentesis, or direct percutaneous needle puncture and aspiration of the lung. Tracheotomy tube specimens provide useful material when the tube is first placed. When it has been in place for a while, there is, inevitably, contamination with oropharyngeal secretions, whether or not there is an inflated cuff.

Voided urine

Voided urine specimens are unsuitable for anaerobic culture because the distal portion of the urethra and the meatus are colonized with a normal flora containing anaerobes that contaminates urine passing through these areas. If a suprapubic bladder catheter or cystostomy or nephrostomy tube is in place, reliable urine specimens are collected from these sites. Endometritis presents a very difficult problem. Anaerobes are clearly very important in this infection. However, most cases of endometritis follow childbirth. It has been demonstrated that in the postpartum period, whether or not there is endometrial infection, significant numbers of anaerobes and other organisms from the cervical and vaginal flora are found in the uterine cavity. There are not even quantitative differences between infected and uninfected patients. This situation can apply in postabortal endometritis, also. Thus, you should obtain blood cultures and culture from better sources of material available. Culture for the *Bacteroides fragilis* group can be useful. Since the presence of the *B. fragilis* group in this type of specimen indicates a poorer prognosis and since this organism is more resistant to antimicrobials than other anaerobes, it makes sense to determine whether it is present.

Fecal contamination

Infections of decubitus ulcers commonly involve anaerobic organisms, particularly when the decubitus is in the vicinity of the anus (sacral decubiti and decubiti of the hips and buttocks). Since these areas are subject to fecal contamination (this is how they become infected), the area must be thoroughly cleansed with an antiseptic agent before cultures are taken. Whenever possible, the physician should aspirate collections of pus from under skin flaps or

from deep pockets, using a syringe and needle. The same considerations apply to other lesions in these areas (i.e., perirectal abscess). Specimens that are normally sterile (such as blood, CSF, and joint fluid) are collected in the usual fashion after thorough skin decontamination.

Best method of collection

In general, material for an anaerobic culture is best obtained using a needle and syringe. The specimen should be collected before administration of antimicrobial agents. All air must be expelled from the syringe and needle. Use of swabs is a poor alternative because of excessive exposure of the specimen to the deleterious effects of oxygen and drying. Swab or culturette anaerobic collection and transport systems can be purchased and are adequate as long as manufacturer instructions are followed.

009. Selection of appropriate specimens

The primary considerations here are the specimen obtained should be representative of the disease process and sufficient material should be collected to assure a complete and accurate examination.

General considerations

For example, a small amount of serous fluid drainage from the surface of a diabetic foot ulcer with underlying osteomyelitis is not likely to yield organisms of the type found in the infected bone; indeed, it may yield no organisms at all. In the example cited, the ideal specimen would be a bone biopsy (to be studied histologically, as well as bacteriologically). Although it is often not feasible to obtain infected tissue, such material is clearly the ideal specimen. Frankly, purulent drainage is next in terms of desirability and is entirely satisfactory. In the case of a spreading lesion of the skin and subcutaneous tissue, such as progressive synergistic bacterial gangrene, material from the active margin of the lesion, rather than from the central portion of the lesion, is most likely to accurately reflect the true bacteriology of the process.

Material obtained on a swab from a sinus tract opening often does not yield the true infecting organisms. A deep biopsy of the sinus tract would be much more reliable. Expecterated sputum, particularly if it is not a good purulent specimen with minimal salivary contamination, presents major problems, especially since pneumonia is often a serious infection. Ways around this problem include obtaining blood cultures, examining pleural fluid when present, screening the sputum specimen under 100x magnification to determine its quality, and transtracheal aspiration. Material from normally sterile sites in the body always provides an excellent specimen, if care is taken to avoid contamination with normal skin flora.

Anaerobic specimens

Anaerobic infections often contain a wide variety of bacteria that vary in their sensitivity to oxygen and pathogenic potential. Whereas we were originally taught the one organism-one disease theory, in reality, anaerobic infections follow a different set of rules more closely related to microbial synergy. Cultures of infections can yield four to five distinct species of organisms, including two or three strains of anaerobic bacteria. The time required to isolate and identify all the organisms in polymicrobial infections can be excessive.

Culture free of contamination

Since anaerobic bacteria can be involved in infections of any type, anywhere in the body, always culture anaerobically specimens of any variety that are free of contamination with

normal flora. Certain specimens are, essentially, always contaminated with normal flora. Therefore, the following are not ordinarily cultured anaerobically—throat swabs, gingival swabs, expectorated sputum, sputum obtained via a bronchoscope, gastric contents, small bowel contents (the latter two types of specimens may yield valuable information on culture in the case of “blind loop” and similar syndromes), large bowel contents of feces (except for *Clostridium difficile* and *C. botulinum*), ileostomy and colostomy effluents, voided or catheterized urine, and vaginal or cervical swabs (except as discussed under specimen collection from patients with endometritis).

Collected through a contaminated area

The majority of anaerobic organisms that we are capable of isolating in laboratories are also found as part of the normal flora in humans. They are found on the mucous membranes of the upper respiratory tract and the vagina, on the skin, and in dominant numbers in the lower intestinal tract. Specimens that must be obtained through a contaminated area are done with a protected swab or by needle aspiration. In general, any type of specimen that is obtained by direct aspiration with a needle and syringe, or tissue removed at surgery, is the best specimen for anaerobic culture.

Collection times

The optimal time for specimen collection is based on both the type of infectious disease process and the ability of the laboratory to process samples.

Late night

Laboratories are usually better staffed during daytime hours to receive specimens. Microbiology laboratories may not be well staffed during evening and late night hours. Samples collected late in the evening often do not produce adequate growth by the next morning. However, provisions must be made to handle urgent samples during “off” hours. Consultation with supervisory personnel is highly recommended.

Timed

Twenty-four-hour specimen collections for culture should be discouraged because of the overgrowth of normal flora. They are accepted only after consultation with the microbiology staff, officer-in-charge (OIC), or pathologist.

Optimum

The first early morning sputum and urine samples are optimal for recovery of acid-fast bacteria, fungi, and other pathogens. Samples collected at other times are acceptable. Early morning secretions are more concentrated and more likely to contain large numbers of the etiologic agent and better yields.

Scheduled

The timing of blood cultures should be determined by the clinical condition of the patient. Physicians should always indicate the collection schedule. Except in acute cases of septicemia, blood cultures should not be drawn more frequently than 30 minutes apart. A total of three cultures per 24 hours is usually sufficient to diagnose most cases of septicemia. In endocarditis, typhoid fever, brucellosis, and other uncontrolled infections, the bacteremia is continuous, thus making timing of collection less critical. In other infections, bacteremia is intermittent and may precede the onset of fever by an hour, making collection timing important. In acute febrile episodes, two draws of at least 10 ml blood each, obtained from separate venipuncture sites, allow immediate initiation of therapy. Samples drawn within 30 minutes can reflect the same bacteremic episode and sequential positive cultures may not be

as valid as those spaced at longer time intervals. The recovery rate after three negative cultures per 24 hours is extremely low, except in cases where a sudden fever spike is observed; then, drawing of an additional blood culture may be indicated. The time interval between the collection of blood cultures is basically determined by clinical circumstances and the urgency to start antibiotics.

Consultation

The following specimens are collected only after consultation with the pathologist or microbiology supervisor and, if tested, the protocol should be published in the OI and Laboratory Guide.

- Viral cultures, unless the tests are done routinely.
- Blood for serum bacteriocidal tests or antibiotic drug assays.
- Darkfield examinations for spirochetes or other bacteria.
- Special blood cultures for recovery of fungi.
- Recovery of Chlamydia, Rickettsia, Leptospira, or other unusual organisms.

These situations often require the use of special laboratory equipment and selection of enriched or selective media. Samples often are collected at specific times or in special ways in order to ensure optimal recovery of microorganisms or to produce results that can be interpreted in relation to therapeutic regimes. Physicians bear the responsibility of informing the laboratory that an unusual infectious disease is suspected. If a physician or someone in the ward is to obtain a specimen, laboratory personnel should be consulted to determine the need for any special techniques or collection instructions.

Collection considerations by type of specimen

Along with the information we have just discussed, we need to understand the collection considerations associated with each type of specimen.

Containers

All specimens, with the exception of stool specimens, are collected in appropriate sterile leak-proof containers. If samples are to be delayed in processing or sent to reference laboratories, a transport medium must be used. If a container is not sterile, results can be erroneous. It is the responsibility of the clinics and wards to see that sterile containers of suitable, leak-proof construction are made available to physicians or ward personnel. Containers for stool cultures should be clean and leak-proof, but need not be sterile.

Aspirated

Anaerobic cultures are best collected by aspirating abscess fluid with a sterile syringe and needle. The aspirated fluid is injected into an anaerobic transport vial. The syringe may be sent to the laboratory only if the needle is removed and a sterile plug is inserted. Swabs from commercial anaerobic transport systems are adequate, but if a regular swab is used, it should be placed immediately into a gassed tube or suitable anaerobic packet. It is important to protect species of anaerobic bacteria from the killing effect of atmospheric oxygen and desiccation. The chance for recovery is enhanced by protecting the specimen from any contact with atmospheric oxygen before inoculation in the laboratory.

Expectorated

Sputum samples contain lower respiratory secretions. Patients are instructed to cough deeply. The mouth is rinsed with water or gargle, and dentures should be removed immediately before the sample is collected. All sputum samples are contaminated to varying degrees with

oropharyngeal secretions. Mechanical rinsing of the mouth immediately before expectoration reduces the number of contaminating bacteria. Induced specimens or transtracheal aspirations are recommended for adult patients who cannot produce sputum.

Induced

Bronchial washings are processed as soon as possible after they are collected. Currently, there is no documentation to support the use of an enrichment medium for delayed transport of such specimens for isolation of *Mycobacterium tuberculosis*. Some microorganisms that can infect the respiratory tract, such as *Haemophilus influenzae*, are susceptible to drying or low temperatures. *M. tuberculosis* specimens should be mailed “unenriched” to a reference laboratory. All in-transit decontaminating solutions developed at the CDC have not been tested with bronchial washings, but have successfully preserved *M. tuberculosis* in sputum specimens for days while killing most contaminating organisms.

Encouraging specimen quality

The collection of clean-catch urine samples must not be left to chance. Ideally, the specimen should be collected by the patient after specific instructions from a nurse or other staff member. There is a high potential for contamination of the periurethral area in females from vaginal or bowel flora. Since most laboratories perform routine colony counts on all urine samples, meticulous care must be taken in specimen collection if valid results representative of bladder urine are to be obtained. If patients are to collect specimens unattended, specific verbal and written instructions help to ensure collection of a good specimen. It may be well to actually read the instructions to the patient, particularly if there is a language barrier. It is recommended that these instructions be printed on a card for the patient to retain during the collection procedure. Instructions should be available in the predominant languages of the area or patient population.

Limitations

Stool specimens submitted for the recovery of acid-fast bacilli should not be processed. It is virtually impossible to recover acid-fast bacilli from fecal material because of the inability to prevent heavy overgrowth with bowel flora.

Swabs from wounds

Surface lesions (wounds) must be sampled carefully. It is imperative the surface lesion be opened and the advancing edge of the lesion firmly sampled. Pus is expressed onto swab. Surface lesions are unsuitable for anaerobic studies. Pus alone may not reveal growth on plating since the encased organisms may be dead. The representative specimen is at the advancing margin of the wound. Never submit a dry swab that has been carelessly rubbed over a surface lesion. Anaerobes are abundant on skin surfaces and are common surface wound contaminants. Scrub or cleanse the area around the wound carefully before sampling.

Anaerobic specimens from wounds

Wound specimens submitted for anaerobic workup must be submitted in an appropriate anaerobic transport medium or anaerobic collection system. Anaerobic transport media are designed to protect the strictest anaerobe. Other methods of transport may preserve some anaerobes for a time, but may not allow optimal recovery of anaerobes. The physician's need for complete anaerobic data is no less than that of the laboratory for a properly selected and submitted specimen in anaerobic transport. The syringe (without the needle) used to collect an aspirate should not be used, if at all possible, unless the syringe can be aseptically plugged.

Source information

Descriptive terms such as “wound,” “eye,” “genital,” or other nonspecific terms are not as helpful to the laboratory as are specific anatomic locations describing the source of specimens along with a diagnosis. Microbiology laboratories should subscribe to the complete Cumitech series published by the American Society for Microbiology. The Laboratory Guide should provide descriptive terms and explanations for your facility.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

008. General considerations concerning specimen collection

1. Why should the laboratory be informed if a culture was obtained after initiation of antibacterial therapy?
2. Why does the skin and mucous surfaces of a patient receiving antimicrobial therapy often become colonized by potential pathogens from the hospital environment?
3. What four special procedures are employed to help distinguish between organisms involved in an infectious process and those representing normal flora or abnormal colonizers that are not actually causing the infection?
4. What is the optimum time to collect a culture from a patient with an intestinal infection due to enteric pathogens?
5. What is the key to obtaining a quality patient-collected specimen such as a clean-catch or midstream urine?
6. What should you do if a specimen that is likely to contain abundant microbial flora can not be processed right away?
7. Why is it essential that contamination with normal flora during the collection of anaerobic specimens be avoided?
8. Why is voided urine unsuitable for an anaerobic culture?

9. How is a reliable urine specimen for an anaerobic culture obtained?
10. When do most cases of endometritis occur?
11. The recovery of what organism indicates a poor prognosis in an endometrial infection?
12. In general, what is the best way of obtaining material for anaerobic culture?

009. Selection of appropriate specimens

1. What are the primary considerations in the selection of a specimen?
2. What is considered the ideal specimen?
3. What is the specimen of choice in the case of a spreading lesion of the skin and subcutaneous tissue?
4. What two organisms are the exception to the rule of not culturing feces anaerobically?
5. What factors determine optimal specimen collection times?
6. When should 24-hour specimen collections for culture be accepted by the laboratory?
7. How should the timing of blood cultures be determined?
8. What specimen is the exception to the rule of using sterile specimen containers?
9. What is the best way to collect anaerobic cultures?

10. If regular swabs are used to collect anaerobic cultures, how should they be handled?
11. How can the number of oropharyngeal contaminating bacteria be reduced when an expectorated sputum sample is collected?
12. Should stool specimens be submitted for the recovery of acid-fast bacilli? Why?

2-2. Specimen Collection and Transport

This lesson explores the collection, transport, receipt, and shipment of specimens. Different types of specimens need to be collected in specific ways. The transport of specimens, although outside the control of the laboratory, is of vital importance to the quality of the data provided to the clinician and for that reason clearly delineated instructions are required in the Laboratory Guide. Once samples arrive at the laboratory, it is your job to receive them into the laboratory workflow. By properly receiving the samples submitted, you greatly enhance the quality of the patient care offered by your laboratory. Although not a function of specimen collection, it is an essential prerequisite that the laboratory be given sufficient clinical information to guide the laboratory technician in selection of suitable media and appropriate techniques. Likewise, it is important for the clinician to appreciate the limitations and potentials of the bacteriology laboratory and to realize that a negative report does not necessarily invalidate the diagnosis. It is essential that close cooperation and frequent consultation among the clinician, nurse, and you, the microbiology technician, is the rule rather than the exception.

010. Collection of specimens

Different methods of collection are used depending on the specimen needed. Let's look at the collection procedures for different groups of specimens.

Collection of blood culture specimens

Although recovery of bacteria from blood during episodes of septicemia is usually not difficult, an understanding of certain concepts helps in the selection of the most appropriate medium and isolation procedure.

Collection procedure

Thoroughly prepare the skin at the anticipated venipuncture site by using the following aseptic technique. Remember you must wear gloves while performing venipunctures!

1. Cleanse the area with 70-percent alcohol using enough friction for mechanical cleansing as well. Start centrally and move out in ever enlarging circles. Repeat this several times, using a new swab each time.
2. Allow to air dry.
3. Starting in the center, apply 2-percent tincture of iodine or providone-iodine; also, move in enlarging circles towards the outside. Make sure the entire area is saturated with iodine.

4. Allow to air dry for at least 1 minute using a watch or timer. Timing is critical in order for the iodine to be effective against spore-forming organisms. Take care not to let excess iodine run between skin folds since prolonged contact with iodine can result in skin burns, particularly in the semi-comatose patient. If the site must be touched again, the gloved fingers used for palpating the anticipated venipuncture site should be disinfected.

Use the same procedure above to prevent contaminating the venipuncture site. Because of the iodine sensitivity of some patients, once the needle is removed and the procedure is finished, cleanse the site with 70-percent alcohol to remove the iodine. Collection of normally sterile body fluids (such as joint, peritoneal, pleural, pericardial, or CSF) by percutaneous needle aspiration should always be preceded by thorough skin decontamination as described here. The introduction of iodine bound to the macromolecular polymer polyvinylpyrrolidone has provided an antiseptic that significantly reduces the toxicity while maintaining the antimicrobial activity of iodine, and is used for those who are hypersensitive to iodine. Such compounds, termed *iodophors*, have been found to be very effective as skin antiseptics prior to venipunctures, administration of parenteral medications, or surgery.

Volume and additives

For each blood culture, a minimum of 10 ml of blood is removed in a syringe. A culture of less than 10 ml of blood for each set of two culture bottles is associated with a significant decrease in recovery of certain bacteria, particularly the *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Preliminary results of various studies have suggested there is an improved recovery of bacteria and yeasts when amounts of up to 30 ml are drawn for each set of blood culture bottles inoculated. Smaller amounts, 1 to 5 ml, can be used for infants and children, depending on their size. Inject approximately 10 ml of blood into each of two bottles that preferably contain at least 100 ml of culture broth. A final dilution of 1:10 is recommended. One culture bottle is used for the recovery of aerobic and facultatively anaerobic bacteria, and the second bottle for the recovery of strictly anaerobic and nutritionally variant streptococci.

In the lab, carefully vent the bottle for aerobic culture by disconnecting the needle from the syringe and equilibrating the vacuum in the culture bottle with the ambient air. Label both bottles to distinguish the vented bottle from the unvented. Commercially available blood culture media with vacuum, CO₂, and sodium polyanetholsulfonate (SPS) have been shown to be entirely satisfactory for the isolation of anaerobic organisms from blood. It is best that a two-bottle system with two different media be utilized, but only one bottle should be vented. The culture medium should include an anticoagulant, and in recent years there has been an increasing preference for SPS. SPS has, in addition to its anticoagulant action, an anticomplementary and antiphagocytic effect to reduce or prevent further intracellular killing of microorganisms by neutrophils following injection of the blood into the culture bottle. SPS has been noted to inhibit the recovery of some species of anaerobic bacteria and occasional strains of *Neisseria meningitidis*. If this appears to be a problem, the addition of a final concentration of 1-percent gelatin to medium counteracts the inhibition of the SPS. Both thiol broth (also known as pyriodoxal hydrochloride or vitamin B) and SPS have been shown to exert some neutralizing effect against certain antimicrobial drugs. Also, a 1:20 dilution of blood to culture broth may be sufficient to eliminate the effect of small amounts of antimicrobial agents in the blood at the time of culture.

Commercial blood culture systems may be used in your MTF and require a different procedure than the one just stated. Always follow the manufacturer's instructions as outlined in the OI for collecting blood cultures in your MTF.

Collection of cerebrospinal fluid and other sterile body fluids

Proper collection of CSF and other sterile body fluids is very important because of their implications in the infectious process. Even one colony of a potential pathogen is significant if cultured from these fluids.

CSF

The examination of CSF from patients suspected of having meningitis represents one of the major emergency procedures faced by personnel in the clinical microbiology laboratory. The reasons for this urgency are bacterial meningitis is a rapidly fatal disease if untreated or inadequately treated, and appropriate antimicrobial therapy often requires prompt identification of the etiological agent. Lumbar puncture and examination of the CSF should be undertaken whenever the physician suspects meningitis or wants to rule it out. It must be remembered that the typical signs of meningeal irritation in an adult (i.e., fever, headache, vomiting, nuchal (back of the neck) rigidity, hyperreflexia, etc.) are usually absent in infants and neonates in whom the clinical manifestations of meningitis are often vague and nonspecific.

An unexplained febrile illness in an irritable infant who is doing poorly should lead the physician to suspect meningitis. Meningitis in children 1 month to 6 years of age is most frequently due to *Haemophilus influenzae* serotype b; over 6 years of age, it is usually due to *H. influenzae*, *N. meningitidis*, or *S. pneumoniae*. With the use of the influenza vaccination, the incidents of *H. influenzae* meningitis is expected to decline. Neonates, on the other hand, are infected with group B streptococci, *E. coli*, other gram-negative bacilli, *Listeria monocytogenes*, and other organisms usually colonized in the birth canal of the mother. The infections from these types of organisms are probably due to the immature immune system of the neonates. Meningitis due to mycobacteria, fungi, leptospires, or protozoa is generally insidious (inconspicuous) in onset. The diagnosis of viral meningoencephalitis is frequently established by exclusion and serological means. Although some viruses can be isolated from CSF, viral isolates are more likely to be obtained from other clinical specimens.

CSF collection procedure

To obtain CSF, a lumbar puncture must be performed by a physician under conditions of strict asepsis since contamination of the specimen can occur readily and confuse the identification of the etiological agent. The aseptic technique used for collecting blood cultures can also be used for collecting CSF and other sterile body fluids. Refer to figure 2-1 while we discuss the procedure. Once the patient is in position, draped, and the site disinfected, the space between lumbar vertebrae L3 and L4 is palpated with a sterile, gloved forefinger. The spinal needle is carefully placed between the spinous processes, through the ligaments, and into the spinal canal, and a few milliliters of fluid is placed in sterile screw cap containers or tubes.

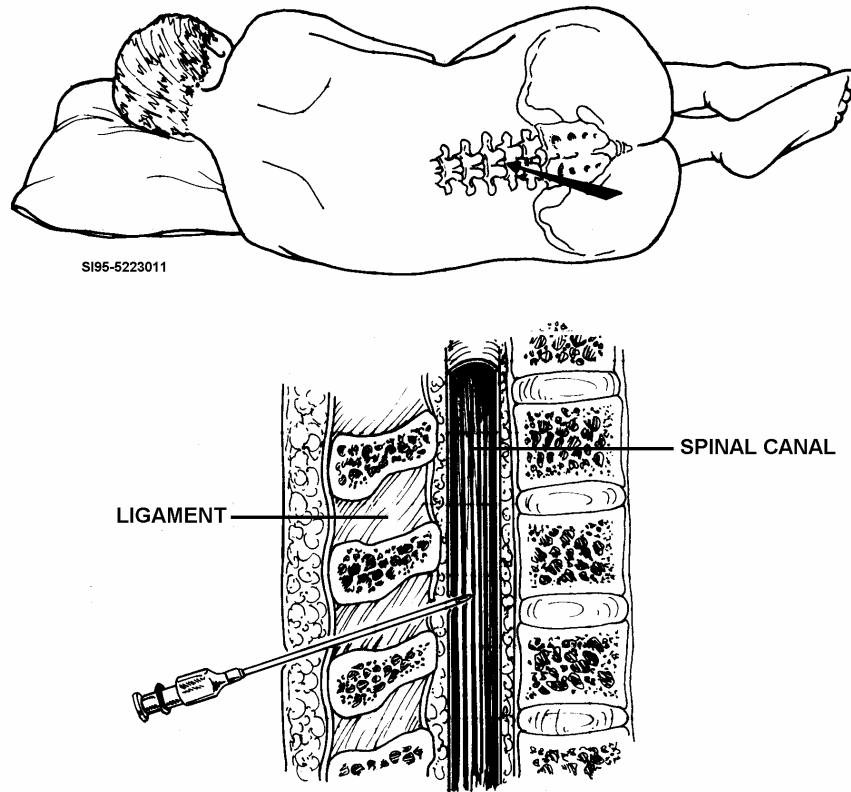


Figure 2-1. Spinal tap collection technique.

Quantity of CSF

Three to four tubes are usually collected. The first tube is for chemistry analysis, the second for microbiology, the third for hematology, and, if there is a fourth tube, it is usually for serological or special chemistry procedures. If only one specimen container is filled, it is submitted to the microbiology laboratory first so it can be opened aseptically, and samples for chemical and cytological (cellular) studies can be removed at the time cultures are inoculated. Since the numbers of microorganisms present are often small, an adequate sample (as much as possible) of CSF should be available for microbiological examination, particularly when a diagnosis of tuberculous or fungal meningitis is considered. Specimens should be collected in sterile containers that can be sealed with a screw cap to preclude leakage and loss or contamination of the contents. Cotton-plugged or rubber-stoppered tubes are not used, and snap-top containers are checked to ensure a tight seal does occur and the contents are not aerosolized on opening.

Quality of CSF

The sterility and absence of microorganisms in CSF specimen containers should be periodically confirmed by culture and also presumptively by Gram stain. To enhance the quality of CSF specimens you should:

1. Suggest the lumbar puncture tray, routinely used in hospitals, be examined to ensure the CSF containers are of satisfactory quality (not all commercially available trays have airtight specimen containers).
2. Attempt to establish a standardized skin preparation.
3. Develop a system whereby the specimen is transported promptly to the laboratory.

Prompt transport of the specimen to the laboratory is mandatory since fastidious organisms such as *H. influenzae* and *N. meningitidis* may not survive storage or variations in temperature. For these reasons, some advocate that smears be prepared and cultures be inoculated at the bedside of the patient when the CSF is obtained. Though such a practice is ideal, it is seldom practical. The microbiology laboratory should process the fluid immediately; if not, the CSF should be incubated or left at room temperature. One exception is viral studies; the CSF is then refrigerated for as long as 24 hours or frozen at -70°C if a longer delay is expected until appropriate media is inoculated.

Methods for the detection of microbial antigens in CSF are indicated now that several types of reagents are available commercially. These approaches are recommended, especially if no organisms are detected microscopically or to confirm the identity of the common etiological agents seen on smears.

Collection of other sterile body fluids

As with the lumbar puncture, the percutaneous needle aspiration of pleural, pericardial, peritoneal, and synovial (joint) fluids is performed aseptically to avoid contamination of the specimen and to prevent the accidental introduction of microorganisms into these anatomical spaces. Since infection of these spaces may be due to anaerobes, it is recommended fluid or pus be collected with a sterile syringe and needle, with any air bubbles present in the syringe expelled. Once collected, the specimen is immediately injected into a sterile tube or vial. Again, as much material as is feasible and practical to collect is submitted to the laboratory. If a large amount of fluid is collected, you can use blood culture bottles and techniques. A small amount of sterile heparin *without* preservatives can be added to the fluid to prevent coagulation since clots may trap microorganisms. Heparin-containing preservatives should not be used since it hinders the recovery of some microorganisms. If heparin is not used, the coagulated material should be emulsified or homogenized to release trapped bacteria and cultured along with a portion of its surrounding fluid. The special procedures and media advocated for the isolation of *Legionella pneumophila* is applied to pleural fluids.

Collection of bone, tissue, abscess, wound, eye, and ear specimens

The culturing of specimens such as these often provides the only reliable or complete information on the cause of a given infection or disease. Several rules usually apply to these types of specimens and are discussed separately. These are general rules and the OI in your laboratory should address the exact collection procedures for your MTF.

Bone

Bone specimens are removed either through surgery or percutaneous biopsy in order to determine the cause of disease or etiological agent of the bone infection (osteomyelitis). Patients develop osteomyelitis from tissue breakdown caused by surgery or trauma, lack of adequate circulation, invasion of bone tissue from nearby infected sites, or by infectious agents delivered through the blood stream (hematogeneous). The most common etiological agent for all ages is *Staphylococcus aureus* that starts out as bacteremia. Many other aerobic and anaerobic, gram-positive and gram-negative organisms, fungi, and mycobacteria have been recovered from bone specimens, although parasites and viruses are rarely, if ever, recovered. Specimens are collected under aseptic conditions and delivered immediately to the laboratory in a sterile, wide-mouth, screw-cap container with or without sterile isotonic saline.

Tissue

Tissue specimens, like bone, are collected aseptically during surgery, from needle or surgical biopsy procedures, or at autopsy. They are also placed in a sterile, wide-mouth, screw-cap container. Anaerobic containers are usually not needed when collecting tissue specimens, because anaerobic bacteria survive within the tissue long enough to be recovered from culture. Smaller tissue samples should be covered with sterile isotonic saline to prevent drying. When culturing sections of lung for *Legionella* spp., sterile saline is *not* used. *Legionella* spp. may be inhibited by saline. Gross surgical specimens submitted for histological procedures are excellent for microbiological procedures if handled aseptically.

NOTE: Tissue specimens placed in a fixative are unsuitable for microbiological procedures! If the site of infection is large, multiple specimens should be collected from different areas of the same site. Any agent of an infectious disease process can cause disease in tissue; therefore, laboratory procedures should be able to recover bacteria, fungi, mycobacteria, and viruses. Also, these procedures should be able to detect the presence of parasites.

Abscesses

An abscess is defined as a localized collection of pus appearing in acute or chronic infections of tissues, organs, or confined spaces. They occur as complications of surgery, trauma, or disease. Abscess specimens are collected aseptically and preferably by needle aspiration. The aspirated internal fluid and, if possible, a portion of the abscess wall is transferred to a leak-proof sterile container. Also, the specimen should be placed in an appropriate anaerobic transport medium or anaerobic collection system. Swab specimens are inferior to needle aspiration, but can be used. If a specimen *cannot* be obtained through needle aspiration, the abscess site is cleansed and a scalpel blade is used to make a small opening. Avoiding the adjacent skin, the tip of the swab is carefully placed deeply into the lesion. Swab specimens should be placed in the appropriate aerobic transport container and anaerobic transport medium. As with tissue, any agent of an infectious disease process can be present in abscesses and wounds.

Wounds

A wound is defined as bodily injury caused by physical means with interruption of continuity. Wound infections are also a complication of surgery, trauma, or disease. They may harbor the causative agent of the infection, or be colonized or contaminated from unrelated microorganisms. Distinguishing between the two is a challenge for the health care provider and microbiologist. Further studies may be needed and your laboratory's OI should address these procedures. As with tissue specimens, fluid collected by needle aspiration is best. If fluid collection is not possible, swab specimens are acceptable. Swab specimens should be placed in the appropriate aerobic and anaerobic transport containers.

Eye and Ear

Infections of the eyes and ears have distinct bacteriology and can be life threatening because of their contact with numerous blood vessels and proximity to the central nervous system. Let's briefly discuss each.

Eye

Depending on the site of infection, specimens submitted from the eye may include swabs, scrapings from lesions, specific tissues within the eye, or, possibly, fluid or fluid-like material. The agents of infection include aerobic and anaerobic bacteria, mycobacteria, fungi,

viruses, chlamydiae, and amebae. Treat these samples as you would other tissue, fluid, or swab specimens. The most common terms associated with eye infections are conjunctivitis and keratitis.

Ear

The external ear and the middle ear are the two most common sites for infection, although soft tissue, cartilage, and bone can be involved. Tympanocentesis is the surgical puncture of the tympanic membrane for removal of fluid from the middle ear. This procedure is rare and reserved for patients with extreme complications. Studies have shown that most middle ear bacterial infections (otitis media) in children are attributed to *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, and group A streptococci. Tympanocentesis is *not* routinely performed on children to obtain a culture. The external ear bacterial infections (otitis externa) are commonly due to *S. aureus*, *P. aeruginosa*, and group A streptococci. Swabs are used in most cases for collecting external ear specimens and transported aerobically.

Collection of upper and lower respiratory tract specimens

The respiratory tract is one of the two major connections between the inside of the body and outside environment; the other is the gastrointestinal tract. This creates a challenge for the microbiologist for determining what is normal flora and the cause of infection or disease. This, in-turn, puts a heavy burden on the collection process. The respiratory tract is divided into two distinct areas. The upper tract contains the oropharynx and nasopharynx (specifically the nose, sinuses, throat, and pharynx). The lower tract contains the larynx, trachea, bronchi, and the alveolar air sacks of the lungs. Let's briefly look at the collection of the most commonly seen upper respiratory tract specimens and look more in-depth at the collection of lower respiratory tract specimens that includes sputum cultures.

Upper respiratory tract

The most common upper respiratory tract specimen you'll encounter in the laboratory is the throat culture. Other specimens include nose, sinus, and pharynx.

Throat and pharynx cultures

Throat cultures are visually obtained by using a tongue depressor and inserting a cotton-, Dacron-, or calcium alginate-tipped swab into the posterior pharynx area of the mouth. Samples are taken from both tonsillar areas, the posterior pharynx, and any areas that show inflammation. It is important to avoid touching the tongue or the walls of the mouth to minimize contamination of the swab with normal mouth flora. See figure 2-2 for the proper throat culture technique.

Nose and Sinus

Cotton-, Dacron-, or calcium alginate-tipped swabs are used for collecting most upper respiratory tract specimens. The swab must remain moist and is best if transported in some kind of transport

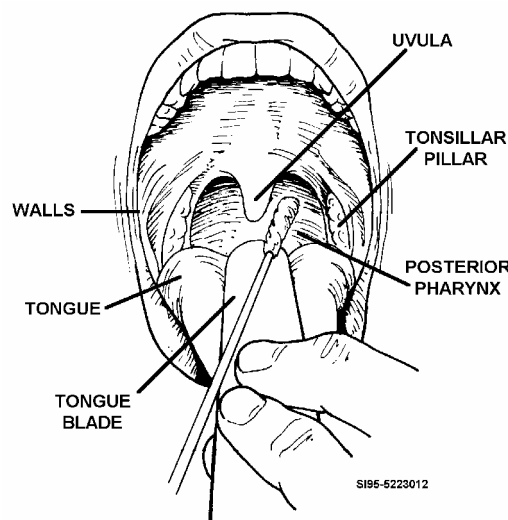


Figure 2-2. Throat culture technique.

medium. These swabs are appropriate for bacterial, fungal, and viral recovery. Other possible specimens include sinus washings, drainage fluid, aspirated nasopharyngeal secretions, and surgical biopsy specimens.

Lower respiratory tract

Lower respiratory tract specimens include sputum, induced sputums, nasotracheal or endotracheal catheter, bronchoscopy bronchoalveolar lavage, protected bronchial brush catheter, and transtracheal aspirates. In some cases, lower respiratory tract or pulmonary infections may be identified by thoracentesis, percutaneous needle aspiration of an abscess, percutaneous transfer needle biopsy, or open lung biopsy. Treat fluid and tissue specimens as mentioned above.

Sputum specimens

The problems encountered in collecting sputum for culture have resulted in the development of several alternative techniques (named above) for its collection. Let's look at the problems you'll encounter in obtaining suitable sputum specimens and the ways around these problems.

Problems

The successful collection of sputum for culture depends on the type and extent of pulmonary disease in the patient. The primary problem with most sputum specimens is they are heavily contaminated with oropharyngeal secretions and bacteria, and may contain only small amounts of secretions from the lower respiratory tract. Many sputum specimens contain only salivary and oropharyngeal secretions, and frequently represent an honest but ineffective effort by patients to raise secretions from the lower respiratory tract. In one study, collection of sputum by expectoration and culture for pneumococci by standard procedures resulted in the isolation of *Streptococcus pneumoniae* in only 55 percent of the patients with clinical pneumonia and pneumococcal bacteremia. Although the reason for the poor recovery of *S. pneumoniae* by culture is not known, there is evidence that interaction with other organisms or products from organisms found in the oropharynx, such as the alpha-hemolytic streptococci, may suppress growth of pneumococci.

Solutions

Partial suppression of the endogenous flora, using gentamicin-containing blood agar, has resulted in a marked improvement in recovery of *S. pneumoniae* from sputum in patients with pneumonia.

Another method for collection of material for culture from the lower respiratory tract is the induction of sputum by ultrasonic or heated saline nebulization. Aerosolization increases the moisture content of the air going to the lower respiratory tract and improves the ability of the tracheobronchial cilia to bring up otherwise thick, viscid, or partially dehydrated secretions. Nebulization is particularly well suited for the recovery of *Mycobacterium tuberculosis*, but can also be used for inducing sputum in patients with other types of pulmonary infection.

Nasotracheal or endotracheal catheter, bronchoscopy bronchoalveolar lavage, or protected bronchial brush catheter are additional methods of collecting specimens from the lower respiratory tract; yet, all these procedures are subject to variable degrees of contamination by the normal oropharyngeal microbial flora. The best way to collect uncontaminated specimens for culture from the lower respiratory tract is translaryngeal aspiration as shown in figure 2-3. In this procedure, a large bore needle is passed percutaneously between the cricothyroid and thyroid cartilage of the larynx, and a small plastic catheter is introduced into the trachea.

Normal saline is administered through the catheter and quickly aspirated along with tracheobronchial secretions.

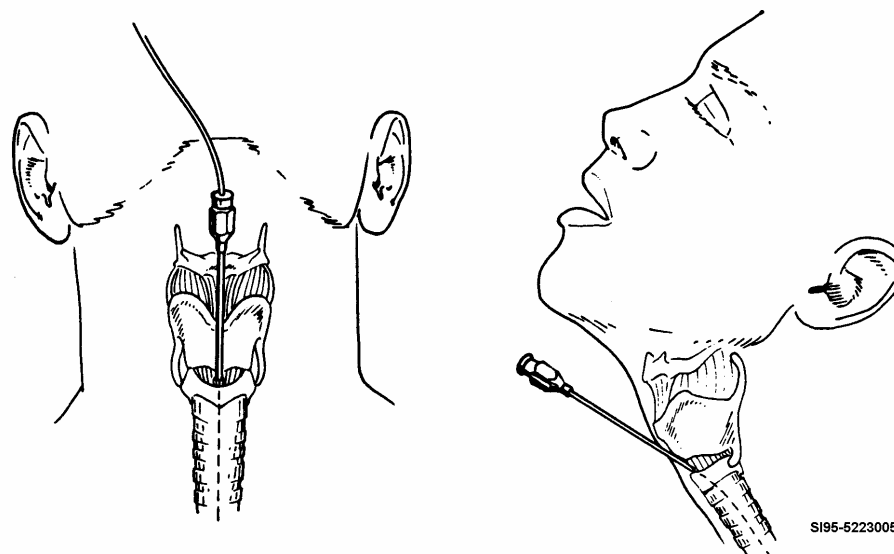


Figure 2-3. Translaryngeal aspiration of lower respiratory tract specimen.

Care should be taken that the saline used for injection does not contain bacteriostatic agents, such as benzyl alcohol or methyl- or propylparaben. These agents are incorporated into saline used for injection of medication to prevent growth of contaminants. They also quickly kill bacteria causing acute pneumonia. Saline should not be used if infection by *Legionella pneumophila* is suspected, as the organism appears to be susceptible to sodium ions and may show a decreased recovery when compared with sodium-free solutions. The tracheobronchial tree below the larynx is normally sterile, and any organisms found usually reflect bacterial or fungal agents associated with infection in the lower respiratory tract. Translaryngeal aspiration can cause considerable discomfort to a patient who is seized with intense coughing spasms following injection of fluid into the trachea. The procedure is done only when clinically indicated and after careful consideration of the potential advantages and disadvantages.

Collection of urine specimens for culture

One of the most common sites of clinically significant infection is the urinary tract. Infection can occur within the interstitial tissue of the renal glomeruli, tubules, kidneys, pelvis (upper tract), or at any point along the ureters, urinary bladder, urethra, or prostate (lower tract).

Terms associated with urinary tract infections and their definitions from *Dorland's Illustrated Medical Dictionary* are:

- Bacteriuria—the presence of bacteria in the urine.
- Pyuria—the presence of pus in the urine.
- Dysuria—painful or difficulty in urination.
- Pyelonephritis—inflammation of the kidney and its pelvis.
- Cystitis—inflammation of the urinary bladder.
- Bacterial cystitis—bacterial infection of the bladder.
- Urethritis—inflammation of the urethra.
- Prostatitis—inflammation of the prostate.

Bacterial growth in urine

Most infections of the kidneys are acquired by two routes—the ascending (bacteria migrating up from the bladder) and the descending (bacteria spread hematogenous to the kidneys). Studies relating clinical infection to the number of bacteria in voided urine have suggested one of the best ways to determine the presence of infection in the upper urinary tract is to demonstrate 10^5 or greater (colony-forming unit (CFU)/ml). Keep in mind that there are possible exceptions to this criterion (e.g., women with bacterial cystitis or dysuria may have as few as 10^2 CFU/ml in a midstream clean-catch urine specimen). If the clinician expects lower colony counts to be significant, the laboratory should be contacted. Bacteriuria can occur from colonization and contamination, as well as infection. Bacteriuria is often accompanied by the presence of pyuria. The measurement of pyuria is a means of detecting the presence of host injury that can differentiate colonization from infection.

Most often found

The microorganisms associated most frequently with urinary tract infection are *Escherichia coli*, *Klebsiella*, and *Proteus* spp. that, under ideal conditions in culture, may double in number every 20 to 30 minutes. Although it is not optimal, urine can be utilized as a culture medium by many bacterial species. Generation times may not always be as short as 20 minutes, but they can be brief, and bacteria can divide every 40 to 50 minutes. The following is a list of factors that influence growth of bacteria in urine:

- pH is significantly higher in women than in men and, therefore, more conducive to bacterial growth in females.
- Osmolality is lower in females than in males and, again, more likely to support bacterial growth in women.
- Urea content—the higher this value, the more inhibitory to bacterial growth.

Best method

Although the rate of replication following collection of a urine specimen can be reduced by refrigeration, the best method of determining the number of bacteria in a freshly voided specimen is prompt inoculation of the specimen to culture medium.

Obtaining the specimen

Collection of a urine specimen should be done in a way that minimizes the possibility of external contamination, which can result in counts higher than those in the bladder at the time the specimen was voided. The procedure least likely to result in contamination of bladder urine is an aseptic suprapubic puncture of the distended urinary bladder with a long aspirating needle as illustrated in figure 2-4. Inasmuch as suprapubic aspiration does not appeal to most patients (or physicians), alternative methods of collecting urine are commonly used. Urethral catheterization of the urinary bladder is not recommended for initial cultures because of the possibility of introducing bacteria into the bladder. The simplest and most practical method is the collection of a midstream urine sample during voiding.

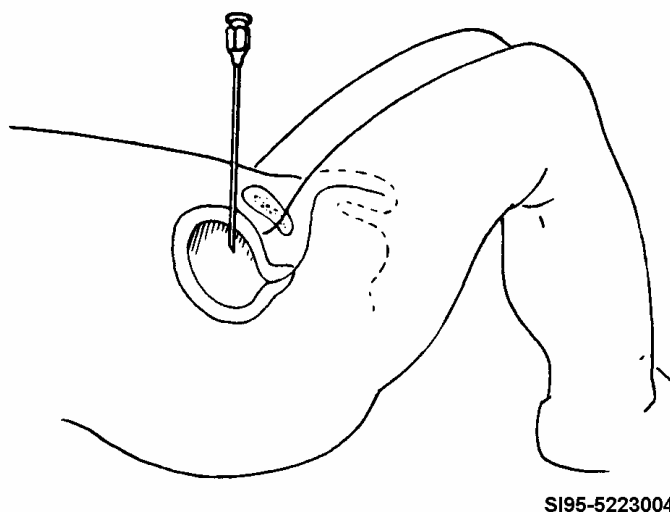


Figure 2-4. Suprapubic puncture of the urinary bladder.

Midstream

Collection of the midstream urine specimen from men involves cleansing the outside area, then starting the urine stream to dislodge and wash out bacterial growth that may have developed in the urethra since the last voiding. Five to ten milliliters is then collected in a sterile container prior to voiding of the latter part of the urine. The specimen should not contain urine collected during the terminal phase of voiding because prostatic secretions or urethral sphincter contractions can dislodge small clumps or microcolonies of bacteria and artificially increase the bacterial count. Although it may not be possible to exclude all contamination during collection, except for suprapubic aspiration, midstream urine specimens probably give the most accurate reflection of the number of bacteria in the bladder.

Special problem

Collection of urine specimens for culture from women poses a special problem because of the increased possibility of contamination. The collection procedure should be carefully reviewed with the patient in order to explain why precautions must be followed. The patient should be instructed concerning the careful cleaning of the labia and external urethral meatus, and means of preventing the labia from contaminating the urine stream during voiding. A midstream specimen with prompt inoculation to the culture medium usually provides an adequate reflection of the number of bacteria in the urine.

Prompt delivery

Prompt delivery of the specimen to the laboratory is important since, all too frequently, the specimen may be left on the ward until a series of specimens are collected and only then taken to the laboratory. If the urine sample cannot be taken directly to the laboratory, it must be refrigerated immediately to retard bacterial growth.

Collection of fecal specimens

Proper collection and preservation of feces is a frequently neglected, but important, requirement for the isolation of microorganisms contributing to intestinal disease.

Procedure

For routine culturing, the stool specimen can be passed directly into a wide-mouth, clean, dry container. Unless the specimen is taken immediately to the laboratory and properly handled on delivery, a number of important microorganisms do not survive the changes in pH or a drop in temperature. This is especially true of most of *Salmonella* spp. When delays are unavoidable, it should be a standard rule that all stool specimens be submitted in a stool preservative, such as 0.033 M phosphate buffer mixed with equal volumes of glycerol, or an enteric transport system. An indicator can be added that assures an approximate pH of 7. The indicator added to the specimen provides visual proof that the pH drop was not inordinate. Stool specimens are introduced into a screw-capped glass container with stool preservative immediately after they are passed. A 0.5 to 2g quantity is sufficient. If sterile swabs are used in obtaining the specimen, they are passed beyond the anal sphincter, carefully rotated, and withdrawn. The swabs are then added to a screw-capped tube containing preservative or culturette and transported to the laboratory for culture. Cary-Blair transport medium is best when culturing for *Campylobacter* and *Vibrio* spp.

Specimen characteristics

If the patient is hospitalized, personnel obtaining the specimen should be instructed explicitly to choose portions of the stool that display either mucus, blood, or both. These areas usually harbor a large number of the organisms involved in the disease process. It cannot be stated too often that the vast majority of bacteria found in feces are anaerobic gram-negative rods. They are usually ignored in the attempt to diagnose infectious intestinal disorders. This is not to imply such organisms may not be involved in disease processes of the intestinal tract. However, to date, such a causal relationship has not been established, with the exception of food-borne *Clostridium perfringens* intoxication and pseudomembranous colitis after antimicrobial therapy resulting in the implantation of *Clostridium difficile*. It is important to remember that the biochemical activities of the anaerobic bacteria contribute to the detriment of *Enterobacteriaceae*. Therefore, the facultative anaerobic enteric bacteria require the type of buffering provided by a stool preservative. There are times when surveys for carriers, as well as institutional outbreaks, indicate a need to use rectal swabs to obtain specimens. Under these circumstances, the swab is not placed in a stool preservative, but into a medium, such as GN broth, especially if the responsible organism was already identified and contacts or carriers are being sought. Enrichments, such as tetrathionate broth or selenite F, can be used, particularly if *Salmonella* spp. are involved.

Diagnostic information

As with most specimens, a single stool specimen with little or no accompanying information is inadequate. The laboratory is much more helpful to the clinician if a brief history is provided. If necessary, the requesting physician should be called for this information. With the information, you can be particularly attentive to culturing the stool on media most likely to yield the responsible bacteria, fungi, or parasites. Without such information, procedures cannot be modified to accommodate as complete a microbiological analysis as is required. Of equal concern are the increased cost and the time lost in searching for impossible organisms. Not all microorganisms involved in gastrointestinal disease may be present in stool specimens, as with *Helicobacter pylori* where gastric biopsy material must be cultured. *H. pylori* is associated with gastritis and peptic ulcer diseases.

Collection of sexually transmitted disease specimens

Until recently, gonorrhoeae dominated the concern in the venereal disease field. With homosexual and increasingly common heterosexual practices of oral-genital and anal-genital intercourse, many other microorganisms can be a significant cause of sexually transmitted

diseases (STD). *Chlamydia trachomatis*, *Gardnerella vaginalis*, *Neisseria gonorrhoeae* and *N. meningitidis*, *Haemophilus ducreyi*, *Treponema pallidum*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, other mycoplasmas, human papillomavirus, herpes virus, human immunodeficiency virus (HIV), protozoa, gastrointestinal pathogens, and other microorganisms are now of great concern for those engaged in sexual activity. Some of these organisms can be symptomatic or asymptomatic and exist without any noticeable pathology.

Common STDs

The HIV is the causative agent of AIDS and eventually leads to death at this time. We discuss HIV in-depth in course 4T051C. Human papillomavirus (HPV) is linked to cervical carcinoma and possibly penile malignancy. In the United States, it is present in epidemic proportions and may be the most prevalent STD today. *U. urealyticum* is related to premature birth, morbidity, and even mortality of the babies of infected females, and may be associated with nongonococcal urethritis in males. In females, it has been associated with acute urethral syndrome and reproductive failure. *M. hominis* has been associated with pelvic inflammatory disease, pyelonephritis, and postpartum fever in females, and with morbidity and stillbirths in infants. *G. vaginalis* is commonly related to bacterial vaginosis (BV), consisting of large amounts of vaginal discharge with an unpleasant odor. It is important in the etiology of premature labor and low birth weight. These are just a few examples of the more common STDs. Specimens for these STDs are probably shipped to a reference laboratory. Let's now discuss the most common requested STD specimens cultured or identified in your laboratory.

Gonorrhoeae

N. gonorrhoeae (the gonococcus) is the causative agent of gonorrhea—a bacterial infection of tremendous public health significance. The clinical spectrum of gonococcal infection includes:

- Uncomplicated urethritis and cervicitis in men and women.
- Anorectal gonococcal infection.
- Oropharyngeal gonococcal infection.
- Ocular gonococcal infection.
- Gonococcal arthritis.
- Acute pelvic inflammatory disease.
- Acute epididymitis.
- Disseminated gonococcal infection.

Symptoms

This fastidious organism is sensitive to temperature extremes and drying, and is transmitted only by intimate sexual contact. In men, the organism generally causes acute urethritis with dysuria (burning on urination) and urethral discharge. The incubation period between acquisition of the organism and onset of symptoms ranges from 1 to 7 days. Most men with gonorrhea have acute symptoms, but asymptomatic infection can result in epididymitis and urethral stricture. In females, the primary infection is endocervical, and dysuria, vaginal discharge, and lower abdominal pain may be present. However, most females with endocervical gonorrhea are asymptomatic. Ascending infection can result in acute pelvic inflammatory disease with scarring of the fallopian tubes.

Characteristics

Gonococci can invade the blood stream, resulting in disseminated gonococcal infection (DGI), which is characterized by fever, hemorrhagic skin lesions, and migratory joint pains. Organisms from the blood stream can localize in one or more joints to cause a purulent and destructive gonococcal arthritis. Oral-genital and anal-genital sexual contacts can result in local oropharyngeal and anorectal gonococcal infections, respectively, and systemic dissemination from infection at these sites has been reported. Ocular gonococcal infections are primarily seen in neonates who acquire the organism during passage through an infected birth canal. Ophthalmia neonatorum is any hyperacute purulent conjunctivitis occurring during the first 10 days of life and is usually contracted at birth from infected vaginal discharge of the mother. It is usually caused by *Neisseria gonorrhoeae*. However, other organisms may be the cause of ophthalmia neonatorum.

Specimen collection

Since this organism can cause infections at a variety of body sites, the collection of specimens for culture and diagnosis is dependent on both the sexual practices of the patient and the clinical presentation. In all cases, specimens from genital sites (urethra and cervix) should be collected. If the patient has a history of oral-genital and/or anal-genital sexual contacts, appropriate oropharyngeal and/or anal-canal specimens are also collected. In suspected cases of disseminated disease, blood cultures and specimens from all local sites are obtained routinely. If gonococcal arthritis is suspected, joint fluid should be aspirated and submitted for culture, and treated like other joint fluid specimens.

Specimen care

Specimens for gonococci isolation should be inoculated immediately, directly onto selective media, such as modified Thayer-Martin medium with carbon dioxide provided by placing a generating tablet in a chamber or plastic bag. Transport of specimens on culture media presents certain advantages, and several systems for this purpose are commercially available. The JEMBEC plate (fig. 2-5) contains a culture medium that is directly inoculated with the specimen. A bicarbonate tablet is placed in a small well along the side of the plate, and the covered plate is placed in a "zip-top" bag. The sealed bag is placed in an ambient air incubator at 35°C. Moisture evaporating from the medium during incubation activates the bicarbonate tablet, and a CO₂-enriched atmosphere is established inside the bag. Incubation for at least 24 hours prior to transport allows outgrowth of organisms and minimizes the loss of viability seen with the swab transport systems.

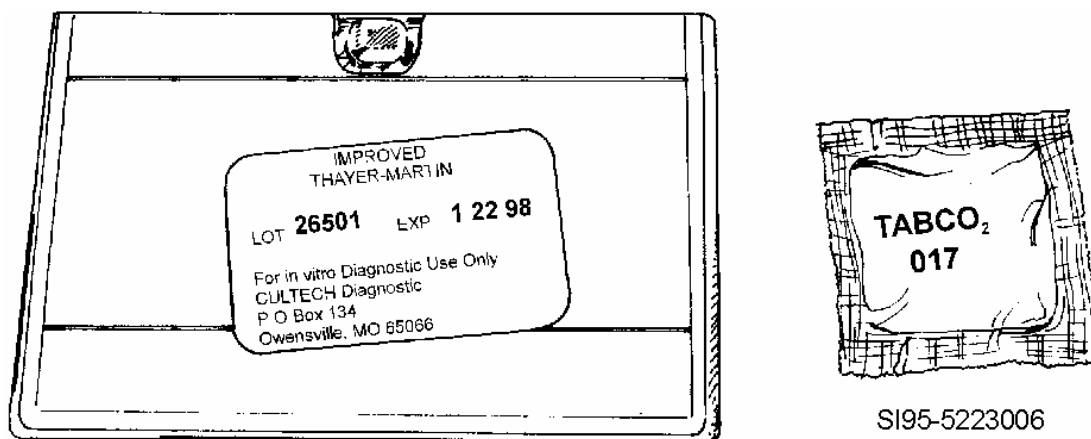


Figure 2-5. JEMBEC plate.

Alternate methods include placing inoculated Thayer-Martin plates immediately into a candle jar (fig. 2-6). If vancomycin-susceptible strains of *Neisseria gonorrhoeae* are suspected, the use of enriched chocolate agar should replace the Thayer-Martin plate.

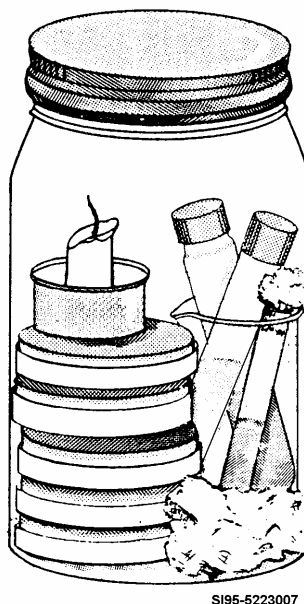


Figure 2-6. Candle jar.

Syphilis

Material from moist lesions is examined for the presence of *T. palladium* by darkfield microscopy. Phase contrast microscopy can be used, but spirochetes are most readily visualized by darkfield microscopy. Since treponemes are very sensitive to drying and oxygen, lesion material is examined as soon as possible after collection. Treponemal lesions are highly infectious; therefore, great care (wearing gloves, etc.) is taken during collection and all materials used discarded appropriately. Primary, secondary, and early congenital syphilis is frequently diagnosed by direct darkfield examination of material from the lesions. Oral lesions are not recommended as sample material for direct examination due to the presence of morphologically similar oral treponemes. A variety of methods exist for the collection of samples for examination. One such method is to clean the surface of the lesion with saline, blot dry, gently abrade the lesion surface until a slight amount of bleeding occurs, apply pressure at the base of the lesion until a clear exudate appears, touch the glass slide to the exudate, cover the material with a coverslip, and examine immediately. If an adequate amount of exudate is not produced, place a drop of saline on the lesion, collect, and examine for *T. palladium*.

Chlamydia

This organism can theoretically infect any tissue comprised of columnar epithelial cells. Thus, infection can occur if the agent is transmitted to the appropriate site where susceptible cells are located. It is essential to obtain an adequate amount of epithelial cells from the infected area to recover *C. trachomatis* in cell cultures. The most productive source for the recovery of *C. trachomatis* from the genital tract of men is the urethra. *Chlamydia trachomatis* conjunctivitis is the leading cause of blindness in the world. *C. trachomatis* is acquired by an infant during passage through the infected vaginal canal at birth. The following table lists other specimens used for the diagnosis of *Chlamydia*:

Genital Infections	
Females	Males
Urethra	Urethra
Cervix	
Fallopian tube (biopsy)	
Respiratory Infections	
Adults	Infants
Sputum	Throat
Conjunctiva	Nasopharyngeal aspirate
Lung tissue	Conjunctiva
	Lung tissue

Urethral swabs

Studies at the Mayo Clinic have indicated urethral swabs are three to five times more productive for yielding the organism than urinary sediments or urine specimens, respectively. Urethral exudate is not useful for the recovery of *C. trachomatis* since viable organisms are predominantly found in epithelial cells lining the urethra.

Number of swabs

Two swabs are required to yield the highest number of chlamydial isolations. One swab is inserted into the endocervix and another into the urethra, yielding an additional 15 percent isolation. Both specimens are extracted into a single vial of 2SP transport medium for delivery to the laboratory. Biopsy or other specimens yielding chlamydial-infected cells rather than exudate are the most productive in revealing infection in the fallopian tube. Nasopharyngeal secretions from children with pneumonia are ideally obtained with the use of an aspirating bulb. Alternatively, a throat specimen is collected with a swab, but it must be extracted into a vial containing 2SP at the time of collection. Specimens from an eye are collected by rubbing the swab over the conjunctiva. Any purulent exudate that is present should be wiped away before collecting the specimen for chlamydial diagnosis. Chlamydial pneumonia in adults appears to be uncommon, except, perhaps, in immunosuppressed hosts in which it was initially detected. Clearly in adults, more studies are needed to establish the role of respiratory tract infections due to *C. trachomatis*.

Calcium-alginate-tipped swabs

Calcium-alginate-tipped swabs are satisfactory for the collection of urethral specimens. The flexible wire shaft and narrow size make this swab more comfortable for patients than other swabs and culturettes since it is inserted 3 to 5 cm into the urethra. It has been reported that calcium-alginate-tipped swabs are inhibitory to *C. trachomatis* in the male urethra, whereas cotton and rayon are not. Other studies did not demonstrate an inhibitory effect of calcium alginate for *C. trachomatis*. As a possible explanation, there may be lot-to-lot and manufacturer variation in the cement used to hold the alginate fibers to the metal swab shaft. Alternatively, sterile, cotton-tipped applicators with plastic shafts can be used for the collection of male urethral specimens for *C. trachomatis* culture. Until routine testing for the presence of chlamydial antigens becomes widely available, adherence to the proper conditions for rapid delivery to the laboratory is necessary.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

010. Collection of specimens

1. Describe how the venipuncture site is prepared prior to drawing a blood culture.
2. Why should you cleanse the site with 70-percent alcohol once the needle is removed?
3. What is the purpose of binding iodine to macromolecular polymers?
4. What is the minimum amount of blood drawn for a blood culture? Why?
5. What is the amount of blood that can be used for infants and children depending on their size?
6. What is the preferred anticoagulant for use in blood culture bottles?
7. What can be done to eliminate the effect of small amounts of antimicrobial agents in the blood at the time of culture?
8. Why is the examination of CSF considered an emergency procedure by clinical microbiology personnel?
9. Do infants usually exhibit typical signs of meningeal irritation?
10. Meningitis in children under 6 years of age is caused primarily by what bacterial agent?
11. Why is a lumbar puncture performed under conditions of strict asepsis for the collection of CSF?

12. Why is an adequate sample especially important when attempting to diagnose a suspected case of tuberculous or fungal meningitis?
13. What type of container is used to collect CSF?
14. What three steps can you take to enhance the quality of the CSF specimens received by the laboratory?
15. If a CSF specimen can not be processed immediately, how should you store the fluid?
16. How are other sterile body fluids usually collected?
17. What is the anticoagulant of choice for use in collecting body fluid?
18. Define osteomyelitis.
19. What is the most common etiological agent of bone infection for all ages and how does it start?
20. Why are anaerobic containers usually not needed when collecting tissue specimens?
21. Why should smaller tissue samples be covered in sterile isotonic saline?
22. Sterile isotonic saline should not be used when culturing for *Legionella* spp. Why?
23. Define abscess.
24. What is the preferred collection method for abscesses?

25. Define wound.
26. What is the biggest challenge for the health care provider and microbiologist concerning wound cultures?
27. Why can eye and ear infections be life threatening?
28. Name the types specimens that can be submitted from the eye.
29. What are the two most common terms associated with eye infections?
30. What is a tympanocentesis and is this routinely performed?
31. Throat culture specimens are taken from what areas of the mouth?
32. Specimens for nose and sinus cultures may include what?
33. Specimens from the lower respiratory tract include what?
34. What is the primary problem encountered with most sputum specimens?
35. What is thought to be the reason for the poor recovery of *S. pneumoniae* from expectorated sputum samples for culture?
36. How is the recovery of *S. pneumoniae* from sputum improved even in the presence of endogenous oropharyngeal flora?
37. How is sputum induced from the lower respiratory tract?

38. Explain how induction of sputum by saline nebulization (aerosolization) works?
39. Nebulization is particularly well suited for the recovery of what organism?
40. What is the best way to collect uncontaminated specimens for culture from the lower respiratory tract?
41. Most kidney infections are acquired by what two routes?
42. What are the microorganisms most frequently associated with urinary tract infections?
43. List the factors that influence the growth of bacteria in urine.
44. What collection procedure is least likely to result in contamination of bladder urine?
45. Why is urethral catheterization of the bladder not recommended for collection of specimens for initial culture?
46. What is the simplest and most practical method of collecting urine for culture?
47. What should be done with a urine specimen if it cannot be taken directly to the laboratory?
48. What bacteria is especially sensitive to the changes in pH that occur with a drop in temperature of a fecal specimen?
49. What is the standard preservative suggested for use with fecal specimens?

50. What quantity of stool is considered a sufficient specimen?
51. What portion of a stool specimen is submitted for culture?
52. What anaerobic gram-negative rods have been associated with food-borne intoxication and pseudomembranous colitis?
53. What enrichments are suggested for use in collecting specimens suspected of containing *Salmonella* spp.?
54. What microorganism is involved in gastrointestinal disease but is not present in stool specimens and what is the specimen of choice?
55. Name at least five sexually transmitted diseases.
56. What types of infections are caused by *N. gonorrhoeae*?
57. What types of specimens are collected for the diagnosis of *N. gonorrhoeae* infections?
58. Why is great care taken during the collection and direct examination of material from primary and secondary lesions of syphilis?
59. Describe the collection procedure used to obtain material for direct darkfield examination of *T. palladium*.
60. What type of tissue is susceptible to infection by *C. trachomatis*?
61. What specimen is obtained from males with urethritis due to *C. trachomatis*?

011. Transporting, receiving, and shipping specimens

No matter how carefully and by-the-book we collect specimens, our efforts are in vain unless we know how to properly transport, receive, and ship the specimens.

How to transport specimens

Inefficiency in transporting specimens to the laboratory after they have been obtained from the patient is a major problem. When a specimen is lost, the loss becomes a convenient excuse for the clinician who left the specimen with allegedly responsible personnel who, in turn, instructed others to transport it to the laboratory. Although this activity is not directly under the jurisdiction of a clinical laboratory, every effort must be made to control this operation. The best possible way to obtain acceptable results is to insist transportation time be as short as possible. Some laboratories refuse to accept specimens if they have been in transport too long. In any event, ensure you properly annotate any aberrations of proper transport on the laboratory report.

Transport media

Microbiological specimens are transported to the laboratory by various means. Certain specimens should be transported in a medium or vehicle that preserves the organisms in the specimen and helps to maintain the ratio of one organism to the other in the specimen. This is especially important for those specimens in which normal flora may get mixed with microorganisms foreign to the location. It becomes even more necessary to use a transport medium if significant microorganisms are present in very low numbers. Nursing supervisors should remind appropriate personnel as often as required that prompt and proper transport of specimens from the clinics or wards to the laboratory is essential. Many laboratories have established a policy of refusing to process specimens that have been handled improperly. The Laboratory Guide should clearly delineate specimen transport requirements. When aberrations in specimen transport occur, this must be properly annotated. You must be ready to assist laboratory management with possible solutions if asked.

Transporting to the lab

A variety of media is used for sending cultures to the laboratory when the specimen cannot be inoculated within a few minutes after collection. Transport media do *not* contain carbohydrates or nitrogen sources necessary for replication, thereby preventing overgrowth of fastidious organisms by contaminating flora. Transport mediums are buffered, most are a semisolid agar, and may contain a variety of compounds designed to favor the survival of specific microorganisms. The main purpose of the medium is to protect the microorganisms from death by drying and to inhibit overgrowth by contaminants. Charcoal is used in certain types of transport media to neutralize the possible effect of toxic lipoproteins on cotton swabs.

Several similar types of transport media are in common use, and one, Cary-Blair, has been used successfully to recover *Shigella* from stool specimens after a 3-week journey from Thailand to Washington, DC. The use of culture swabs in plastic tubes containing a small ampule of a transport medium has improved the recovery of bacteria from cultures sent to the laboratory. Various commercially prepared transport systems are used and made for either aerobic or anaerobic specimens. Ordinary nutrient broth or anaerobic broth can be used when swabs or aspirates are involved. Certain specimens should be plated directly by the clinician (i.e., gonococcal specimens are immediately plated onto modified Thayer-Martin), then transported to the laboratory. For the isolation of *Bordetella pertussis*, direct inoculation of Bordet-Gengou agar is advocated. Many laboratories do not accept stool specimens for culture unless they are transported in various buffered preservatives.

Transport problems

When problems arise concerning transport of specimens, refer to your OI or listed references. *Manual of Clinical Microbiology*, published by ASM Press, or *Bailey & Scott's Diagnostic Microbiology*, published by Mosby, may be used.

Suitable transport media

It is important culture specimens be processed as soon as possible after collection, preferably within 2 hours for most specimens. If longer delays are unavoidable, a suitable transport medium/system or refrigeration must be used. Here are a few exceptions to the 2-hour rule:

- CSF should be delivered within 15 minutes.
- Corneal scrapings should be delivered within 15 minutes.
- Fluids, including gastric washing and amniotic, should be delivered within 15 minutes.
- Tissue specimens and tissue specimens in anaerobic transport systems should be delivered within 15 minutes.
- Feces should be delivered within 1 hour if unpreserved.

Guidelines

The following table lists guidelines concerning specimen transport and storage:

Specimens	Transport System	Transport and Storage Temperature
Autopsy tissue, bronchial wash, intravenous (IV) catheter, CSF for viral agents, lung biopsy sample, pericardial fluid, sputum, and urine, <i>except</i> suprapubic.	Sterile, leak-proof container with no preservative.	4°C
CSF for bacterial agents and synovial fluid.	Sterile, leak-proof container with no preservative.	25°C
Abdominal fluid, amniotic fluid, bile, cul-de-sac, deep lesion, lung aspirate, sinus aspirate, surgical tissue, transtracheal aspirate, suprapubic urine, and any other anaerobic culture.	Anaerobic transport system.	25°C
Blood cultures, corneal scrapings, cultures for <i>Bordetella</i> spp., gonorrheal specimens (JEMBEC), and vitreous humor.	Directly inoculated to appropriate medium.	25°C
Burn wound biopsy sample, <i>Campylobacter</i> spp., external ear sample, <i>Shigella</i> spp., <i>Vibrio</i> spp., and <i>Yersinia</i> spp.	Transport medium (See OI for specific transport mediums for individual cultures).	4°C
Bone marrow, <i>Bordetella</i> spp., cervical swab, conjunctive (eye) swab, <i>Corynebacterium</i> spp., internal ear swab, genital culture, nasopharyngeal swab, <i>Neisseria</i> spp., <i>Salmonella</i> spp., and upper respiratory culture.	Transport medium (See OI for specific transport mediums for individual cultures)	25°C

Vulnerability

Many species of bacteria are vulnerable to delays in processing, temperature changes, and decreased moisture. During prolonged transport, rapidly growing bacteria can overgrow more fastidious pathogens. For example, if urine samples are delayed, they should be refrigerated, inoculated to primary isolation medium before transport, or transported in preservative solution. Colony counts on urine samples are not valid if not processed within 1 hour of receipt because of the rapid doubling time of many urinary tract pathogens. If the urine is not

cultured within 1 hour, refrigerate the specimen (typically not more than twenty-four hours – follow your local policy). Boric acid can also be used for preservation of bacterial elements. Refrigerated transport or use of an acceptable urine preservative is recommended if the specimen is to be shipped.

Direct delivery

It is also well known that, if left at room temperature, a pathogen present in a specimen containing either normal flora or other organisms inadvertently introduced during collection is most likely to become overgrown by those organisms, thereby reducing the probability of its recovery. Hence, the cardinal rule is all specimens should be dealt with as expeditiously as possible. You are not measuring chemicals, enzyme levels, or body cells, but living, replicating organisms; therefore, the laboratory central processing section must deliver these specimens without undue delay.

Preserving by ability

If a delay in transport is anticipated or if cultures are sent to a reference laboratory, Stuart's, Amies, or Cary-Blair transport medium is normally used. Check the guide or OI of each referral laboratory you use to determine the appropriate measures to take. Dry swabs are unacceptable. A transport medium is formulated to maintain the viability of bacteria and allow only a slow rate of replication. Fastidious strains, however, may not survive the nutritionally poor medium. Some bacterial populations can double within 1 hour if body fluids are present.

Anaerobic specimen transport

The collection of specimens for anaerobic culturing poses a special problem—the conventional methods previously described do not lead to optimal recovery of these air-intolerant microorganisms. A crucial factor in the final success of anaerobic culturing is the transport of clinical specimens. With anaerobic transport, the lethal effect of atmospheric oxygen on these organisms is nullified until the specimen has been processed anaerobically in the laboratory. The use of a double-stoppered collection tube or vial, gassed out with oxygen-free CO₂ or N₂, and containing an agar or broth indicator system, as shown in figure 2-7, is recommended for anaerobic specimens. You inject the specimen (pus, body fluid, or other liquid material) through the rubber stopper after first expelling all air from the syringe and needle. In the laboratory, the material is aspirated from the transport container by needle and syringe, inoculated to media, and incubated under anaerobic conditions. The syringe, with a sterile plug, can be used for direct transport to the laboratory if the specimen can be processed within 30 minutes of collection. Various types of commercial anaerobic transport systems can also be purchased. Anaerobic transport specimen vials with an anaerobic indicator are available from BBL (Cockeysville, MD) or GIBCO Laboratories (Lawrence, MA). Anaerobic transport systems for swabs are available from BBL and Marion Scientific (Kansas City, MO). Some anaerobic culture swabs generate their own anaerobic atmosphere and contain small amounts of transport medium to prevent dehydration.

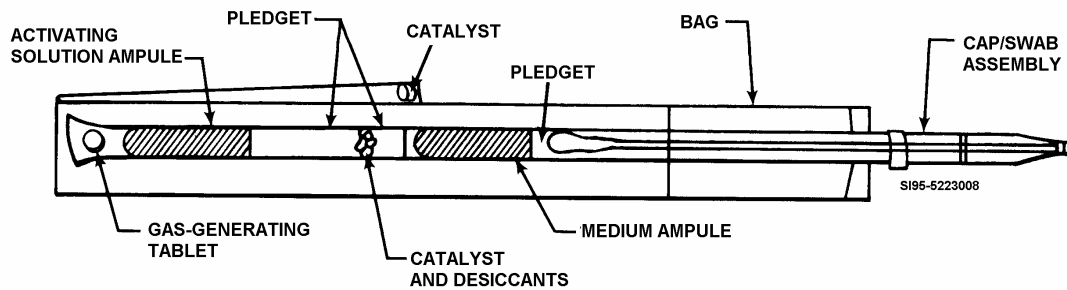


Figure 2-7. Anaerobic culture swab.

Bioenvironmental bags

Bioenvironmental bags, as shown in figure 2-8, that generate an oxygen-free environment are used to transport specimens or directly plated specimens anaerobically. They are not suitable for swabs because no specific means are incorporated to maintain a moist environment, nor is there a holding medium. They can be used to transport specimens in plastic syringes and smaller specimens aspirated (through an attached syringe) into small-gauge sterile plastic tubing. It can also be used for tissue specimens placed in a sterile container, then placed into the bag, if a long delivery time is anticipated.

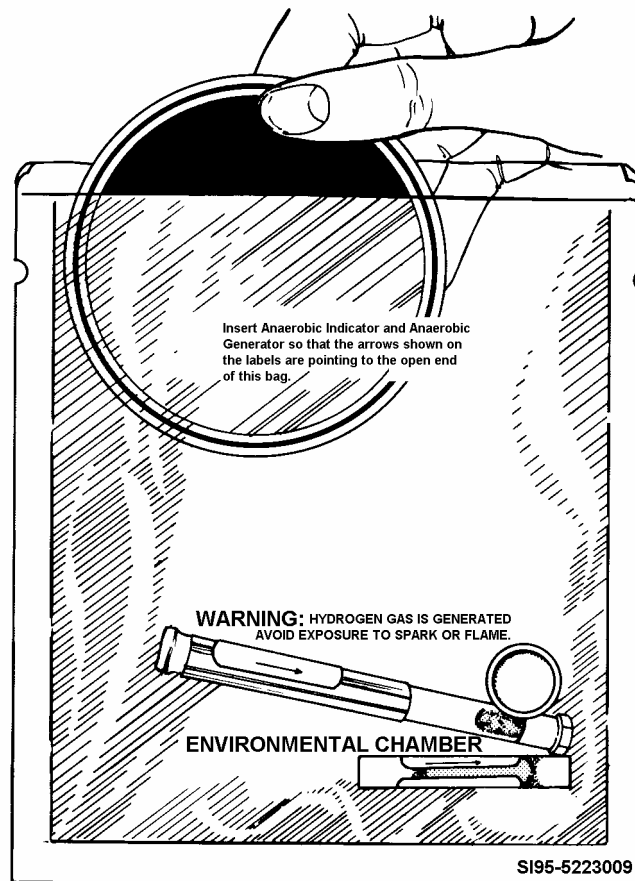


Figure. 2-8. Bioenvironmental bag.

Transport materials

It is important to note the materials recommended for anaerobic transport actually constitute ideal universal transport systems since all types of microorganisms should survive well in them. It should be stressed that, although the swab is the most widely used transport vehicle, it is preferable to submit a larger specimen whenever possible (i.e., a filled syringe, tube, or tissue). When organisms are scarce (as in some forms of tuberculosis), the larger the specimen, the better.

Material not in supportive environment

All laboratory personnel must be aware that submitted material not in a supportive environment must be handled immediately, especially spinal fluid, urine, pus, or any material not transported in a preservative. All smears should be fixed and stained immediately. In addition, laboratory personnel doing the primary culturing of specimens should acquaint themselves with the clinical diagnosis and any other pertinent laboratory findings, especially with select specimens such as blood spinal fluid, wounds, burns, and so forth. Thus, hematological and chemical data, immunochemical findings on levels of immunoglobulins, complement, and so forth, are frequently helpful in interpreting the findings of the laboratory and planning for the isolation of certain specific microorganisms. Laboratory personnel must also remember to handle all clinical specimens and cultures with appropriate attention to laboratory safety for themselves and coworkers.

Receiving and rejecting specimens

Although the laboratory is not responsible for specimen collection, the person receiving such specimens has the responsibility of ensuring the laboratory is given sufficient clinical information to process the specimen correctly.

Personnel

The most important skill personnel receiving and processing specimens should possess is the ability to communicate with other staff members, as well as the general public. Additionally, they should also possess good memories and the ability to work quickly and without constant supervision. Training of new personnel to fill positions in the receiving area should be given top priority. The training should be thorough and conducted by experienced technicians. Training emphasis should be not only on the development of technical skills, but also on human relations skills. In order to accomplish the job of interpreting and correlating results accurately, specific questions must be constantly addressed, such as those discussed here, by laboratory personnel.

Has the specimen submitted for culture been properly collected or obtained?

Although, whether the culture has been properly collected or obtained, is often out of the direct control of laboratory personnel, they must assume some responsibility in seeing it is carried out correctly. By voicing your concern that collection procedures are done correctly, you can have an impact on decisions that ensure proper emphasis is placed on this aspect of laboratory quality control. Remember, the proper collection of specimens for culture is the most important step in the recovery of microorganisms responsible for infectious disease.

Has the specimen been properly delivered to the laboratory?

Prompt delivery of all specimens to the laboratory is essential in providing quality health care to patients. Once collected, specimens should be processed as soon as possible. A delay in the transport of a specimen to the laboratory can diminish the success of a diagnostic investigation. The diagnosis of an acute infection becomes more difficult with time since

some pathogens are extremely delicate and sensitive to slight changes in environmental conditions. Therefore, it is your responsibility to emphasize its importance in your verbal and written communications with the professional staff responsible.

Is the request clearly understood?

If the request is not clearly understood, communication is the answer. Call the person transcribing the doctor's orders to clarify the request. Once the discrepancy has been resolved, process the specimen as soon as possible. If the discrepancy cannot be immediately resolved, a judgment call on your part must be made as to what procedures are being requested and the process carried out. A note in the specimen log explaining the problem and the basis for your decision is warranted in such a situation and for all intents and purposes is required.

Does the necessary minimal patient information and history appear on the request?

Complete, legible patient information is essential for proper identification and accessioning of all specimens received. Good quality control demands every effort be made to identify each specimen in such a way the chance of confusion between it and any other specimen being processed is kept to an absolute minimum. A laboratory computer-generated accession number, unique to each specimen, in combination with the patients' name and social security number (SSN), ensures proper identification of each specimen, as well as providing a means of monitoring the specimen's progress throughout the diagnostic loop.

Has the specimen been taken from a site that is normally sterile?

Sterile body fluids should be collected and sent to the laboratory in a sterile, screw-capped tube or anaerobic vial. An adequate volume of fluid is at least from 3 to 5 ml of CSF and 10 ml of other body fluids. When this amount is not available, the physician should send as much as possible. Surgically obtained tissue should be placed in a sterile, wide-mouthed jar and should represent the pathologic process. Specimens to be cultured for anaerobes are placed in anaerobic transport systems in which air has been replaced with an oxygen-free gas. Swabs are less satisfactory for anaerobic culture; however, if they are used, they must be placed in a pre-reduced anaerobically sterilized (PRAS) transport medium. Anaerobic cultures are set up immediately. If there is a delay in processing, the specimens are kept at room temperature for no longer than 2 hours.

Specimen accessioning and labeling

Centralized processing of all laboratory specimens is the current trend. The two primary driving forces toward centralized receiving and processing are the application of computer technology to specimen accessioning and an increased emphasis on quality control. The primary advantage of centralized processing is the development of an audit trail, enabling the laboratory to track a sample as it progresses through the workflow. The ability to track a sample can provide a great deal of valuable technical and administrative information to both laboratory and hospital staff at a moment's notice. In addition, data concerning quality control, turn around times, and workload recording is available. All specimens received in the laboratory must be properly labeled and accompanied by the correct laboratory requisition. Standard Forms (SF) 553 and 554, Microbiology I and II, respectively, are used, or you use forms unique to your laboratory. Local forms can simplify requisitioning and mesh with your particular specimen processing system whether it is computerized, manual, centralized, or decentralized. All forms accompanying a specimen should have the appropriate blocks filled in with the pertinent information. Properly completed forms, with attention paid to possible diagnosis and antibiotic therapy, aid you in isolating pathogenic agents with a minimum loss of time and greater efficiency.

You label each specimen container in such a way that, if the request form is separated from the specimen, the two can be quickly identified as belonging to each other. The label is completed by ward or clinic personnel and shows the name of the patient, the register number, the ward location and bed number, patient status, and any other significant data. Once an acceptable specimen reaches the receiving area, laboratory labels are affixed, the requisition is time stamped, and Uniform Chart of Accounts (UCA) and common procedural terminology (CPT) data are recorded. The specimen is then transported as soon as possible to the microbiology section for further processing.

Rejecting Criteria

Laboratory personnel must reject specimens not obtained in a proper manner and should be supported in this position by infectious disease clinicians and pathologists. In rejecting specimens, of course, the reasons should be explained to the requesting physician. Some examples of situations where specimens may be rejected are no label, prolonged transport, specimens unsuitable for request, improper or leaking container, and duplicate specimens on same day for the same request, except blood. Specimens should never be arbitrarily discarded before a discussion with the requesting clinician. Some specimens, such as those taken at the time of surgery, are difficult or impossible to replace. Specimens that cannot be replaced are Gram-stained and interpreted as carefully as possible. Guidelines for specimen rejection criteria should be spelled out in the OI.

How to ship specimens

At some point in your career, you may be required to ship a specimen to a reference laboratory using the United States Postal Service (USPS). Shipping specimens requires not only a knowledge of the specimen itself, but also of current directives and postal regulations concerning shipment.

Shipment of specimens

All microbiology specimens to be transported through the USPS have to be packaged under strict regulations formulated by the Public Health Service. A complete list of etiologic agents included under these regulations is available on request from the CDC and is also included with a presentation of several recommended standard procedures published by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards or NCCLS).

Packaging of specimens

Package specimens properly to protect them in transit, and also to protect the personnel handling them. Never mail specimens in Petri dishes. Do not place dry ice inside a thermos or enclose in hermetically sealed containers. Pressure builds up as CO₂ is liberated and could explode the thermos or containers.

Regulation

Part 72 of Code of Federal Regulation (CFR) 42, *The Public Health and Welfare*, defines etiologic agents and provides guidelines for transportation of such specimens. You must follow these rules without deviation. Since most bacteriological specimens are shipped in volumes less than 50 ml, the rules for such quantity are discussed in the following paragraph.

Volumes less than 50 ml

When volume is less than 50 ml, the material is placed in a securely closed, watertight container (primary container—test tube, vial, etc.) that is enclosed in a second, durable watertight container (secondary container). Several primary containers are enclosed in a

single secondary container if the total volume of all primary containers so enclosed does not exceed 50 ml. The space at the top, bottom, and sides between the primary and secondary containers must contain sufficient non-particulate absorbent material to absorb the entire contents of the primary container(s) in case of breakage or leakage. Each set of primary and secondary containers are then enclosed in an outer shipping container constructed of corrugated fiberboard, cardboard, wood, or other material of equivalent strength. Following these guidelines protects the specimen from damage and also protects personnel handling the package in case of unexpected damage.

Labeling

Mailing containers have labels affixed on the outside that read like that shown in figure 2-9. The label, required on shipments of etiologic agents, conforms to specifications published in CFR 42, Part 72, Subpart 72.2.

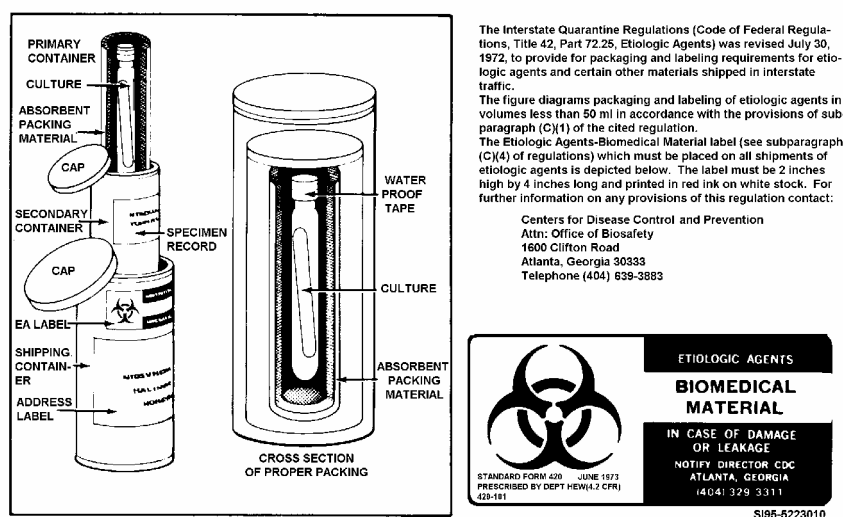


Figure 2-9. Packaging biomedical material.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

011. Transporting, receiving, and shipping specimens

1. For what types of specimen is it especially important to use transport medium?
2. How are transport media formulated? Why?
3. What are the main purposes of transport media?
4. What function does charcoal play in certain types of transport media?

5. What type of transport medium is recommended for gonococcal specimens?
6. What agar is suggested for the isolation of *B. pertussis*?
7. When is the best time for specimens to be processed?
8. If urine samples are delayed in transit to the laboratory, what should be done?
9. What are the three transport mediums normally used?
10. Although swabs are the most widely used transport vehicle for anaerobic specimens, what is the preferred specimen?
11. What is the most important skill that personnel receiving and processing specimens should possess?
12. What are some of the questions laboratory personnel should ask themselves in order to accomplish the job.
13. What is the current trend in processing laboratory specimens?
14. What are the two primary driving forces toward centralized receiving and processing?
15. What is the primary advantage of centralized processing?
16. What are some of the reasons specimens may be rejected?
17. You should never discard a specimen before a discussion with whom and why?

18. Where should rejection criteria be spelled out?
19. Shipping specimens requires not only a knowledge of the specimen itself but also of what?
20. Where can you find a list of the etiological agents and the regulations?
21. Specimens should be packaged properly for what two brief reasons?
22. What could happen if dry ice is placed inside a thermos or enclosed in a hermetically sealed containers?
23. What federal regulation defines etiologic agents and provides guidelines for transport of such specimens?

Answers to Self-Test Questions

008

1. So that specific counteractive measures, such as adding penicillinase or merely diluting the specimen, can be carried out.
2. Resistant organisms colonize as normal flora is suppressed.
3. (1) Collect specimens using aseptic techniques.
(2) Bypass areas of normal flora.
(3) Culture only for specific pathogens.
(4) Quantify culture results.
4. During the acute phase or diarrheal stage.
5. Patient education.
6. In most instances, hold the specimen at 4°C in a refrigerator for several hours before culturing, but always process it by the end of the shift upon which it arrived.
7. Because normal flora anaerobes are often present in such large numbers that even minimal contamination of a specimen with normal flora can give very misleading results.
8. Because the distal portion of the urethra and the meatus are colonized with a normal flora containing anaerobes.
9. Suprapubic bladder catheter, cystostomy, or nephrostomy tube.
10. Following childbirth.
11. *Bacterioides fragilis*.

12. By using a needle and syringe.

009

1. The specimen obtained must be representative of the disease process, and a sufficient amount of material must be obtained to assure a complete and accurate examination.
2. Infected tissue.
3. Material from the active margin of the lesion.
4. *Clostridium difficile* and *Clostridium botulinum*.
5. Type of infectious disease process and the ability of the laboratory to process samples.
6. Only after consultation with the microbiology staff, OIC, or pathologist.
7. By the clinical condition of the patient.
8. Stool specimens.
9. Aspiration with a sterile syringe and needle.
10. They are immediately placed in a gassed tube or suitable anaerobic packet.
11. Mechanical rinsing of the mouth immediately before expectoration.
12. No, it is virtually impossible to recover acid-fast bacilli from fecal material because of heavy overgrowth with bowel flora.

010

1. (1) Cleanse the area with 70-percent alcohol using enough friction for mechanical cleansing as well.
(2) Start centrally and move out in ever enlarging circles. Repeat this several times, using a new swab each time.
(3) Allow to air dry.
(4) Starting in the center, apply 2-percent tincture of iodine or providone-iodine; also, move in enlarging circles towards the outside. Make sure the entire area is saturated with the iodine.
(5) Allow to air dry for at least 1 minute using a watch or timer.
2. Because many patients are sensitive to iodine.
3. Creation of an antiseptic with reduced toxicity while maintaining its antimicrobial activity.
4. 10 ml, the culture of lesser amounts is associated with a significant decrease in recovery of certain bacteria.
5. 1–5 ml.
6. Sodium polyanetholsulfonate (SPS).
7. A 1:20 dilution of blood to culture broth.
8. Bacterial meningitis is a rapidly fatal disease if untreated or inadequately treated.
9. No.
10. *Haemophilus influenzae* serotype b.
11. Contamination could confuse the identification of the etiological agent.
12. Very few organisms may be present.
13. Sterile screw cap containers or tubes.
14. (1) Suggest that the lumbar puncture tray, routinely used in the hospital, be examined to ensure that the CSF containers are of satisfactory quality.
(2) Attempt to establish a standardized skin preparation.
(3) Develop a system whereby the specimen can be transported promptly to the laboratory.
15. The CSF should be incubated or left at room temperature; refrigerated or frozen for viral studies.
16. By percutaneous needle aspiration performed aseptically.
17. Sterile heparin without preservatives.

18. Infection of the bone.
19. *Staphylococcus aureus* that starts out as bacteremia.
20. Because anaerobic bacteria survive within the tissue long enough to be recovered from culture.
21. To prevent drying.
22. Sterile saline may inhibit growth of *Legionella* spp.
23. A localized collection of pus appearing in acute or chronic infections of tissues, organs, or confined spaces.
24. Needle aspiration.
25. A bodily injury caused by physical means with interruption of continuity.
26. Distinguishing between the causative agent of the infection or the colonization or contamination from unrelated microorganisms.
27. Because of their contact with numerous blood vessels and proximity to the central nervous system.
28. Swabs, scrapings from lesions, specific tissues within the eye, or, possibly, fluid or fluid-like material.
29. Conjunctivitis and keratitis.
30. A surgical puncture of the tympanic membrane for removal of fluid from the middle ear; no.
31. Both tonsillar areas, the posterior pharynx, and any areas that show inflammation.
32. Nasopharyngeal swabs, sinus washings, drainage fluid, aspirated nasopharyngeal secretions, and surgical biopsy specimens.
33. Sputum, induced sputums, nasotracheal or endotracheal catheter, bronchoscopy bronchoalveolar lavage, protected bronchial brush catheter, and transtracheal aspirates.
34. They are heavily contaminated with oropharyngeal secretions and bacteria and may contain only small amounts of secretions from the lower respiratory tract.
35. Evidence suggests that interaction with other organisms or products from organisms found in the oropharynx suppresses the growth of pneumococci.
36. By using blood agar containing gentamicin.
37. By ultrasonic or heated saline nebulization.
38. Increases the moisture content of the air going to the lower respiratory tract and improves the ability of the tracheobronchial cilia to bring up secretions.
39. *Mycobacterium tuberculosis*.
40. Translaryngeal aspiration.
41. Ascending (bacteria migrating up from the bladder) and descending (bacteria spread hematogenous to the kidneys).
42. *Escherichia coli*, *Klebsiella*, and *Proteus* spp.
43. pH, osmolality, and urea content.
44. Suprapubic puncture.
45. Because of the possibility of introducing bacteria into the bladder.
46. Midstream urine during voiding.
47. It should be refrigerated.
48. *Salmonella* spp.
49. 0.033 M phosphate buffer mixed with equal volumes of glycerol.
50. 0.5–2g.
51. Portions displaying mucus, blood or both.
52. *Clostridium perfringens* and *Clostridium difficile*.
53. Tetrathionate broth and selenite F.
54. *Helicobacter pylori*; gastric biopsy material.

55. Any five of the following:
- (1) Human immunodeficiency virus (HIV).
 - (2) Human papillomavirus (HPV).
 - (3) Chlamydia.
 - (4) *Gardnerella vaginalis*.
 - (5) Gonorrhoeae.
 - (6) Syphilis.
 - (7) *Ureaplasma urealyticum*.
 - (8) *Mycoplasma hominis*.
56. Uncomplicated urethritis and cervicitis in men and women, anorectal gonococcal infection, oropharyngeal gonococcal infection, ocular gonococcal infection, gonococcal arthritis, acute pelvic inflammatory disease, acute epididymitis, and disseminated gonococcal infection.
57. The organism may be recovered from local genital sites (cervix, urethra), extra-genital sites (oropharynx, rectum), or systemic sites (blood, skin lesions, joint fluid) in the case of disseminated disease.
58. Treponemal lesions are highly infectious.
59. Wearing gloves, clean the surface of the lesion with saline, blot dry, gently abrade the lesion surface until a slight amount of bleeding occurs, apply pressure at the base of the lesion until a clear exudate appears, touch the glass slide to the exudate, cover the material with a coverslip, and examine immediately.
60. Any tissue comprised of columnar epithelial cells.
61. Urethral swabs.

011

1. Specimens in which normal flora may get mixed with microorganisms foreign to the location, and even more necessary if significant microorganisms are present in very low numbers.
2. They are formulated without carbohydrates or nitrogen necessary for replication, thereby stopping or slowing down replication and preventing the overgrowth of fastidious organisms.
3. To protect the organism from death by drying and to inhibit overgrowth of contaminants.
4. It neutralizes the possible effect of toxic lipoproteins on cotton swabs.
5. Modified Thayer-Martin.
6. Bordet-Gengou.
7. As soon as possible or preferably within 2 hours.
8. They should be refrigerated, inoculated to primary isolation medium before transport, or transported in preservative solution.
9. Stuart's, Amies, and Cary-Blair.
10. A filled syringe, tube, or tissue.
11. The ability to communicate with staff members, as well as the general public.
12. (1) Has the specimen submitted for culture been properly collected or obtained?
(2) Has the specimen been properly delivered to the laboratory?
(3) Is the request clearly understood?
(4) Does the necessary minimal patient information and history appear on the request?
(5) Has the specimen been taken from a site that is normally sterile?
13. Centralized processing.
14. Computer technology and increased emphasis on quality control.
15. Development of an audit trail.

16. No label, prolonged transport, specimens unsuitable for request, improper or leaking container, and duplicate specimens on same day for the same request, except blood.
17. The requesting clinician; some specimens, such as those taken at the time of surgery, are difficult or impossible to replace.
18. The OL.
19. Current directives and postal regulations concerning shipment.
20. On request from the CDC and is also included with a presentation of several recommended standard procedures published by the National Committee for Clinical Laboratory Standards.
21. To protect them in transit and to protect the personnel handling them.
22. The thermos or container could explode.
23. CFR 42, Part 72.

Do the unit review exercises before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter.

21. (008) The *most important* step in the recovery of microorganisms responsible for infectious disease is
 - a. proper specimen collection.
 - b. prompt specimen delivery to the laboratory.
 - c. distinguishing normal flora from pathogens.
 - d. the correct choice of primary plating media.
22. (008) Not only does refrigeration preserve the viability of most pathogens, it also
 - a. produces anaerobic conditions.
 - b. prevents drying out of the specimen.
 - c. minimizes overgrowth of normal flora.
 - d. provides for an artificial culture medium.
23. (008) What specimen is suitable for anaerobic culture?
 - a. Voided urine.
 - b. Coughed sputum.
 - c. Bronchoscopic specimens.
 - d. Transtracheal aspiration specimens.
24. (009) The *optimal* times for specimen collection are based on both the type of
 - a. microbiology equipment and the availability of the doctor.
 - b. infectious disease process and the availability of the doctor.
 - c. microbiology equipment and the ability of the laboratory to process samples.
 - d. infectious disease process and the ability of the laboratory to process samples.
25. (009) Containers used for most specimens must be sterile, *except* with
 - a. stool specimens.
 - b. sputum specimens.
 - c. wound culture specimens.
 - d. cerebrospinal fluid (CSF) specimens.

26. (010) What is the *minimum* amount of specimen collected for each bottle in a two-bottle blood culture resulting in a final recommended dilution ratio of 1:10 on an adult patient?
- 5 ml.
 - 10 ml.
 - 15 ml.
 - 30 ml.
27. (010) What is *not* a characteristic of sodium polyanethosulfonate (SPS)?
- Antifungal.
 - Anticoagulant.
 - Antiphagocytic.
 - Anticomplementary.
28. (010) What dilution of blood-to-culture broth is sufficient to eliminate the effect of small amounts of antimicrobial agents in the blood at the time of culture?
- 1:5.
 - 1:10.
 - 1:20.
 - 1:30.
29. (010) To obtain cerebrospinal fluid (CSF), the spinal needle is carefully placed between lumbar vertebrae
- L1 and L2.
 - L2 and L3.
 - L3 and L4.
 - L4 and L5.
30. (010) How many tubes of cerebrospinal fluid (CSF) are usually collected and which tube is sent for culture?
- 2–3; first tube.
 - 2–3; third tube.
 - 3–4; second tube.
 - 3–4; fourth tube.
31. (010) If the cerebrospinal fluid (CSF) *cannot* be processed immediately for bacterial studies, it is
- incubated or frozen at -70°C .
 - refrigerated or frozen at -70°C .
 - incubated or stored at room temperature.
 - refrigerated or stored at room temperature.
32. (010) The anticoagulant of choice for the collection of body fluids is
- sodium citrate.
 - heparin with preservatives.
 - heparin without preservatives.
 - ethylene diamine tetra-acetic acid (EDTA).
33. (010) The *most common* etiological agent of bone infections for all ages that starts out as bacteremia is
- S. aureus*.
 - C. perfringens*.
 - S. pneumoniae*.
 - N. gonorrhoeae*.

34. (010) What antibiotic is added to blood agar to partially suppress endogenous oropharyngeal flora and improve the recovery of *S. pneumoniae*?
- Penicillin.
 - Ampicillin.
 - Gentamicin.
 - Tetracycline.
35. (010) The *best* way to collect uncontaminated specimens for culture from the lower respiratory tract is
- bronchoscopy.
 - bronchial brushing.
 - tracheal cannulation.
 - translaryngeal aspiration.
36. (010) The *simplest, most practical* method for collecting a urine sample is
- urethral catheterization.
 - midstream collection.
 - suprapubic puncture.
 - 24-hour collection.
37. (010) What transport medium is *best* when culturing for *Campylobacter* and *Vibrio* spp.?
- Amie's.
 - Stuart's.
 - Cary-Blair.
 - Bordet-Gengou.
38. (010) What portion of a stool specimen is cultured for *optimum* recovery of pathogens?
- Discolored portions.
 - Portions displaying mucus or blood.
 - Interior portions of formed specimens.
 - Exterior portions of formed specimens.
39. (010) What anaerobic bacteria found in stool specimens is the etiological agent of pseudomembranous colitis?
- Bacteroides fragilis*.
 - Bacteroides uniformis*.
 - Clostridium difficile*.
 - Clostridium perfringens*.
40. (010) What sexually transmitted disease (STD) is associated with cervical carcinoma and possibly penile malignancy, and is present in epidemic proportions in the United States?
- Gonorrhoeae.
 - Bacterial vaginosis.
 - Human papillomavirus (HPV).
 - Human immunodeficiency virus (HIV).
41. (010) What is *usually* the causative agent of ophthalmia neonatorum?
- Treponema pallidum*.
 - Neisseria gonorrhoeae*.
 - Herpes simplex type II.
 - Chlamydia trachomatis*.

42. (010) In suspected cases of disseminated gonococcal infection, besides the urethral and cervical specimens, the *specimen of choice* is
- blood.
 - joint fluid.
 - throat fluid.
 - pleural fluid.
43. (010) What type of tissue can *C. trachomatis* infect?
- Squamous epithelial.
 - Columnar epithelial.
 - Caudate epithelial.
 - Extant epithelial.
44. (010) What specimen is obtained from men with urethritis for the isolation of *C. trachomatis*?
- Metal swab.
 - Urethral swab.
 - Serum sample.
 - Urine sediment.
45. (011) What ingredient is added to certain types of transport media to neutralize the possible effects of toxic lipoproteins on the cotton swab?
- Nitrogen.
 - Charcoal.
 - Carbohydrates.
 - 0.033 M phosphate buffer.
46. (011) A crucial factor in the final success of anaerobic culturing is the transport of clinical specimens
- in a culturette.
 - in the presence of atmospheric oxygen.
 - without the presence of atmospheric oxygen.
 - on ice to keep the specimen at refrigerator temperature.
47. (011) *Never* discard a specimen until you have discussed it with the
- NCOIC.
 - pathologist on call.
 - nursing supervisor.
 - requesting clinician.
48. (011) What specimen transport apparatus is *not* acceptable to mail?
- Plastic screw-cap vial.
 - Glass test-tube.
 - Petri dish.
 - GasPak.

Please read the unit menu for unit 3 and continue ➡

Unit 3. Microbiology Media, Media Selection, and Isolation and Cultivation of Bacteria

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BACTERIA had been grown in culture broths for some time before Robert Koch, the German physician, who began experimenting with solid media in the 1800s. He first used 2- to 5-percent gelatin that worked well in cool temperatures or when the bacteria did not hydrolyze the gelatin. Frau Hesse, wife of one of his investigators, suggested agar-agar, a seaweed preparation commonly called agar. Agar came to European kitchens from Java and was used in preparing jellies. This new material seemed inert to the action of bacteria.

3–1. Microbiology Media

Since Robert Koch's introduction of solid culture media for growing and isolating bacteria, the field of bacteriology has expanded and prospered. A great deal of research has been done to prepare an artificial medium that is similar to the environment each organism prefers in nature. In order for bacteria to build up their cells and reproduce, the elements necessary for the various cell structures and functions must be supplied in the immediate environment.

012. Microbiology media general information and storage

The proper preparation and storage of a medium is as important as its selection. We should reemphasize here that in preparing any medium from commercially manufactured ingredients, you always follow the manufacturer's directions explicitly. Literally hundreds of different media have been formulated, each with its distinct recipe. Each is designed to meet the nutritional and other needs of the organism or organisms it was designed to support.

General classes of media

No single culture medium is satisfactory for cultivating all bacteria. A general-purpose or basal culture medium has sources of carbon, nitrogen, and inorganic salts; but the addition of other substances, such as blood, serum, amino acids, or vitamins, are required for growth of pathogenic organisms. The basic energy sources are supplied by water extracts, or infusions of meat, vegetables, or yeast. Other complex energy sources include hydrolysates or digests of protein, that yield the nitrogen containing amides, amino acids, peptides, peptones, and proteoses. Additional energy sources are supplied by carbohydrates, alcohols, organic acids, glycosides, or similar materials. Carbohydrates, especially glucose, are often incorporated into media for the primary isolation of pathogens. When studies for hemolysis are performed,

carbohydrates are excluded from blood agar, for they tend to inhibit the bacterial production of certain hemolysins. Culture media is either liquid or solid. You prepare a solid medium by adding agar to a liquid nutrient medium, which you then heat with added agar powder or flakes to melt the agar, and then sterilize it. At a temperature of 96°C, agar melts and goes into solution. When it cools to 40°C, the agar solidifies and congeals the medium. You generally use agar in 1.5- to 2.0-percent concentrations in preparing solid media. Lower concentrations of 0.5 to 1 percent give a semi-solid medium, ideal for performing motility checks of pure cultures.

Background/historical information regarding media preparation

As previously mentioned, agar is an inert, purified extract of *Gelidium*, a type of seaweed not metabolized by bacteria. Your main purpose in culturing bacteria is to produce pure cultures on a solid medium. Therefore, a very important factor in media preparation is sterility. Media must be free of bacteria before inoculation so growth of the organism from a clinical specimen is not masked or inhibited by contaminants. The most common means of sterilizing media is by the autoclave, in which steam under pressure is used to reach the necessarily high temperature of about 121°C. However, caution must be used—prolonged heating at high temperatures can change the composition of a medium. For example, excessive heat chemically breaks down lactose broth, and sometimes demonstrates a positive lactose fermentation (false positive) by organisms that do not ferment lactose. Increased heat also makes a medium more acidic than desired or forms an undesirable precipitate that is visible in the medium. The gelling ability of the medium can also be destroyed. The vast majority of laboratories commercially purchase growth media, preparing just a handful of select media in-house.

Media quality control

Media quality control can be as simple as a visual check of commercially prepared and purchased media to in-depth quality control (QC) checks for media prepared in-house. Your laboratory should subscribe to the Clinical and Laboratory Standards Institute (formerly the NCCLS) publications that contain the approved standard *Quality Assurance for Commercially Prepared Microbiological Culture Media; M22-A2*, for media QC. All media QC should address sterility, ability to support growth, produce characteristic colony morphology, and, as appropriate, selectivity, inhibition, and/or biochemical response. With purchased media, as long as the manufacturer complies with the Clinical and Laboratory Standards Institute's standards and is in the table listed in the standard, you do not have to perform QC, except for visual checks. However, Chocolate agar, *Campylobacter* media, and media for the selective isolation of the pathogenic *Neisseria* should be monitored just as the media prepared in-house.

Visual checks

Visual checks include looking for cracked or broken Petri dishes or tubes and contamination (due to microorganisms, dirt, water, etc.). Also, you should look at the color and consistency (bubbles or lumps) of the media. Color changes can occur if the media gets too hot or cold during shipment, or is allowed to sit out on the "back dock," or in the supply receiving area too long before refrigeration. We know that red blood cells hemolyze if frozen or heated, so what do you think happens to the 5-percent sheep's blood agar plate (SBAP) if exposed to extreme temperature changes? You're right! The red blood cells within the agar hemolyze and the agar can become clear or darken. It is difficult to identify a hemolytic organism like *Streptococcus* group A on clear SBAP. If the conditions as stated above are present, you should not use the media, but discard it and request a replacement.

In-house media

If you prepare in-house media, your laboratory's OI should address the appropriate QC for that specific media. QC procedures, organism maintenance, and tables listing appropriate QC organisms can be found in the Clinical and Laboratory Standards Institute's *M22-A2* and a number of other good references (newer editions may be available); some examples are:

- Miller, J. M., *Quality Control in Microbiology*, Centers for Disease Control, 1987.
- Miller, J. M., Chapter 120 in the *Manual Clinical Microbiology*, 6th ed., American Society for Microbiology (ASM), 1995.
- Sewell, D. L., Quality Control in the *Clinical Microbiology Procedures Handbook* Vol. 2., ASM, 1992.

Storing prepared media

Once you prepare and pour the medium, and it is ready for storing, you must observe the following rules.

- Most commonly used media are refrigerated.
- Thioglycollate broth and semi-solid media are stored at room temperature. If the thioglycollate broth shows a pink or red layer at the surface, heat the tubes of media in a hot water bath before inoculation to drive off the excess oxygen before use. Heat the tubes only once; additional heatings make the broth unsuitable for bacterial growth.
- All plates are incubated at room temperature in an inverted position for at least 18 hours or overnight to check for sterility. If there is no growth, wrap or tape the plates in stacks and refrigerate. Additionally, a small percentage of plates are placed in an incubator for 18 hours as a further check of their sterility.
- When removed from the refrigerator, plates are allowed to warm to room temperature before use. Inoculation of a cold medium lengthens the lag phase of the growth curve and can even kill some organisms.
- Media containing a dye are protected from light during storage because some dyes break down under strong light.
- Surface areas of media must be moist (not wet or dry) for best results. Condensation of moisture on tubes and Petri dishes due to refrigeration is likely to increase the chance of contamination of the medium in those particular tubes and dishes. A wet surface makes it difficult to obtain isolated colonies and heightens the chances of contaminating an area of the laboratory while you are streaking the plate with a wire loop.
- If media are to be stored for more than 5 days, they must be protected from dehydration by enclosing them in suitable containers, such as plastic bags. A dried medium, manifested by the medium retracting from the edge of the tube or plate, or by a dry, dull surface, is not to be used because inaccurate results and abnormal growth is seen.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

012. Microbiology media general information and storage

1. What constituents are found in a general-purpose or basal culture medium?
2. What substances are added to a basal medium in order to grow pathogenic organisms?
3. Are carbohydrates included in blood agar when studies for hemolysis are to be performed? Why?
4. What concentration of agar is used most often in preparing solid media?
5. What is agar, and is it metabolized by bacteria?
6. What is the approved standard that specifically addresses media quality control?
7. What functions should all media QC address?
8. How are thioglycollate broth and semisolid media stored?
9. What is done to thioglycollate broth showing a pink layer at the surface?
10. How are plate media checked for sterility?
11. Why are plate media allowed to reach room temperature before use?
12. What effect does a wet surface on an agar plate have on isolating colonies?

13. How are plate media stored if they are not to be used for more than 5 days?

013. What factors influence the media to be used?

It is important laboratory personnel keep their selections of media to a minimum to avoid costly duplication of purpose. The number of media available for initial plating is endless, and the choice should be based on the judgment of an experienced technician. The choice is influenced by factors, such as cost, preference, and commercial availability. Culture media is selected for general and specific purposes.

Basic nutrient media or supportive media

There are numerous media of this type. Initially, basic nutrient media in broth or solid form support the growth of many organisms. Most basic nutrient media are used for growing non-fastidious organisms that comprise the majority of bacteria. Trypticase soy broth (TSB), nutrient agar, and brain-heart infusion agar are good general mediums, but they have limitations. With TSB, some fastidious organisms do not grow. This is due to its glucose content that is metabolized to acid end products by certain bacteria, such as pneumococci, retarding growth. Fluid thioglycollate medium supports the growth of a wide variety of anaerobes. However, it should not be relied on too strongly, especially in the area of anaerobic bacteriology. The basic nutrient media are modified to promote or inhibit growth by addition of substances—enrichment, differential, and selective media are produced.

Enrichment media

It is frequently necessary to use an enrichment medium in the examination of fecal material for the presence of pathogenic bacteria. Enrichment media, because of their chemical composition, inhibit or destroy the normal intestinal flora, such as the coliforms (commensals), and promote the growth of *Salmonella* and *Shigella*. These organisms, if present in small numbers, are allowed to grow almost unrestricted, thus enriching the population of such forms. Examples of enrichment media are Tetrathionate broth, Selenite broth, and GN broth, which are used primarily in enteric bacteriology. In contrast, enriched media contain an added nutritive supplement. They are usually prepared by adding blood, serum, or other nutrients to the basic nutrient medium for the primary isolation and subculture of fastidious organisms, usually pathogens.

Differential media

Differential media are designed to separate various organisms, depending on carbohydrate utilization, fermentation, or oxidation, or enzyme activity. Some media are both differential and selective, such as eosin methylene blue (EMB) agar, MacConkey agar, and xylose lysine desoxycholate (XLD) agar. For example, XLD agar contains the carbohydrate (lactose) and indicator (phenol red). On this medium, *Salmonella* produces red, black-centered colonies. *Shigella* and *Providencia* produce red colonies, and *Escherichia*, *Citrobacter*, *Klebsiella*, *Proteus*, and *Enterobacter* produce yellow colonies. The lactose and phenol red are used to differentiate between lactose and nonlactose fermenters. If the lactose is fermented, an acid byproduct is produced that gives a visible (yellow) color change to the medium. Another example is the green metallic sheen produced by *E. coli* on EMB.

Selective media

Selective or inhibitory media inhibit the growth of certain organisms while permitting others to grow (i.e., *Salmonella* and *Shigella* (SS) agar, mannitol salt agar (MS), and phenylethyl alcohol agar (PEA) used for growing staphylococci and gram-positive cocci, respectively). It

must be remembered that these selective media are relative in their action and can suppress what they are designed to show. Phenylethyl alcohol does slightly suppress gram-positives, and Lester-Martin medium or Transgrow does suppress gonococci. Thus, after initial growth on selective media, the organism is transferred to more compatible media. In some cases, a selective medium can distinguish between genera. SS agar grows *Salmonella* and *Shigella* species, but brilliant green (BG) agar grows *Salmonella* and inhibits *Shigella*.

014. The general applications and purposes of media additives

There are many different reagents that have been added, or you can add to media for special purposes. Some reagents neutralize antibiotics that might be present in the specimen from a patient being treated for an infection, while others promote growth of specific organisms having unusual nutritional needs. Still others give instant identification of bacteria by color. Let's take a look at some examples you'll run across in your work in bacteriology.

Para-aminobenzoic acid (PABA)

This substance neutralizes the bacteriostatic effect of sulfonamides in culture media. Any medium inoculated with a sulfonamide-containing specimen can require the addition of 0.0002 percent PABA. If PABA is not available, you can use a similar concentration of procaine hydrochloride. This requirement for PABA particularly pertains to urine, blood, and fecal cultures from patients who may have received sulfonamide therapy.

Sodium thioglycollate

This is incorporated in media not only to permit the growth of anaerobic bacteria (because of its reducing action), but also to neutralize the bacteriostatic effect of mercurial and silver disinfectants. Mercury compounds are often used as preservatives in plasma, serum, biologicals, and pharmaceuticals intended for parenteral injection. You use thioglycollate media to test the sterility of such products. Resazurin is also added to thioglycollate broth to indicate the presence of oxygen, which would make the medium unsuitable for culturing anaerobes.

Antibiotic inhibitors

These are substances that neutralize the activity of antibiotics. They are added to culture media when clinical material is suspected of containing the corresponding antibiotics. For instances, you use the enzyme penicillinase to inactivate penicillin and also to inactivate streptomycin to some degree. In culturing blood or other body fluids containing penicillin, you add 1 ml of penicillinase to each 100 ml or less of sterile medium. This amount of the enzyme inactivates 50,000 units of penicillin, the highest level of antibiotic that could be expected in 10 ml of blood or other body fluids. One milliliter also inactivates up to 1,000 units of streptomycin. Penicillinase does not interfere with the sulfa-inactivating action of PABA that can also be added to the medium. Keep penicillinase refrigerated during storage because this reagent, like many enzymes, loses activity at room temperature.

Dyes

Dyes incorporated in media have selective bacteriostatic effects, but they are generally more active against gram-positive bacteria. Crystal violet, BG, and basic fuchsin are used in well-known selective media. Thionin and basic fuchsin are used in differentiating species of *Brucella*. If you are trying to isolate streptococci in the presence of staphylococci, the latter is effectively inhibited if you add a 1:500,000 dilution of crystal violet to the broth of agar medium. Add 1 ml of autoclaved aqueous 1:25,000 solution of the dye to each 20 ml of medium.

Sodium desoxycholate and other bile salts

In proper combination with other substances in the media, bile salts inhibit the growth of gram-positive bacteria. The addition of this inhibitor is useful when you try to isolate gram-negative bacteria from clinical material in which both gram-positive and gram-negative bacteria are present. On desoxycholate agar, the motility of all flagellated bacteria is temporarily suppressed, and the swarming of certain species of *Proteus* is considerably inhibited. If citrates are also present, as in desoxycholate-citrate agar or SS agar, the bacteriostatic effect extends to some of the gram-negative bacteria, notably the nonpathogenic enteric bacilli. Bile salts are not used successfully in media to isolate the gonococcus, meningococcus, or species of *Hemophilus*.

Potassium tellurite

When added to culture media in a final concentration of 0.01-percent potassium tellurite retards the growth of most gram-negative bacteria and permits the growth of *Streptococcus* species and other gram-positive organisms. A 0.03-percent concentration of tellurite inhibits most *Streptococcus* species, but *Staphylococcus* species and the *Corynebacteriae* are still able to grow. Potassium tellurite is most useful in agar for the isolation of *Corynebacterium diphtheriae*. You can add it to broth to isolate gram-positive bacteria, notably *Staphylococcus* species from mixed culture material overgrown by species of *Proteus* or other rapidly growing gram-negative bacilli.

Chloral hydrate

If added to nutrient agar in a final concentration of 0.1 percent, chloral hydrate has little or no inhibitory effect on either gram-positive or gram-negative bacteria. It does, however, prevent the swarming of species of *Proteus*, rendering them temporarily non-motile. Unlike bile salts and tellurite, chloral hydrate does not “lake” blood (lyse red blood cells), and so you can use it in blood agar plates without interfering with the development of characteristic zones of hemolysis by streptococci and other organisms.

Media containing antibiotics

In appropriate concentrations, these media are inhibitory to some organisms and non-inhibitory to others. Antibiotic media are used in the selective isolation of certain pathogens from specimens containing many contaminating microorganisms. For example, you incorporate penicillin in Bordet-Gengou agar in a concentration of 1 unit per ml of medium. The antibiotic restricts growth of some gram-positive organisms of the throat and allows isolation of *Bordetella pertussis*, the cause of whooping cough. Penicillin and streptomycin in combination, or chloramphenicol alone, is incorporated in media for the isolation of certain fungi from bacteria-contaminated specimens.

Sodium azide

This additive, which inhibits the growth of gram-negative organisms, is used in culture media to help isolate hemolytic streptococcus species from the respiratory passages. Azide blood agar base is a selective medium for isolation of streptococci and staphylococci from materials of sanitary importance, such as sewage, swimming pool waters, food, and other sources containing a mixed flora.

015. The nature of primary plate and broth media

The following table lists the wide variety of primary plating media used to meet the bacteriological requirements of various organisms. This list is not all inclusive. Reference books should be consulted for a more in-depth study. The text following the table provides additional information concerning the uses of particular media in culturing specific organisms.

Aerobic Cultures — Primary Plate Media	
1. Enriched	Purpose
a. 5-percent SBAP.	Supports the growth of most medically significant bacteria (not <i>Haemophilus</i> or <i>Neisseria gonorrhoeae</i>). Distinguishes among 3 types of hemolysis produced by the streptococci.
b. Chocolate agar plate (CAP).	Supports the growth of <i>Haemophilus</i> and <i>Neisseria gonorrhoeae</i> .
2. Differential	Purpose
a. Bile esculin agar (BEA).	Distinguishes between group D streptococci.
b. MacConkey (MAC).	Supports the growth of <i>Enterobacteriaceae</i> and some non- <i>Enterobacteriaceae</i> . Distinguishes between lactose-positive and lactose-negative, and gram-negative rods.
c. EMB.	Isolation of gram-negative enteric bacilli. Distinguishes the pathogenic enteric bacilli, <i>Salmonella</i> and <i>Shigella</i> , from those that ferment either or both of the sugars lactose and sucrose.
3. Differential and Selective	Purpose
a. MS.	Isolation of coagulase-positive staphylococci. Distinguishes between mannitol fermenters and mannitol non-fermenters.
b. Bismuth sulfite.	Isolation of <i>S. typhi</i> and other <i>Salmonella</i> (inhibits <i>Shigella</i>).
c. BG.	Detects H ₂ S production. Isolation of <i>Salmonella</i> (inhibits <i>Shigella</i>).
d. Hektoen enteric (HE).	Isolation of enteric pathogens especially <i>Shigella</i> . Detects H ₂ S production.
e. SS.	Isolation of enteric pathogens, especially <i>Salmonella</i> and <i>Shigella</i> . Detects H ₂ S production.
f. XLD.	Isolation of enteric pathogens, especially <i>Shigella</i> . Detects H ₂ S production.
g. Thiosulfate citrate bile salts (TCBS).	Isolation of <i>Vibrio</i> spp.
4. Selective	Purpose
a. Cefsulodin-irgasan-novobiocin (CIN).	Isolation of <i>Y. enterocolitica</i> .
b. Columbia colistin-nalidixic acid (CNA).	Isolation of <i>S. aureus</i> and streptococci (inhibits <i>S. epidermidis</i> and <i>Micrococcus</i> species).
c. PEA.	Isolation of gram-positive cocci and anaerobic gram-negative bacilli.
d. Modified Thayer-Martin (MTM).	Isolation of pathogenic <i>Neisseria</i> , <i>N. gonorrhoeae</i> , and <i>N. meningitidis</i> (also <i>N. lactamica</i>).
e. Transgrow.	Isolation of <i>N. gonorrhoeae</i> . Transport media.

Aerobic Cultures — Primary Plate Media	
f. New York City Medium (NYC).	Isolation of pathogenic <i>Neisseria</i> .
g. Streptococcal selective agar (SSA).	Isolation of <i>Streptococcus pyogenes</i> and <i>S. agalactiae</i> .
h. Skirrow agar.	Isolation of <i>Campylobacter</i> , and recommended for <i>Helicobacter pylori</i> .
i. Buffered charcoal yeast extract agar (BCYE).	Isolation of <i>Legionella</i> spp.
j. Bordet-Gengou agar.	Isolation of <i>Bordetella pertussis</i> .
k. Campylobacter (CAMPY).	Isolation of <i>C. jejuni</i> and "related campylobacters" (strains that grow at 42°C but not at 25°C).
Anaerobic Cultures — Primary Plate Media	
1. Enriched	Purpose
CDC anaerobic blood agar (AnBAP).	Supports growth of all anaerobes and enhances growth of peptostreptococci.
2. Selective	Purpose
a. Cycloserine-cefoxitin egg yolk fructose agar.	Isolation of <i>Clostridium difficile</i> .
b. Kanamycin-vancomycin laked blood agar (KVLB).	Isolation of most <i>Bacteroides</i> spp.
c. PEA.	Supports the growth of aerobic gram-positive cocci and anaerobic gram-negative bacilli.
3. Differential and Selective	Purpose
<i>Bacteroides</i> bile esculin agar (BBE).	Isolation of <i>Bacteroides fragilis</i> group (presumptive identification).
Primary Broth Media	
1. Enrichment	Purpose
a. Brain heart infusion (BHI).	Cultivation of pathogenic cocci and other organisms.
b. Cooked meat (also called chopped meat) (CM).	Cultivation and maintenance of anaerobes.
c. Thioglycollate (THIO).	Cultivation of aerobic, microaerophilic, anaerobic, and fastidious microorganisms.
d. TSB.	Supports growth of many types of organisms (general use in bacteriology).
2. Enteric Enrichment	Purpose
a. Gram-negative (GN).	Enrichment for gram-negative bacilli especially <i>Salmonella</i> and <i>Shigella</i> .
b. Selenite F or Selenite cystine.	Enrichment for <i>Salmonella</i> .
c. Tetrathionate.	Enrichment for <i>Salmonella</i> and <i>Shigella</i> .
d. Alkaline Peptone water.	Enrichment for <i>Vibrio</i> spp.

Primary plate media for aerobes

A widely used medium is 5-percent sheep blood agar that supports the growth of most medically significant bacteria, except for *Haemophilus* spp. and *N. gonorrhoeae*. Sheep blood agar provides red blood cells for detection of hemolysins. Peptones are a source of amino acids, carbon, and nitrogen; sodium chloride provides electrolytes.

Chocolate agar

CAP, prepared by heating red cells, supports the growth of *Haemophilus* spp. and *N. gonorrhoeae*. It satisfies the hemoglobin (X factor) and nicotinamide adenine dinucleotide (NAD or V factor) requirements of these organisms. The NAD is released from the red blood cells during the heating process. The medium contains an *N. gonorrhoeae* (GC) agar base that includes peptones, corn starch, and salts.

MacConkey agar

Media that differentiate between lactose-positive and lactose-negative gram-negative rods are types of differential agar. An example is MAC agar that supports the growth of the *Enterobacteriaceae* and other gram-negative rods. The bile salts and crystal violet differentiate between lactose and non-lactose fermenting organisms while completely inhibiting gram-positive organisms. When lactose is fermented, the medium is acidified, bile salts are precipitated, and the dye complex (crystal violet and neutral red) is pulled out of solution in the medium. The colonies turn pink when they absorb the precipitated dye. Bacteria that do not ferment lactose appear as clear colonies.

Eosin methylene blue

EMB medium is also used for the isolation and differentiation of gram-negative enteric bacilli. Other organisms are inhibited by the methylene blue and eosin dye content. On EMB, colonies of potentially pathogenic enteric bacilli can be differentiated from those of organisms capable of rapidly fermenting either or both of the sugars lactose and sucrose. Organisms that ferment lactose or sucrose acidify the medium, decreasing the solubility of methylene blue and eosin with the subsequent absorption of the precipitated dye into the colony. *Salmonella* and *Shigella* are distinguished from *Escherichia coli* by appearing as a transparent amber color or colorless, while the *E. coli* is blue-black and has a metallic sheen when viewed by reflected light. Other coliform bacilli form mucoid, brownish colonies.

Desoxycholate

Desoxycholate is a differential plating medium for the cultivation of gram-negative enteric bacilli. Chemicals, citrates, and sodium desoxycholate inhibit gram-positive organisms. Lactose allows differentiation of the enteric bacilli. Organisms that utilize lactose form red colonies, while those that do not ferment lactose form colorless colonies.

To select for media

Media formulated to “select for” medically significant bacteria, in addition to “differentiating among” related species, are both selective and differential. MS, used to isolate coagulase-positive staphylococci, distinguishes between mannitol fermenters and those that do not ferment mannitol. Staphylococcal species are tolerant of high salt concentration (75 grams per liter), while most other bacteria are inhibited by these salt concentrations. Acid production on MS with phenol red indicator by presumptive coagulase-positive staphylococci produces colonies surrounded by bright yellow zones. Coagulase-negative staphylococcal colonies have reddish-purple zones indicating a mannitol-negative reaction.

Bismuth sulfite

Bismuth sulfite, used to isolate *Salmonella typhi* and other species of *Salmonella*, is inhibitory to all other organisms. In this medium, freshly precipitated bismuth sulfite acts, together with BG, as a selective agent by suppressing the growth of coliforms, while permitting the growth of *Salmonellae*. Sulfur compounds provide a substrate for hydrogen sulfide (H_2S) production; metallic salts stain the colony and surrounding medium black or brown in the presence of H_2S .

Brilliant green agar

A highly selective plating medium used to isolate the pathogenic *Salmonellae* is BG agar. The brownish medium becomes red during incubation of the plates. Typical *Salmonella* colonies appear reddish, pink, or nearly white, depending on the cultures and length of incubation. Lactose or sucrose fermenting organisms produce yellow or green colonies surrounded by a bright yellow-green area of the medium. BG agar is not used to culture for *Shigella* because dysentery bacilli grow poorly or not at all on this medium.

Hektoen enteric agar

HE agar is a differential, selective medium for the isolation of *Salmonella* and *Shigella* from enteric specimens. An increased carbohydrate and peptone content and a lower toxicity of the bromthymol blue indicator system overcome the inhibitory effect of the medium, allowing good recovery of *Shigella*. The medium contains bile salts to inhibit gram-positive bacteria, and lactose, sucrose, and salicin to detect organisms that ferment these carbohydrates. Commensal organisms are usually temporarily inhibited on HE by the dyes and appear as yellow colonies due to the acid produced from fermentation and the absorption of the precipitated bile-dye complex into the colonies. Prolonged incubation results in these organisms turning pink or salmon in color. A non-fermenting colony increases the pH of the medium by producing ammonia during peptone utilization, resulting in green colonies. Sodium thiosulfate provides a source of sulfur for the detection of H_2S production that combines with the iron in ferric ammonium citrate to form iron sulfide (FeS). H_2S -producing organisms have black centers due to the formation of the FeS.

Salmonella-Shigella agar

SS agar is a differential, selective medium for the isolation of *Salmonella* and *Shigella* from enteric specimens. Gram-positive and coliform organisms are inhibited by a bile salts mixture. Nonlactose fermenters form colorless colonies; an occasional coliform or other lactose fermenters produce pink or red colonies. The mechanism of action is similar to the one described for MAC agar; H_2S detection is the same as described for HE agar.

Xylose lysine desoxycholate medium

XLD medium is a satisfactory medium for the isolation and presumptive identification of *Salmonella* and *Shigella*. It relies on xylose fermentation, lysine decarboxylation, and the production of H_2S for the primary differentiation of *Salmonella* and *Shigella* from nonpathogenic bacteria. Xylose is included in the medium so *Shigella* species can be identified by a negative reaction. Failure to ferment xylose results in red colonies caused by alkalization of the medium during peptone utilization. Xylose fermenters cause the phenol red indicator to turn yellow due to acid production. *Salmonella* species use xylose, and also decarboxylate lysine, resulting in an alkaline pH. The high acid level produced by fermentation of lactose and sucrose prevents lysine positive coliforms from reverting the pH to alkaline and, since the nonpathogenic H_2S producers do not decarboxylate lysine, any acid produced from sugar utilization is not masked by alkaline products. The acid level also prevents blackening by these organisms until after 18 to 24 hours. Sodium desoxycholate allows for the inhibition of coliform bacilli without inhibiting *Salmonella* and *Shigella*.

Several selective media

Several selective media are available for direct isolation of *Yersinia enterocolitica*. CIN, with a peptone base, yeast extract, and mannitol and bile salts with neutral red and crystal violet as indicators is used for most *Yersinia* spp. and can be useful for isolation of *Aeromonas* species.

Thiosulfate citrate bile salts

TCBS with sucrose and bromthymol blue as an indicator was formulated for the isolation of the Vibrios. Its alkaline pH of 8.6 makes it an ideal selective medium for this group of organisms. The growth of most *Enterobacteriaceae* is totally suppressed for at least 24 hours. Fermentation of sucrose distinguishes among those vibrios that cause dysentery-like diarrhea, food poisoning, and cholera. After 24 hours of incubation at 37°C, *Vibrio cholera* appears as yellow-brown colonies; a yellow color in the medium is caused by sucrose fermentation. *Vibrio parahaemolyticus* colonies are colorless with green centers; sucrose is not fermented. When slight growth of enteric flora occurs, the colonies are minute, and there is no yellow color in the medium.

Selective agar

Selective agar can contain antimicrobial agents that kill or inhibit the growth of certain organisms and allow others to grow. Columbia blood agar with CNA inhibits gram-negative bacteria, but allows gram-positive cocci to grow. Streptococci are isolated on Columbia blood agar containing polymyxin B and nalidixic acid (PBNA). Staphylococci grow after 48 hours of incubation, but growth is very poor. Colistin (polymyxin E) disrupts the cell membrane of gram-negative organisms, making it effective against many gram-negative bacteria.

Phenylethyl alcohol agar

Aerobic gram-positive cocci and anaerobic gram-negative bacilli grow well on PEA agar, which causes aerobic gram-negative bacteria to elongate into filamentous forms. The inhibition of the DNA synthesis of gram-negative bacteria is reversed when the PEA is removed. While all three types of media (CNA, PBNA, and PEA) are inhibitory to aerobic gram-negative bacilli, *Proteus* spp. and *Pseudomonas* spp. may show pinpoint growth.

Thayer-Martin

The pathogenic *Neisseria* is isolated on Thayer-Martin (TM) and its modifications, Martin-Lewis (ML), Transgrow, and NYC media. Vancomycin, colistin, and nystatin are added to a GC agar base to formulate the selective media. Vancomycin inhibits gram-positive bacteria by interfering with cell wall synthesis. Colistin, which is active against gram-negative organisms, disrupts cell wall permeability, causing osmotic leakage of essential molecules.

Modified Thayer-Martin

Modified Thayer-Martin (MTM) also contains trimethoprim lactate used specifically to inhibit *Proteus*. ML contains the same ingredients as MTM, except anisomycin has been substituted for nystatin since anisomycin is more effective than nystatin in inhibiting yeast.

New York City

NYC media consist of a protease peptone-cornstarch-agar-buffered base. Additional ingredients include horse plasma and hemoglobin solution, glucose, and yeast dialysate. Vancomycin, colistin, nystatin, or amphotericin and trimethoprim lactate are added to make the media selective.

Transgrow

Transgrow serves not only as a culture medium but as a good transport medium as well. The increased agar concentration allows the bottles to be prepared with agar slants for transporting the culture in a carbon dioxide atmosphere. If possible, the bottle should be incubated in an upright position at 35°C for 16 to 18 hours before transport. This allows enough growth before transport so the organisms have a better chance for survival and can be identified when delivered to the laboratory. If the bottle is kept in an upright position and the

cap tightened, there should be no carbon dioxide loss. *Campylobacter* selective medium is designed to isolate *Campylobacter jejuni* and “related campylobacters.” A selective culture medium for *Campylobacter* includes the addition of vancomycin, polymyxin, trimethoprim, cephalothin, and amphotericin to either Trypticase soy agar (TSA) or *Brucella* agar with 5-percent sheep blood. An addition of these antimicrobial agents to Thioglycollate or *Brucella* broth can also serve as an enrichment medium. The addition of cefazolin has been found to be more inhibitory to *Pseudomonas* spp.

Primary plate media for anaerobes

Freshly prepared plate media are recommended for the recovery of anaerobic bacteria. The following is a list of primary media for cultivating anaerobic bacteria:

1. CDC AnBAP to support growth of facultative and strict anaerobic bacteria; contains TSA base with 5-percent sheep blood; supplemented with yeast extract, hemin, vitamin K₁, and L-cystine.
2. BBE for the selective growth and presumptive identification of *Bacteroides fragilis* group and for occasionally growth of *Fusobacterium mortiferum* and *F. varium*, *Klebsiella pneumoniae*, *Enterococcus* species, and yeasts. This media consists of 20 percent bile and 100 µg/ml of gentamicin.
3. KVLB for the selection of *Bacteroides* spp. and *Prevotella* spp. Yeast and other kanamycin-resistant gram-negative bacilli sometimes grow on this agar. This medium contains the same ingredients as AnBAP with the addition of 75 µg/ml of kanamycin and 7.5 µg/ml vancomycin. Antibiotic amounts may vary depending on manufacture.
4. PEA sheep blood agar supports most gram-positive organisms and inhibits enteric gram-negative bacilli. This medium also contains the same ingredients as AnBAP with the addition of 2.5 g/liter PEA.
5. CNA blood agar supports the growth of anaerobic and facultatively anaerobic gram-positive bacteria, and inhibits most gram-negative bacteria.
6. Cycloserine cefoxitin egg yolk fructose agar is inoculated if clostridia are suspected. This media consist of trypticase soy or proteose peptone base containing fructose, neutral red indicator, 500 mg/liter cycloserine, and 16 mg/liter cefoxitin.
7. THIO broth medium (without indicator) supplemented with 5 µg/ml hemin and 0.1 µg/ml of vitamin K₁ used to backup plate media.
8. CM broth is used to support anaerobic bacteria and used to backup plate media.

Primary broth media

Broth media are used in aerobic and anaerobic bacteriology because, not only do they support the growth of many types of organisms, some also serve as enrichment for pathogenic bacteria. Enrichment is a general term used to describe media that contain specific nutrients for the purpose of enhancing the growth of one or a group of organisms.

Brain heart infusion

BHI broth is used to cultivate a variety of organisms, including the pathogenic cocci. CM broth is excellent for growing anaerobic organisms. It initiates growth of minute inocula that makes it valuable for use in the primary culture of clinical specimens in which the causative agent is present in small numbers.

Thioglycollate

THIO broth is used to grow strictly anaerobic and microaerophilic organisms, as well as aerobes. TSB has general use in bacteriology since it supports the growth of many types of organisms and is a common broth used for blood cultures.

Gram-negative

GN broth used in stool bacteriology is an enteric enrichment medium that promotes growth of *Salmonella*, supports growth of *Shigella*, but suppresses growth of *Enterobacteriaceae* for 6 hours. Selenite F and selenite cystine were designed primarily for the isolation of *Salmonella*. The sodium selenite is more toxic to other bacterial flora that might inhabit the intestine than to *Salmonella*. Prolonged incubation of the broth results in enhanced growth of the coliform bacilli over the *Salmonella*. Tetrathionate broth was also designed for enrichment of *Salmonella*. A mixture of bile salts kills or inhibits coliform organisms and permits *Salmonella* to grow. All enteric enrichment broths are subcultured to differential media after 12 to 24 hours of incubation. Alkaline peptone water at a pH of 8.4 to 8.5 is used for enrichment of *Vibrio cholerae* and other *Vibrio* spp.

016. What are some reasons for using biochemical media?

There is a wide variety of media to test for biochemical reactions. These reactions are valuable tools for getting more specifically identifying information on an organism isolated in pure culture. Let's learn about the reasons for using biochemical media and explore examples of differential carbohydrate and carbohydrate fermentation media.

Differential carbohydrate media

The ability of a specific organism to attach and break down a particular carbohydrate is a useful characteristic, and this is accompanied by identifiable by-products. Thus, by carefully selecting the test carbohydrates, you obtain a pattern of fermentation reactions characteristic of specific organisms. Carbohydrates, including many of the 6- and 12-carbon sugars, such as glucose and sucrose, respectively, are excellent sources of energy for microorganisms. However, there are great differences in the ability of bacteria, though closely related taxonomically, to use one or more of these numerous compounds for energy and growth. This feature is particularly helpful to you in distinguishing among the morphologically similar facultative anaerobes that inhabit the human intestinal tract (i.e., the enteric bacilli, especially *Salmonella typhi*, and the *Salmonellae* of food poisoning). For example, differentiation of enteric bacteria is achieved through the incorporation of lactose and sucrose in some brands of EMB agar. Differentiation is possible since the organisms that attack lactose form colored colonies and those that do not appear as colorless colonies. Such media as EMB agar and MAC agar contain carbohydrates and chemicals inhibitory to many gram-positive bacteria.

Glucose, but not lactose

The ability of an unknown organism to ferment glucose, but not lactose, provides valuable information. The breakdown of sugars is usually accompanied by the formation of identifiable byproducts. That is, you can test for the production of organic acids and certain gases that form in the culture during breakdown of a carbohydrate medium.

Kligler's Iron agar and Triple Sugar Iron agar

Two other examples of differential media commonly used to distinguish between closely related organisms are Kligler's Iron agar (KIA) and Triple Sugar Iron agar (TSI). These media are useful because they take advantage of fundamental differences in the physiology of bacteria that seem identical from outward appearance.

KIA

KIA contains lactose, dextrose, nutrient agar, and phenol red indicator.

TSI

TSI contains sucrose, in addition to lactose and dextrose. Color changes in these media, caused by selective fermentation of the carbohydrates, form the basis for genus and species identification.

KIA and TSI

KIA and TSI also contain an iron compound that indicates whether or not an organism can produce H_2S . As fermentation proceeds, the medium is blackened through the reaction of H_2S and iron to form FeS .

Inconclusive

The study of reactions in KIA and TSI differential media usually leads only to tentative or presumptive identification of an organism. This situation comes about because bacteria belonging to related but different genera sometimes give identical results in one or more of the reactions seen in KIA and TSI media. For example, *Enterobacter cloacae*, *Escherichia coli*, and *Klebsiella pneumoniae* yield both a yellow (acid) slant and butt in a TSI culture tube. The presence of gas bubbles and H_2S production is often used as distinguishing characteristics, but, in general, reaction on differential carbohydrate media serves as a preamble to further steps in identification.

Confirmation by other means

Identity has to be confirmed by other means such as the fermentation of additional sugars and sugar alcohols, or amino acids, or by serological procedures.

Carbohydrate fermentation media

To study carbohydrate fermentation, you must choose a proper basal medium. One such medium is trypticase agar base. Trypticase agar base permits accurate fermentation determinations. Since small amounts of acids are not readily dispersed throughout the medium, positive reactions can often be determined more quickly than in liquid media. Carbohydrate taxo discs or appropriate carbohydrates can be added for fermentation studies. This medium is particularly recommended for use with clostridia, bacilli, common micrococci, enteric bacilli, and other organisms not generally considered to be nutritionally fastidious. Cystine trypticase agar (CTA) is another such medium for detection of motility. With added carbohydrates for fermentation reactions of fastidious organisms, including *Neisseria*, *Pasteurella*, streptococci, *Brucella*, corynebacteria, and vibrios, it may be readily cultivated on CTA medium. Carbohydrates can be in the medium, or carbohydrate taxo discs can be conveniently selected and added as needed to tubes of plain CTA medium. Both CTA and trypticase agar base contain phenol red as the indicator. CTA contains sodium chloride, and trypticase agar base does not. These media have a pH of about 7.3 to 7.4 to detect carbohydrate fermentation. In the presence of an indicator, a change of pH due to accumulation of metabolic products gives a visual indication of fermentation. Specific reactions on differential and carbohydrate media are shown later in our study of individual genera of the pathogenic microorganisms.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

013. What factors influence the media to be used?

1. Why is it important for personnel to keep their selection of media to a minimum?
2. What factors influence the type of media used?
3. What is the purpose of basic nutrient media?
4. What reaction causes the retarding of growth in fastidious organisms when Trypticase soy broth is used as a basic nutrient medium?
5. For what purpose is enrichment media used in enteric bacteriology?
6. What are some examples of enrichment media?
7. How does an enriched media differ from an enrichment medium, and how is an enriched medium prepared?
8. What is the purpose of the differential media?
9. What are three examples of differential media?
10. What constituents are incorporated in the XLD agar to differentiate between the lactose and nonlactose fermenters?
11. What is the purpose of the selective media?
12. What two selective media are used for growth of staphylococci and other gram-positive cocci?

13. Why are organisms grown on selective media, such as Phenylethyl and Transgrow, transferred after initial growth to a more compatible media?

014. The general applications and purposes of media additives

1. Match each media additive or related item in column B with the statement in column A by placing the letter of the column B item beside the number of the column A item that most nearly describes it. Each element in column B may be used once, more than once, or not at all.

<i>Column A</i>	<i>Column B</i>
____ (1) Substance neutralizes the bacteriostatic effect of sulfonamides in culture media.	a. Dyes.
____ (2) Incorporated in media not only to permit the growth of anaerobic bacteria, but to neutralize the bacteriostatic effect of mercurial and silver disinfectants.	b. Crystal violet.
____ (3) Substance is used to inactivate penicillin and, also, to inactivate streptomycin to some degree.	c. Penicillinase.
____ (4) Does not interfere with sulfa-inactivating action of para-aminobenzoic acid.	d. Sodium desoxycholate.
____ (5) Incorporated in the media have selective bacteriostatic effects, but are generally more active against gram-positive bacteria.	e. Chloral hydrate.
____ (6) Used in differentiating species of <i>Brucella</i> .	f. Potassium tellurite.
____ (7) Salt that inhibits growth of gram-positive bacteria and also suppresses the motility of flagellated bacteria.	g. Sodium azide.
____ (8) Retards growth of most gram-negative bacteria and is especially useful when trying to isolate <i>C. diphtheriae</i> .	h. Penicillin.
____ (9) Prevents the swarming of species of <i>Proteus</i> , rendering them temporarily nonmotile.	i. Chloramphenicol.
____ (10) Used in blood agar plates without interfering with the development of characteristic zones of hemolysis of streptococci and other organisms.	j. Para-aminobenzoic acid.
____ (11) Incorporated in Bordet-Gengou agar in a concentration of 1 unit per ml in medium.	k. Sodium thioglycollate.
____ (12) Used alone, incorporated in media for the isolation of certain fungi from bacteria contaminated specimens.	l. Thionin and basic fuchsin.
____ (13) Additive that inhibits the growth of gram-negative organisms and is also used in culture media to help isolate hemolytic <i>Streptococcus</i> species from the respiratory passages.	m. Sodium chloride.

015. The nature of primary plate and broth media

1. Match the organism in column B with the enriched or selective media in column A used to culture it. Each item is used once.

Column A

- ___ (1) Bismuth sulfite.
- ___ (2) MacConkey.
- ___ (3) Kanamycin-Vancomycin (laked blood).
- ___ (4) Mannitol salt.
- ___ (5) Blood agar with polymyxin B and nalidixic acid.
- ___ (6) Martin-Lewis.
- ___ (7) Thiosulfate citrate bile salts.
- ___ (8) Chocolate.
- ___ (9) Xylose lysine desoxycholate.
- ___ (10) Cefsulodin-irgasan-novobiocin.

Column B

- a. Staphylococcus spp.
- b. Shigella spp.
- c. Haemophilus spp.
- d. S. typhi.
- e. Y. enterocolitica.
- f. Enterobacteriaceae.
- g. Neisseria.
- h. Streptococcus spp.
- i. Vibrio spp.
- J. Bacteroides spp.

016. What are some reasons for using biochemical media?

1. How can you use carbohydrate fermentation studies as an aid in identifying genera and species of bacteria?
2. What two carbohydrates are incorporated in some brands of EMB for differentiation of enteric bacteria?
3. Why are different media, such as KIA and TSI, useful in the identification of enteric organisms?
4. Which of the sugars contained in TSI medium is lacking in KIA medium?
5. What sugars does TSI medium contain?
6. As fermentation proceeds, TSI is blackened through the reaction of H₂S and iron to form what other compound?
7. What genera of organisms commonly produce both yellow (acid) slant and butt in a TSI tube?

8. What other characteristics of TSI are often used as distinguishing characteristics?
9. What two basal media are used for non-fastidious and fastidious organisms, respectively?
10. Why can positive reactions on trypticase agar base be more quickly determined than in liquid media?
11. What are some organisms for which the use of Trypticase agar base is recommended?
12. What are some organisms that may readily be cultured on CTA medium?
13. What causes the change in pH and subsequent visual indication of fermentation on the medium?

3-2. Isolation and Cultivation of Bacteria

Pure cultures are essential to the study of colony characteristics, biochemical properties, morphology, staining reaction, immunological reactions, and susceptibility of a microbial species to antimicrobial agents. The major problem in diagnostic bacteriology is to effectively separate pathogenic microbes from harmless organisms with which they are closely associated in nature. From the moment of birth, the human body is heavily populated with bacteria representing many different genera. Some of these microbial populations are *transient*, while others are *resident* in the sense that they can almost always be recovered in large numbers from the skin, the body orifices, and the intestinal tract—the so-called normal flora. Once you immobilize a single-celled microorganism on a solid culture media suitable for growth and reproduction, its progeny provide (within the space of a few hours) the material needed for cultural studies—an isolated colony visible to the naked eye. Beyond this point, different procedures (developed over the years for subculturing the colony) enable you to select the condition of growth best suited to bring out the morphological and physiological characteristics that experience has shown establishes the identity of unknown organisms.

017. Factors to consider when cultivating bacteria

Clinical microbiology is constantly evolving and changing. New methods involving non-growth techniques for the detection of infectious agents are coming to the forefront of future testing procedures. These include automated instrumentation, immunological procedures, and even DNA probes. The cultivation and isolation for identification of pathogenic microorganisms is still the “gold standard” for the diagnosis of infectious diseases. To

cultivate bacteria in the laboratory, you must consider several important factors. These factors are very similar to those involved in the life processes of other plants, animals, and humans. They are:

- Nutrition (proper food).
- Moisture.
- Acidity/alkalinity (pH).
- Temperature.
- Gas exchange.

Nutrition

The proper foods include a source of carbon (organic or inorganic), nitrogen, inorganic chemical salts, and other growth-promoting substances. Most media contain peptone, a breakdown product from protein, that provides an available source of carbon and nitrogen. Certain pathogens need serum or blood in order to reproduce on culture media. Carbohydrates are needed by some organisms, but not by others, as a source of energy. You can add calcium, magnesium, potassium, and sodium salts, as well as certain other minerals and even dyes, to culture media to either assist or inhibit bacterial growth, depending on your purpose in cultivation.

Moisture

Moisture in the medium and in the atmosphere is essential for growth. There is usually sufficient moisture in a culture medium, particularly in a broth. You can add moisture to closed containers, such as candle jars, by soaking a piece of gauze or cotton with water and placing the dampened material in the container beside the plates of medium. Incubators usually contain a pan you fill with water and place in the bottom, and it is your responsibility to keep it filled. Newer models may have a system incorporated into the incubator that measures moisture and keeps it at a constant level automatically. Depending on the model and manufacture, during equipment installation, water is added to the designated area. The water is replaced yearly as part of the quality control. The moisture level can be adjusted with the needs of the microorganisms being cultured.

Acidity/alkalinity

The proper pH must be maintained during the life of bacteria. Just as you die if your body pH changes very much, so does bacteria. Media are usually prepared to a specific pH in the range of 6.8 to 7.2. However, as bacteria grow, they utilize the food available to them and, in so doing, change the pH of the medium because of the accumulation of their waste products. To counteract this change, buffering agents are incorporated into culture media. Buffer substances retard changes in the pH of the growing culture, thus permitting more abundant growth before a limiting acidity or alkalinity is reached.

REMINDER: Disc susceptibility testing utilizing the Kirby-Bauer method is not done in a CO₂ atmosphere for most organisms because the pH of the surface of the agar is too low, producing falsely increased and decreased antimicrobial activity.

Temperature

Bacteria vary considerably with respect to their temperature requirements for growth. We classify all bacteria into three broad temperature groups as shown in the following table:

Classification	Temperature Range	Optimum Range
Psychrophilic (cold-loving)	-5°C to 30°C	10°C to 20°C
Mesophilic	10°C to 45°C	20°C to 40°C
Thermophilic (heat-loving)	25°C to 80°C	50°C to 60°C

The majority of human pathogens are mesophilic, growing best in vitro at 35°C. Incubation temperatures must be between 35 and 37°C. Authorities have found 35°C is adequate for most pathogens. Incubator temperatures must be checked and recorded daily.

Gas exchange

Gas exchange between bacteria and their environment is vital. While most bacteria encountered in medical bacteriology grow under conditions of normal atmospheric O₂, certain others need more CO₂ than is found in air. Still others obtain their oxygen only from chemicals incorporated in the medium. Since some pathogenic forms use only one of several different systems of respiration found among bacteria in general, you must furnish the specific atmospheric environment those disease agents require. Otherwise, your isolation attempts almost always result in failure. Most organisms grow better when CO₂ tension is slightly increased. *Campylobacter* spp. are unique in their need for a mixture of different gases. *Campylobacter* spp. require a microaerobic atmosphere containing approximately 5 percent O₂, 10 percent CO₂, and 85 percent N₂ for recovery. Microaerobic gas generator packs, jar- or bag-type systems, are available through commercial manufacturers for routine use.

Physiology

Respiration is a process by which bacteria generate energy for growth. In basic chemistry, you learned energy is released through a coupled reaction in which one compound is oxidized while another compound is simultaneously reduced. You'll recall oxidation represents a loss of electrons and reduction a gain of electron. In bacterial respiration, electron transport is accompanied by the transfer of hydrogen atoms. So, for practical purposes, the energy-yielding, oxidation-reduction reaction is pictured as a transport of hydrogen atoms from one compound to another. In other words, an oxidized compound within the cell (hydrogen donor) gives up one or more hydrogen atoms to a second compound (hydrogen acceptor), which is thereby reduced by its acceptance of these same hydrogen atoms. Bacteria utilize a wide assortment of organic and inorganic compounds in generating energy for cell functions. In general terms, if members of a given species couple hydrogen atoms with atmospheric O₂ (the hydrogen acceptor) as the final link in a chain of oxidation-reduction reactions, then we speak of this process as "aerobic" respiration. If some chemical substance other than free O₂ serves as the hydrogen acceptor, you term this respiration "anaerobic." In bacterial classification, microorganisms are divided into five major categories on the basis of their requirements for atmospheric oxygen as the hydrogen acceptor:

- Aerobic.
- Facultatively anaerobic.
- Anaerobic.
- Microaerobic.
- Capnophilic.

Aerobic

An aerobe growing on solid medium must have O₂ in the atmosphere surrounding the culture. Broth culture must contain dissolved O₂ throughout the liquid for aerobic respiration to proceed. Quite often, the diffusion of O₂ into the broth is accelerated by agitating (shaking) the medium during incubation. In most aerobic organisms, the hydrogen atoms arising from oxidation-reduction reactions in the cell are joined to atmospheric O₂ to form hydrogen peroxide (H₂O₂) as the end product in respiration. Since H₂O₂ is toxic to biologic systems, the aerobes produce an enzyme, catalase, to break down the harmful compound into water and O₂.

Facultatively anaerobic

This type of bacterium is able to use either atmospheric O₂ or some other substance as a hydrogen acceptor. By this process, a facultative organism adapts to grow in either an aerobic or an anaerobic environment. Many of the bacteria that normally live in the human intestine are facultative anaerobes that show rapid growth aerobically on laboratory media. They adapt to anaerobic conditions when the supply of O₂ in the culture is exhausted. Most of the pathogenic organisms you'll study are facultative anaerobes.

Anaerobic

An obligate anaerobe does not grow in a culture exposed to atmospheric O₂. Free O₂ is toxic to the anaerobe's respiratory mechanism, and hydrogen atoms are usually transferred only between certain organic compounds fabricated by the cell from constituents of the culture medium. In effect, the anaerobic forms do not possess the respiratory enzymes necessary to couple hydrogen atoms with O₂, the hydrogen acceptor commonly used by the aerobes and facultative anaerobes. Many of the obligate anaerobes of interest to you in the clinical laboratory, such as certain gangrene organisms of the genus *Clostridium*, survive an O₂ atmosphere by forming resistant spores. These spores remain metabolically inactive until an environment free of O₂ stimulates their germination.

Microaerobic

Between the strict aerobes and the obligate anaerobes, you find some interesting gradations among bacteria with respect to requirements for atmospheric O₂. For example, the microaerobes (formerly called microaerophils) are forms for which O₂ is toxic in normal atmospheric concentration. These bacteria are not true anaerobes because a need for O₂ does exist, although the amount required is extremely small. Then, there are so-called indifferent microbes that grow either in the presence or absence of O₂. The gas is not toxic to these anaerobic organisms, but atmospheric O₂ is not used as a hydrogen acceptor in respiration.

Capnophilic

Organisms that require increased CO₂ concentrations than found in room air are called capnophilic. You produce an atmosphere containing 2 to 3 percent CO₂ by incubating cultures in a candle jar. Any wide-mouthed vessel with an airtight lid will suffice, provided it is large enough to accommodate several cultures. Place the inoculated tubes or plates of media in the vessel; then place a lighted candle in the container above the cultures, and securely tighten the lid. The burning candle is extinguished when about 2 to 3 percent CO₂ accumulates. Be certain to place the candle as high as possible in the candle jar to permit accumulation of CO₂ that extinguishes the flame. (The CO₂ is heavier than air and fills the bottom first.) Jar- and bag-type systems with appropriate generator envelopes and CO₂ incubators provide a CO₂ atmosphere of 5 to 7 percent. The above requirements are needed for all bacteria, but in

varying degrees, depending on the bacteria to be cultivated and the source of the specimen. Detailed growth requirements are discussed in the specimen processing section and in Volume 2 that discusses the bacteria species.

018. Considerations in the macroscopic examination of bacterial morphology

Once the growth requirements have been met and you actually have bacteria growing on a plate, it is identification time! One of the initial steps in bacterial identification is the systematic macroscopic examination of colony morphology or characteristics. In this lesson, let's learn about the factors you must consider and the terminology used to describe various morphological features of bacterial colonies.

Colony morphology or characteristics

In a suitable environment, a bacterium grows and multiplies. As a result, where originally there was one organism, descendants (progeny) of that organism begin to build up around the original cell. Within a short period of time, there are enough organisms in one area to be seen macroscopically. As these organisms reproduce, they form groups, or colonies, that possess qualities that aid in their identification. The characteristics are referred to as colony size; shape or form; elevation; margin; texture; and color, pigmentation, and hemolysis on blood agar. Recognition of typical colonies of each genus is essential as a step to identification.

Colony size

The size of a colony is labeled as pinpoint, small discrete, large, or spreading, and can be reported as millimeter (mm) in diameter. Under normal cultural conditions, size is an aid in identification. Significance is often attached to minute (dwarf) colonies in they are apt to be variant colonies having a degree of virulence different from larger colonies of the same organism. As with the shape of the colony, size is affected by the factor of motility. Distortion in size is also caused by the presence of too much moisture on the medium surface. This is one reason why you should not inoculate media surfaces with bacteria while water droplets, arising from condensation, are still present on the culture plate. The resulting growth may not take the form of isolated colonies needed as an aid to identification.

Colony shape or form, elevation, and margin

Colony shape refers to the whole outline of the colony, along with its elevation above the medium, and the outer edge or margin. When examining colony growth, you must observe individual colonies that have not been distorted or influenced by colonies growing in close proximity. Certain terms are used to describe the colony shape in relation to its outline, such as punctiform, circular, filamentous, irregular, rhizoid, and spindle. Some colonies are irregular and appear spread out. You can see examples of colony shape in figure 3-1.

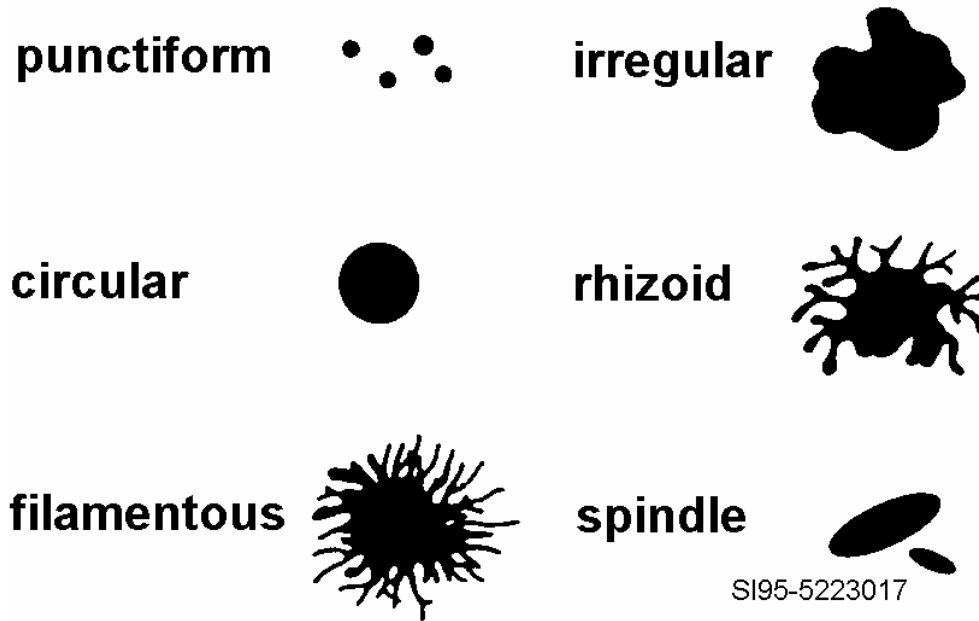


Figure 3-1. Colony shape or form.

Area of the colony

The more motile an organism, the greater the area of the colony. For example, *Proteus vulgaris* can produce filamentous or spreading colonies that cover the entire plate and give the appearance of a thin film of bacteria on the plate—H-type colonies. *Proteus* also grow as a small discrete colony—O-type colonies. The H and O designations are derived from the German words, Hauch and Ohnehauch, denoting spreading and non-spreading forms. The H-type colony is due to active flagellar motion, and the O-type colony indicates a nonflagellated and, therefore, non-motile organism. It is significant that the presence of flagella correlates with the antigenic properties of the organism. Antigens are chemical components of the bacterial cell that stimulate the formation of specific protective substances, “antibodies,” in the bloodstream of man and animals. You’ll later learn how to capitalize on this phenomenon by using serological techniques to detect differences in antigenic structure of closely related microorganisms. Sometimes, you can distinguish morphologically identical species only by means of analysis of their antigens.

Description of colony elevation

Colony elevations are described by such terms as flat, raised, convex, pulvinate, umbonate, and umbilicate (fig. 3-2). To best view these characteristics, you should use oblique light and a hand lens to see shadow effect. Older colonies tend to flatten and yield non-typical features; always be aware of the time factor when observing colony morphology.

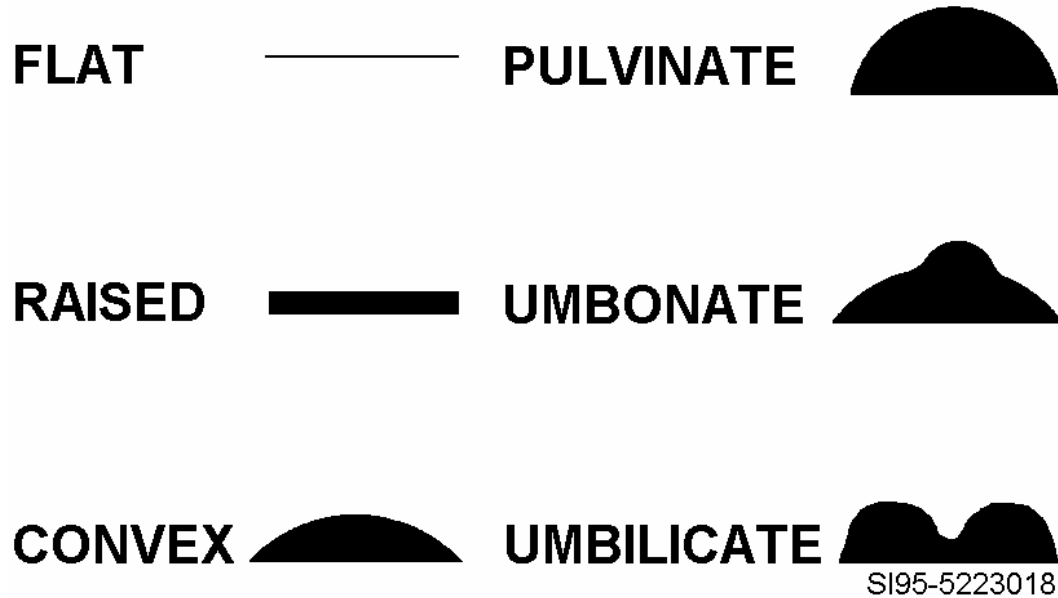


Figure 3-2. Colony elevation.

Description of colony margins or edges

Colony margins or edges are described using terms such as entire, undulate, lobate, erose, filamentous, and curled, as you can see in figure 3-3.

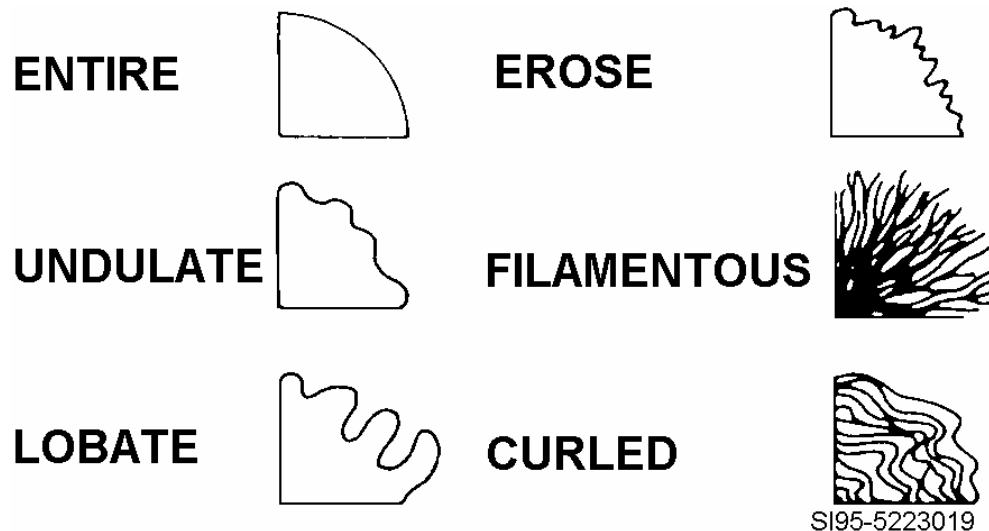


Figure 3-3. Colony margin.

Colony texture

Another useful aid to identification is the texture of the colony. Texture is described by such terms as rough, smooth, creamy, and mucoid. Some colonies are so mucoid that, if you touch them with a metal loop, you see a long string of sticky material adhering to the loop as you withdraw it. Mucoid colonies can result from heavy capsule formation by individual cells or by secretions produced by bacteria in response to an external stimulus. Creamy colonies have more of a “peanut butter”-like consistency.

Smooth (S) and rough (R) colonies are also frequently encountered. Many bacteria produce both the S- and R-type colonies. The S-type colony is generally smooth, translucent, convex, glistening (moist), circular, and "butter-like" in consistency. The S-type bacteria are usually encapsulated and, as a rule, produce a smooth growth in a liquid medium or broth. Loss of virulence or capsule can cause the bacteria to show a variation in colonial growth; for example, instead of an S colony, an R colony is produced. An R-type colony is generally dull in appearance, less translucent (more opaque) than the S colony, and may have an irregular circumference, and a wrinkled or rough surface. Bacteria from R colonies, when grown in broth, may form flakes or granules. They may settle to the bottom or form a surface scum or pellicle. Stained slides made from the broth or from surface colonies from the plated medium usually reveal long, filamentous organisms. Glistening, dull, frosted glass, and shiny are used to describe the surface of a colony, whereas opaque, translucent, and transparent are used to describe density.

Color, pigments, and reaction on blood agar

In addition to size, shape, and texture, bacterial colonies can develop certain characteristic colors, pigmentations, and reactions with blood agar that can aid in identification.

Color

Colors are produced in a wide range, depending on the type of medium used, time and temperature of incubation, age of culture, and other environmental factors. On a dye-free medium, the colonies of most bacteria are white to gray, but can be black or buff. With the pigmented media, a colony can be a bright yellow on one medium and a dull yellow, orange, or white on another. Some bacteria produce color naturally, while others obtain their color from the dyes within the media.

Pigments

A number of pigments that can be helpful in making a species identification are produced by nonfermenters. Water-insoluble pigments include carotenoids that are yellow-orange, violacein that are violet, and phenazines that are red, maroon, and yellow in color. Water-soluble and diffusible pigments include fluorescein, pyorubin, melanin, pyocyanin, and other pigments—byproducts that discolor the culture medium. Pigment production can be enhanced by growing organisms in milk-, potato-, or gelatin-containing media, and incubating at room temperature. We'll discuss color and pigmentation in greater detail with the study of individual bacteria.

Hemolysis on blood agar

Hemolysis reactions on blood agar are divided into four basic groups:

1. **Gamma** — no change or lysis of the red blood cells in the media surrounding the colony.
2. **Beta** — a complete zone of red blood cell lysis with the media surrounding the colony. Hemolysis can be in various degrees or so complete you can see through the agar.
3. **Alpha** — a partial hemolysis of blood cells around the colony that gives a green discoloration of the media.
4. **Alpha prime** — gives a halo effect of incomplete hemolysis immediately around the colony, and a second zone of complete hemolysis at the edge or periphery.

Characterization of colony morphology is useful in basic or routine identification, and is the first step in moving toward more detailed methods.

019. The purpose of the streak plate technique and the reasons for subculturing

In almost every case, clinical specimens submitted to the laboratory contain more than one kind of organism. The organisms may all be pathogens, or one or more pathogens may be found among several saprophytes. In order to consider macroscopic examinations as a means of identification, a single colony, separated from other colonies in mixed cultures, is a prerequisite. A single or pure colony is also a must for all other detailed methods of identification. There are several ways of obtaining pure cultures from mixed populations and, sometimes, more than one method may have to be used in processing a single specimen. The choice of methods, or combination of methods, depends on the nature of the specimen. Plating or streak plates and subculturing are the most commonly used procedures for the cultivation and isolation of bacteria.

Streak plate

The streak plate technique, when effectively performed, is probably the most practical and useful method for obtaining discrete or single colonies. Methods of streaking may vary from one laboratory to another, but the following technique is used for inoculating any type of agar plate. Streaking is accomplished in the sequence of steps shown in figure 3-4. Although this procedure has been designed for an inoculated plate from a swab, it is also used for cultures from, ground tissue, broths, and agar slants.

Streaking

Using a sterilized wire loop proceed as indicated below and in figure 3-4.

1. Place the swab near the periphery of the plate and roll back and forth over a small area of the plate.
2. Using the incinerator, sterilize the loop and spread the inoculum until approximately one-fourth of the plate surface is covered.
3. Sterilize the loop again, allow it to cool, then overlap the previous streak and streak another fourth of the plate.
4. Repeat previous step.
5. Sterilize your loop and streak the center of the plate in a zigzag motion. As you continue the streaking, fewer and fewer cells are dispersed by the loop, and finally single cells are spread across the agar.

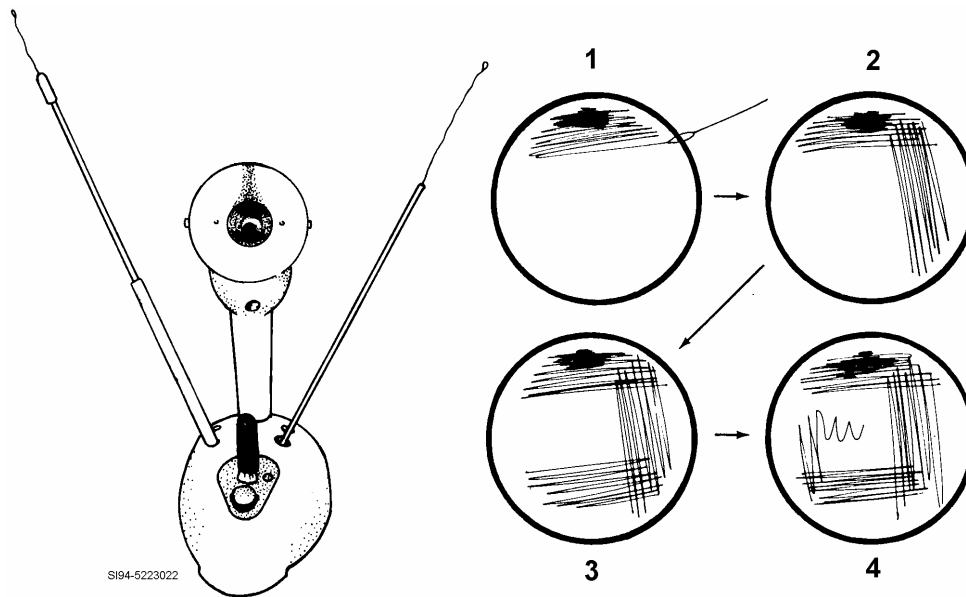


Figure 3-4. Streak plate procedure.

Growing cells

Each cell grows into a visible colony under suitable environmental conditions. When using blood agar plates for throat cultures for strep screens, make multiple stabs in the areas of inoculation to aid in the identification of beta hemolytic streptococci. You may notice distinct colonies in the central portion of the plate. However, additional information concerning hemolytic activity can be obtained from the effect of reduced oxygen tension of the organisms stabbed into the agar. In some instances, beta hemolytic streptococci appear to be alpha hemolytic on the surface. The “O” hemolysin of beta hemolytic streptococci are active only under reduced oxygen tension, such as those provided the organisms stabbed into the agar. However, in cases where different types of media are used and the atmospheric growth condition consists of reduced oxygen tension, the stabbing can be omitted.

Incubating

After completing the above procedure, turn the plate upside down to prevent contamination by water condensation. Incubate as indicated by the specimen source, type of culture, or the organisms in which you want to recover. After *routine* incubation at 35 to 37°C for 18 to 24 hours in the inverted position, isolated colonies are examined macroscopically and microscopically for characteristics of various genera and species.

The reasons for subculturing

After you obtain isolated colonies by the streak plate technique, you can “pick” the pure colonies by touching the center of a colony with a straight wire or loop and proceed with additional testing procedures. But what happens when there are not enough isolated colonies on the plate for additional testing, or they are not completely isolated? The next step would be to “subculture” or “re-streak” to increase the number of bacterial colonies and for isolation.

Re-streaking to plated agar media

Re-streaking from one plate to another is one form of subculturing to obtain isolated growth from mixed flora. Touch the center of a colony with a sterile wire or loop and, using the streak plate technique, subculture the material to a suitable agar media. This provides

numerous isolated colonies for additional tests or isolates heavily overgrown plates for more subculturing. Only when the colony is isolated and you are confident you have a pure culture, then you are ready to proceed with additional test for identification. These tests may include Gram stain, tubed media for biochemical or motility test, differential media, immunoassay procedures, or automated instrumentation.

Tubed media

There are several different configurations in tubed media, each developed for a specific purpose, but we'll limit our discussion to the following:

- Slant cultures.
- Stab cultures.
- Slant and stab cultures.
- Liquid broth cultures.

Slant cultures

Slant cultures are basically used to maintain stock cultures in the laboratory. “Slants” are tubed agar media that have been allowed to harden in a near-horizontal position to give a large surface area for bacterial growth. You prepare them by streaking an inoculum over the slant surface from bottom to top. If the slant contains water or condensation at its base, drain the moisture out by inverting the tube before inoculation. Do not spread the water over the surface of the slant because the resulting growth will not give a characteristic appearance.

Stab cultures

Stab cultures are usually semi-solid media (not slanted) that hardened in a vertical position to give great depth and a small surface area. These cultures are used mostly for motility studies. Using a straight sterile wire, touch a pure colony and carefully stab straight and evenly into the media approximately two-thirds of the depth of the medium. Avoid tearing, bubbles, or a jagged stab. These may later interfere with correct interpretation of motility test.

Slant and stab cultures

Slant and stab cultures are used almost exclusively for biochemical tests. Using a straight sterile wire, touch a pure colony and stab deep into the tube of solid media and streak over the slanted agar surface as in figure 3-5. You should center the stab line and extend it approximately two-thirds of the depth of the medium. Insert the inoculating wire straight and evenly to avoid tearing the medium and creating bubbles. Later, these bubbles could be mistaken for gas production.

Liquid broth cultures

These cultures are used to maintain the viability of an organism by successive transfer to fresh medium and to grow a large volume of liquid culture for inoculation to differential or fermentation media. To inoculate a broth culture, use a wire loop to pick a colony from a plate. Using a colony, emulsify the

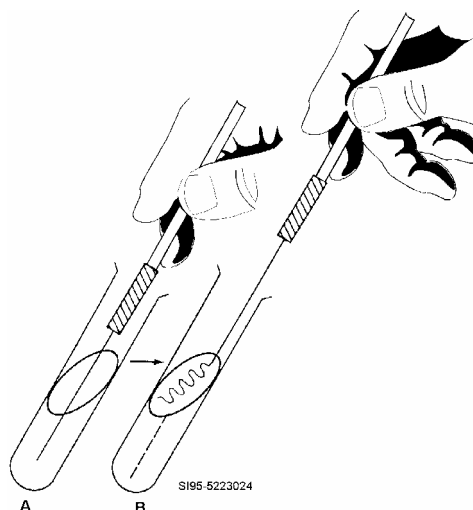


Figure 3-5. Inoculating an agar slant.

material on the moist wall of the tube just above the liquid level and wash down the solid matter by gently tilting the tube. If the inoculum is liquid instead of growth from a colony, simply tilt the tube, place a loopful just above the broth, and replace the tube upright as seen in figure 3-6.

NOTE: Do not swirl the loop or swab in the liquid. Droplets escape from the tube and contaminate the surrounding air. The resulting aerosol can infect you and your fellow workers.

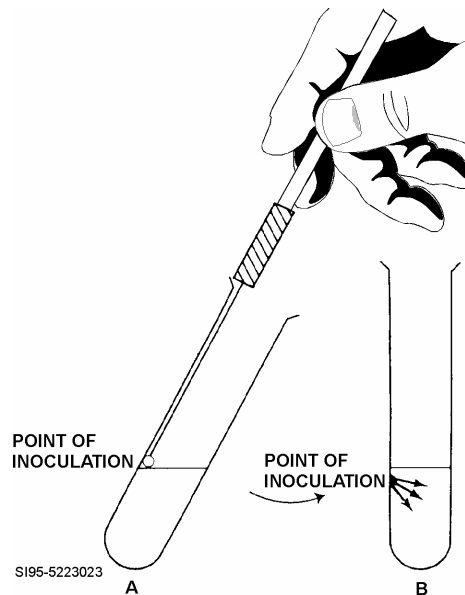


Figure 3-6. Inoculating liquid broth.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

017. Factors to consider when cultivating bacteria

1. What are five factors that you must consider in cultivating bacteria?
2. What do some of the proper food-providing bacterial nutrition include?
3. Most media contain what essential nutrition and what does it provide?
4. Serum or blood is needed by certain pathogens for what purpose?

5. What purpose do buffering agents serve when incorporated into culture media?
6. Which form of bacteria grows best at a temperature of 50 to 60°C?
7. The majority of human pathogens grow best at 35°C and are called what type of form, in terms of growth temperature?
8. In bacterial respiration, electron transport is accompanied by the transfer of what type of atoms?
9. In most aerobic organisms, the hydrogen atoms arising from oxidation-reduction reactions in the cell are joined to atmospheric oxygen. What substance is formed as the end product in respiration?
10. What enzyme do the aerobes produce to break down H_2O_2 into water and oxygen?
11. What is a facultative anaerobe?
12. What effect does oxygen have on the respiratory mechanism of obligate anaerobes?
13. What essential ability do the aerobes and facultative anaerobes have that the obligate anaerobes do not possess that enables them to survive the presence of oxygen?
14. What sort of structures are produced by obligate anaerobes, such as certain organisms of the genus *Clostridium*, that enable them to survive an atmosphere of oxygen?
15. What are microaerobic conditions?

16. What are capnophilic conditions?

018. Considerations in the macroscopic examination of bacterial morphology

1. In examining colonies of bacteria, what physical macroscopic characteristics do you observe?
2. What are some terms used to describe colony size?
3. Colony shape refers to the outline of the colony along with what other characteristics?
4. What are some terms used to describe colony shape in relation to its outer edge?
5. An H-type colony is the result of active flagellar motion. Would you expect this type of colony to be filamentous or pinpointed?
6. Colony elevations are described by what terms?
7. The terms "rough," "smooth," "creamy," and "mucoid" refer to what characteristic of the colony?
8. What two conditions can result in the production of mucoid colonies?
9. Loss of virulence or capsule causes bacteria to show what type of colony texture?
10. Color production is dependent on what factors?
11. What are two types of pigments?

12. What are the four basic groups of hemolysis reactions on blood agar and what are their definitions?

019. The purpose of the streak plate technique and the reasons of subculturing

1. The streak plate technique is practical and most useful for what reasons?
2. How does this surface streaking technique used on plate cultures help in the isolation of a bacterium?
3. At what portion of the plate would you most likely find information concerning the hemolytic activity of beta hemolytic streptococci and why?
4. Why are the plates turned upside down after streaking?
5. How can you “pick” pure colonies from the culture?
6. When can you proceed with additional test for identification?
7. What is the basic purpose of slant cultures?
8. What would result if the condensed water over the slant surface is not allowed to drain before inoculation?
9. For what general purpose are stab cultures used?
10. How should the stab be made in stab cultures?
11. What is the purpose of slant and stab cultures?

12. What are some uses of liquid broth cultures?
13. What technique is used to inoculate a broth culture with a colony from a plate?
14. What can happen if you swirl the loop too vigorously while inoculating a broth?

Answers to Self-Test Questions

012

1. Sources of carbon, nitrogen and inorganic salts.
2. Blood, serum, amino acids, or vitamins.
3. No; they tend to inhibit the bacterial production of certain hemolysins.
4. 1.5 to 2.0 percent.
5. An inert, purified extract of *Gelidium*, a type of seaweed; no.
6. The Clinical and Laboratory Standards Institute publication: *Quality Assurance for Commercially Prepared Microbiological Culture Media; M22-A2*.
7. Sterility, ability to support growth, produce characteristic colony morphology, and as appropriate, selectivity, inhibition, and/or biochemical response.
8. At room temperature.
9. Heat the tubes of media in a hot water bath before inoculation.
10. Leave it at room temperature in an inverted position for at least 18 hours and also incubate a small percentage for 18 hours.
11. So that the lag phase of organisms plated on it is not increased and so cold sensitive organisms don't die.
12. It makes it difficult to isolate colonies.
13. It must be protected from dehydration by placing it in a plastic bag.

013

1. To avoid costly duplication of purpose.
2. Cost, judgment or preference of an experienced technician, and commercial availability.
3. To support the growth of many organisms (nonfastidious).
4. The glucose content is metabolized to acid end products by certain bacteria.
5. It is used to inhibit or destroy the normal intestinal flora and promote the unrestricted growth of pathogens such as *Salmonella* and *Shigella*.
6. Tetrathionate broth, Selenite broth, and GN broth.
7. An enriched medium contains an added nutritive supplement and is prepared by adding serum, blood or other nutrients to basic nutrient medium.
8. To separate various organisms depending on carbohydrate utilization, fermentation or oxidation, or enzyme activity.
9. (1) EMB agar.
(2) MacConkey agar.

- (3) XLD agar.
- 10. The carbohydrate (lactose) and the indicator (phenol red).
- 11. Inhibit the growth of certain organisms while permitting others to grow.
- 12. Mannitol Salt agar and Phenylethyl Alcohol (PEA) agar, respectively.
- 13. These media suppress the organisms they are designed to show after some time of growth.

014

- 1. (1) j.
- (2) k.
- (3) c.
- (4) c.
- (5) a.
- (6) l.
- (7) d.
- (8) f.
- (9) e.
- (10) e.
- (11) h.
- (12) i.
- (13) g.

015

- (1) d.
- (2) f.
- (3) j.
- (4) a.
- (5) h.
- (2) g.
- (3) i.
- (4) c.
- (5) b.
- (6) e.

016

- 1. The ability of a specific organism to attach and break down a particular carbohydrate is a useful characteristic, and this is accompanied by identifiable byproducts. Thus, by carefully selecting the test carbohydrates, you obtain a pattern of fermentation reactions that are characteristic of specific organisms.
- 2. Sucrose and lactose.
- 3. They are able to differentiate the organisms on fundamental differences other than outward appearances.
- 4. Sucrose.
- 5. Lactose, dextrose, and sucrose.
- 6. Iron sulfide.
- 7. *Escherichia*, *Enterobacter*, and *Klebsiella*.
- 8. The presence of gas bubbles and H₂S production.
- 9. Trypticase agar base and CTA media.

10. Small amounts of acids are not readily dispersed throughout the media.
11. Clostridia, bacilli, common micrococci, and enteric bacilli.
12. *Neisseria*, *Pasteurella*, streptococci, *Brucella*, corynebacteria, and vibrios.
13. Accumulation of metabolic products.

017

1. (1) Nutrition (proper food).
(2) Moisture.
(3) Acidity/alkalinity (pH).
(4) Temperature.
(5) Gas exchange.
2. A source of carbon (organic or inorganic), nitrogen, and inorganic chemical salts.
3. Peptone, a breakdown from protein; it provides an available source of carbon and nitrogen.
4. For the organism to reproduce on culture media.
5. Buffering substances retard changes in the pH of the growing culture, thus permitting more abundant growth before a limiting acidity or alkalinity is reached.
6. Thermophilic.
7. Mesophilic.
8. Hydrogen.
9. Hydrogen peroxide (H₂O₂).
10. Catalase.
11. A facultative anaerobe is one that prefers to grow as an anaerobe, but adapts to aerobic conditions. The vast majority of medically significant bacteria are in this category.
12. It is toxic.
13. They produce the respiratory enzyme necessary to couple hydrogen atoms with oxygen, the hydrogen acceptor commonly used by both of these types of organisms.
14. Resistant spores.
15. Oxygen is toxic, except in minute amounts.
16. Organisms require increased CO₂ concentrations than found in room air.

018

1. Colony size, shape or form, elevation, margin, texture, color, pigmentation, and hemolysis on blood agar.
2. Pinpoint, small discrete, large, or spreading.
3. Its elevation and margin.
4. Punctiform, circular, filamentous, irregular, rhizoid, and spindle.
5. Filamentous.
6. Flat, raised, convex, pulvinate, umbonate, and umbilicate.
7. Texture.
8. Heavy capsule formation by individual cells or by secretion produced by bacteria in response to an external stimulus.
9. The R-type colony is produced with dull appearance, less translucent (more opaque) than the S-type colony, and may have an irregular circumference and a wrinkled or rough surface.
10. Type of medium used, time and temperature of incubation, age of culture, and other environmental factors.
11. Water-insoluble and water-soluble.

12. Gamma, beta, alpha, and alpha prime. Gamma is no change or lysis of the red blood cells of the media surrounding the colony. Beta is a complete zone of red blood cell lysis within the media surrounding the colony. Alpha is a partial hemolysis of blood cells around the colony that gives a green discoloration of the media. Alpha prime gives a halo affect of incomplete hemolysis immediately around the colony and a second zone of complete hemolysis at the edge or periphery.

019

1. Obtaining discrete or single colonies.
2. As the wire loop is streaked back and forth across the plate, fewer and fewer organisms are deposited on the medium surface. Eventually, only single cells are deposited.
3. The stabbed sections into the agar; the “O” hemolysins of beta hemolytic streptococci are active only under reduced oxygen tension.
4. To prevent contamination by water condensation.
5. By touching the center of a colony with a straight wire or loop.
6. Once the colony is isolated and you are confident you have a pure culture.
7. Maintain stock cultures.
8. The resulting growth does not give a characteristic appearance.
9. Motility studies.
10. Carefully stab straight and evenly into the media approximately two-thirds of the depth of the medium.
11. Biochemical tests.
12. To maintain the viability of an organism by successive transfers to fresh medium and to grow a large volume of liquid culture for inoculation to differential or fermentation media.
13. Emulsify the material on the moist wall of the tube just above the liquid, and wash down the solid matter by gently tilting the tube.
14. Droplets escape from the tube and contaminate the surrounding air. The resulting aerosol can infect you and other technicians.

Do the unit review exercises before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter.

49. (012) What is *not* a constituent of a basal culture medium?
- a. carbon.
 - b. nitrogen.
 - c. organic salts.
 - d. inorganic salts.

50. (012) Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards or NCCLS) publication *M22-A2* directs microbiology media
- quality control.
 - preparation.
 - disposal.
 - storage.
51. (012) Thioglycolate (THIO) broth is stored
- frozen.
 - refrigerated.
 - in a water bath.
 - at room temperature.
52. (012) Many prepared bacteriological plating media can be stored for an extended time if
- moisture is retained.
 - condensation is prevented.
 - the media is kept in the dark.
 - repeated warming and cooling is restricted.
53. (013) *Most* basic nutrient media are used for growing
- fastidious organisms that comprise the minority of bacteria.
 - fastidious organisms that comprise the majority of bacteria.
 - nonfastidious organisms that comprise the minority of bacteria.
 - nonfastidious organisms that comprise the majority of bacteria.
54. (013) What media is *not* an example of an enrichment media?
- Gram-negative (GN) broth.
 - Tetrathionate broth.
 - Selenite broth.
 - Basal broth.
55. (013) The purpose of selective media is to
- select the lactose fermenters among nonpathogens.
 - differentiate between the lactose and nonlactose fermenter.
 - inhibit the growth of certain organisms while permitting the growth of others.
 - inhibit the growth of all lactose fermenters while permitting the growth of others.
56. (014) What media additive(s) inhibits growth of Gram-positive bacteria and also suppresses the motility of flagellated bacteria?
- Sodium azide.
 - Chloral hydrate.
 - Potassium tellurite.
 - Sodium desoxycholate and bile salts.
57. (015) Failure to ferment xylose in xylose lysine desoxycholate (XLD) agar results in
- yellow or white colonies.
 - blackening of the colonies.
 - alkalinization of the medium.
 - acidification of the surrounding medium.

58. (015) What serves as *both* a culture medium and a good transport medium?
- Loeffler medium.
 - Transgrow medium.
 - Trypticase agar base.
 - 5-percent sheep blood agar.
59. (016) What does cystine trypticase agar (CTA) contain that trypticase agar base does not?
- phenol red.
 - carbohydrate.
 - sodium chloride.
 - carbohydrate taxo discs.
60. (017) Bacteria that grow best at a temperature of 20°C to 40°C are called
- mesophilic.
 - thermophilic.
 - pleomorphic.
 - psychrophilic.
61. (017) Free oxygen is toxic to
- aerobes.
 - anaerobes.
 - facultative anaerobes.
 - microaerobic bacteria.
62. (018) Pinpoint, small discrete, large, and spreading describe what colony morphology or characteristics?
- Size.
 - Color.
 - Shape.
 - Texture.
63. (018) H-type colonies of *Proteus* spp. are due to
- active flagellar motion.
 - Ohne-hauch forms.
 - urease production.
 - capsular material.
64. (018) What basic group of hemolytic reactions on blood agar is *best* described as no change or lysis of the red blood cells in the media surrounding the colony?
- Alpha.
 - Alpha prime.
 - Beta.
 - Gamma.
65. (019) Plates are turned upside down *after* streaking in order to
- prevent overgrowth by pathogens.
 - increase the logarithmic growth phase.
 - decrease the lag phase of the growth curve.
 - prevent contamination by water condensation.

66. (019) The *basic* use for slant and stab cultures is
- a. for motility.
 - b. for biochemical tests.
 - c. to maintain stock cultures.
 - d. to grow a large volume of bacteria.

Please read the unit menu for unit 4 and continue ➔

Unit 4. Specimen Processing

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AS YOU learned in Unit 2, the first step in properly identifying microorganisms begins with collection, a process that is basically out of the hands of the laboratory. Once the specimen is collected, however, it is in your hands! Correctly processing the specimen is the second critical step in eventual isolation and identification of bacterial agents of disease. The physician can only treat what is identified. This unit outlines general guidelines for processing the more common clinical specimens.

4-1. Processing Clinical Specimens

Each laboratory establishes its guidelines for handling specimens through OIs. The decisions whether or not to do direct smears and employ a variety of selective or differential media depends upon budget, patient population, geographic location, and other parameters. It is the responsibility of the laboratory to make the physician fully aware of what organisms will be recovered by its routine culture procedures. The laboratory must be notified when the physician suspects bacterial agents that require additional selective media for primary isolation. In this section, we will review general guidelines for processing various specimens.

020. Specimen processing guidelines

When you are processing microbiology specimens there are many factors to keep in mind. Some are general guidelines, while others are specific to the specimen you are processing. We'll present some of those in this lesson.

General microbiology specimen processing guidelines

Ensure your laboratory has provided the clinics with the procedures for proper collection and timely delivery to the microbiology department. In addition, you must ensure, when aberrations to any collection or delivery procedures occur, that consultation with the clinical staff is conducted. You must also annotate the end-result of these consultations in the patient's result file. Failure to document these annotations can have serious legal repercussions for the laboratory not to mention the omission to the patient's medical record. Often you will find that specimens with a questionable collection or delivery history, which wasn't to the letter, may still be acceptable to the provider in order to minimize patient discomfort. Just remember, this is always the decision of the provider even if similar instances have occurred before. The delivery of care for each patient is different.

Be aware of all safety requirements and follow them to the letter – you don't want to get sick from not following safety guidelines. Be aware of pathogens that are reportable to the state health department or equivalent. The bottom line is to make sure you are able to conduct your work towards quality patient care in a safe and efficient manner.

Processing blood culture specimens

The laboratory's processing OI for blood cultures is decided by the blood culturing system employed by your lab and MTF. These systems range from manual or conventional, semiautomated, to completely automated. The system chosen by a laboratory is based on the number of blood cultures routinely performed, patient population seen, and the lab's resources (personnel, space, and budget). Each system will be briefed in this lesson.

Blood culture media

Any general-purpose, commercially available nutrient broth medium can be used for the blood culture depending on the system used at your MTF. Soybean casein digests, (Tryptic (Difco) or Trypticase (BBL soy broth)), Columbia broth, and BHI broth have been found to be satisfactory. Commercially available liquid media are generally bottled under vacuum with CO₂ and contain 0.025 percent SPS as an anticoagulant. To this extent, they are satisfactory for cultivation of anaerobes from blood. Bottles containing 50 to 100 ml of media are employed. If only one blood culture bottle is used, do not use a vented one. However, it is best to use two bottles—one that is not vented (for anaerobes) and the other vented. The routine addition of penicillinase to blood culture media does not appear to be justified, except in selected cases where the patient is receiving high doses of a penicillin or cephalosporin at the time of collection. The isolation of *Brucella* is performed by inoculation of the blood into Castaneda's double medium or into *Brucella* broth, which must be subcultured at least twice weekly, for four weeks. Prolonged increased CO₂ incubation is required for the recovery of *Brucella*. Retain cultures for 28 days before reporting them as negative.

Incubation and examination of conventional blood cultures

Cultures are incubated at 35°C and inspected on the same day of collection and daily thereafter for at least seven days. Constantly agitating aerobic bottles for the first 24 hours of incubation will enhance the growth of most bacteria. You are looking for evidence of turbidity, hemolysis, gas bubbles present in the media, or the appearance of small colonies in the broth or on the settled red blood cells (RBC). If any one of the above is observed, prepare Gram-stained smears and cultures immediately, and report the microscopic examination of the smear by phone to the patient's physician, clinic, or ward as soon as possible. Suspected positive blood cultures are usually subcultured to 5-percent sheep blood agar, chocolate, MAC agar, and supplemented anaerobic blood agar.

Perform routine "blind" subcultures of grossly negative culture between 6 and 12 hours after blood collection. For a "blind" subculture from aerobic bottles, place the inoculum onto quadrants of chocolate agar plate and incubate at 35°C in 5- to 10-percent CO₂ for 48 hours before examination. If there is no evidence of bacterial growth in the bottles after 48 hours, repeat the "blind" subculture procedure, or the acridine orange stain may be used. If all cultures, subcultures, and stained smears are negative, the blood culture is reported as "no growth aerobic or anaerobic after 7 days incubation."

Semiautomated methods of performing blood cultures

We reviewed the conventional method; now, let's look at several semiautomated methods for performing blood cultures.

Biphasic bottle procedure

Another modified method for the rapid isolation of bacteria and fungi from blood cultures involves preparation of a bottle for culture containing both liquid and solid media. Such bottles, called "biphasic" bottles, allow for easy isolation of microorganisms on solid agar slants. Following inoculation of 10 ml of blood to the laboratory's preferred blood culture medium, the culture is incubated for 6 to 18 hours, and a plastic paddle or slide containing agar or several different agar culture media is then attached to the primary blood broth bottle. Subculture is easily performed by inversion of the primary blood culture bottle with the attached plastic paddle containing agar culture media. This simple procedure doesn't require needles or opening the bottles. A daily subculture is then carried out by inversion of the unit and observation for growth on the agar. This procedure provides isolated colonies after short incubation periods and reduces the inhibition of growth of microorganisms by cellular and humoral elements in the original blood specimen obtained for culture. The biphasic systems do not recover anaerobic bacteria sufficiently.

Lysis-centrifugation procedure

An approach to the separation and rapid removal of bacteria and fungi from the antimicrobial activity of blood is the blood cell lysis-centrifugation procedure. The commercial lysis-centrifugation system available is the Isolator by Wampole Laboratories, Cranbury N. J. This method requires a lysing agent, saponin, to destroy both red and white blood cells. SPS is added as an anticoagulant; polypropylene glycol to decrease foaming; ethylene diamine tetra-acetic acid (EDTA) to chelate calcium ions and, in-turn, inhibit the complement cascade and coagulation; and a small amount of an inert fluorochemical to cushion and concentrate the microorganisms. The mixture of blood, lysing agent, and microorganisms is centrifuged for 30 minutes at 3000 x g to sediment the bacteria or fungi on the cushion. Following centrifugation, the lysing agent, hemolyzed blood, and serum are aspirated, and the remaining fluid, with any sedimented microorganisms, is then withdrawn and inoculated directly to primary isolation media for bacteria and fungi. Preliminary evaluation of the lysis-centrifugation procedure has shown significantly reduced time requirements for the isolation of yeasts, such as *Candida albicans* and gram-positive cocci (*Staphylococcus aureus*). It is postulated this improved recovery of microorganisms correlates best with removal of bacteria and fungi from the hostile cellular and humoral factors of blood. This results in the more rapid isolation of colonies on selective or enriched agar, which can then be identified by several different methods. Disadvantages to the system include a high rate of plate contamination that requires the procedure to be done inside a laminar flow biosafety cabinet, and the failure to recover certain bacteria, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Listeria monocytogenes*, and anaerobic bacteria, as well as conventional systems do.

Automated methods

There are numerous automated methods used to perform blood cultures. We will look at some of the more popular instruments found in most laboratories.

The Bactec System

The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993.

“Automation was developed to reduce the handling time and shorten the time required to detect the presence of microorganisms. The Bactec System was a pioneer analyzer in clinical microbiology and became the first automated instrument in many clinical laboratories. With the wide acceptance of this method advances have been made that further decrease labor time and reduce the time needed to detect microbial growth. The newest blood culture instruments feature walk-away automation.

The Bactec 460 (originally Johnston Laboratories, currently Becton Dickinson Diagnostic Instruments) was the first widely used automated blood culture system. The next generation of instruments is more automated and computerized, although the detection principle has remained the same: analysis of the air space in blood culture vials for the presence of CO₂ elaborated as a microbial metabolic byproduct. The Bactec 460 system uses bottles that are filled with measured amounts of media containing ¹⁴C. Because the weak beta radiation in the media cannot penetrate the glass, the bottles are safe to handle. However, the use of radioactive materials does require adherence to safety guidelines for handling and disposal that may require additional training. Several media types are available for growth of aerobic and anaerobic organisms, including optional resins for antimicrobial inactivation, hypertonic (10 percent sucrose) media, high volume, and pediatric volume vials. The detection of organisms is based on the use of ¹⁴C-labeled glucose and other substrates with subsequent release of ¹⁴CO₂ in a vial.

Once the vial tops are wiped with alcohol, up to 15 racks of bottles are placed on the sample charger and the instrument automatically tests all vials present. The instrument begins its test operation by producing a partial vacuum in the ion chamber, at the same time heating two 18-gauge needles. The head valve then closes, lowering the movable head down onto the vials, and driving the needles through the rubber septum on top of the test vial. The atmosphere above the broth level is flushed into the ion chamber by closing the outlet valve and opening the inlet valve, drawing gas from a reserve tank through a filter and the culture vial. The radioactive gas passes through the chamber and changes the impedance between the two poles. The impedance is converted into a numeric value, or growth index (GI), that is printed on tape or datalog card. If this value exceeds a reset threshold, the vial is determined positive for bacterial growth and a red light corresponding to the location of the vial will be seen. The measurement system is cleansed before the next reading cycle.

The Bactec 460 can also be employed for the detection, identification, and susceptibility testing of mycobacteria. The system requires a different instrument hood and uses special Bactec vials, with 7H12 broth containing ¹⁴C palmitic-1 acid. The labeled CO₂ released by metabolism of palmitic acid is used to detect growth. Specimens are decontaminated, concentrated, and injected into culture vials. The vials are read on the instrument with positive cultures detected in 7 to 12 day.

The Bactec 460 system has been superseded by the NR-660, NR-730, and NR-860 systems. All of these models use infrared detection of microbial metabolic CO₂ byproduct, thus eliminating the problems of working with radioactive material. The test and detector systems on all three instruments are the same. Infrared light is used to measure the amount of CO₂ present in the bottle headspace. Vials are automatically disinfected with ultraviolet light.”

The BACTEC 9240 is the newest system from Becton-Dickinson Diagnostic Instrument that is similar to the Organon Teknika BacT/Alert (the first of its kind). This system incorporates bar-code bottle identification and the in-bottle indicator chamber using fluorogenic pH indicators.

The Organon Teknika BacT/Alert

The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993.

“The BacT/Alert (Organon Teknika) is a self-contained instrument with total walk-away automation that continually agitates and monitors blood cultures. Microbial growth is detected through noninvasive monitoring of the level of CO₂ within each sample bottle by a colorimetric sensor attached to the bottom of the bottle. Each bottle cell is scanned at 10-minute intervals or 144 times/day. The culture bottles contain an internal colorimetric sensor to detect microbial growth. Production of CO₂ causes the sensor to change color from green to yellow. The BacT/Alert uses solid-state reflectometers to monitor the sensors. Excited light is directed toward the sensor, that is reflected back to a light-absorbing photodiode. The instrument measures the amount of light returned and compares this with an initial threshold or the sample’s own past performance (rather than a common threshold). A sample is determined positive when one of three criteria is met: (1) a sustained increase in the rate of change, (2) an acceleration of CO₂ production (how the rate has changed from baseline), or (3) amplitude of signal (the threshold that is applied on initial entry). Background CO₂ produced by blood components is differentiated from microbial CO₂ production.”

Concerns

As with everything in life, nothing is perfect. Both automated methods have advantages and disadvantages. Advantages include a faster detection time for most pathogens, the ability to handle large numbers of blood culture bottles automatically, and the ability to monitor growth without hands-on visual inspections. The ability for the Bactec to be modified to successfully detect mycobacteria, and the availability of resin-containing broth media for the enhanced recovery of fungi and fastidious bacteria are other advantages. Disadvantages with the Bactec system include false-positives because of carryover from positive bottles to others due to inadequately sterilized sampling needles and inappropriate setting of threshold values. False-negatives are caused by the failure of certain organisms to metabolize enough substrate. A problem with the Bactec 460 system is the regulations governing disposal of radioactive material that vary from state to state. The current disadvantage with the BacT/Alert is the few media or broths available, none of which contain resins. The advantages of the BacT/Alert is it is noninvasive (no carryover), has nonradioactive material, and each bottle has its own baseline.

What happens with positive bottles?

These automated systems identify growth, not microorganisms. Once a positive bottle is identified, subculturing is required. Treat vials from automated systems just like positive conventional cultures.

Cerebrospinal fluid and other sterile body fluids

Processing CSF for Gram stain and culture is a priority in the laboratory. A child or an adult with acute meningitis caused by *Haemophilus influenzae* or *Neisseria meningitidis* may have only hours to live if antibiotic therapy is not started right away. As you can see, a preliminary Gram stain result is essential in treating acute meningitis. Other sterile body fluids are processed very similar to CSF, but are usually not as critical.

CSF

As stated earlier in Unit 2, the second tube collected is used for microbiology studies. The specimen is delivered to the microbiology laboratory as soon as possible (ASAP) and processed immediately. Fluid volume and concentration of bacteria are critical in CSF studies for Gram stain and culture. Visually examine the CSF for color and clarity. Process the grossly purulent or cloudy specimens by using a sterile pipette to directly pipette fluid onto a slide for Gram stain. The Gram stain smear is read ASAP and results reported to the physician, clinic, or ward. For routine cultures, inoculate on 5-percent sheep blood agar, chocolate agar in an enrichment broth, and possibly a MAC, EMB, or anaerobic agar as indicated by Gram stain. Concentration of bacterial cells is a concern with CSFs that are macroscopically clear; therefore, it is important to concentrate these specimens.

A preferred method for concentrating clear specimens for Gram-staining is the cytocentrifugation technique. This requires a cytocentrifuge with special containers. Another method is through centrifugation. Centrifuge the CSF tube at 1500 x g for 15 minutes. The supernatant fluid can be used for direct-antigen testing or can be shared with chemistry or immunology departments (if only a small amount of specimen was collected). The pellet is then mixed and pipetted onto a slide for Gram stain and inoculated to the media mentioned above. The agar plates are incubated at 37°C in 5- to 10-percent CO₂ and examined daily for at least 72 hours. The broth is incubated in air at 37°C for at least 5 days and the broth cap should be loose to allow air exchange. Broths are examined daily for growth. If growth is present, prepare a Gram stain and inoculate agar plates as indicated.

Direct antigen testing

As mentioned above, acute meningitis can be fatal and, as a result, early identification is critical. Along with the Gram stain result, direct antigen detection procedures aid the laboratory in faster turn-around times for preliminary results. Direct antigen detection systems use the principle of a purified antibody-coated particle, such as latex or erythrocytes that bind to a specific antigen (capsular polysaccharide) found naturally on the surface of the bacterial cell. The latex (or erythrocyte) is needed to provide the visible evidence of the bound antibody and antigen. Direct antigen detection systems are commercially available for the identification of *Haemophilus influenzae* group B, *Neisseria meningitidis* groups A, B, C, Y, and W-135, *Streptococcus pneumoniae*, group B streptococci, and *Cryptococcus neoformans* in CSF. All antigen testing is followed up with standard culturing procedures, except for *C. neoformans*. As with all procedures, follow the manufacturer's instructions, know the procedures' limitations, and recommend follow-up or confirmatory testing procedures.

Other sterile body fluids

Most sterile body fluids are processed like CSF. Volume and concentration are also important and the same techniques as above apply. Centrifuge at 1500 x g for 15 minutes decant or using a sterile pipette aspirate supernatant leaving approximately 1ml of fluid to resuspend the pellet. Vortex or mix the pellet with the remaining fluid.

Safety reminder: The vortex or pellet mixing procedure.

For routine specimens, inoculate 5-percent sheep blood agar, chocolate agar, MAC or EMB agar, enrichment broth, and anaerobic plate media, incubate as required, and prepare a smear for Gram stain. Smears can also be prepared by cytocentrifugation. If a clot is present, it should be homogenized (broken up) to release trapped bacteria and mixed with supernatant. Keep in mind sterile body fluids are collected from numerous sites and may need special

handling and processing procedures. The media inoculated is dependent on the site, disease process, and concerns of the clinician. If enough fluid is collected, conventional and automated blood culture bottles can be inoculated and processed as blood cultures.

Bone, tissue, abscess, wound, eye and ear specimens

Bone, tissue, abscess, wound, eye, and ear specimens can be plated to the same routine media as the sterile body fluids or to unique media, depending on the infectious agent the clinician is suspecting. The basic culturing techniques and media for each will be briefly discussed. Unique media requirements will be discussed in the lessons that discuss the individual microorganism.

Bone and tissue specimens

Processing bone and tissue specimens for culture is unique since bone and tissue are usually solid. Bone specimens should be ground using a sterile mortar and pestle with a sterile broth to form a suspension. The suspension is inoculated to 5-percent sheep blood agar, chocolate agar, MAC or EMB agar, enrichment broth, and anaerobic media, and a smear is prepared. Tissue specimens are homogenized using a sterile mortar and pestle, or in a sterile plastic bag by using a commercial tissue grinder. The suspension fluid can be a sterile broth or saline. Inoculate the same as with bone specimens. Aspirate specimens are treated like other sterile fluids. If not enough bone or tissue specimen for grinding or homogenizing is submitted, place the whole specimen in an enrichment broth.

Abscess and wound specimens

With abscess and wound specimens, the site of culture is very important to the laboratory. This information is taken into account for plating appropriate media, culture conditions, and when interpreting Gram stains. Most abscesses will be inoculated to 5-percent sheep blood agar, chocolate agar, MAC or EMB agar, enrichment broth, and anaerobic media, and a Gram stain smear is prepared. Deep wound specimens are plated the same as abscesses. Superficial wounds, cellulitis, burns, bites, and so forth are plated on 5-percent sheep blood agar, chocolate agar, and MAC or EMB. The presence of anaerobic normal flora and other normal flora found on the skin make it difficult to culture these specimens anaerobically or inoculate an enrichment broth that will be overgrown with normal flora. If specimens are collected properly, anaerobic cultures can be performed.

Eye and ear specimens

As with wound specimens, great care must be taken during collection in order to avoid normal flora. Most eye specimens are inoculated to 5-percent sheep blood agar, chocolate agar, and an enrichment broth (THIO or CM). The enrichment broth is very important to the recovery of fastidious organisms. Ear specimens are inoculated to 5-percent sheep blood agar, chocolate agar, MAC or EMB, and possibly CNA agar or PEA agar.

Upper and lower respiratory tract specimens

Upper and lower respiratory tract specimens propose their own set of problems and concerns. Most of these problems are presented during collection, but also affect processing procedures. Because of the normal flora present and the specific infectious agents involved, we will discuss in more detail the processing procedures for respiratory tract specimens.

Upper respiratory tract specimens

Cultures of the upper-respiratory tract must be interpreted cautiously because of the microflora normally present in the nose, oral cavity, and pharynx, and because of the frequency of nosocomial acquisition of potentially pathogenic microorganisms by seriously

ill patients. Since potential pathogens, such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*, and yeast, may be present in the oropharynx, their isolation from cultures of the respiratory secretions does not represent evidence of their etiological role in respiratory infections.

Nasopharyngeal (nose and sinus)

Nasopharyngeal cultures are performed to detect carrier states of *Neisseria meningitidis*, *Corynebacterium diphtheriae*, and *Streptococcus pyogenes*. In addition, such cultures aid in the diagnosis of whooping cough and croup. Nasopharyngeal specimens are obtained with a Dacron, cotton, or calcium alginate swab on a flexible wire that is gently passed through the nose into the nasopharynx, rotated, removed, and placed into a suitable transport medium for isolation. Nasopharyngeal aspirates are superior to swabs for isolating *Bordetella pertussis* from children suspected of having whooping cough. *H. influenzae* type B can cause a form of croup known as acute epiglottitis. The distinctive appearance of the epiglottitis and the rapidly progressive and fulminating course, which can lead to death within 24 hours, demand prompt initiation of therapy. Nasopharyngeal and blood specimens are obtained and cultured for *H. influenzae* once an airway has been assured.

Routine nasopharyngeal cultures are inoculated to 5-percent sheep blood agar and chocolate agar. These plates are incubated at 37°C in 5- to 10-percent CO₂. Cultures for specific organisms are inoculated to special selective and enrichment media, and incubated, as needed, for recovery.

Although nasopharyngeal cultures are sometimes recommended in comatose patients unable to expectorate a sputum sample, the frequency of aspiration and subsequent anaerobic pleuropulmonary infection in these patients, and the misleading results provided by oropharyngeal contamination make percutaneous transtracheal aspiration mandatory. Keep in mind that orolaryngeal involvement is often common in both acute and chronic disseminated forms of histoplasmosis and blastomycosis, in tuberculosis, and in leishmaniasis. Cultures for these agents and biopsy with histological demonstration of the organisms are necessary to establish the correct diagnosis. It is imperative the clinician communicate his or her clinical suspicions to the microbiology technician immediately. Otherwise, valuable time and specimens may be lost.

Throats

Throat cultures are obtained most frequently for the diagnosis of streptococcal pharyngitis and less commonly for the diagnosis of pertussis, diphtheria, and pharyngitis due to gonococci or viruses. There is no need to identify other microorganisms in routinely submitted throat cultures since there is little or no evidence to document their role in producing pharyngitis. Exudative pharyngitis, enlarged cervical nodes, headache, nausea, vomiting, and abdominal pain are commonly associated with streptococcal pharyngitis, whereas cough, rhinorrhea, and hoarseness are commonly associated with viral pharyngitis. However, many viral infections are confused clinically with streptococcal pharyngitis. Acute tonsillopharyngitis with vesicles or shallow ulcers on the anterior fauces, palate, and buccal mucosa is usually due to herpes simplex virus or to Coxsackie virus A.

A history of an incomplete immunization series or no immunization during childhood should alert the clinician to the possibility of infection with *B. pertussis* or *C. diphtheriae*, particularly when incomplete immunization is coupled with the characteristic signs and symptoms of these diseases. Gonococcal pharyngeal infection should be suspected in patients with gonococcal infections at other sites. Plate the specimen on selective and enrichment media, as needed. Plate routine throat cultures on 5-percent sheep blood agar, and make 2-3

stabs in the media to recover oxygen-labile and oxygen-stable streptococcal hemolysins. Once plated and incubated overnight, follow identification procedures discussed in Volume 2.

Direct detection on throat swabs for group A streptococcal antigen

Rapid methods for the direct antigen detection of group A streptococci are becoming accepted practices in many laboratories and allow the clinician to start antibiotic therapy immediately. Major improvements have been made in procedures using latex agglutination, enzyme immunoassay (EIA), and gene probe technologies. Commercially manufactured kits using these techniques are numerous and available through many different companies. Research into specificity, sensitivity, cost per test, total technician time vs. manpower, and confirmatory testing procedures should be taken into account when deciding on which kit to use in your laboratory. All the methods require the extraction of group A streptococci antigens from the swab, so this must also be taken into account.

Once the antigen is extracted, one of the above procedures is used for identification. A problem encountered by some of the procedures is the false negative results due to low colony counts of beta hemolytic streptococci. It is, therefore, recommended by some that two swabs should be collected—one for direct antigen testing and, if the direct antigen test is negative, the other swab can be used for standard plating procedures.

CDC guidelines for processing throat and nasopharyngeal cultures

The following are CDC guidelines for processing throat and nasopharyngeal cultures:

- Throat cultures are routinely processed for the recovery of beta hemolytic streptococci only. Organisms other than beta hemolytic streptococci do not cause primary acute pharyngitis. Staphylococci can cause tonsillar abscesses, *H. influenzae* causes acute constrictive epiglottitis, and *Corynebacterium diphtheriae* causes a membranous pharyngitis.
- Attempts to recover *H. influenzae* routinely from throat cultures are the prerogative of each laboratory. A high percentage of healthy adults and children harbor *Haemophilus* species in their oropharynx. Physicians must inform the laboratory if *Haemophilus* infection is suspected.
- Antibiotic susceptibility testing is not performed on bacterial isolates recovered from throat cultures. Beta hemolytic streptococci are universally susceptible to penicillin. Other organisms are not considered pathogenic in the absence of specific complications.
- Coliform bacilli in throat cultures are not usually reported. Coliform bacilli do not normally cause pharyngitis but can colonize the throat and serve as a reservoir for lower respiratory infections. Hospitalized patients tend to colonize with coliform organisms that are resistant to many antibiotics used in that hospital. Susceptibility tests are not performed except on request or for nosocomial purposes.

Lower respiratory tract specimens

Sputum specimens are screened for epithelial and white blood cells (WBC) before acceptance for culture. Microscopic examination of sputum is an accepted practice to detect specimens that are contaminated with oral and nasopharyngeal flora. There are several sets of published guidelines available for use in screening sputum specimens.

Four criteria for grading sputum specimens

The four common criteria for grading sputum specimens are listed below. You may want to consider one of these for use in your laboratory. The following criteria indicate oropharyngeal contamination and suggest the sputum specimen may not be representative of the lower-respiratory tract:

1. Greater than 10 squamous epithelial cells per 100X total magnification field.
2. Less than 25 polymorphonuclear leukocytes per 100X field.
3. Greater than 10 epithelial cells and less than 25 polymorphonuclear leukocytes per 100X field.
4. Greater than 25 squamous epithelial cells per 100X field.

Other methods

Other published methods assign a positive number to the quantity of WBCs observed, and a negative number to the quantity of epithelial cells present. By adding the positive and negative values gathered from a sputum smear, any negative sum would be a criterion for rejection. When sputum screen results are provided to the physician, cellular constituents can help interpret the culture report. Specimens graded as unsatisfactory are not plated and the physician is notified by phone and in writing.

Uses

Microscopic examination is not used to determine the acceptability of bronchoscopy specimens and transtracheal aspirates for bacterial culture. Transtracheal or intralaryngeal aspiration is a rapid method of obtaining lower-airway secretions that do not contain oropharyngeal contamination. Bronchoscopy specimens can contain some oropharyngeal contamination introduced during instrument passage.

Sputum processing rationale

Bacterial culturing of sputum is fraught with error, and clear-cut results are seldom obtained. Specimens are frequently collected haphazardly by personnel who are not aware of the necessity for a fresh, clean specimen resulting from a deep cough, and fail to transport the specimen to the laboratory promptly. Expecterated sputum is frequently contaminated with oropharyngeal flora, and it is difficult to determine which of the many different potential pathogens isolated is responsible for pulmonary infection. Rarely, a potential pathogen is isolated in pure culture and can be presumed to represent the etiological agent. There is no completely satisfactory method for isolating bacterial pathogens from expecterated sputum at present. Under no circumstances is anaerobic bacterial culturing of sputum performed. A Gram stain preparation precedes all sputum culture efforts, as noted above.

Routine processing

Routine culturing for sputums, bronchoalveolar washings, and tracheal aspirates include 5-percent sheep blood agar, chocolate agar, and MAC or EMB, incubated at 37°C in 5- to 10-percent CO₂. When culturing a transtracheal aspirate or lung biopsy, add an anaerobic media. If the clinician is looking for a specific infectious agent, the laboratory should be notified by the clinician or on the request slip. Depending on the agent, special stains and media are required. Other guidelines to assist you in processing sputum specimens are:

- Sputum samples of less than 2 ml in volume should not be processed unless the material is obviously purulent. With the exception of legionellosis, most respiratory bacterial infections cause copious amounts of sputum to be expectorated. Small quantities of a clear, thin material usually represent saliva.

- Only one sputum sample per 24 hours should be submitted, except for postbronchoscopy specimens. If more than one specimen is received in series, the first morning specimen or the one with no microscopic evidence of contamination should be selected for processing. One sputum sample per 24 hours is usually adequate to reflect the respiratory secretion pool. Postbronchoscopy specimens usually represent the most ideal deep-cough specimens that can be obtained.
- A direct Gram stain is performed on all routine sputum specimens to assess their quality as representative of lower respiratory secretions. Some criteria must be used to determine the quality of the specimen. Samples that are representative of “spit” rather than true lower-respiratory secretions produce insignificant results. The reporting of a potentially pathogenic bacterium in a nonrepresentative sputum sample could be misleading, particularly in cases of clinical pneumonitis.
- Each smear is stained and several fields of view are examined and evaluated. Published criteria are available, as noted earlier, that describe methods to predict the absence of infection or significant contamination with oral secretions.

Contaminated specimens

Squamous epithelial cells are derived from the oral mucosa and their presence in sputum samples represents contamination with “spit.” If the specimen is contaminated, another specimen is requested. If a contaminating organism from the oropharynx is incorrectly considered the pathogen causing the pneumonitis, therapy may be misdirected. For this reason, it is recommended the Gram stain results be reported along with the culture results. Grading criteria are not applicable to sputum specimens submitted for isolation of *Mycobacterium tuberculosis* or mycotic agents.

Urine specimens for culture

Urine cultures comprise the largest number of specimens in most routine clinical microbiology laboratories. Estimates as to what percentage of these specimens submitted for culture are “negative” (no growth) or bacterial counts below levels that are clinically significant range from 60 percent to as high as 80 percent—hence, the need for screening procedures.

Urine screening methods

The purpose of urine screening is twofold:

1. To provide accurate and timely information to the clinician for prompt patient care.
2. To rapidly detect urine specimens lacking significant bacteriuria.

By application of appropriate technologies, the screening purpose can be achieved and substantial savings of technician time and media expense can be realized since specimens with negative screening results need not be cultured. However, you are cautioned that a negative screening result does not absolutely rule out significant bacteriuria. The decision as to when a negatively screened specimen should be cultured deserves careful consideration. Methods for the detection of bacteriuria range from the manual gram staining of uncentrifuged urine, to the semiautomated colorimetric filtration methods, to the fully automated photometric methods. With the exception of the few fully automated systems that also provide identification techniques, the screening methods only detect the presence of bacteria and must be followed by routine culturing procedures. First, we will look at brief descriptions of the various methods, which will be followed by the routine culturing procedure for urine specimens.

Microscopic methods

Two microscopic screening methods are gram staining and acridine orange staining. A Gram stain of uncentrifuged urine is an accurate indication of significant bacteriuria. Two or more organisms per oil-immersion field from uncentrifuged urine correlates with a culture of greater than or equal to (\geq) 10^5 CFU/ml. Variables that affect this correlation are methods of specimen collection and transport, improperly prepared slide (e.g., organisms not evenly dispersed on slide), and patients receiving antimicrobial therapy. This method is the most rapid, reliable, and least expensive for estimating bacteriuria at $\geq 10^5$ CFU/ml. It is less sensitive than most of the semi-automated or fully automated procedures that result in false negatives. Reading numerous smears at one time can be quite labor intensive.

The acridine orange staining method for urine screening has been studied by different investigators with mixed results. Some investigators report it is predictive for cultures with $\geq 10^4$ CFU/ml and, therefore, is more sensitive than the Gram stain method. Others report it is comparable to the Gram stain method.

Enzymatic methods

Let's look at two enzymatic methods of screening urine specimens—catalase and glucose oxidase. These screening procedures are simple and fast, but not specific for bacteria. The catalase enzyme is present in many organisms causing urinary tract infections. Urine is mixed with hydrogen peroxide and oxygen is released (visual bubbling) that results in a positive reaction. False positives occur because the catalase may be positive due to RBCs, WBCs, or kidney cells.

In the glucose oxidase test, bacteria metabolize the glucose normally present in urine (2 to 10 mg/100 ml); therefore, a positive result is indicated by the absence of glucose. False negatives occur from diabetic patients because the glucose level in these patients is so high the bacteria cannot metabolize enough to give a positive result.

Dipstick methods

Two rapid diagnostic tests used to detect urinary tract pathology are the nitrate reductase (Greiss) test and leukocyte esterase (LE) test. In the nitrate reductase method, nitrate is reduced to nitrite by the enzyme nitrate reductase, which is present in gram-negative bacilli. The LE method detects the enzyme LE present in pyuria because of the WBCs. Both tests consist of a dry chemistry strip dipped into a well-mixed, room-temperature urine specimen. The strip is visually compared to a color chart. These tests are variable in regards to sensitivity—false-negatives and positives are common. Combination strips have been produced and are more sensitive than when the test strips are used alone, but the sensitivity is still too low to be used alone as a screening method.

Colorimetric filtration methods

The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993.

“The Bac-T-Screen (Vitek Systems, Inc.) is a 2-minute urine screen requiring 1 milliliter of well-mixed urine. The urine is poured into an active barrel, the start button is pushed, and the specimen is mixed with 3ml of diluent. The specimen is then vacuumed or suctioned through a piece of filter card. Bacteria or white cells are attracted to and subsequently adhere to the filter. This step is followed by the addition of 3ml of safranin dye, that after a 30-second period, is vacuumed through the filter. Bacterial cells, white cells, and background fibers are stained by the

safranin. The filter is then decolorized twice by vacuuming 3ml of solution through the filter. The decolorization step provides selective removal of the dye from the filter but not from the bacteria and white cells. Stained bacterial and white cells produce a pink to red pigmentation of varying intensity on the filter. The filter card may be read visually or with a test card reader. The test card reader provides a more objective interpretation and may simplify interpretation of questionable results. The light-emitting diode (LED) printout reports results in relative absorbance units. Clogs in the barrel are detected and aspirated by a probe in the dispenser cap.”

There are semiautomated and manual systems for screening urine via colorimetric filtration. The semiautomated system is a sensitive screening test for identifying clinically significant bacteriuria.

A manual filtration method recently developed, FiltraCheck-UTI (Meridian Diagnostics), uses a disposable filter disk and a color chart for interpretation of results. Studies show it is comparable to the Bac-T-Screen and the Gram stain. As with the Bac-T-Screen, a high number of false positives are identified due to the sensitivity to low levels of bacteria and the presence of increased WBCs. Both methods detect bacteriuria and pyuria.

Bioluminescence methods

According to *Dorland's Illustrated Medical Dictionary*, bioluminescence is defined as chemoluminescence occurring in living cells, especially the emission of light as a result of cellular oxidation of a heat-stable substrate (luciferin) in the presence of a heat-sensitive enzyme (luciferase). An example of this principle is found in the tail of the firefly. The glowing cold light is the end product of the chemical reaction in which luciferin is converted to oxyluciferin and light by the enzyme luciferase driven by the removal of a phosphate group from the organic molecule, adenosine triphosphate (ATP). The light formed by this reaction can be measured directly with a luminometer.

One system used in bioluminescence screening is the Los Alamos Diagnostics (LAD) 633. The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993.

“The Los Alamos UTIscreen LAD 633 is a fully automated instrument that uses the UTIscreen bacterial test kit to estimate the quantity of bacteria and yeast present in a urine specimen. This rapid screening test uses luciferin/luciferase to detect bacterial or yeast ATP. The assay involves three steps: an initial incubation step for the release and destruction of urinary somatic cellular ATP; a second step for the release of ATP from living microbial cells; and a final step for the detection of light emitted as a by-product on the addition of luciferin/luciferase to any microbial ATP present. The LAD 633 will process up to 25 samples in a single run that takes less than 10 minutes to complete. This instrument is fully automatic and programmable, features walk-away automation, contains a full-function computer that can interface with other lab computer systems, and prints results for a permanent record.

Minimal technician time is required to operate the instrument. Twenty-five μL of urine are dispensed into tubes in a carousel and loaded into the instrument that automatically injects reagents, performs calibrations, incubates, interprets, and displays results on the monitor screen. Test results are automatically printed at the end of the test run. Results obtained with the LAD 633 luminometer and UTIscreen test kits are comparable with other rapid bacteriuria screens, including microscopic,

enzymatic, and filtration methods. The advantages offered by the fully automated urine screening instrument over other screening methods and standard culture include ease of performance and more rapid and objective results.”

Other systems using bioluminescence principles include the Lumac Bacteriuria Screening Kit (formerly marketed by 3M company), and the Monolight system (formerly marketed by Analytical Luminescence Laboratory, Inc.). Bioluminescence techniques have been more successful in industrial applications and are usually more expensive than other screening procedures.

Impedance and conductivity methods

Several instruments have been described that detect microbial growth by monitoring changes in the impedance or conductivity of a liquid medium in which organisms are growing. The microorganisms metabolize substrates and eliminate products of different electrical mobility during growth. The changes in impedance and conductivity are detectable at higher concentrations of microorganisms. The time taken to reach the threshold of detection depends on the concentration of organisms; the lower the concentration, the longer the detection time. Detection time varies with growth rate and the metabolic state of the organisms. The Bactometer (Bactomatic Inc.) monitors impedance, while the Malthus System (Malthus Instruments Ltd.) monitors conductance. These instruments have not worked well in practice, but are used in industrial processing applications for monitoring bacterial contamination.

Photo-optic detection of growth

The systems that apply automated photometry to detect bacteriuria include the AutoMicrobic System (AMS) (Vitek Systems Inc.), Sensititre Microbiology System ARIS, and Microscan autoSCAN-W/A (Baxter Diagnostics Inc.). These systems are used primarily for identification and susceptibility of bacteria that have been isolated from primary culture. However, these systems have additional capabilities for urine screening. For example, the AMS urine screen card detects, enumerates, and identifies nine of the most frequent organisms that cause urinary tract infection. The system consists of a monitor, a printer, and 3 modular components: the filler/sealer, the reader/incubator, and a computer. Plastic cards divided into microchambers contain basic components that inhibit all but one organism or group of organisms. The lyophilized medium in each microchamber is reconstituted with diluted urine by the AMS sample injector. Reactions in each microchamber cause the photo-optics in the AMS reader-incubator to sense the presence or absence of a clinically significant number of organisms. Five enumeration wells contain an enriched medium that supports the growth of most aerobic and facultative anaerobic microorganisms. Based on the theory of most probable numbers, at least three of the five enumeration wells will show growth when the urine sample contains 1×10 organisms per milliliter. Each instrument will be discussed in detail in a separate lesson. Before deciding on which instrument or procedures to implement in your laboratory, a careful literature search and comparative study should be done. Time, cost, patient population, and level of bacteriuria that must be detected are all taken into consideration.

The purpose of calibrated loops and pour plates

Most of the urine screening instruments detect the presence of bacterial growth, but do not identify the bacteria. Calibrated loops and pour plates are manual ways to perform quantitative and semi-quantitative bacterial colony count analysis on urine.

Calibrated loop

Once a urine specimen is identified as “positive” using a screening method, it is cultured for isolation. In most laboratories, the calibrated loop-direct streak method is commonly used to determine the degree of bacteriuria. Calibrated loops are designed to deliver a known volume of 0.01 or 0.001 ml of urine. The larger volume is recommended for specimens collected at surgery, and suprapubic and symptomatic females with pyuria. The loops can be made of plastic (disposable), platinum, or other material. QC for calibrated loops is described in *Clinical Microbiology Procedures Handbook* and *Cumitech 2A*, and includes monthly testing for wire loops and every lot number for plastic loops for proper volume delivered.

The procedure for inoculating urine with a calibrated loop is as follows:

1. Sterilize and cool a calibrated wire loop. Aseptically remove plastic loops.
2. With the cap or top still on the container, mix the urine thoroughly.
3. Remove the top and insert the loop vertically into the urine. The loop must be inserted vertically or it will pick up more urine than desired.
4. Spread a loopful of urine across the center of the plate with a single streak. The inoculum is then spread at right angles to the primary inoculation (fig. 4-1).
5. Without re-sterilizing, insert the loop into the urine again for the second plate. Repeat step 4.

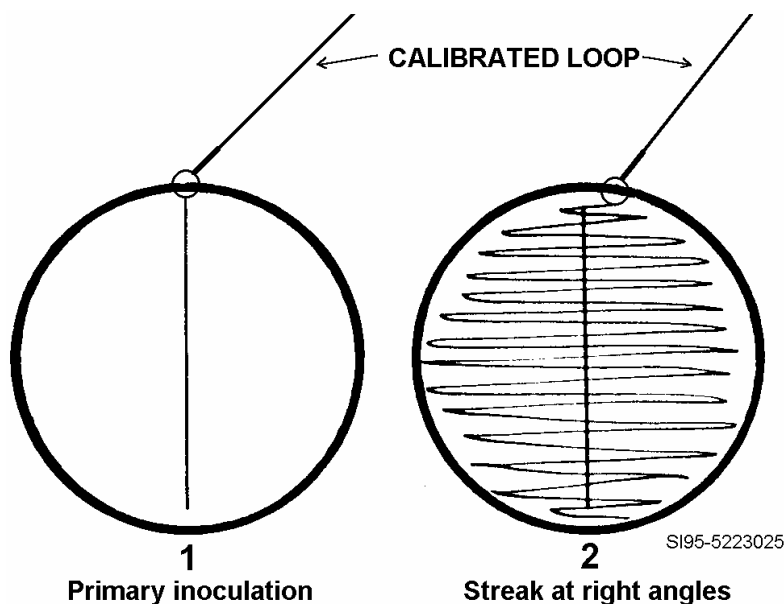


Figure 4-1. Urine culture inoculation for semiquantitative bacterial colony counts (Outline of a typical test card used by the Vitek Auto Microbic System).

The choice of media is determined by your laboratory, but the use of 5-percent sheep blood agar and a MAC agar is most common. After streaking, the plates are incubated for at least 24 hours at 35 to 37°C in air. The number of colonies present is counted and CFUs multiplied by 100, if a 0.01 ml loop is used, or by 1000, if a 0.001 ml loop is used. Plates with no growth or tiny colonies are reincubated for an additional 24 hours before discarding. Interpretive criteria have changed over the years. Specimens that show growth of 10^4 CFU/ml or less of two or more probable pathogens, is usually considered contamination unless other clinical information is given. In these cultures, the organisms are minimally identified and quantified. Susceptibility tests are not routinely performed, and the plates are held in case the clinician should request more definitive studies. Growth of greater than 10^4 CFU/ml of a single

organism is usually considered clinically significant and a full workup is performed. Growth of less than 10^4 CFU/ml of one organism may indicate an infection. In males, a CFU count of 10^3 may be significant, and a count $\geq 10^2$ CFU/ml in symptomatic women with pyuria should be reported. Organisms are identified and susceptibility test are not usually performed unless requested by the clinician. *Staphylococcus aureus* in pure culture is thought to be significant regardless of CFU count. Yeast in any number is also reported and a pure culture may be identified as to species. In urine, all isolates should be counted and reported regardless of final workup.

Pour plates

Pour plates are normally used to determine the approximate number of viable organisms in a liquid medium, such as water, milk, urine, or broth culture. They are reported as the number of CFUs per milliliter of inoculum. Pour plates are frequently used to determine the hemolytic activity of deep colonies of some bacteria, such as streptococci, by using blood agar. The pour plates also provide excellent study of pure cultures, especially when the components of a mixed culture are to be separated. Distinctive features, such as size, shape, and color of the colonies are recognized.

Method

The method requires the preparation of serial dilutions of the specimen in sterile water or saline. These dilutions are pipetted into sterile Petri dishes. Melted agar medium is added and evenly distributed by tilting the plate and cooling. After incubation at 35 to 37°C, the colonies are examined as surface or subsurface colonies for differences, or they are counted. The melted agar medium cannot exceed 50°C and the dilution should be adequately mixed.

Diluting

In the description of a pour plate technique, we touched on the advisability of diluting the inoculum from a clinical specimen to ensure well-isolated colonies would grow out on the plate. You can also use dilution cultures to estimate the number of bacteria in a specimen. A urine sample, for instance, can contain so many bacteria that it would be impossible to obtain separate and pure colonies by plating the undiluted urine. Diluting this specimen, say 1:1000 in saline, not only assures isolation of colonies, but also allows you to make a colony count. Multiplying the number of colonies on the plate by the dilution factor tells the physician the number of bacteria per ml of urine. In this calculation, each colony is assumed as the progeny of a single bacterium present in the original specimen.

Fecal specimens

The most common symptom of pointing to an infection of the lower gastrointestinal tract is diarrhea. Diarrhea can be a nonpainful, profusely watery diarrheal syndrome or be accompanied by painful abdominal cramping. Dysentery is a common term used in association with diarrhea and according to *Steadman's Medical Dictionary 25th ed.*

Illustrated. "Dysentery is defined as a disease marked by frequent watery stools, often with blood and mucus, and characterized clinically by pain, tenesmus, fever, and dehydration."

Dysentery can be serious and lead to septicemia and in severe cases death. Dysentery is due to many different microorganisms and it is important to distinguish between the causative agents, due to the differences in treatment.

Collecting a fecal specimen is relatively easy, but the processing may not be as easy. The choice of media is dependent on the pathogen sought from a specimen that is teeming with bacterial normal flora. This requires communication between the clinician and the laboratory. The laboratory's geographic location and patient population must also be considered.

Selection of media for routine cultures

Generally for all fecal specimens, the primary culture media should be able to recover *Campylobacter*, *Salmonella*, and *Shigella* spp. This includes a CAMPY-blood agar plate, MAC or EMB agar, and two moderately selective enteric differential media, such as HE and XLD agar. In most laboratories, an enrichment broth, such as GN broth, is also inoculated. The CAMPY-blood agar plate is incubated at 42°C for 72 hours in a reduced oxygen atmosphere produced by an appropriate generator envelope. The other routine medias are incubated at 35° to 37°C in air for 48 hours and examined daily. The broth is subcultured after 24 hours to the same routine media initially inoculated.

Special media considerations

Special media must be inoculated in order to recover certain organisms. General considerations and a few examples follow.

Yersinia spp.

For recovery of *Yersinia enterocolitica*, CIN agar, also known as *Yersinia* selective agar, is recommended. MAC agar may be used if CIN is not available. After inoculation, the plate is incubated at 32°C for 24 hours, or 22 to 25°C (room temperature) for 48 hours.

Vibrio spp.

The selective-differential media TCBS may be a routine culture media for laboratories located near coastal areas or where *Vibrio* infections are endemic. If TCBS media is not available, a MAC or sorbitol-MAC may be used.

Clostridium difficile

Clostridium difficile is associated with pseudomembranous colitis and nosocomial diarrhea, and is a major cause of antibiotic-associated diarrhea. Stool samples may be submitted for culture or toxin studies. For culture, specimens are inoculated to cycloserine cefoxitin egg yolk fructose agar, incubated anaerobically for 48 hours, and then examined.

Enterohemorrhagic E. coli

This newly recognized enteric pathogen produces a bloody diarrhea, and is associated with major outbreaks at fast-food restaurants. Specimens are inoculated onto sorbitol-MAC agar, incubated at 35 to 37°C, and examined at 24 hours.

Gastric biopsies for Helicobacter pylori

Helicobacter pylori can be identified in histologic-stained smears through the production of urease in a special medium, or by culture (that will only be discussed here). The gastric biopsy specimen is homogenized in 0.9 percent saline, and inoculated onto Marshall medium containing vancomycin, nalidixic acid, and amphotericin B. The plate is incubated microaerophilically at 35 to 37°C for up to 7 days.

Sexually transmitted disease specimens

For these specimens, we will primarily discuss culture specimens for *Neisseria gonorrhoeae*. The other common STDs (i.e., HIV, *Treponema pallidum* (Syphilis), and *Chlamydia trachomatis*) will be discussed in detail in their respective areas.

Processing cultures for Neisseria gonorrhoeae

Symptomatic urethral gonorrhea in males is easily diagnosed by the examination of a Gram-stained smear of urethral exudate. The discharge seen with gonorrhea is generally thick, white to greenish-yellow, and easily expressed from the penis. This material is collected on a swab,

rolled onto a glass slide, and inoculated onto media selective for *N. gonorrhoeae*. The appearance of typical GN diplococci inside polymorphonuclear leukocytes on the smear is diagnostic for gonococcal urethritis in males. While the Gram stain of urethral discharge is highly sensitive and specific for diagnosis of gonorrhea in males, this technique detects only 40 to 70 percent of endocervical gonococcal infections. Therefore, culture is mandatory for diagnosis of genital infection in females. Cultures are also required for the diagnosis of gonococcal infection at both oropharyngeal and rectal sites.

Media

A variety of enriched selective media for culture of *N. gonorrhoeae* is available and includes MTM, ML, and NYC media. All of these have antimicrobial agents added that inhibit other microorganisms, but allow the growth of both *N. gonorrhoeae* and *N. meningitidis*. Vancomycin and colistin, antimicrobials found in all three formulations, inhibit gram-positive and GN bacteria, respectively. Trimethoprim is also added to inhibit the swarming of *Proteus* species present in rectal or cervicovaginal specimens. Nystatin, anisomycin, or amphotericin B is added to inhibit yeasts and molds. MTM and ML media are supplemented with growth factors for fastidious microorganisms and are chocolate agar-based, while NYC medium is a clear peptone agar-based medium containing yeast extract, horse serum, and lyse horse erythrocytes. The NYC medium also supports growth of genital mycoplasma. All of these media allow selective recovery of *N. gonorrhoeae* from body sites harboring a large endogenous bacterial flora.

Processing

Ideally, specimens for culture of gonococci are collected, inoculated directly onto growth media, and incubated immediately. Specimens collected on swabs are rolled in a “Z” pattern on selective media and cross-streaked with a bacteriologic loop. The JEMBEC plates are removed from their plastic bags and the plate is streaked with a sterilized loop. The plates are placed in a CO₂ incubator or candle jar (discussed in Unit 2) at 35 to 37°C. Candles for candle jars are composed of white wax or bee’s wax only. Scented or colored candles may produce substances on extinction in the sealed jar that are inhibitory for *N. gonorrhoeae*. Although direct inoculation and immediate incubation is preferable, this technique is not always practical, particularly in busy clinics or hospital emergency rooms. For these situations, various specimen collection and transport systems are commercially available. Stuart’s or Amie’s media are used for the nongrowth transport of swab specimens for *N. gonorrhoeae*. These systems are easy to use, available in most clinic and hospital situations, and require no special equipment. Specimens sent to laboratories in these systems are processed within 6 hours of collection because there is a rapid decrease in numbers of viable organisms after this time. With these systems, long delays in transport and exposure to extremes in temperature can severely compromise successful recovery of the organisms.

Identification

Neisseria isolates from genital sites are presumptively identified using a minimal number of cultural characteristics. On primary isolation medium, *N. gonorrhoeae* grows as tiny, gray, glistening, umbonate colonies. Other larger and flatter colony types are also observed. When exposed to oxidase reagent (tera-methyl phenylenediamine dihydrochloride), the colonies produce a positive purple-color reaction within 10 seconds. A Gram-stain of the colonies from the plate will show GN diplococci typical of *Neisseria*. Presumptive cultural identification of *N. gonorrhoeae* isolates from genitourinary sites of males and females is based on the growth of typical colonies on selective media that are oxidase positive and have GN diplococcal morphology. However, isolates from extra-genital sites are identified by standard methods. Confirmatory identification of *N. gonorrhoeae* and other *Neisseria* species

are performed by a variety of methods. Modifications of older techniques, as well as entirely new and innovative test systems, now allow rapid identification of some of these organisms. Identification methods for *Neisseria* fit into three basic categories—carbohydrate degradation tests, immunologic tests, and chromogenic enzyme substrate tests.

Processing specimens for other genital tract infections

Although the organisms most commonly associated with genital tract infections are agents of STDs, the laboratory must be able to identify other organisms that may cause infection and are unique to men and women. For example, women may be screened for group B *streptococci* because of its ability to cause meningitis and septicemia in the newborn. The isolation of *Staphylococcus aureus* in patients with toxic shock syndrome is common. *Candida albicans* and *Trichomonas vaginalis* are isolated in vulvovaginitis. Routine genital swabs are inoculated onto 5-percent sheep blood agar and chocolate agar, and incubated at 35 to 37°C in 5- to 7-percent CO₂.

Anaerobic specimens

All anaerobic specimens are processed using the same basic techniques regardless of specimen source or site and media used for recovery or isolation. This lesson will look at the procedures for processing the majority of anaerobic specimens.

Microscopic examination

Once received in the laboratory, the specimen is processed as expeditiously as possible. At this point, the microscopic examination of the specimen is the single, most important step. Most often, this procedure is the Gram stain. This procedure provides some early information to the physician, who uses it for the empirical choice of antimicrobial therapy. It also serves as a QC in the laboratory because the final culture results, aerobic and anaerobic, are compared to the original Gram stain. The direct smear is mentioned here, although it is usually done following the inoculation of plates to avoid contamination of specimens, particularly if they are swabs. A routine or general aerobic procedure for facultative and aerobic organisms is performed. Other optional cultures for fungi and mycobacteria, if indicated or requested, are also done. Last, but not necessarily in the order presented, the anaerobic culture procedure is performed. Microscopic examination of the smear is extremely important. The Gram stain of the transtracheal aspiration from the patient with necrotizing pneumonia demonstrates slender, pointed GN bacilli and polymorphonuclear leukocytes, the former being commensurate with the appearance of fusobacteria. Gram stain of the exudate from cellulitis can show long chains of gram-positive cocci, with the culture yielding peptostreptococci. A methylene blue preparation of a yellow granular fleck in the aspirate from the patient with an enlarged jaw shows a granule with pleomorphic clubbing on the periphery, pathognomonic of actinomycosis. Large gram-positive bacilli present in the exudate from gas gangrene suggest the presence of *Clostridium perfringens*. More often, however, the Gram stain of an anaerobic process, ulcer or abscess, appears with all morphologic types of gram-positive and GN organisms present. This is consistent with the synergistic nature of anaerobic infections.

Anaerobic media

For optimal recovery of anaerobes, enriched and selective media are required, since most anaerobes are fastidious and mixed with faster-growing aerobes. Primary plates should be freshly prepared and used within two weeks, or media produced under anaerobic conditions should be used. Media produced under anaerobic conditions is called prereduced anaerobically sterilized (PRAS) media. PRAS media, both tube and plate, are available from commercial sources. See Unit 3 for a review of primary isolation media for the recovery of

anaerobic bacteria from clinical specimens. Inoculate/streak plates and immediately place in anaerobic environment for 48 hours at 35 - 37°C. The combination of plates used is decided by your laboratory's OIC or technical supervisor, and stated in the OIs.

Anaerobic environment techniques

Large laboratories may elect to utilize a glove box or anaerobic chamber, as shown in figure 4-2. These are commercially available as flexible or formed plastic units. Using a mixture of gases containing nitrogen, hydrogen, and CO₂ controls the atmosphere in the unit. Supplies and specimens transferred into the chamber are passed through a double-door port. All manipulations, including streaking, subculturing, incubation, reading of plates and storage of media, are carried out within the chamber. Conventional plates and reading techniques are utilized and the continuous anaerobic incubation provides for early recognition of colonies and identification of organisms. The major drawbacks are the significant amount of laboratory space dedicated to this equipment and the need for an alternative technique in the event of equipment failure.

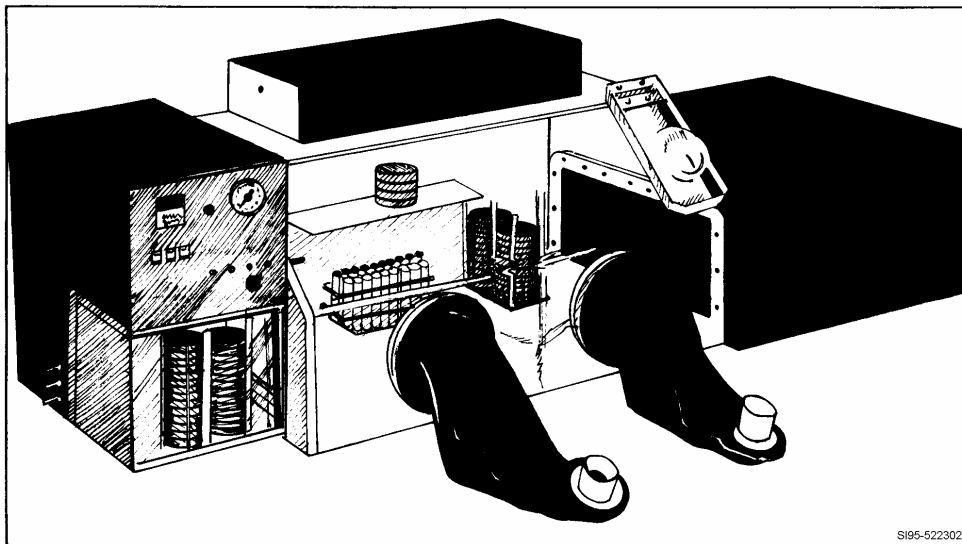


Figure 4-2. Anaerobic chamber or glove box.

Common method

The most common method of achieving anaerobiosis is the self-contained anaerobic system (GasPak or BBL). The GasPak jar is shown in figure 4-3. The unit is used with a disposable hydrogen and carbon dioxide generator envelope and a disposable anaerobic indicator. This polycarbonate plastic anaerobic jar, used with the disposable hydrogen generator, has no external connections, thereby eliminating the need for manometers and the like. It uses a room temperature catalyst, palladium-coated alumina pellets, that eliminates the need for an electrical connection to heat the catalyst. The inoculated media or tubes are placed in the jar, along with one hydrogen generator envelope with a top corner cut off and a methylene blue anaerobic indicator. Ten ml of water is introduced with a pipette into the envelope; then, the cover is immediately placed in position. The clamps are screwed hand-tight. The hydrogen reacts with the oxygen in the presence of the catalyst. An anaerobic condition is thus produced. As the anaerobic atmosphere is achieved, condensed water appears as a visible mist or fog on the inner wall of the jar, and the lid becomes warm. The methylene blue indicator should appear colorless and the jar will be under a slight positive pressure after incubating overnight.

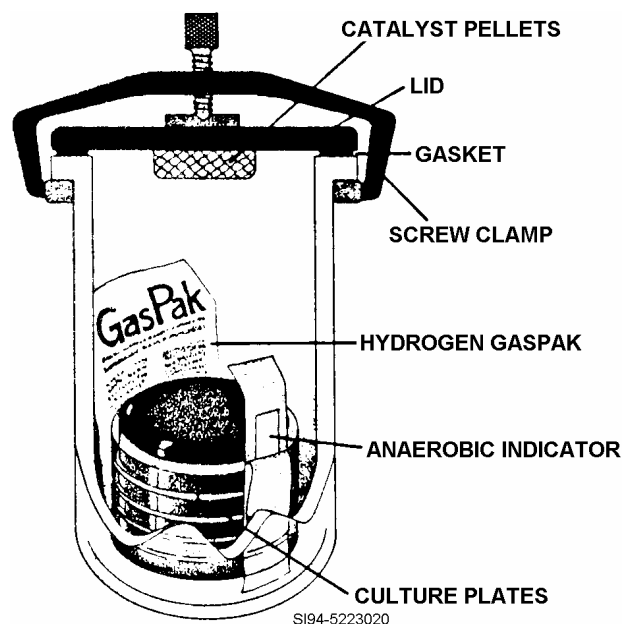


Figure 4-3. GasPak anaerobic jar.

Modification

A modification of this system utilizes the earliest described technique for achieving anaerobiosis—evacuation-replacement. GasPak or similar units can be exhausted of their atmosphere, flushed with nitrogen, and filled with a final mixture of nitrogen, hydrogen, and CO₂. Any remaining oxygen is catalyzed in the unit by the use of a palladium-coated aluminum catalyst. The evacuation-replacement system is felt to be the most economical for any size laboratory and has proven to be very satisfactory for the isolation of those anaerobes of clinical significance to man. Every precaution must be taken to prevent a laboratory accident when using the GasPak unit because hydrogen is an explosive gas.

NOTE: When using this method, observe the following:

1. Any open flame within proximity must be extinguished.
2. Inspect all jars for cracks and discard if faulty.

Review

The following table is a review of the primary media used for processing specimens. Keep in mind, your laboratory may use a different combination--these are general guidelines.

ABBREVIATION KEY			
BAP	Blood agar plate	ANA	Anaerobic agar
CAP	Chocolate agar plate	CNA	Colistin-nalixidic acid agar
MAC	MacConkey	THIO	Thioglycollate broth
XLD	Xylose lysine desoxycholate	GN	Gram-negative broth
TM	Thayer-Martin	CM	Cooked or Chopped Meat

A REVIEW FOR PRIMARY MEDIA SELECTION				
Specimen Source	Plate Media	Broth Media	Gram Stain	Comments
Abscesses	BAP, CAP, MAC, ANA	THIO	Yes	
Autopsy specimens	BAP, CAP, MAC, ANA	THIO	Yes	
Biopsy or tissue samples	BAP, CAP, MAC, ANA	THIO or CM	Yes	
Bone marrow	BAP, CAP, MAC, ANA	THIO or CM	Yes	
Body fluids:				
—CSF	BAP, CAP	THIO	Yes	Some labs may plate to a MAC.
—Other fluids	BAP, CAP, MAC, ANA	THIO	Yes	All fluids are a little different and other media requirements may exist in your lab.
Catheter tip	BAP	THIO		Using sterile forceps, roll segment back and forth across the agar and then place tip into THIO.
Ear	BAP, CAP, MAC, maybe CNA or PEA			Tympanocentesis fluid plate to ANA, THIO, and Gram stain.
Eye	BAP, CAP	THIO		
Genital tract:				
—Urethra	BAP, CAP, MAC, TM		Yes	TM for <i>N. gonorrhoeae</i> cultures only.
—Cervix, vaginal	BAP, CAP, TM			TM for <i>N. gonorrhoeae</i> cultures only.
—Amniotic fluid, prostatic fluid, cul de sac, Bartholin abscess	BAP, CAP, MAC, TM	THIO	Yes	
—Screen for group B streptococci	CAN	Enrichment broth for group B streptococci		
Intestinal tract:				
—Rectal swab, stool	MAC or EMB, XLD, HE, or SS, CAMPY	GN or other enteric enrichment broth		MAC with 1 percent sorbitol depending on geographical area or routinely for bloody stools.
—Bile, gallbladder	BAP, MAC or EMB, XLD, HE, or SS			
—Screen for <i>Yersinia</i> spp.	CIN			
—Screen for <i>Vibrio</i> spp.	TCBS			
—Screen for <i>Clostridium difficile</i>	Cycloserine cefoxitin egg yolk fructose agar			Associated with pseudomembranous colitis.

A REVIEW FOR PRIMARY MEDIA SELECTION				
Specimen Source	Plate Media	Broth Media	Gram Stain	Comments
—Screen for Hemorrhagic <i>E. coli</i>	MAC with 1percent sorbitol			
—Gastric biopsies for <i>Helicobacter pylori</i>	Marshall medium			Homogenize specimen in 0.9 percent saline.
Respiratory tract*:				
—Routine throat	BAP			
—Routine nasopharyngeal	BAP, CAP			
—Routine sputum	BAP, CAP, MAC		Yes	Screen sputum for epithelial cells before plating.
—Transtracheal aspirate, lung biopsy	BAP, CAP, MAC, ANA	THIO or CM	Yes	Homogenize lung biopsy specimen.
Urine				
—Clean catch, catheterized	BAP, MAC			
—Suprapubic	BAP, MAC			ANA if anaerobic culture requested.
Wound:				
—Deep	BAP, CAP, MAC, ANA	THIO	Yes	
—Superficial	BAP, CAP, MAC			Your laboratory may add a THIO broth.

*Respiratory cultures for specific microorganisms follow laboratory OI for primary media and incubation requirements.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

020. Specimen processing guidelines

1. Who establishes procedures for proper collection and timely delivery to the microbiology department?
2. How do you correct errors in microbiology specimen collection or delivery?
3. List three media used for blood cultures.
4. Under what condition does the routine addition of penicillinase to blood culture media appear to be justified?

5. For isolation of *Brucella*, what media is used and about how long is the culture usually retained before reporting as negative?
6. After blood culture specimens are taken and incubated at 35°C, at what times are they normally inspected for growth?
7. What characteristics are observed to indicate evidence of growth in the blood culture media?
8. What must be done in processing suspected positive cultures?
9. In processing a “blind” subculture from the blood culture bottle, what medium and atmospheric growth conditions are recommended?
10. List four alternate procedures for performing blood cultures.
11. What is meant by the phrase “biphasic blood culture bottle?”
12. How is daily subculturing achieved using biphasic blood culture bottles?
13. What are some of the disadvantages of the lysis-centrifugation procedure?
14. How are the organisms detected in a positive blood culture using the Bactec system?
15. What type of broth is used when testing for mycobacteria?
16. How is microbial growth detected with BacT/Alert?

17. What are the disadvantages with the Bactec system?
18. What is the disadvantage with the BacT/Alert system?
19. What do you do with positive blood cultures from these automated systems?
20. How do you process grossly purulent or cloudy specimens?
21. What is the preferred method for concentrating clear specimens for Gram-staining?
22. If you don't have a cytocentrifuge, what is another method used for concentrating the CSF?
23. Once the pellet is mixed and inoculated to plates and the broth, how is the broth incubated?
24. What is the principle of direct antigen testing?
25. What organisms can be detected using direct antigen testing in CSF?
26. If a clot is present in other sterile body fluids, what should you do?
27. What should be done to bone specimens before plating?
28. On what media is the suspension inoculated?
29. How are tissue specimens processed before plating?

30. What fluids can be used when processing tissue specimens?
31. What should you do if you do not receive enough bone or tissue for processing?
32. What are the different media used in processing deep wounds versus superficial wounds?
33. What selective media may be used for ear specimens?
34. Why should cultures of the upper-respiratory tract be interpreted cautiously?
35. Nasopharyngeal cultures are performed to detect carrier states of what organisms?
36. What type of specimen is considered superior to swabs for isolating *Bordetella pertussis* from children?
37. What organism causes an acute epiglottitis that can lead to death within 24 hours?
38. Throat cultures are obtained most frequently for the diagnosis of what condition?
39. A history of no immunizations increases the possibility of infection with what two organisms?
40. Why don't you perform antibiotic susceptibility testing on isolates recovered from throat cultures?
41. For what reason should susceptibility testing be performed on coliform bacilli recovered from the throat?

42. What is done to all sputum specimens submitted for routine culture?
43. How are specimens graded as unsatisfactory for culture to be handled?
44. What is usually the minimum acceptable volume of sputum for culture?
45. What type of sample is the exception to the rule of submitting only one sputum sample per 24 hours?
46. What types of lower-respiratory specimens are *not* examined microscopically to determine their acceptability for culture?
47. What is the purpose of screening urine for bacteriuria?
48. What savings can be realized by screening urines for cultures?
49. In the Gram-staining method, how do two or more organisms per oil-immersion field correlate with culture results?
50. What are the two rapid dipstick tests?
51. What is used to stain bacteria attached to the filter card in the colorimetric filtration method?
52. What does the decolorization step provide in the same method?
53. What substance is detected by bioluminescence methods for bacteriuria?

54. What do impedance conductivity methods detect and how?
55. The AutoMicrobic System (AMS) consists of different components. What are they?
56. How and where are the reactions in the AMS card microchambers read?
57. What are the two known volumes of urine calibrated loops deliver inoculation of urine cultures?
58. What is the multiplication factor for each size loop?
59. What happens when a growth of greater than 10^4 CFU/ml of a single organism from a urine culture is shown?
60. For what purpose are pour plates used?
61. What are four examples of specimens from which pour plates might be made?
62. For what purpose would pour plates be used in relationship to streptococci studies?
63. Generally for all fecal specimens, the primary culture media should be able to recover what organisms?
64. What temperature and atmosphere, and for how long are CAMPY plates incubated before examination?
65. What are the plating and incubation requirements for *Yersinia* spp.?

66. What media are used for incubating *Vibrio* spp.?
67. What is *Clostridium difficile* associated with?
68. If you suspect *C. difficile* in stool specimens, what media do you inoculate and under what conditions?
69. What is the newly recognized organism that produces bloody diarrhea and is associated with major outbreaks at fast-food restaurants, and what media is used for recovery?
70. What is the specimen of choice for recovering *Helicobacter pylori*?
71. How is symptomatic urethral gonorrhea in males easily diagnosed?
72. What microscopic morphology of Gram-stained urethral exudate is diagnostic for gonococcal urethritis in males?
73. List three enriched selective media for the culture of *N. gonorrhoeae*.
74. Why must specimens sent to the laboratory on Stuart's or Amie's media be processed within 6 hours?
75. What cultural characteristics are used to presumptively identify *N. gonorrhoeae*?
76. Name the three basic categories of identification methods for *Neisseria*.
77. What information does the Gram stain of anaerobic specimens provide?

78. Why is the direct smear usually done following the inoculation of plates?

79. Match each organism or condition in column B with the statements in column A by placing the letter of the column B item beside the number of the column A statement that suggest its presence in a specimens. Each item in column B may only be used once.

<i>Column A</i>	<i>Column B</i>
____ (1) Slender, pointed gram-negative bacilli in a transtracheal aspiration from a patient with necrotizing pneumonia.	a. <i>Clostridium perfringens</i> .
____ (2) Cellulitis exudate showing long chains of gram-positive cocci.	b. Actinomycosis.
____ (3) Granule with pleomorphic clubbing.	c. Peptostreptococci.
____ (4) Large gram-positive bacilli present in the exudate from gas gangrene.	d. <i>Fusobacteria</i> .

80. For optimal recovery of anaerobes, what is required and why?

81. What does PRAS stand for?

82. How are anaerobic specimens or plates incubated?

83. What are the two methods of achieving anaerobiosis?

84. What are the major drawbacks to using an anaerobic chamber?

85. Which method is the most common for achieving anaerobiosis?

86. What is the catalyst used with the GasPak system?

87. What color will the indicator show and what type of pressure is noted after a complete anaerobic atmosphere has been achieved and the GasPak is incubated overnight?

4-2. Automation and Molecular Detection

With the arrival of computers, automation in the laboratory has become commonplace and the microbiology laboratory is not exempt. Instrumentation in microbiology is used for various procedures including streaking, screening, identifying, and antimicrobial susceptibility testing. In addition to the arrival of automation, new diagnostic technologies in microbiology have appeared on the scene.

021. Automated instruments for identification and susceptibility

There are numerous manufacturers and distributors with a variety of equipment for the microbiology laboratory. It is virtually impossible to review all the instrumentation available to the microbiologist. This lesson will narrow the information to a few instruments that perform identification and antimicrobial susceptibility testing. Before we discuss the principle of these instruments, we need to review a few chemistry definitions.

Definitions

We use definitions from *Stedman's Medical Dictionary 25th Ed. Illustrated* for the following terms:

- Photometry — the measurement of intensity of light.
- Spectrometry — the procedure of observing and measuring the wavelengths or energy of light.
- Colorimetry — a procedure for quantitative chemical measurement based on comparison of the color developed in a solution of test material with that in a standard solution; the two solutions are observed at the same time and quantitated on the basis of the absorption of light.
- Turbidimetry — a method for determining the concentration of a substance in a solution by the degree of cloudiness of turbidity it causes or by the degree of clarification it induces in a turbid solution (measures the decreased amount of transmitted light).
- Nephelometry — a technique for estimation of the number and size of particles in a suspension by measurement of light scattered from a beam of light passed through the solution.
- Fluorometry — an analytic method for determining fluorescent compounds, using a beam of ultraviolet light that excites the compounds and causes them to emit visible light.

Some of these words and definitions are used in the following text and others are for comparison and understanding the basic principles.

Vitek AMS

The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993. Figure 4-4 illustrates a plastic card used in the disposable test kit.

“The Automicrobic System (AMS) (Vitek Systems, Inc.) consists of a monitor, a printer, and 3 modular components: the filler/sealer, the reader/incubator, and a computer. The system is fully automated from sample loading to results printout and provides same-day identification and susceptibility testing of most aerobic gram-negative and gram-positive bacterial isolates. In addition the AMS offers enteric pathogen and urine screen capabilities, overnight identification of yeast

isolates, and rapid enzymatic identification of anaerobic and fastidious gram-negative isolates. Test inoculum is prepared from primary isolation plates. Several isolated colonies are picked and suspended in 0.85 percent sterile saline in an uncapped tube. A nephelometer, available from Vitek, is used to adjust the density of the test inoculum to a McFarland standard specified for the type of test card.

Disposable test kits consist of clear plastic cards about the size of a playing card. Each test card contains 30 test wells connected by a series of capillaries and is sealed on both sides with clear tape. The wells contain lyophilized biochemicals or specific dilution of antimicrobial solutions. Filling stands are configured to hold up to 10 test cards and corresponding inoculum tubes. The prepared inoculum is placed adjacent to the test card desired and connected with a transfer tube. The filling stand is placed in the chamber of the filling module, the door is closed, and air is evacuated from the chamber. During this process air is forced out of the card wells via the transfer tube. When the vacuum is subsequently released, the pressure difference between the interior and exterior of the card forces specimen through the capillary channels into the wells containing media. The tray stand is removed from the chamber, each transfer tube is cut, and the card is sealed using the sealing unit.

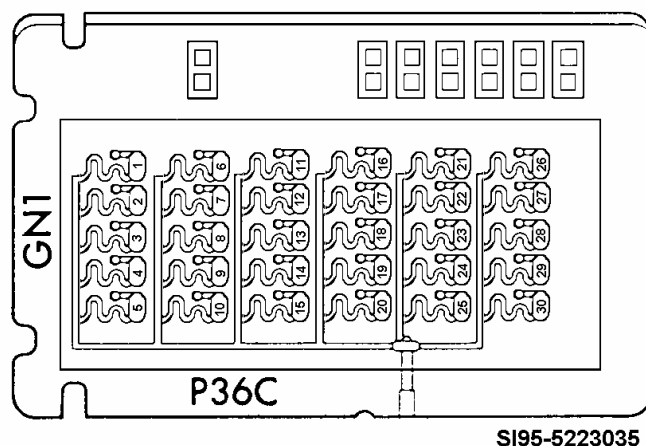


Figure 4-4. Vitek AutoMicrobic System (AMS) typical test card.
(Reprinted with permission from bioMérieux, Inc.)

Filled and sealed test cards are loaded into the reader/incubator module. The interior of this module contains a revolving carousel that holds 4 trays. Each tray contains up to 30 test cards stacked vertically. During the test period each card is scanned hourly by the reader head; reading time is 12 seconds/card or 7 minutes/tray of 30 cards. Vertical drive mechanisms move the reader head down the tray as it is read. An electro-optical detection system aligns the reader with each card. The reader extracts the card with a horizontal drive mechanism, moves it through the optical system, and returns the card to the tray. The optical system consists of 12 LEDs that emit light of 660 nm through the test card. Any attenuation of light is measured by phototransistor detectors. Card type and specimen number are also read by the optical detectors. Incubation time required for identifications and/or susceptibilities range from 4 to 18 hours. Data readings

are stored in the reader module and the computer module. It (computer) directs the reader/module functions and data analysis and automatically sends final reports to the printer.

The growth rate in the presence of specified dilutions of antimicrobial agents in susceptibility cards is monitored and compared with the growth rate in the positive control well to determine the minimal inhibitory concentration (MIC) of each antimicrobial. Biochemical reactions in test well of identification cards are compared with known reactions and probabilities. The two closest patterns are compared, the identification probabilities are computed, and subsequent identifications are reported as normalized percent probabilities. The yeast identification card requires off line incubation.”

Sensititre Microbiology System: ARIS

The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993.

“Same-day (5 hour) or overnight (18 hour) bacterial identification and susceptibility testing may be performed with an automated instrument manufactured by Radiometer America Inc. The Sensititre Microbiology System has two separate modules, the Automatic Inoculator and the AutoReader, that are interfaced with a Digital Professional 386 computer with P/OS operating system that drives the readers, collects and stores data, and a software package for complete analysis and epidemiologic evaluations of data.

The Sensititre system uses fluorogenic technology to detect and correlate bacterial enzyme activity to antibiotic susceptibility testing. Specifically defined fluorescent probes for detection of carbohydrate metabolism, deamination of tryptophan, presence of four bacterial enzymes, use of four carbon sources, decarboxylase reactions, and other tests for urea and esculin hydrolysis are used for identification of bacterial organisms.

The test inoculum is prepared by emulsifying several well-isolated colonies directly from the primary isolation plate and emulsifying them in sterile, demineralized water to achieve a turbidity equivalent to a 0.5 McFarland standard. The Automatic Inoculator includes a built in nephelometer to achieve this final concentration. A strip containing fluorescent product from a fluorogenic (nonfluorescent) substrate is then added for 5-hour testing. Enzymatic action of the test organism surface enzymes on the fluorescent label releases the fluorescent product, that is detected by the autoreader. The amount of fluorescence is directly related to bacterial growth. In some cases incubation must be extended overnight. The addition of the fluorogenic marker to the inoculum does not interfere with overnight, nonfluorogenic reading.

The Sensititre Automatic Inoculator is a rapid, microprocessor-controlled dispensing instrument that automatically delivers inoculum to a 96-well microtiter tray. Each prepared inoculum tube is fitted with a disposable dosehead to dispense the preprogrammed volume of inoculum into each microtiter plate well and clamped into place on the bridge of the automatic inoculator. The labeled microtiter plate is placed into the plate holder, and the technologist selects the desired dosing pattern desired with the keypad. Inoculum is dispensed into the microtiter tray wells through positive

displacement action occurring in the dosehead. After inoculation, the plate is removed and undergoes off-line incubation in a standard 35° to 37°C incubator for 5 hours or overnight.

After the off-line incubation, plates are loaded onto the Sensititre AutoReader for reading and interpretation. The reader contains a single excitation/detection wavelength fluorometer to measure the intensity levels of fluorescence emitted by the test well. As the plate is transported over the reading optics, raw fluorescence values are counted and transmitted to a microcomputer. For susceptibility testing, the presence or absence of bacterial growth is determined by the amount of fluorescence in each well. Optics consists of interference filters and a beamsplitting cube with wavelength selective coatings in conjunction with lenses to focus the light onto the sample and the detector. The light source is a boardband xenon flash lamp that generates microsecond pulses of high intensity light. The detector is a photomultiplier tube that provides the means for measuring low levels of fluorescence. The autoreader transports the microtiter plate over the excitation/detection optics while infrared LEDs and phototransistors relay binary signals to the microprocessor for exact well-positioning information.

Susceptibility panels are available for determination of full-range minimum inhibitory or breakpoint concentrations of antimicrobials for testing gram-positive, gram-negative, and urinary isolates. Auto identification panels provide organism identification for aerobic gram-negative bacilli. Each plate is designed to test 3 organisms by repeating 32 biochemical tests 3 times across the 96 well plate. Stabilized, dried plates are sealed with foil seals and wrapped individually in foil pouches containing a desiccant.

After auto identification panels are read by the autoreader, the raw fluorescence data is transferred to the DEC PRO 380 computer where the final identification is determined by two methods. Initially a biocode match is sought in the biocode library. If there is not a match in the library, the final identification is calculated by the computer, using probability and data matching routines. The auto identification system will identify over 140 taxa comprising Enterobacteriaceae, oxidase-positive fermenters, pseudomonads, and other nonfermenters.

Final reports may be printed in selected formats displaying desired patient and specimen information, isolate identification, and susceptibility results with interpretations.

The Sensititre Aris automated reading and incubation system is a module that can be retrofitted to the Sensititre AutoReader module. This system features on-line incubation, bar-coding technology, and automatic reading on completion of the required incubation period. The Aris holds up to 64 panels and is a strictly fluorogenic system.”

Microscan autoSCAN-W/A

The following passage is quoted by permission from *Principles of Laboratory Instruments*, Chapter 22, Automation in Microbiology by Barbara Lewis and Kathy Ristow, Mosby-Year Book, Inc., 1993.

“The autoSCAN-W/A (Baxter Diagnostics Inc., Micro-Scan Division) is a fully automated instrument capable of providing identifications within 2 hours and susceptibilities within 3.5 to 7 hours with rapid panels or performing overnight

testing with traditional panels. The autoSCAN-W/A system consists of an incubator/processor and a data management system (DMS). In addition, the RENOK Rehydrator/Inoculator is used to inoculate the dried identification and susceptibility panels.

The autoSCAN-W/A uses rapid fluorescence technologies (fluorogenic and fluorometric) for rapid panels and turbidity and/or color development in standard identification and susceptibility panels. Rapid bacterial identifications are based on bacterial enzymatic activity detected using synthetic fluorogenic substrates and fluorescent pH indicators. Rapid susceptibility testing measures growth or inhibition of growth in the presence of fluorogenic substrates and antimicrobials. The autoSCAN-W/A contains a fluorometer optics module that consists of a section for excitation of the fluorophore and a section for detection of the subsequent fluorescence.

The rapid chromogenic identification panels use colorimetric optics to read each well at six different wavelengths during each read cycle. Subsequently, the computer selects the wavelength reading that best discriminates the reaction for each well. Antimicrobial susceptibility wells are read at two different wavelengths.

The preparation of test inoculum begins by picking several morphologically similar colonies from primary isolation plates and emulsifying this growth in a specified inoculation liquid to achieve a 0.5 McFarland turbidity. The fluid is poured into the ID section of the inoculator set transfer seed trough. This is followed by the addition of either inoculated MIC broth (for combo panels) or 25 ml of uninoculated inoculum water with Pluronic-D. The transfer lid is replaced, the RENOK rehydrator/inoculator is attached, and incubation is completed.

Before loading panels into the autoSCAN-W/A, patient demographics are entered in the computer. Bar codes are then printed and affixed to the panels (either before or after panel inoculation). Panels are loaded (and subsequently removed) through the operator's door at the center top of the instrument. Inside the instrument is a carousel holding 8 towers, each of which has 12 shelves to hold test trays. A bar-code reader, the spectrophotometer, the fluorometer, reagent dispenser, and panel-accessing apparatus surround the carousel. The reagent compartment contains reagents that are automatically added after an appropriate incubation period.

After loading labeled panels into the instrument and initiating the autoSCAN-W/A, the operator's hands-on work is completed. The bar-code reader scans all trays to create an internal map of each panel, and the computer schedules the processing and reading of all panels and begins operation independent from the computer. On completion of each panel of any type, results are automatically uploaded to the data management system where a variety of report printing options are available to the user (e.g., automatic or batch).

Rapid fluorogenic panels provide 2-hour identification of aerobic gram-negative and gram-positive bacteria. Rapid chromogenic panels are available for 4-hour identification of fastidious gram-negative bacteria, yeasts, and anaerobes. Rapid fluorogenic susceptibility panels are available to determine minimal inhibitory concentrations for gram-negative and gram-positive bacteria and also to determine breakpoint susceptibilities of gram-negative and gram-positive isolates.

The data management system features both unidirectional and bi-directional laboratory interface options that allow for cost-effective and rapid reporting of

patient test results. Reporting algorithms may be used to selectively report the most cost-effective and/or formulary antibiotics. The epidemiology program provides capability for multiple-parameter searching as specified by the user. Repeat isolates are automatically excluded from epidemiology reports to prevent skewing of the data.

The autoSCAN-W/A can incubate and process up to 96 test panels simultaneously. Rapid and conventional panels may be tested during the same run. A smaller version of the autoSCAN-W/A, the WalkAway-40 Microbiology System, has a maximum capacity of 40 rapid or conventional panels.”

022. Molecular detection and immunoassays

The past few decades have witnessed an explosion of new technology to aid the laboratory diagnosis of infectious diseases that would have been unthinkable in the days of Pasteur. Five specific areas have seen the greatest changes:

1. Bacterial identification.
2. Antimicrobial susceptibility.
3. Detection of bacteremia and fungemia by instrumentation.
4. Immunodiagnostic methods for the detection of both antigens and antibodies.
5. Nucleic acid probe technology.

We have discussed the first three in detail in the prior lesson and introduced the fourth. This lesson will briefly discuss the last two.

Molecular detection

DNA! DNA! DNA! This seems to be the new buzzword. We hear it on the news; it is used as evidence in criminal cases, in the identification of remains from fallen soldiers, and in the identification of new strains of virus and other microorganisms. So, what is DNA? Well, we know from Unit 1 it is deoxyribonucleic acid, it is present in all life forms (with the exception of some viruses), and it is used in bacterial taxonomy. But, what else is there? To begin to understand the basics of DNA technology, we should start with a few definitions. According to *Dorland's Illustrated Medical Dictionary*, 27th ed.:

- Molecular is pertaining to, or composed of molecules. We know a molecule is the smallest substance that can exist alone.
- Gene, is a segment of a DNA molecule that contains all the information required for synthesis of a product (polypeptide chain or RNA molecule), including coding and noncoding sequences. It is the biological unit of heredity, self-producing, and transmitted from parent to progeny.
- Genetics is the study of genes and their heredity.
- Molecular biology is the study of molecular structures and events underlying biological processes, including the relation between genes and the functional characteristics they determine.
- DNA is a nucleic acid that constitutes the genetic material of all cellular organisms and the DNA viruses.
 - Single-stranded DNA is a linear polymer of deoxyribonucleotides in which the β -D-deoxyribofuranose residues are connected by 5' 3' phosphate linkages to form the backbone of the molecule, and the purine bases (adenine (A) and

guanine (G)) and the pyrimidine bases (cytosine (C) and thymine (T)) are attached as side chains, one to each deoxyribose (a pentose sugar) residue.

- In double-stranded DNA, each base in one strand is hydrogen bonded to its complementary base in the other strand, and the strands are twisted to form a double helix, as shown in figure 4–5. Right about now you are probably saying to yourself “What did you say?” It is important to have basic definitions so we can start from a common ground and build up.

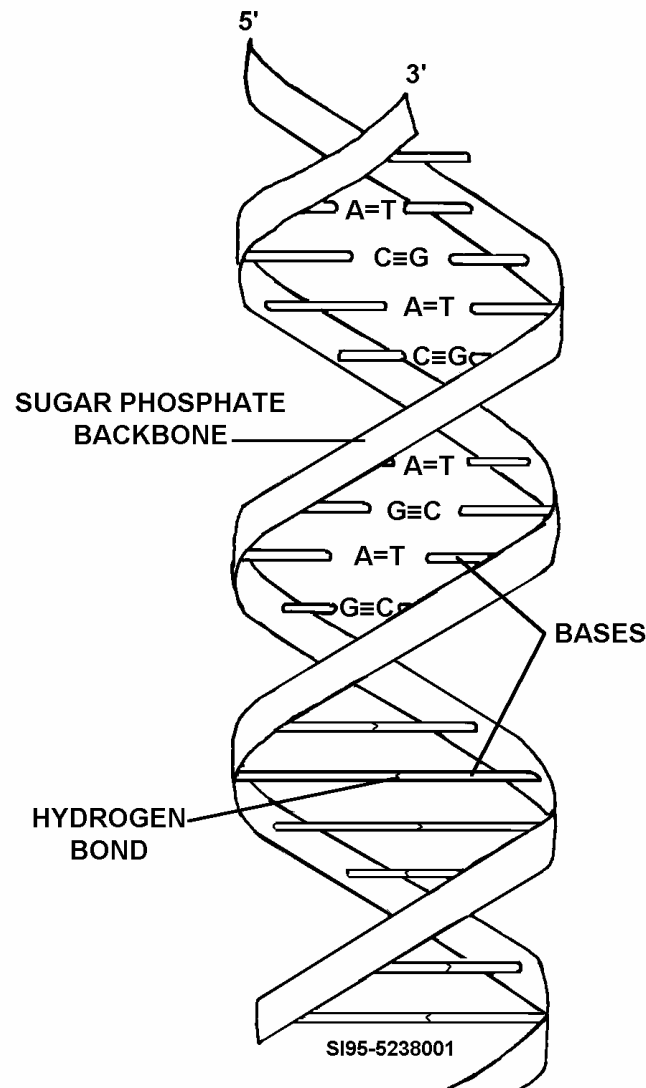


Figure 4–5. Double-helix DNA.

Where is DNA?

DNA is contained in the nucleus of almost every cell. Although the cell nucleus is microscopic, the amount of information contained in it is amazingly large. This information is coded in the very long molecules of DNA. As mentioned in Unit 1, the DNA in *Escherichia coli*, a GN rod measuring 2–6 μm long, has been reported to be approximately 1,400 μm in length. The library of information contained in DNA enables the nucleus of a single cell to designate instructions for the formation and structure of an entire, tremendously complex

organism, such as the human body. In humans and most other organisms, genetic information is encoded in a linear sequence of the 4 nucleotide bases (abbreviated A, T, G, and C) as mentioned above. These bases occur in a group of three called triplets or codons. The sequence of these bases spells out the genetic code. Therefore, the sequence ACG/TCA/ATC/CGC has a different set of instructions than the sequence TGC/AAC/TAC/TCG. DNA exists in the form of 2 linear strands, side by side, bound together by hydrogen bonds. The two or double strands (called helix) are twisted together and look like a spiral staircase. An important detail to remember concerning the strands of the DNA helix is, if you know the sequence of the bases on one of the two DNA strands, you can predict the base sequence of the other. This is because A on one strand always binds to T on the other, and G always binds to C. No other pairing relationship is normally possible.

Characterizing genes

This complementary pairing provides the basis for some of the techniques used to detect and characterize genes. These techniques employ short strands of known sequence as “probes” to detect strands with the complementary sequence. Nucleic acid probes are shorter segments of DNA that precisely match the bases in the strand of DNA that is their target. Probes will only combine with DNA they match precisely. If we send in a probe for *Neisseria gonorrhoeae*, and it hooks up with a target base pair, you can detect the sequence or combination and identify your piece of DNA as being from that bacterium. Under the conditions used in most assays, double strands must share at least 16–20 consecutive bases of perfect complementary to form a stable hybrid. The probability of such a match occurring by chance is less than one in a billion. Thus, nucleic acid probes possess an extraordinary degree of specificity. Even more than a fingerprint, cellular DNA is unique to each person, animal, and microorganism.

Tagging DNA

DNA probes can easily be tagged with radioisotopes, fluorochromes, or enzymatic markers prior to use and can act as molecular strains that recognize and bind only to the exact complementary sequence. For example, let’s say *E. coli* has a sequence of ACG/ATC/AGG/CAA/TCC. The probe would look like this TGC/TAG/TCC/GTT/AGG. The basic procedure for molecular testing is to isolate the cellular DNA from blood, tissue, or bacteria by a chemical process. The strands or the chain is separated by heat or exposed to alkali that is called denaturation. It is then followed by an enzymatic treatment to remove traces of contaminating RNA or protein. Fragments of the DNA strand is incubated with a known “labeled” probe that binds only to the fragment bearing its complementary sequence. The labeled probe is measured and the unknown strand is now identified. Nucleic acid probes commercially available at this time and manufactured by Gen-Probe, Inc., San Diego, CA, are *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Legionella pneumophila*, *Gardnerella vaginalis*, Group A *Streptococcus*, *Trichomonas vaginalis*, *Candida* spp., and Human papillomavirus.

Immunoassays

There are two general approaches involved in the diagnosis of infectious agents of disease by immunoassays. They are testing for antigen-specific antibodies and testing for specific antigens (i.e., direct antigen testing). The values of these tests are measured in terms of sensitivity, specificity, and predictive value, and are given in percentages. The immunoassay methods used in microbiology include EIA, radioimmunoassay (RIA), immunofluorescence, agglutination assays, complement fixation test, and monoclonal antibody immunoassays. The principles of these methods will be described in detail in courses A and C. You need to be aware that these techniques are used in the clinical microbiology laboratory and can be studied in detail in the civilian technical reference available in your laboratory.

Polymerase chain reaction

This recently developed method is quite useful although expensive, and has been brought into the popular consciousness via television programs that deal with forensic examinations and legal dramas. The method takes very small amounts of nuclear material (sometimes as low as one part per million) and amplifies (multiplies) the sample to detectable levels. Some methods use RNA while most use DNA, and can be used to test any nuclear cell material. Some of the various polymerase chain reaction (PCR) methods include the Northern, Southern, and Western blots. PCR methods have a high degree of specificity. A source of error in the procedure is that during amplification the nuclear strands may become airborne and contaminate other specimens resulting in false positive reactions. For this reason, the amplification area must be separate from the testing area.

The procedure involves the denaturation of DNA using heat. A single DNA strand acts as a primer and template for the DNA to bind to and build through a process called annealing. The heating and annealing procedure is performed many times in succession until amplification of the sample reaches a detection threshold. It is reported that 30 cycles will produce 1 billion parts.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

021 Automated instruments for identification and susceptibility

1. In the Vitek system, how is the test inoculum prepared?
2. What do the test card wells for the Vitek system contain?
3. After the test cards are filled and sealed, where are they loaded?
4. When are the cards scanned and what is the reading time?
5. What is the range of incubation time required for identification and/or susceptibilities?
6. What is the time frame for bacterial identification and susceptibility testing with the Sensititre Microbiology system?
7. What is the technology used by the Sensititre system?

8. How is the test inoculum prepared for this system?
9. Where is the inoculum dispensed?
10. After “off-line” incubation, what happens to the trays or plates?
11. What is the light source and the detector?
12. What are the components of the Microscan autoSCAN-W/A system?
13. What technology does this system use for rapid panels?

022. Molecular detection and immunoassays

1. How has DNA analysis been used?
2. What is molecular biology?
3. Name the two purine bases and two pyrimidine bases of the DNA strand.
4. What is encoded in the linear sequence?
5. What does the double-stranded DNA helix look like?
6. What “tags” can be attached to DNA probes?
7. What are the two general approaches involved in immunoassays?

4-3. Stains for Bacteriology

It is almost axiomatic (self-evident) that microscopic examination of a stained slide is the first task we undertake in identifying bacteria. Staining is necessary because most of the smaller internal and external structures of the cell are otherwise invisible. Indeed, a certain amount of these structures become visible only after stains are applied in conjunction with intense heat or strong chemicals. It is not surprising, then, that most staining procedures prove lethal to bacteria. So-called vital or supravital dyes are available to stain cells internally without destroying life functions, but these dyes have only limited usefulness in routine work. The staining techniques you most frequently use begin with a fixation process that quickly kills all cells on the slide. Fixation not only makes the cells adhere to the slide during staining, but it also reduces the hazard of handling exposed smears of living pathogenic organisms. A few circumstances require you to observe bacteria in the living state. The circumstance most often encountered in clinical laboratory practice is detection of motility. Later in this section, we describe the wet mount or hanging drop technique of studying unstained bacteria. But first, we must review some precautions in the proper preparation of smears, discuss certain fundamentals of stain technology, and explain some of the routine and special staining procedures performed by the bacteriologist daily.

023. Staining preparation, and reagents, categories and modes of action

Just as important as the previous information is knowing how to prepare for staining, and the reagents, categories and modes of action of staining.

Staining preparation

There are three important points to keep in mind when you prepare smears for staining:

1. Always use clean slides.
2. Make more than one slide of a specimen.
3. Air-dry the smear completely.

It is essential to use only clean, unmarred glass slides because oily deposits, scratches, and residues of previous smears lower the quality of the stained slide. You generally make two or more smears from the same specimen for a number of reasons: the first slide might stain imperfectly; a special stain could be called for after initial microscopic observation; and multiple smears give a more representative picture of the bacteria present in the specimen. Complete air drying of the smear lays the groundwork for a uniform stain. An electric slide warmer is used to hasten the drying of the smear. It is also permissible, when time is short and an electric slide warmer is not available, to place the wet slide on the laboratory bench near the base of an enclosed incinerator so heat radiating downward from the device accelerates drying.

Preparing smears from specimens

There are also several things to remember when transferring a specimen to a slide. Pus and serous exudates take the stain better if you spread them as a thin film over a large area of the slide. Moreover, masses of leukocytes and red cells are apt to obscure any bacteria present if the smear is too thick. For smears of sputum or feces, select small flecks of mucus or blood-tinged particles. These elements are more likely to yield organisms on microscopic examination. If the specimen to be stained is a cloudy liquid, you can use a sterile loop or pipette to place some of the specimen directly onto the slide. If the material is solid, you can use a sterile loop or applicator stick to collect the specimen. The specimen is then emulsified in a drop of sterile water or saline on the slide. Swabs can be gently rolled back and forth on the slide. Remember, once the swab touches the slide, it is no longer sterile. Culture media

should always be inoculated before a smear is made. If the swab contains dried material or a meager specimen, a drop of sterile water or saline can be added to the slide before “rolling” the specimen.

We have already noted that specimens of urine, spinal fluid, and other body fluids yield positive results more often if sediments are collected from the liquids by centrifugation. Transfer some of the sediment to a slide using a sterile loop or pipette. Spread the sediment to make an even film. In working with sediment and other specimens, except those in which blood and tissue cells present a complicating factor, make a thicker smear, as a rule, than you ordinarily would in staining bacterial cells from broth or solid media. The concentration of microbes in clinical material is generally much lower than in laboratory cultures.

Preparing smears from broth cultures and solid media

Smears of liquid (broth) cultures are prepared by depositing a loopful of the medium on a glass slide. If the broth is highly turbid, spread the droplet over an area slightly smaller than the size of a dime. When growth is scant, you may find it necessary to use 2 or 3 loops of broth to prepare a concentrated film. Bacterial growth on solid media presents a different problem. Pick a minute amount of material from a pure colony and emulsify it in a drop of sterile water or saline on a glass slide. Figure 4-6 is an example of a slide with a wax pencil circle that maintains the material in a small area. It is also easier to locate when looking through the microscope. Picking is best done with a straight inoculating wire or loop. If the colonies are very tiny, a sterile wooden applicator stick can be used to touch the colony and place it directly on the slide without water or saline. The suspension of organisms should be only slightly cloudy; otherwise, the smear will be too thick for microscopic observation of individual cells.

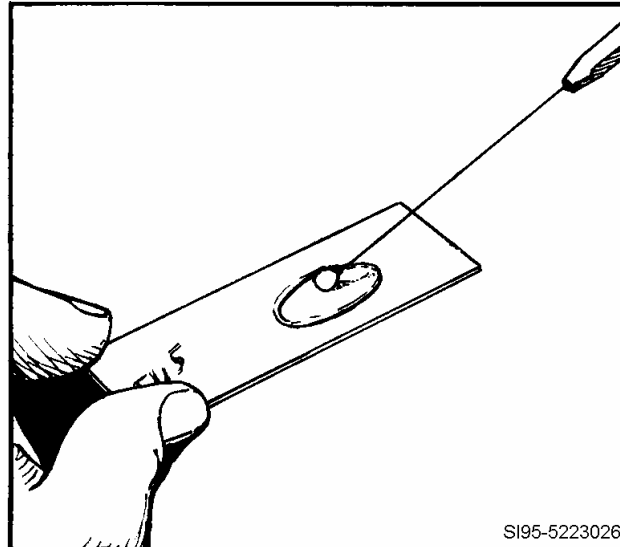


Figure 4-6. Preparing a smear for Gram-staining.

Fixing the material to be stained

After you make smears and allow the slides to dry in the air, you must “fix” them by placing the slide on a slide warmer to about 60°C or by using alcohol. If the slide is to be heat fixed, it should be heated until it feels slightly warm but not hot to the touch. If the slide gets too hot, the bacteria morphology is altered. When the slide has cooled, the smear is ready for staining. Methanol fixation is useful for examining blood specimens or blood cultures because it preserves the morphology of the RBCs, as well as the bacteria. Slides are allowed

to soak in 95-percent methanol for one minute, removed from the coplin jar, and air dried before staining. Fixing causes the smear to adhere so it is not washed off the slide during the staining process. If a number of routine cultures are to be examined using the same kind of stain, it is a practical idea to prepare several smears on a single slide. Areas are marked off with a wax pencil and a “map” of the slide is drawn to avoid confusion during the microscopic examination.

SAFETY REMINDER: Fixing the material to a slide does not always have a bacteriocidal effect on the bacteria; the slides must be considered potentially infectious. Handle slides carefully and discard with other contaminated material or waste.

Reagents

Bacteria are colorless and transparent in their natural state. For the most part, fine structures are not visible unless you accentuate them by imparting a color to the cell. The staining process serves a dual purpose. The dye in the stain provides a visual contrast between various components of the cell or between the cell and its background. Second, the dye coating expands the dimensions of the smaller structures and thus improves the resolution of the light microscope.

Dye chemistry

From a chemical standpoint, a *dye* is an organic compound, usually structured of benzene rings and side groups that are specific for each dye. One such chemical group, the *chromophore*, gives the dye molecule its characteristic color. If the chromophore is an anion (negatively charged) in solution, the dye is said to be an acid dye. Conversely, a cationic chromophore (positively charged ion) denotes a basic dye. Eosin, one of the dyes incorporated in the familiar EMB agar for coliforms, is typical of acid dyes. Methylene blue, crystal violet, basic fuchsin, and safranin are commonly used basic dyes.

Additional reagents

In addition to the application of a *dye* to the smeared specimen, other reagents are usually employed as adjuncts in the staining process. A *mordant* is a substance that fixes or chemically bonds the dye to the cell wall and thereby prevents the dye from being removed by decolorizers. The iodine reagent in the Gram stain is a prime example of a mordant. Iodine combines with crystal violet to prevent washing out of the dye during decolorization of gram-positive organisms with acetone-alcohol solution. A *decolorizer*, in this instance acetone-alcohol, is a chemical solvent used deliberately to remove a dye from the material being stained. The decolorization step distinguishes the gram-positives from the gram-negatives. A *counterstain* refers to a secondary dye that follows in sequence the application of (or removal of) the principal dye in a staining procedure. Counterstains are useful in developing a color contrast and in highlighting special structural features of a cell.

Categories of stain

For the sake of convenience, staining procedures can be classified into four different categories:

1. Direct stains.
2. Indirect stains.
3. Differential stains.
4. Selective stains.

In *direct staining*, a dye is applied to bacteria to bring into view the general characteristics of cell morphology, such as shape, size, and groupings. *Indirect stains* color the background so

the cells stand out in contrast, but the bacteria, themselves, do not take the dye. India ink preparations exemplify this type of staining. *Differential stains* are used to distinguish between morphologically similar organisms on the basis of the relative affinity of cells, or cellular components, for a given dye. The Gram stain, which we discuss shortly, is perhaps the best-known example of a differential bacteriological stain. *Selective stains*, as the term implies, bring out specific structural features, such as flagella, capsules, or spores.

Modes of dye action

The exact mechanism of dye action is not fully understood in each case, but we do know some dyes undergo a firm chemical union with cellular components. With other dyes, the staining involves only a physical absorption or simple coating action at the cell surface. In general, the acid dyes combine with basic elements of the cytoplasm to give an evenly stained appearance. But many bacteria, and notably the genus *Yersinia*, exhibit “bipolar” staining because cellular constituents concentrated at both ends of the cell stain more intensely or accumulate dye to a greater extent than other areas. Basic dyes demonstrate gross morphology, as well as certain internal structures. Reagents used to stain bacteria can be a source of error if not properly prepared or applied. The concentration of the dye, concentration of bacteria, and the time during which the dye is in contact with the microorganisms determine what the final color pattern will be. You must check each set of stains for the proper timing sequence if you are to achieve optimum results. Also, knowledge of culture conditions will often aid you in interpreting staining reactions. As an example, old cultures, particularly broth cultures, contain dead, dying, or atypical cells that do not stain uniformly. Bacteria grown in a carbohydrate broth may not yield typical results, particularly if the carbohydrate has been fermented with the production of acid.

024. Gram-staining principles, techniques, and sources of error

With the foregoing information as background, we can touch on several of the more important differential and selective stains. Two of these, the Gram and acid-fast staining procedures, are performed several times daily in a busy medical laboratory. The Gram stain will be discussed in this lesson; the acid-fast stain and fungal stains will be discussed in Volume 3 in the Mycobacterium and Mycology sections, respectively.

Gram-staining

Most bacteria are classified into one of two categories on the basis of the *Gram-staining reaction*—gram-positive or gram-negative.

Gram-positive

If the cells of a culture retain a crystal violet dye (cells are purple or blue) after washing them with alcohol or acetone-alcohol mixture, these bacteria are said to be “gram-positive.”

Gram-negative

Cells that release crystal violet under treatment with decolorizer and subsequently stain red or pink with a safranin counterstain are termed “gram-negative.” The knowledge of whether an organism is gram-negative or gram-positive is critically important because this information limits the number of genera that must be considered in identifying an unknown bacterium.

Mechanism of Gram-staining

The mechanism of the Gram stain is not fully known. The property of being gram-positive appears to be associated with the thick peptidoglycan part of the cell wall that contains numerous teichoic acid cross-linkages. These teichoic acid cross-linkages seem to resist alcohol decolorization. The gram-negative cell wall consists of a thin layer of peptidoglycan

and a thick external coat of lipopolysaccharides, which seems to account for the differences in the Gram stain characteristics. The gram-negative forms are stained red or pink by the safranin counterstain that follows the decolorizing step. Although most bacteria are clearly either gram-positive or gram-negative, some species exhibit a definite tendency to display both positive and negative cells in a stained preparation. These organisms are called “gram-variable.” But whether positive, negative, or variable, the gram reaction is species specific when properly performed and interpreted.

Procedure of Gram-staining

The Gram-staining procedure is not unduly sensitive to variations in technique, but there are certain precautions you must watch in order to get a good stain consistently. One of these is the age and condition of the specimen to be stained. Gram-positive organisms become gram-negative as a result of autolysis, aging, acidity of the culture medium, improper temperature of incubation, or the presence of toxic substances (drugs, metabolic wastes, etc.). For best results, prepare Gram stains on cultures 18- to 24-hours old.

Quality of Gram-staining

The quality of the smear is equally important. If you prepare films unevenly or too thickly, dense deposits of material will retain crystal violet on decolorization regardless of the Gram reaction. Under these conditions, falsely gram-positive clumps of bacteria are seen in an otherwise gram-negative smear. Smears must be completely dry before heat-fixing, or else protein carried over into the smear from culture media or specimens will be precipitated. The background of the smear is then difficult to decolorize and can contain debris and misleading artifacts.

Decolorization

Decolorization of Gram-positive cells can result if you use an iodine (mordant) solution that has deteriorated. Gram's iodine solution remains stable for long periods when protected from light by storing it in a dark bottle. When the iodine solution fades in color from brown to light amber or yellow, it is no longer suitable for use. Overly enthusiastic treatment with the decolorizing solution also results in a false gram-negative reaction. *Immediately* after the washings become clear during treatment of the smear with alcohol or acetone-alcohol mixture, you must rinse the slide with water to prevent overdecolorization.

QC

QC is performed on each new batch of stains and on a weekly basis thereafter. Use *Streptococcus pyogenes* American Type Culture Collection (ATCC) 19615 and *Escherichia coli* ATCC 25922 as known gram-positive and gram-negative organisms for controls. Prepare a mixed suspension of the two strains, place a drop on a slide, and stain using the same procedure as used for test or specimen slides. Expected results are purple cocci in chains (streptococci) and pink or red rods (*E. coli*).

025. Wet mount and fluorescent staining techniques

A wet mount of an unstained preparation, while not done routinely, is an integral part of the identification process for certain microorganisms. The wet mount procedure and fluorescent staining techniques will be discussed in this lesson.

Wet mount unstained preparations

Occasionally, the microscopic examination of wet, unstained preparations aids in gross morphology of microorganisms, such as fungal hyphae, endospores, protozoan trophozoites, and helminth eggs and larvae. In the basic saline wet mount procedure, a drop of warm

(37°C), 0.85-percent aqueous sodium chloride (NaCl) is added to a slide. A drop of specimen is added to the saline and gently mixed together. A coverslip is then placed over the mixture. The slide is examined with light microscope at 100 x and 1,000 x for characteristic morphology, motility, and for reactions to certain chemicals if added.

The hanging drop method is generally used to detect motility of living bacteria in pure culture. This method serves the same purpose as the saline mount, but there is less distortion from the weight of the coverslip and a deeper field of focus can be achieved. The technique for preparation and microscopic observation of hanging drop mounts is illustrated in figure 4-7. Deposit a drop of broth culture containing the cells on a coverslip and invert it over a thick glass slide with a center concave well. You see flagellar motion as directional movement of individual cells. Do not confuse motility with Brownian movement, which is a vibratory type of motion of the bacterial cells due to molecular bombardment.

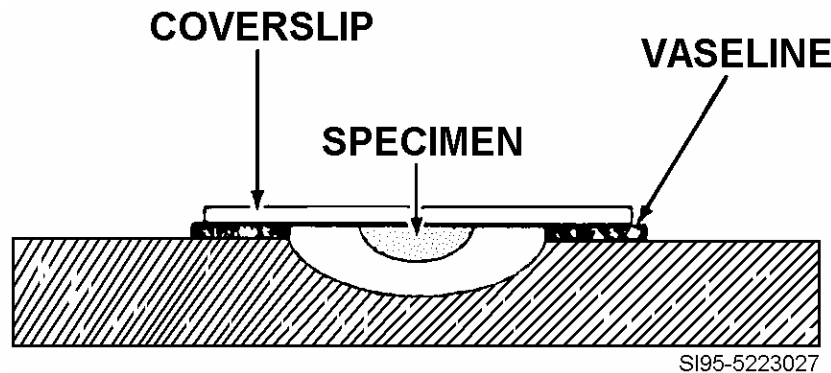


Figure 4-7. Hanging drop slide.

Fluorescent staining techniques

Fluorescent stains are becoming more prevalent in microbiology laboratories that possess a fluorescent microscope. Advantages include a more sensitive method for identifying organisms in blood, CSF, and tissue specimens; ability to detect low numbers of microorganisms; thick or bloody smears can be used; and rapid screening of specimens at lower magnification is allowed; and may be more sensitive for acid-fast bacilli. The principle of fluorescence is certain dyes called fluors or fluorochromes have the property of becoming excited or raised to a higher energy level after absorbing UV light, which is of a shorter wavelength than visible light. As the excited molecules return to their normal state, they release the excess energy in the form of visible light of a longer wavelength than that which first excited them. This reaction causes them to become self-luminous. Modern microscopic methods have been developed to use the improved detection possible with this system.

Acridine orange

The fluorochrome acridine orange is intercalated or bound to nucleic acid, either in the native or the denatured state. Bacterial and fungal DNAs fluoresce orange, and mammalian DNA fluoresces green with UV light. Acridine orange staining for identifying bacteria in blood culture media is widely accepted, and some studies show it to be as sensitive as blind subcultures for the initial detection of positive cultures. The procedure is:

1. Fix the smear with heat or methanol as with the Gram stain procedure.
2. Flood the smear with acridine orange stain that can be purchased from a number of microbiology suppliers. The stain is allowed to remain on the surface for two minutes. Do not let the stain dry out.

3. After two minutes, rinse the slide with tap water and air dry by leaning it upright to drain.
4. Examine the slide microscopically under UV light. Use the same light source as used for fluorescein.

QC

QC is to be done with the new reagents and each time the stain is used. A positive blood culture is saved, and a smear is prepared and ran with the test or unknown sample.

Rhodamine-Auramine

Rhodamine-Auramine is another fluorescent stain used specifically for acid-fast bacilli.

Fluorescein-conjugated stains

Fluorescein-tagged antibodies to specific bacteria were developed and are used in both research and clinical microbiology laboratories to identify bacteria. Antibodies bound to the fluorochrome fluorescein isothiocyanate (FITC) are used to visualize bacteria in direct specimens. The most widely used application of fluorescein-tagged antisera is for the identification of group A beta-hemolytic streptococci or *Streptococcus pyogenes*. Fluorescent antibody stains are also employed in the identification of enteropathogenic strains of *Escherichia coli*, *Neisseria gonorrhoeae*, and a number of *Salmonella* species. The use of specific, fluorescein-tagged antisera provides a rapid, sensitive technique for identifying *Legionella pneumophila* in lung biopsies, pleural fluids, and other specimens. It remains one of the most rapid and specific methods of identifying *Legionella* species in both tissue sections and cultures.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

023. Staining preparation, and reagents, categories, and modes of action

1. What are three important points to remember when you prepare smears for staining?
2. In transferring pus serous exudates, why should they be spread as a thin film over a large area of the slide rather than making the smear too thick?
3. What portions of the sputum or feces are most favorable for a smear and why?
4. How are the smears of urine, spinal fluids, and other body fluids made?
5. When preparing smears of broth cultures when the growth is scant, how is a concentrated film made?

6. How are smears from bacterial growth on solid media prepared?
7. When heat-fixing the slide, what can happen if the slide gets too hot?
8. When is methanol fixation useful and why?
9. What two purposes does the dye serve in the staining process?
10. From a chemical standpoint, what is a dye?
11. What is the chemical group that gives the dye molecule its characteristic color?
12. What are some basic dyes?
13. What is the purpose of a mordant?
14. What purpose does iodine serve in the Gram stain?
15. What is the essential purpose of counterstains?
16. What is direct staining?
17. What is the difference between a direct and an indirect stain?
18. The India ink preparation is an example of what type of staining?
19. What type of stain does the Gram stain exemplify?

20. What category of stains is used to bring out specific structural features, such as flagella, capsules, and spores?
21. How do acid dyes result in an evenly stained appearance of the organism?
22. Bipolar staining shows what feature of an organism?
23. What are three major factors that influence the final color pattern on a properly stained smear?

024. Gram-staining principles, techniques, and sources of error

1. Why is determining whether an organism is gram-positive or negative of critical importance?
2. What is the counterstain used in the Gram stain procedure?
3. What term is used to identify bacteria that exhibit both positive and negative cells in a stained preparation?
4. What are some of the reasons that gram-positive organisms stain gram-negative?
5. Material for Gram-staining are taken from cultures that are how old?
6. What is the reason for false gram-positive clumps in an otherwise gram-negative smear?
7. What is the quality control procedure for Gram stains?

025. Wet mount and fluorescent staining techniques

1. What type of gross morphology can you expect to see in wet, unstained preparations?

2. How is the slide examined and what are you looking for?
3. What is the purpose of hanging drop method?
4. What are some of the advantages of fluorescent stains?
5. The acridine orange stain is widely accepted for what use?
6. How are smears for this stain fixed?
7. What is the quality control procedure for the acridine orange stain?
8. What is the most widely used application of fluorescein-tagged antisera?
9. What is one of the most rapid and specific methods of identifying *Legionella*?

Answers to Self-Test Questions

020

1. The laboratory.
2. Consultation with the clinical staff; annotate the end result of these consultations in the patient's result file.
3. Soy bean casein digests, such as Tryptic (Difco) or Trypticase (BBL) soy broth, Columbia broth, and Brain Heart Infusion broth.
4. In selected cases, when a patient is receiving high doses of a penicillin or cephalosporin at the time of collection.
5. Castaneda's double medium and *Brucella* broth; they are retained for 28 days.
6. On the same day and daily, thereafter, for at least 7 days.
7. Turbidity, hemolysis, gas bubbles present in the media, or the appearance of small colonies in the broth or on the settled red blood cells after the 24 hour agitation.
8. Prepare Gram-stained smears and cultures immediately and report the microscopic examination of the smear by phone to the patient's physician, clinic, or ward as soon as possible.
9. Quadrants of chocolate agar plate; incubate at 35°C in 5 percent to 10 percent CO₂ for 48 hours before examination.
10. (1) Biphase bottle.

-
-
- (2) Lysis-centrifugation.
 - (3) The Bactec System.
 - (4) The Organon Teknika BacT/Alert.
 11. One containing both liquid and solid media.
 12. By inversion of the unit and observation for growth on the agar.
 13. A high rate of plate contamination that requires the procedure to be done inside a laminar flow biosafety cabinet, and the failure to recovery certain bacteria, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Listeria monocytogenes*, and anaerobic bacteria, as well as conventional systems do.
 14. The use of ^{14}C -labeled glucose and other substrates with subsequent release of $^{14}\text{CO}_2$ in a vial.
 15. Special Bactec vials with 7H12 broth containing ^{14}C palmitic-1 acid.
 16. Through noninvasive monitoring of the level of CO_2 within each sample bottle by a colormetric sensor attached to the bottom to the bottle.
 17. False-positives because of carryover from positive bottles to others due to inadequately sterilized sampling needles and inappropriate set threshold values.
 18. The few media or broths available, none of which contain resins.
 19. Subculturing is required; treat vials from automated systems just like positive conventional cultures.
 20. Using a sterile pipette, directly pipette fluid onto a slide for Gram stain. The Gram stain smear is read ASAP and results reported to the physician, clinic, or ward. For routine cultures inoculate on 5 percent sheep blood agar, chocolate agar, in an enrichment broth, and possibly a MAC or eosin methylene blue EMB, or anaerobic agar as indicated by Gram stain.
 21. The cytocentrifugation technique.
 22. Centrifuge the CSF tube at $1500 \times g$ for 15 minutes.
 23. The broth is incubated in air at 37°C for at least 5 days and the broth cap should be loose to allow air exchange.
 24. A purified antibody-coated particle, such as latex or erythrocytes, that bind to a specific antigen (capsular polysaccharide) found naturally on the surface of the bacterial cell.
 25. *Haemophilus influenzae* group B; *Neisseria meningitidis* groups A, B, C, Y, and W-135; and *Streptococcus pneumoniae*, group B streptococci and *Cryptococcus neoformans*.
 26. It should be homogenized (broken up) to release trapped bacteria and mixed with supernatant.
 27. Should be ground using a sterile mortar and pestle with a sterile broth to form a suspension.
 28. 5-percent sheep blood agar, chocolate agar, MAC or EMB agar, an enrichment broth, and anaerobic media, and a smear is also prepared.
 29. Homogenized using a sterile mortar and pestle, or in a sterile plastic bag by using a commercial tissue grinder.
 30. Sterile broth or saline.
 31. Place the whole specimen in an enrichment broth.
 32. Enrichment broth and anaerobic media are used for deep wounds.
 33. Colistin-nalixidic acid (CNA) agar or phenylethyl alcohol (PEA) agar.
 34. Because of the microflora normally present and the frequency of nosocomial acquisition of potentially pathogenic microorganisms.
 35. *N. meningitidis*, *Corynebacterium diphtheria*, and *S. pyogenes*.
 36. Nasopharyngeal aspirates.
 37. *H. influenzae* type B.
 38. Streptococcal pharyngitis.
 39. *B. pertussis* and *C. diphtheriae*.

40. Beta hemolytic streptococci are universally susceptible to penicillin. Other organisms are not considered pathogenic in the absence of specific complications.
41. For nosocomial purposes.
42. Screened for epithelial and white blood cells.
43. They are not plated and the requesting physician is notified by phone and in writing.
44. 2 ml unless the specimen is obviously purulent.
45. Postbronchoscopy specimens.
46. Sputum specimens submitted for isolation of *Mycobacterium tuberculosis* or mycotic agents.
47. (1) To provide accurate and timely information to the clinician for prompt patient care.
(2) To rapidly detect urine specimens that do not contain significant bacteriuria.
48. Technician time and media expense.
49. Culture of greater than or equal to (\geq) 10^5 CFU/ml.
50. The nitrate reductase (Greiss) test and leukocyte esterase (LE) test.
51. Safranin dye.
52. Selective removal of the dye from the filter, but not from the bacteria and white cells.
53. Bacterial or yeast adenosine triphosphate (ATP).
54. Microbial growth by monitoring changes in the impedance or conductivity of a liquid medium in which organisms are growing.
55. A monitor, a printer, and 3 modular components: the filler/sealer, the reader/incubator, and a computer.
56. By photometry (photo-optics) in the AMS reader-incubator.
57. (1) 0.01 ml.
(2) 0.001 ml.
58. Multiply by 100 if a 0.01 ml loop is used or by 1000 if a 0.001 ml loop is used.
59. The count is considered to be clinically significant and a full workup is performed.
60. To determine the approximate number of viable organisms in a liquid medium.
61. (1) Water.
(2) Milk.
(3) Urine.
(4) Broth culture.
62. To determine the hemolytic activity of deep colonies.
63. *Campylobacter*, *Salmonella*, and *Shigella* spp.
64. 42°C for 72 hours in a reduced oxygen atmosphere produced by an appropriate generator envelope.
65. Specimen is inoculated to cefsulodin irgasan novobiocin (CIN) agar and incubated at 32°C for 24 hours, or 22° to 25°C (room temperature) for 48 hours.
66. Thiosulfate citrate bile salts sucrose (TCBS) is recommended, but a MAC or sorbitol-MAC may be used.
67. Pseudomembranous colitis, nosocomial diarrhea, and is a major cause of antibiotic-associated diarrhea.
68. Cycloserine cefoxitin egg yolk fructose agar and incubated anaerobically for 48 hours.
69. Hemorrhagic *E. coli*; sorbitol-MacConkey agar, incubated at 35° to 37°C, and examined at 24 hours.
70. Gastric biopsy specimens.
71. By examination of a Gram-stained smear of urethral exudate.

72. Typical gram negative diplococci inside polymorphonuclear leukocytes on the smear.
73. (1) Modified Thayer-Martin (MTM).
(2) Martin-Lewis (ML).
(3) New York City (NYC).
74. Because there is a rapid decrease in numbers of viable organisms after this time.
75. Tiny, gray, glistening, umbonate colonies.
76. Carbohydrate degradation tests, immunologic tests, and chromogenic enzyme substrate tests.
77. Some early information to the physician, who uses it for the empirical choice of antimicrobial therapy. It also serves as a quality control in the laboratory.
78. To avoid contamination of specimens, particularly if they are swabs.
79. (1) d.
(2) c.
(3) b.
(4) a.
80. Enriched and selective media are required, since most anaerobes are fastidious and are mixed with faster growing aerobes.
81. Prereduced anaerobically sterilized.
82. Immediately placed into an anaerobic environment and incubated at 35° to 37°C for 48 hours once inoculated/streaked.
83. Glove box or anaerobic chamber and the GasPak jar.
84. The significant amount of laboratory space dedicated to this equipment and the need for an alternative technique in the event of equipment failure.
85. The self-contained anaerobic system (GasPak or BBL).
86. Palladium-coated alumina pellets.
87. Colorless; slight positive pressure.

021

1. Several isolated colonies are picked and suspended in 0.45% sterile saline in an uncapped tube.
2. Lyophilized biochemicals or specific dilution of antimicrobial solutions.
3. Into the reader/incubator module.
4. Hourly by the reader head; 12 seconds/card or 7 minutes/tray of 30 cards.
5. 4 to 18 hours.
6. Same-day (5 hour) or overnight (18 hour).
7. Fluorogenic.
8. By emulsifying several well-isolated colonies directly from the primary isolation plate and emulsifying them in sterile, demineralized water to achieve a turbidity equivalent to a 0.5 McFarland standard.
9. Into a 96-well microtiter tray.
10. Loaded onto the Sensititre AutoReader for reading and interpretation.
11. A broadband xenon flash lamp; photomultiplier tube that provides the means for measuring low levels of fluorescence.
12. An incubator/processor and a data management system (DMS). In addition, the RENOK Rehydrator/Inoculator is used to inoculate the dried identification and susceptibility panels.
13. Fluorescence (fluorogenic and fluorometric).

022

1. As evidence in criminal cases, in the identification of remains from fallen soldiers, in the identification of new strains of virus and other microorganisms, and for bacterial taxonomy.
2. The study of molecular structures and events underlying biological processes, including the relation between genes and the functional characteristics they determine.
3. Adenine (A) and guanine (G); cytosine (C) and thymine (T)
4. Genetic information.
5. Spiral staircase.
6. Radioisotopes, fluorochromes, or enzymatic markers.
7. Testing for antigen-specific antibodies and testing for specific antigens.

023

1. (1) Always use clean slides.
(2) Make more than one slide of a specimen.
(3) Air-dry smear completely.
2. Helps take the stain better and because masses of leukocytes and red cells are apt to obscure any bacteria present.
3. Small flecks of mucus or blood-tinged particles; these elements are more likely to yield organisms on microscopic examination.
4. Using a sterile loop or pipette, spread the sediment to make an even film, and make a thicker smear, as a rule, than you ordinarily do when staining bacterial cells from broth or solid media.
5. Use 2 or 3 loops of broth.
6. Pick a minute amount of material from a pure colony and emulsify it in a drop of sterile water or saline on a glass slide.
7. The bacteria morphology is altered.
8. Examining blood specimens or blood cultures; because it preserves the morphology of the red blood cells, as well as the bacteria.
9. (1) The dye provides a visual contrast between various components of the cell or between the cell and its background.
(2) The dye coating expands the dimensions of the smaller structures and thus improves the resolution of the light microscope.
10. An organic compound, usually structured of benzene rings and side groups that are specific for each dye.
11. Chromophore.
12. Methylene blue, crystal violet, basic fuchsin, and safranin.
13. It fixes or chemically bonds a dye to a cell wall, thereby preventing the dye's removal from the cell by decolorizers.
14. Mordant.
15. Useful in developing a color contrast and in highlighting special structural features of a cell.
16. Dye is applied to bacteria to bring into view the general characteristics of cell morphology, such as shape, size, and grouping.
17. In direct staining, the bacteria take the dye; in indirect staining, the bacteria do not take the dye.
18. Indirect.
19. Differential stain.
20. Selective stains.
21. They combine with the basic elements of the cytoplasm.
22. Cellular constituents are concentrated at both ends of the cells.

23. (1) Concentration of dye.
- (2) Concentration of bacteria on the slide.
- (3) The length of time that the stain is in contact with the bacteria.

024

1. This information limits the number of genera that must be considered in identifying an unknown bacterium.
2. Safranin.
3. Gram-variable.
4. Autolysis, aging, acidity of the culture medium, improper temperature of incubation, or the presence of toxic substances.
5. 18- to 24-hours old.
6. If you prepare films unevenly or too thickly, dense deposits of material will retain crystal violet on decolorization regardless of the Gram reaction.
7. Quality Control is performed on each new batch of stains and on a weekly basis thereafter. Use *Streptococcus pyogenes* American Type Culture Collection (ATCC) 19615 and *Escherichia coli* ATCC 25922 as known gram-positive and gram-negative organism for controls. Prepare a mixed suspension of the two strains, place a drop on a slide, and stain using the same procedure as used for test or specimen slides. Expected results are purple cocci in chains (streptococci) and pink or red rods (*E. coli*).

025

1. Gross, such as fungal hyphae, endospores, protozoan trophozoites and helminth eggs and larvae.
2. With light microscope at X100 and X1,000 for characteristic morphology, motility, and for reactions to certain chemicals if added.
3. It is generally used to detect motility of living bacteria in pure culture.
4. (1) A more sensitive method for identifying organisms in blood, CSF, and tissue specimens.
- (2) Ability to detect low numbers of microorganisms.
- (3) Thick or bloody smears can be used.
- (4) Allows rapid screening of specimens at lower magnification.
- (5) May be more sensitive for acid-fast bacilli.
5. Identifying bacteria in blood culture media.
6. With heat or methanol.
7. Quality Control is to be done with the new reagents and each time the stain is used. A positive blood culture is saved; a smear is prepared and ran with the test or unknown sample.
8. The identification of group A beta-hemolytic streptococci or *Streptococcus pyogenes*.
9. The use of specific, fluorescein-tagged antisera.

Do the unit review exercises before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter.

67. (020) When performing *blind* subcultures, what agar is inoculated?
- a. Phenylethyl alcohol (PEA).
 - b. 5-percent sheep blood.
 - c. MacConkey (MAC).
 - d. Chocolate.
68. (020) The Bactec 460 uses what radioactive detection label to demonstrate bacteremia?
- a. ^3H .
 - b. ^{57}CO .
 - c. $^{14}\text{CO}_2$.
 - d. $^{125}\text{CO}_2$.
69. (020) If a clot is present in a sterile body fluid, it should be
- a. stained.
 - b. discarded.
 - c. centrifuged.
 - d. homogenized.
70. (020) What can be used as a suspension fluid when grinding tissue specimens?
- a. water or saline.
 - b. water or broth.
 - c. sterile water or saline.
 - d. sterile broth or saline.
71. (020) Sputum specimens graded as *unsatisfactory* for culture are handled by
- a. requesting an additional specimen be submitted.
 - b. discarding the specimen and annotating the specimen log.
 - c. not plating the specimen and notifying the physician by phone and in writing.
 - d. plating the specimen and notifying the physician that the specimen was contaminated.
72. (020) The method of determining the degree of bacteriuria *most commonly* used is
- a. pour plate.
 - b. agar dilution.
 - c. saline dilution
 - d. calibrated loop.
73. (020) The campylobacter (CAMPY)-blood agar plate is incubated at 42°C in a reduced oxygen atmosphere for
- a. 24 hours.
 - b. 48 hours.
 - c. 72 hours.
 - d. 96 hours.

74. (020) Trimethoprim is added to enriched selective media for the culture of *N. gonorrhoeae* to inhibit
- gram-positive organisms.
 - Proteus* spp.
 - yeasts.
 - molds.
75. (021) After inoculation in the Sensititre Microbiology System rapid procedure, the plate is removed and undergoes off-line incubation in a standard 35°C to 37°C incubator for
- 2 hours.
 - 3 hours.
 - 4 hours.
 - 5 hours.
76. (022) In the deoxyribonucleic acid (DNA) strand, the purine bases are attached to a backbone made of
- phosphate and sugar.
 - phosphate and protein.
 - hydrogen and sugar.
 - hydrogen and protein.
77. (022) Cellular deoxyribonucleic acid (DNA) is unique to each person just like
- genes.
 - eye color.
 - a fingerprint.
 - a tissue type.
78. (023) For *best* results when preparing smears from sputum or feces, select
- dark areas.
 - green areas.
 - areas exhibiting froth.
 - areas with mucus or blood.
79. (023) The two ways to fix a slide before staining are
- heating or using 95-percent methanol.
 - heating or using 75-percent methanol.
 - air drying or using 95-percent methanol.
 - air drying or using 75-percent methanol.
80. (023) What is *not* considered a basic dye?
- Eosin.
 - Safranin.
 - Crystal violet.
 - Methylene blue.
81. (023) What category of stains colors the background, but *not* the bacteria themselves?
- Direct.
 - Indirect.
 - Selective.
 - Differential.

82. (024) For *best* results, Gram stains are performed on cultures
- a. 9- to 15-hours old.
 - b. 10- to 16-hours old.
 - c. 18- to 24-hours old.
 - d. over 24 hours old.
83. (025) The *most widely* used application of fluorescein-tagged antisera is for the identification of
- a. *Pneumocystis carinii*.
 - b. *Legionella pneumophila*.
 - c. hemoflagellates in blood smears.
 - d. group A beta-hemolytic streptococci.
84. (025) What technique is used for the rapid identification of *Legionella pneumophila* in lung biopsies?
- a. Quellung reaction.
 - b. Periodic acid-Schiff stain.
 - c. Fluorescein-tagged antisera.
 - d. Negative staining using methylene blue.

Please read the unit menu for unit 5 and continue ➔

Unit 5. Antimicrobial Agents, Drug Resistance, and Susceptibility

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PROIR to World War I, a noteworthy statement of the principles of chemotherapy was made. Ehrlich defined the essential qualities needed for a useful chemotherapeutic drug as two in number: (1) an *affinity* between some part of the drug and a receptor possessed by the parasite, and (2) a *toxic potential* of the drug that is capable of destroying the (viability of the) parasite once the binding has occurred. Although the language used by Ehrlich is somewhat old-fashioned by today's standards, his analysis is as fresh and compatible with current biology as anything now being written. The disciplines of biochemistry, microbiology, and pharmacology, with their subdivisions into molecular biology, biophysics, and so forth, needed to be developed before we could understand the action of drugs. Our understanding of drug action, however, has been limited not so much by any major deficiency in our concepts as by our knowledge of biochemical detail. In this unit, we will show how representative antimicrobial agents act against bacteria and examine some of the resistance mechanisms displayed by microorganisms. Keep in mind that this unit does not cover all the antimicrobial agents available. Finally, we will discuss the basics of antibiotic sensitivity testing.

5–1. Antimicrobial Agents

For our purposes, the term “antimicrobial agent” embraces an assortment of therapeutic organic chemicals, including substances synthesized in the laboratory and those derived from living organisms. The widely prescribed sulfonamides typify drugs of the synthetic group. By common usage, the term “antibiotic” is reserved for chemical substances produced by living forms—compounds like the penicillins—that, in low concentration, suppress the growth of microbes or kill them outright. But since the underlying principle of sensitivity testing and assay is identical for most synthetic and naturally occurring chemotherapeutic agents, we will not emphasize the differences in the source of the drug.

026. Antibiotic effects and modes of actions

There are thousands of known antimicrobial agents derived from natural selection procedures or from chemical routes. Of these, a large number have been tested for potential clinical application. Unfortunately, the vast majority has not proven useful, often because the agent is

too toxic to be employed as a selective chemotherapeutic agent. In pharmacological terms, this means the therapeutic index (ratio of the curative to the toxic dose) is unfavorable. Many of the toxic antibiotics have been thoroughly studied by those interested in the mechanisms of microbial physiology and chemotherapy. The organization of this lesson follows current opinion regarding the major affects and target of action of the antimicrobial agent. You must remember the presence of a drug causes both primary and secondary biochemical changes in a microbial cell, and it is often difficult to separate one group of changes from the other.

What is an antibiotic?

An antibiotic is a chemical substance derived from, or produced by, various species of microorganisms that is capable in minute concentrations of inhibiting the growth of other microorganisms. Antibiotics are widely distributed in nature and play a significant part in regulating the microbial population of soil, water, sewage, and compost. They differ significantly chemically and in their modes of action. Thus, there exists little or no relation between the antibiotics other than their ability to adversely affect the life processes of certain microorganisms. Several hundred antibiotics have been purified, but only a few have been sufficiently nontoxic to be of use in medical practice. Those currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Bacillus*, *Penicillium*, *Cephalosporium*, *Micromonospora*, and *Streptomyces*.

Desirable properties of antibiotics

An essential property of a chemotherapeutic agent is selective toxicity. The pathogen must be destroyed or inhibited without injury to the host. The ideal antibiotic is bacteriocidal rather than bacteriostatic in its effect. Antimicrobial substances are usually characterized as either *bacteriostatic* or *bacteriocidal*, although some of them possess both bacteriostatic (inhibitory) and bacteriocidal (lethal) properties, depending on drug concentration and the type of bacterial pathogen involved. This difference in effect is understandable if you examine the nature of the cell function that is altered. For instance, an agent is clearly bacteriocidal if its action so weakens the main supporting structure—cell wall—that the high internal osmotic pressure of the cytoplasm causes lysis of the cell. On the other hand, a drug that upsets nucleic acid synthesis can prevent the cell from reproducing even though the functions of respiration and growth remain intact. The result of this inhibitory, or bacteriostatic, effect is multiplication of the pathogen is suppressed, and the natural defenses of the host are given an opportunity to overcome the infection.

Categories

If we group antimicrobial substances in terms of their effect on microbial physiology, three major categories can be recognized. Drugs that interfere with:

- Genetic replication of the cell (transcription).
- Expression of genetic information (translation).
- Assembly or function of cell components (cell walls and membranes).

Basic principles of antibiotic action

Before an antibiotic can act, it must first interact with some part of a parasite or a pathogenic microorganism in a human or animal host. This interaction may be less specific than the binding implied by Ehrlich's definition. The interaction is initiated by a specific active transport process of the cell that serves to increase the intracellular "free" concentration of the antibiotics above that achieved by passive diffusion. The balance of influx and efflux determines the intracellular concentration of the antibiotic, and no specific binding of the

drug to any intracellular components need be assumed. The consequences of the high intracellular concentration of the antibiotic are eventually expressed by a specific interaction of the drug molecule with some enzyme, a subcellular component of the cell, and so forth.

Toxicity

The explanation of how any given antibiotic acts ultimately involves one or more very specific biochemical or biophysical events in the invading bacterial or fungal cell. Successful chemotherapy requires the metabolic process to be attacked in the microorganism be as different as possible from that of the animal host. Obviously, it is a clinical failure to kill or inhibit the growth of the microorganism at the cost of the patient's life or continued well-being. Aminoglycosidic antibiotics are the most toxic to the host, as well as to the microorganism, and the real or potential damage to the patient by the antimicrobial agent must be balanced against the degree of danger to the patient's life posed by the microorganism.

Resistance

A discussion of the biochemical basis of the *mode of action* of the clinically used antibiotics depends on understanding some fundamental processes in the human host. Resistance to the effect of an antibiotic, either as a constitutive property of the microorganism before it infects a host, or as a complication that suddenly appears during a course of therapy with an antibiotic, is also explicable in highly specific biochemical terms. Resistance to specific antimicrobial agents is reviewed further on. The purpose of this unit is to discuss the biochemical basis of susceptibility (sensitivity) and resistance to some commonly used antibiotics.

An antimicrobial agent inhibits a microorganism in a variety of specific ways. These are reviewed later in this unit. However, an organism *resists* an antibiotic in only three general ways, and these constitute the basic reasons why a particular antimicrobial agent does not inhibit the growth of a microorganism that is isolated from a patient.

1. The antibiotic may be unable to reach the potential target site of its action. In a mutant, some change in the physiology of the cell following a biochemical change to that cell can decrease the ability of the drug to reach the site of action.
2. The pathogenic agent may possess some biochemical mechanism (enzyme) that acts to reduce or eliminate the toxic potential of the antibiotic. In a mutant, an increased level of enzymatic activity or even a new mechanism for inactivating the drug can develop. Examples are (1) beta-lactamases that cleave penicillins and cephalosporins to inactive components, (2) acylases that acetylate chloramphenicol to yield inactive derivatives, and (3) enzymes that inactivate aminoglycosides by phosphorylation, adenylation, acetylation, and so forth.
3. The pathogenic agent may have evolved biochemically in such a way that the target site for the antibiotic as determined with other cells no longer accommodates the drug, and no productive (toxic) interaction occurs. In the mutant cell, this change in the biochemistry of the target site occurs during the period of observation or treatment of the patient. Examples are cells that become resistant to erythromycin, lincomycin, streptomycin, and other agents.

All three mechanisms for blocking the action of an otherwise active antibiotic or for acquiring resistance to it are known. To understand them, it is necessary to know the biochemical basis for the mode of action of the drug. In particular, this is essential if

resistance is related to a change at the cellular target of the antibiotic. It is necessary to consider a number of the salient facts known about the antibiotics currently useful in medicine, and they are discussed in the remaining portion of this unit.

The general organization follows the known (or presumed) major biochemical target for each antibiotic or class of antibiotic, if a large number of chemically related drugs have the same or similar targets. The ways in which an antibiotic can disturb the physiology of a parasitic or pathogenic agent are almost infinite. Any single enzyme or structure in the living cell can potentially be affected and rendered incapable of fulfilling its normal function. In spite of this tremendous range of potential target sites for antibiotics, those most used in human and veterinary medicine fall into well-defined categories of action, and only a few critical areas of microbial physiology are affected. These areas are mostly concerned with the genetic replication of the cell (transcription), the expression of that genetic information into functional proteins (translation), and the assembly or function of critical cell components, such as the bacterial cell wall and membranes.

It should be noted that there is a major difference between human eukaryotic cells and the usual bacterial or fungal antagonist. In contrast to the latter, the human cell possesses mitochondria, and although the argument as to their origin is not settled, these semiautonomous organelles have many of the characteristics of bacteria. Their nonchromosomal genetic material is similar to that of bacteria, and their apparatus for transcription and translation of mitochondrial DNA and RNA is similar to that of bacteria. Thus, although many of the useful antibiotics to be described attack some part of the bacterial or fungal cell that is fundamentally different from anything in the human host, there is a real potential for some damage to human cells via the mitochondria they possess. The toxic action of chloramphenicol on mammalian cells is perhaps related to its ability to permeate the mitochondrial membrane and interfere with mitochondrial protein synthesis. A chemically different drug, erythromycin, which has a very similar target site and mode of action, is seemingly unable to cross the mitochondrial membrane, and, thus, erythromycin shows little, if any, toxicity for human cells. Subtle factors of this sort account for many of the anomalies connected with the practical use of antibiotics.

The effects of two or more antimicrobial agents in combination

There are several reasons why it is advantageous to treat an infection with a combination of antimicrobials. These include providing broad spectrum coverage in the case of polymicrobial infections or infections where the etiologic agent is not known, decreasing the potential for the development of resistance to the individual antimicrobials as in the treatment of tuberculosis, decreasing dosage and minimizing the potential of toxic side effects, increasing bactericidal rates, and treating an infection that cannot be cured by a single agent.

Interactions

Antimicrobial combinations may demonstrate synergism, antagonism, or additivity. In synergism, the combined effect of drugs is greater than their independent effects when measured separately. Antagonism implies the combined effect of the drugs is less than the sum of their independent effects when measured separately. Finally, additivity means the combined effect of the drug is equal to the sum (or partial sum) of their independent effects, when measured separately.

Synergism

A case in which two antibiotics with different modes of action seem to act synergistically is that of the aminoglycosides and the beta-lactams. The latter causes defects in the cell-wall structure that permit increased entry of the aminoglycosides and increased inhibition of ribosome-dependent protein synthesis results. Another example, also involving beta-lactam antibiotics, seems to concern inactivation of enzymes that destroy penicillins and cephalosporins (beta-lactams) by lactamase-resistant structures capable of binding tightly to these enzymes. For instance, cloxacillin potentiates the activity of lactamase-sensitive penicillins.

Antagonism

As mentioned previously, antagonism is occasionally noted between antibiotics. One example is between penicillin and chlortetracycline when both are used in the treatment of pneumococcal meningitis. Much higher mortality has been noted when both antibiotics are used in combination than with the use of penicillin alone. The explanation may be tetracycline antibiotics produce reduced cell growth and division, and the penicillin thus has reduced potential for its target—the inhibition of cell wall formation in rapidly dividing cultures.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

026. Antibiotic effects and modes of actions

1. What is an antibiotic?
2. Antibiotics differ in terms of what two characteristics?
3. Most antibiotics currently used are derived from which genera of organisms?
4. What is meant by selective toxicity of a chemotherapeutic agent?
5. What term is used to describe an antimicrobial substance that inhibits the growth of an organism?
6. In what three major categories is microbial physiology affected by the action of antibiotics?

7. What is a prerequisite for successful chemotherapy?
8. What is the disadvantage of aminoglycosidic antibiotics?
9. What are the three general ways a microorganism resists an antibiotic?
10. What are some examples of biochemical mechanisms that act to reduce or eliminate the toxic potential of an antibiotic?
11. What is a major difference between human eukaryotic cells and bacterial cells?
12. Where does the potential for damaging human cells through the use of antibiotics lie?
13. For what reasons is it advantageous to treat an infection with a combination of antimicrobials?
14. What are the three possible outcomes of combination antimicrobial therapy?
15. What are two of the synergistic modes of action demonstrated by aminoglycosides and beta-lactams?
16. What is the result of treating pneumococcal meningitis with penicillin and chlortetracycline?

027. Inhibitors of bacterial cell function and protein synthesis

Antimicrobial agents act as inhibitors to bacterial cell function and protein synthesis. Let's look at exactly how this happens.

Inhibitors of bacterial cell function

An antimicrobial agent can affect either the function or structure of a bacterial cell. Although the function of a structurally normal cell is inhibited in almost an infinite number of ways, it is convenient to categorize the inhibitors as inhibitors of (1) nucleic acid synthesis or function, (2) protein synthesis, (3) the normal functioning of the plasma membrane, (4) the function of a specific microbial enzyme or enzyme system, or (5) cell wall formation. There are useful antimicrobial agents that fit into each of these five categories.

Antimicrobial agents that affect DNA and RNA

There are many known drugs that are potent inhibitors of some phase of transcription and translation—the complex series of reactions by which the DNA of a parent cell is copied prior to cell division and directs the formation of RNA species, which are needed for the synthesis of cellular proteins, cofactors, substrates, and so forth. There is a large number of known potent antibiotics that inhibit transcription. However, relatively few antibiotics of this type are in clinical use, probably because the biochemistry of transcription in bacteria is not sufficiently different from that in the human host cells to permit a very favorable therapeutic index with this class of drug. Agents such as the actinomycins are too toxic to be of much use as antimicrobial drugs in humans. They are used occasionally as antitumor agents, but are cited here as an example of drugs showing the high toxicity associated with so many inhibitors of transcription.

Rifampin (Rifampicin)

There are a large number of rifamycin antibiotics, mostly of semisynthetic origin. The class comprises other chemically similar structures and has been given the generic name *ansamacrolide*. This term should *not* be confused with the macrolides (i.e., erythromycin). The two classes of antibiotics are chemically very different, and their modes of action differ as well. The original rifamycins isolated from *Streptomyces mediterranei* were not well absorbed and were not very active. Chemical modification of the structures has led to several series of semisynthetic derivatives with high antimicrobial potency and excellent therapeutic indices. Rifampin is marketed as a rather specific inhibitor of *Mycobacterium tuberculosis*. The availability of rifampin has greatly improved the current therapy for tuberculosis.

The rifamycins (rifampin) are very specific inhibitors of RNA polymerase. Moreover, it is specifically the bacterial DNA-dependent RNA polymerase that is affected. Neither DNA-dependent DNA synthesis in bacteria nor nuclear DNA-dependent RNA synthesis by eukaryotic cells are inhibited. This explains the selective toxicity of rifampin toward bacterial cells. Rifampin can inhibit RNA synthesis in the mitochondria of human cells, but the drug appears to be relatively impermeable to the mitochondrion. Rifampin occasionally causes hepatotoxicity, which could be related to an effect on mitochondrial RNA replication. Rifampin specifically inhibits bacterial RNA polymerase by binding to the *beta subunit* of the core enzyme of the polymerase. This drug blocks the initiation phase of RNA synthesis, and if RNA synthesis was begun prior to the addition of the rifampin, there is no inhibition of the remaining steps in the synthesis of RNA.

Nalidixic Acid (NegGram)

Nalidixic acid (fig. 5-1), a rather simple compound made by chemical means, is an inhibitor of DNA replication in bacterial cells. It fails to inhibit DNA transcription in mammalian cells. Apparently, it can inhibit mitochondrial DNA replication, but the drug is usually relatively nontoxic, perhaps because it fails to reach significantly high concentrations in plasma and other body compartments. It is, however, concentrated in the urine, where it reaches effective therapeutic levels. The exact target of nalidixic acid action is still in dispute. It is safe to say its primary effect in the intact living bacterium is a prompt, selective, and reversible blocking of DNA synthesis. This block occurs with many types of DNA replication, including normal duplication of DNA as necessary for cell division, the repair of DNA that normally occurs after damage by ultraviolet light, and the replication seen with some viruses.

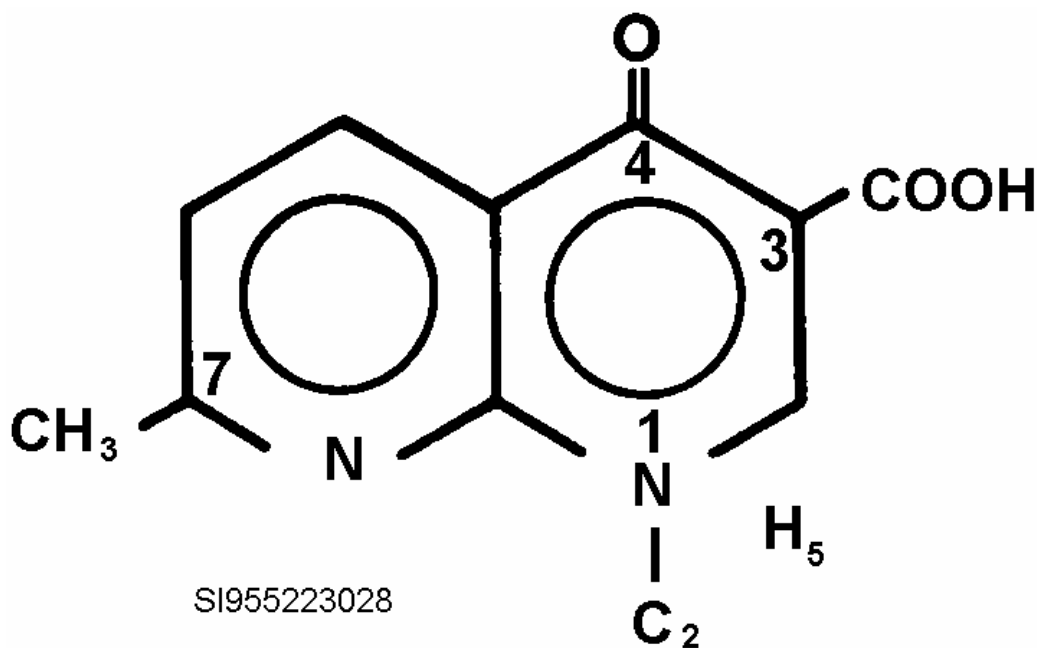


Figure 5-1. Structure of Nalidixic Acid.

Quinolones

Quinolones, biochemically related to nalidixic acid, are potent antibiotics that target DNA gyrase. Gyrase is an enzyme essential for DNA replication and, in turn, inhibits bacterial DNA synthesis. Widespread development of bacterial resistance has limited clinical applications of nalidixic acid and its early analogs (oxolinic acid and cinoxacin). The original two-ring quinolone nucleus was modified with different side chain substitutions to synthetically produce the newer quinolones. The fluoroquinolones are new agents that contain a fluorine atom attached to the nucleus at position 6. The quinolones available in the United States are cinoxacin, norfloxacin, ciprofloxacin, enoxacin, ofloxacin, and lomefloxacin. These antibiotics are well absorbed after oral ingestion and have good penetration into the prostate, extravascular body fluids, bone, muscle, lungs, kidneys, and intestinal wall. Low concentrations are found in the CSF from patients with meningitis. Quinolones are excellent against *Salmonella*, *Listeria*, *Brucella*, and *Mycobacterium* spp.

Sulfonamides and Trimethoprim

Since the 1930s, sulfonamides have been used in the United States as an effective systemic antimicrobial agent. They were found to be active against a wide range of bacteria, but their main success was in the treatment of streptococcal infections and pneumococcal pneumonia. The antibiotics discovered since then have a greater use in chemotherapy because of their higher potency and the fact that bacteria readily develop resistance to sulfonamides. However, sulfonamides are still widely used in the treatment of urinary tract infections and certain forms of meningitis, and in veterinary medicine. The structure of representative sulfonamides is shown in figure 5-2. In 1968, trimethoprim (TMP)-sulfamethoxazole (SMX), also called co-trimoxazole, was clinically introduced and was highly effective against many different infections.

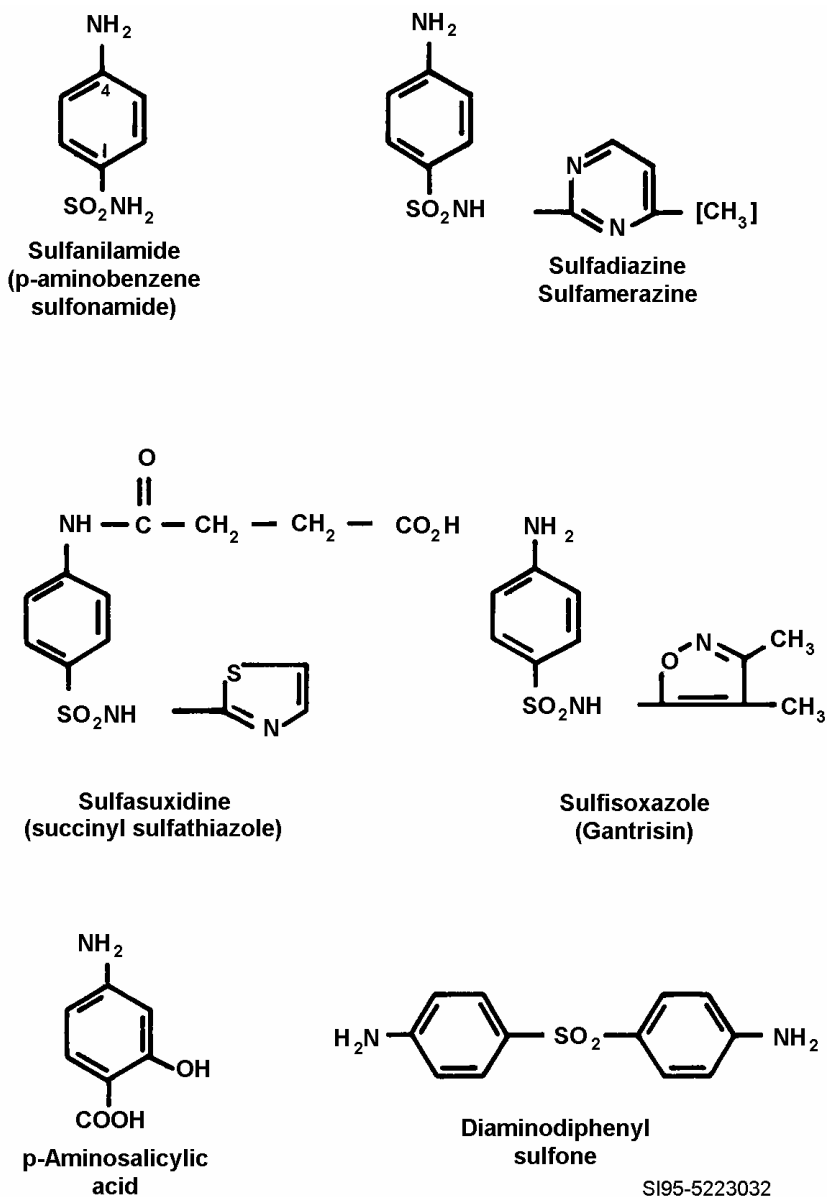


Figure 5-2. Structure of Sulfonamides and other analogs.

Sulfonamides are derived from sulfanilamide, and trimethoprim is a pyrimidine analog. Sulfonamides and trimethoprim work together to inhibit folate metabolism and that action ultimately prevents the synthesis of bacterial DNA. Each blocks (or hinders) a different site of the bacterial folic acid metabolic pathway; therefore, they increase the antibacterial activity of one another and act synergistically. They are used orally, as a topical ointment, and intravenously (although rare). They can be used in combination or separately, and in different chemical forms (i.e., sulfadiazine, sulfisoxazole, and sulfamethoxazole). A common fixed combination of TMP-SMX is widely distributed throughout the body. Separately or in combination, these agents have been used in the treatment of: bacteria infections due to gram-positive and gram-negative organisms (e. g., conjunctivitis, otitis media, sinusitis, acute bronchitis, and pneumonia), toxoplasmosis, *Nocardia asteroidis* infections, and for *Pneumocystis carinii* pneumonia in immunocompromised patients with leukemia, AIDS, or organ transplants.

Nitrofurantoin

Nitrofurantoin belongs to a class of compounds consisting of a primary nitro group joined to a heterocyclic ring. The mode of action is unknown, but is thought to inhibit bacterial enzymes and damage DNA. This drug is taken orally and used almost extensively for urinary tract infections. Nitrofurantoin is available in microcrystalline (Furadantin) and macrocrystalline (Macrochantin) forms, and is a broad spectrum antibiotic against gram-positive and gram-negative bacteria.

Inhibitors of protein synthesis

A relatively large group of useful antimicrobial agents interfere with protein synthesis in bacteria. Because this process differs in a few essential details from that in eukaryotic (mammalian) cells, it is a good target for chemotherapy. In particular, the ribosomal subunits involved in messenger RNA translation in bacterial systems are smaller (30S and 50S) than those involved in mammalian translation (40S and 60S, respectively).

Aminoglycosidic and aminocyclitol antibiotics

A large number of antibiotics are included under the term *aminoglycosides*. Those currently available (gentamicin, sisomicin, and netilmicin) are derived from *Micromonospora* spp., and streptomycin, neomycin, kanamycin, tobramycin, and paromomycin are derived from *Streptomyces* spp. The differences in their suffixes “micin” versus “mycin” are related to the difference in their origin. Their common denominator, structurally, is the presence of a cyclohexane ring with basic (amino or guanidino) groups in the one and three positions. Streptomycin, neomycin, tobramycin, kanamycin, and gentamicin are naturally occurring. In naturally occurring structures, the positions have hydroxyl groups, with the exception of C₂, which sometimes has only hydrogen atoms. Different residues (sugars, including basic sugars) are attached to the free hydroxyl groups; thus, a great variation in overall structure is possible (for example, the streptomycin group differs greatly from the more recently introduced gentamicins and tobramycins). Amikacin is a semisynthetic derivative from kanamycin and netilmicin is derived from sisomicin.

The mode of action of each aminoglycoside is believed to be roughly similar and is discussed here in terms of streptomycin; the longest-used aminoglycosidic antibiotic and the substance whose action has been the most intensely studied. Streptomycin is one of the oldest antibiotics known (introduced in 1944), and is still used extensively today. It has a broad spectrum of action, but resistance in bacteria readily develops, as will be discussed. In addition, this and other aminoglycosidic antibiotics are selectively toxic to the eighth cranial

nerve, which is responsible for hearing and equilibrium. The various actions of streptomycin on *in vivo* and *in vitro* protein synthesis in bacteria are well described. A large number (10 or more) of different effects of streptomycin have been noted, and the problem is to distinguish between primary and secondary actions. The primary effect of streptomycin is thought to be interference with 30S ribosomal unit function and inhibiting protein synthesis. Neomycin is available only for oral and topical use due to its severe toxicity with IV administration. Even in the presence of inflammation, aminoglycosides penetrate poorly into the CSF, vitreous fluid of the eye, prostate, tracheobronchial secretions, and biliary tract. Aminoglycoside antibiotics are active against aerobic gram-negative bacilli and *S. aureus*.

Tetracycline antibiotics

Aureomycin (7-chlorotetracycline) was isolated in 1948, and terramycin (5-oxytetracycline) was isolated two years later. Since then, several clinically useful tetracycline-type antibiotics have been developed, including chlortetracycline, oxytetracycline, and tetracycline, which are short-acting; demeclocycline and methacycline, which are intermediate-acting; and doxycycline and minocycline, which are long-acting compounds. All these tetracyclines are amphoteric substances that tend to form insoluble complexes with many anions (Ca^{++} , Mg^{++} , etc.). Variable and relatively poor absorption of the tetracyclines from the gastrointestinal tract are primary disadvantages and can reflect the formation of complexes, especially during or after ingestion of food. The mode of action of the tetracyclines has been studied extensively, and quite a large number of metabolic effects have been observed. However, it is generally accepted that the primary effect of the tetracyclines is produced by the binding of the antibiotic to the 30S ribosomal subunit that, through a complex process, prevents bacterial polypeptide synthesis.

Resistance to the tetracyclines is quite common and often associated with the acquisition of plasmids by the formerly sensitive bacterium. A major factor in the inherent or acquired resistance to tetracyclines is bacterial cell membrane function. The “normal” accumulation of tetracycline by naturally resistant bacteria can be below that required for bacteriostatic or bactericidal action. After acquisition of a plasmid bearing new genetic information, a naturally susceptible bacterium also can exhibit resistance to the tetracyclines that are related to an alteration in a protein moiety of the 30S ribosomal unit. In these examples, there is a reduced binding affinity between the antibiotic and the ribosomal unit. Tetracyclines obtain excellent penetration in tissue, but are in low concentrations in CSF. Because they also cross the placenta and are incorporated into fetal bone and teeth, they must be used with caution during pregnancy.

Macrolide antibiotics

The so-called macrolides are a very large group of antibiotics produced primarily by the genus *Streptomyces*. Their common structural denominator is a large lipid ring (inner ester or lactone, chemically termed a *macrolide*). The 14- and 16-membered macrolide antibiotics are the macrolides most studied and used in both human and veterinary bacterial chemotherapy. They are different because of the number of atoms (14 to 16) and the pattern of the lactone ring. Erythromycin is naturally occurring, and oleandomycin, spiramycin, and josamycin are other natural analogs, which are quite similar in structure and are examples of the 14-membered group. Clarithromycin and azithromycin are 14- and 15-membered semisynthetic macrolides, which offer significant advantages over erythromycin. Currently, the 16-membered macrolide antibiotics are not used in clinical practice in the United States. However, cross-resistance between some of them (i.e., tylosin) and erythromycin has been noted, and the very heavy use of tylosin in veterinary medicine poses a potential problem for

human therapy. Consequently, the use of tylosin has been severely restricted in the United Kingdom, although no such action has been taken in the United States. The 16-membered macrolides are also used for the treatment of humans in Japan and the Orient. Therefore, one should be aware of their existence and the possibility that exposure of the microbial population to these agents, even if in animals used for food, can increase clinical resistance to erythromycin. Macrolides are relatively broad-spectrum against gram-positive and some gram-negative bacteria, mycoplasmas, treponemes, rickettsiae, and chlamydiae.

It is essential to note that, in addition to the so-called ansamacrolides already discussed, a second and quite different type of antimicrobial agent is also classified with the macrolides. These other macrolide drugs are antifungal agents and have no antibacterial effects. They are structurally quite different from erythromycin and have a very different mode of action. Three of them—amphotericin B, candicidin, and nystatin—are used in human chemotherapy.

Erythromycin

Erythromycin A (erythromycin) is one of the four closely related antibiotics of the macrolide class that are produced by *Streptomyces erythraeus*. Erythromycin, whose structure is shown in figure 5-3, has been in clinical use since the 1950s. It is a nontoxic antibiotic effective mainly against gram-positive bacteria and certain strains of mycoplasma, and is structurally very similar to oleandomycin.

All the studies done on the mode of action of erythromycin lead to the conclusion its primary action is to inhibit RNA-dependent protein synthesis in bacteria. Protein synthesis in so-called resistant bacteria (e.g., gram-negative microorganisms, such as *Escherichia coli*) is also sensitive to erythromycin, but these insensitive strains of bacteria are limited in their ability to accumulate erythromycin at the target site of its action. The exact stage of protein synthesis inhibited by the bound erythromycin is not completely understood. However, the observed effect is a slowdown or stoppage in the translation of messenger RNA into functional protein molecules. The effect of erythromycin is reversible; thus, if the concentration of the drug is low and the duration of contact with the bacterium is short, a bacteriostatic action is seen. The bacterial cell is killed if contact is prolonged and the concentration of erythromycin is high. Studies of bacterial resistance to erythromycin have failed to give any evidence that bacteria can enzymatically change the structure of the antibiotic. However, there is some evidence to show that resistant strains of bacteria are impaired in their ability to accumulate erythromycin.

Chloramphenicol

Chloramphenicol is a broad-spectrum antibiotic produced by *Streptomyces venezuelae* and, more practically, by total chemical synthesis. It has a simple structure (fig. 5-4), but possesses two relatively unusual features—an aromatic nitro group and a dichloroacetyl side chain. Of four possible stereoisomers, only one possesses antibacterial activity. Both the nitro group and the dichloroacetyl residue can be replaced by some other groupings with substantial retention of biologic activity. Chloramphenicol is a bacteriostatic agent that binds to the peptidyltransferase component of the 50S ribosomal subunit and, through a complex process, inhibits protein synthesis.

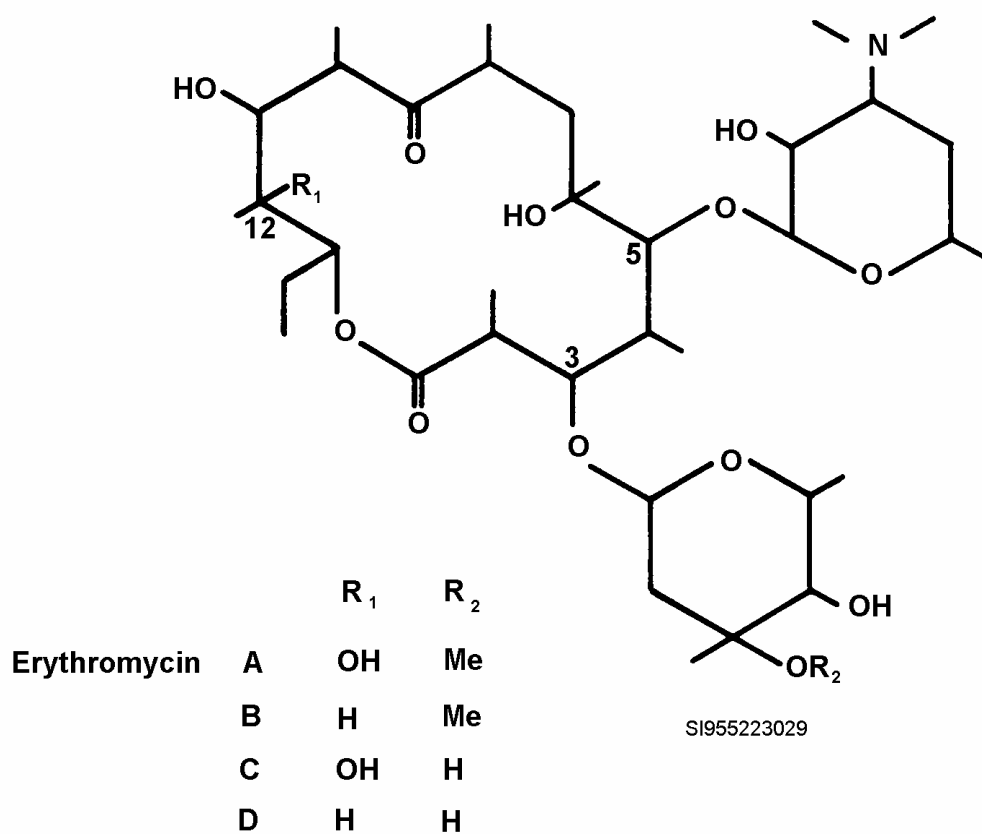


Figure 5-3. Structure of Erythromycin.

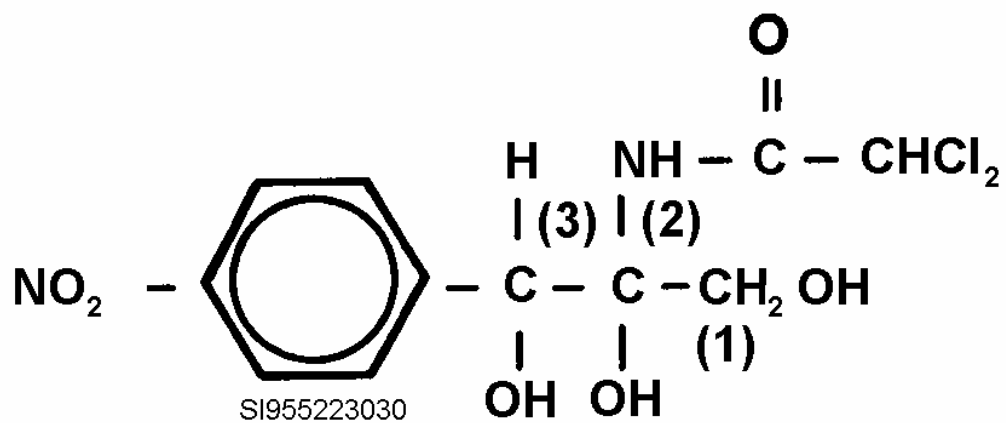


Figure 5-4. Structure of Chloramphenicol.

Chloramphenicol has always been a popular antibiotic and still enjoys a large degree of support from certain clinicians, especially for hospital use in seriously ill patients. It is probably the most controversial antimicrobial drug in common use because of its known tendency to produce highly toxic symptoms, including blood dyscrasias, aplastic anemia, and depression of bone marrow function. These dangers must be balanced against the broad spectrum of activity of chloramphenicol and its ability to inhibit the growth of most bacteria, rickettsiae, and chlamydiae (microorganisms of the psittacosis-lymphogranuloma group). It must be used with great caution, and most physicians believe it should be administered only under adequate hospital supervision, including frequent tests for changes in the hematopoietic system. It can be bactericidal against common meningeal pathogens, such as *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*. Resistance to chloramphenicol is well documented and related to enzymes (constitutive or induced) that inactivate the drug primarily by acetylation of the two free hydroxyl groups present in the molecule.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

027. Inhibitors of bacterial cell function and protein synthesis

1. Why are only a few antibiotics that inhibit transcription in use?
2. What generic name has been given to the rifamycins?
3. From what organism were the original rifamycins isolated, and what disadvantages did they have?
4. Under what name, and for what purpose, is the most useful rifamycin marketed?
5. Specifically, how do rifamycins inhibit bacteria?
6. Why doesn't rifampin inhibit RNA synthesis by the mitochondria of human cells?
7. What does nalidixic acid inhibit in bacterial cells?
8. In what body fluid is nalidixic acid concentrated?

9. What does the quinolones specifically target?
10. What quinolones are available in the United States?
11. What is TMP-SMX?
12. What is the mode of action of sulfonamides and trimethoprim?
13. What are the two forms of Nitrofurantoin?
14. What are the aminoglycosides derived from *Micromonospora* spp.?
15. What are the aminoglycosides derived from *Streptomyces* spp.?
16. Why is there a difference in their suffixes?
17. What aminoglycoside has been used the longest, and what disadvantages does it share with other aminoglycosides?
18. What is the primary effect of streptomycin?
19. What characteristics do all tetracyclines share in common?
20. What are the primary disadvantages of tetracyclines?
21. How is the primary effect of the tetracyclines on bacteria produced?

22. What event is the development of resistance of bacteria to tetracyclines often associated with?
23. What genus primarily produces the macrolides?
24. What organisms are macrolides mainly effective against?
25. What is erythromycin's primary mode of action?
26. Chloramphenicol is produced by what organism?
27. What is the most controversial antimicrobial drug in common use?
28. What toxic symptoms are caused by chloramphenicol?
29. What organisms are inhibited by chloramphenicol?

028. Antimicrobial agents that affect bacterial cell-wall synthesis

Perhaps the best-known antibiotics that selectively inhibit an infectious cell without harming the human host are some of those that affect the formation of the bacterial cell wall. Mammalian cells completely lack a cell wall, which is a semirigid (basket-like) structure possessed by most bacteria. Thus, an agent that prevents the normal synthesis of a bacterial cell wall does not directly inhibit or affect any similar biochemical process in the human host. The natural and semisynthetic penicillins are remarkably effective antibiotics, and they act in this way. They selectively inhibit bacterial cell wall formation without an appreciable concomitant toxic effect on the human host. After the structures and some properties of these inhibitors of cell-wall biosynthesis are reviewed, the modes of action of each with respect to the formation of cell walls are discussed.

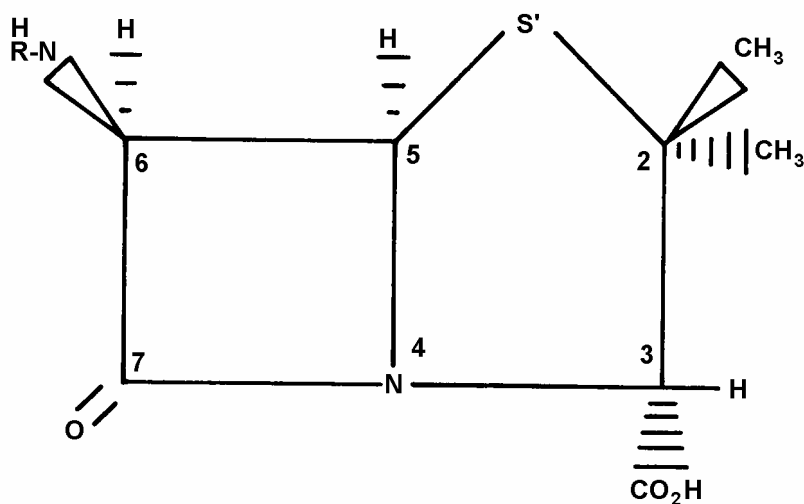
Beta-lactam antibiotics

It is important to note antibiotics that act by inhibiting cell wall formation are effective only with growing populations of bacteria. Mature cells are not inhibited. This property of penicillin has long been recognized. Fortunately, however, in most clinical infections, the causative bacteria must multiply in order to endanger the host; thus, the possibility that only

mature cells are present is not a real concern. The antibiotics in this group are, therefore, effective in preventing rapid or uncontrolled proliferation of the pathogenic bacteria. Let's start with the beta-lactams.

Penicillins

The discovery of penicillin marked a milestone in the history of chemotherapy. The detailed story of the original finding of penicillin by Fleming is well known and beyond the scope of this unit. However, by 1942, the work on the determination of its structure (fig. 5-5) was begun at Oxford and developed into a giant Anglo-American joint effort soon thereafter. By the end of World War II, penicillin was being used for chemotherapy, and the basic structure of its beta-lactam nucleus was established. The fermentation of the producing *Penicillium* species was affected by the composition of the growth medium employed, and various penicillins were obtained following supplementation of the medium with certain organic acids. For example, such acids contributed to the development of the R group of penicillins.



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Figure 5-5. Structure of the penicillins.

The addition of phenylacetic acid in 1953 led to the isolation of nearly homogeneous benzyl penicillin (penicillin G). In the search for additional natural antibiotics of the penicillin type, a new substance known as cephalosporin C was discovered. It proved to have a beta-lactam moiety fused onto a sulfur-containing ring different from that of the penicillins. The resistance of cephalosporin C to inactivation by beta-lactamases brought much attention to this antibiotic in spite of its inherently low activity.

The penicillin structure is now known to be more complex. It contains the chemical nucleus 6-aminopenicillanic acid, which includes a beta-lactam ring fused to a thiazolidine ring. Changes in the side chain may modify the pharmacokinetic and antibacterial properties of the drug, whereas the substitutions at position 6 make the difference between the penicillins. The mode of action for the penicillins is from their ability to inhibit different bacterial enzymes or penicillin-binding proteins (PBP), which are essential for peptidoglycan synthesis. Their ability to trigger membrane-associated autolytic enzymes that destroy the cell wall results in

their bacteriocidal effect. The inhibition of bacterial enzymes involved in cell growth is another minor mode of action. The penicillins are a group of natural and semisynthetic antibiotics, as shown in the following table:

Natural:	Semisynthetic:
Benzylpenicillin (penicillin G) Phenoxymethyl penicillin (penicillin V)	Penicillinase resistant –Methicillin –Nafcillin –Isoxazoyl penicillins —Cloxacillin —Dicloxacillin —Oxacillin
Penicillin + β -lactamase inhibitor:	Extended spectrum:
Ampicillin-sulbactam (Unasyn) Amoxicillin-clavulanate (Augmentin) Ticarcillin-clavulanate (Timentin) Piperacillin-tazobactam (Zosyn)	Aminopenicillins –Ampicillin –Amoxicillin –Bacampicillin Carboxypenicillins –Carbenicillin –Ticarcillin Ureidopenicillins –Azlocillin –Mezlocillin –Piperacillin

The penicillins have antibacterial activity against many gram-negative and most gram-positive aerobic bacteria and numerous anaerobic organisms.

Cephalosporins

Cephalosporins are derivatives of the fermentation products of *Cephalosporium acremonium*, and, like penicillins, they bind to PBPs of susceptible organisms, which interferes with the synthesis of peptidoglycan of the bacterial cell wall. There are numerous variations to the chemical structure, which consists of a beta-lactam ring fused to a dihydrothiazine ring with a 7-aminocephalosporanic acid nucleus.

Semisynthetic beta-lactam antibiotics

The naturally occurring penicillins and cephalosporins (e.g., penicillin G and cephalosporin C) have several handicaps as useful chemotherapeutic agents. Although penicillin G is an agent of choice when an infectious agent is susceptible to it, such is often not the case. Many gram-positive bacteria produce hydrolases (beta-lactamases) that destroy the penicillin, and most gram-negative organisms are not susceptible to penicillin G. Although cephalosporin C has a much greater inherent resistance to inactivation by beta-lactamases than does penicillin G and a useful spectrum of antibacterial action, it is not potent enough to be very useful in chemotherapy. Useful alterations in these two types of antibiotics need to yield one or more of the following:

1. Increased resistance to the inactivating effect of beta-lactamases produced by an infectious bacterium.
2. An alteration in the spectrum of action, mainly in the direction of gram-negative organisms.

3. Increased potency or action, or improved dissemination into the tissues following oral administration.

All the semisynthetic penicillins and cephalosporins introduced in recent years have at least one of these desired properties.

Other beta-lactam antibiotics

We will now review the other beta-lactam antibiotics.

Aztreonam

Aztreonam is the only monobactam antibiotic, and it disrupts bacterial cell wall synthesis of gram-negative aerobes by binding to PBP 3. It is the only monobactam in clinical use at this time and is given intravenously. It is distributed to the body tissues and fluids, and crosses inflamed meninges to be potentially therapeutic to meningitis caused by most *Enterobacteriaceae*, *Neisseria*, and *Haemophilus* spp.

Carbapenems

Carbapenems are broad-spectrum antibiotics that bind to PBP 1 and PBP 2 causing cell elongation and lysis of gram-positive and gram-negative bacteria. They are the most potent beta-lactam against anaerobes.

β -Lactamase inhibitors

Let's now look at β -lactamase inhibitors—clavulanic acid, sulbactam, and tazobactam.

Clavulanic acid

Clavulanic acid is a weak antimicrobial agent, which is naturally occurring and found in cultures of *Streptomyces clavuligerus*. It inhibits β -lactamase from staphylococci and many gram-negative bacteria, and acts as a “suicide inhibitor” by forming an irreversible acyl enzyme complex with the β -lactamase. This leads to a loss of activity of the enzyme. Clavulanic acid acts synergistically with various penicillins and cephalosporins against many β -lactamase-producing bacteria. Clavulanic acid, along with ampicillin, amoxicillin, or ticarcillin combination, is active *in vitro* against *Mycobacterium tuberculosis*.

Sulbactam

Sulbactam is a semisynthetic, 6-desaminopenicillin sulfone with weak antibacterial activity, but also acts synergistically with penicillins and cephalosporins. It inhibits certain plasmid and chromosomally mediated β -lactamases of *S. aureus*, *M. catarrhalis*, *H. influenzae*, *Neisseria* spp., many *Enterobacteriaceae*, *Legionella* spp., *Bacteroides* spp., and *Mycobacterium* spp. Sulbactam is combined with ampicillin, which penetrates body tissues and fluids as with clavulanic acid and amoxicillin. It also enters the CSF in the presence of inflamed meninges, but clavulanic acid with amoxicillin penetrates inflamed meninges very poorly.

Tazobactam

Tazobactam is a penicillanic-acid sulfone derivative structurally related to sulbactam and is a suicidal β -lactamase inhibitor. As with the above compounds, it is synergistic with the penicillins, and is most active with piperacillin against β -lactamase producing aerobic and anaerobic gram-negative bacilli.

Glycopeptides and polypeptides

Glycopeptides and polypeptides are another group of antibiotics involved in inhibiting bacterial cell wall synthesis.

Vancomycin

This bactericidal agent is a complex glycopeptide with unknown structure, and is obtained from *Streptomyces orientales*. It was initially used against penicillin-resistant staphylococci and it has been most useful against methicillin-resistant staphylococci. It is also used in patients allergic to penicillins or cephalosporins. Glycopeptides inhibit peptidoglycan synthesis in the bacterial cell wall. Glycopeptides are used mainly against aerobic and anaerobic gram-positive organisms. Toxicity, when noted, is quite variable and includes “renal irritation.” When impaired renal function exists in a patient receiving vancomycin, higher-than-normal levels may be responsible for toxic effects, including damage to the eighth cranial nerve.

Polymyxin

Polymyxins are cyclic basic polypeptides derived from *Bacillus polymyxa* and only two are available for human use—polymyxins B and E (colistin). These antibiotics interact with the phospholipids of the bacterial cell membrane, which increases cell permeability and disrupts osmotic integrity. This process results in leakage of intracellular constituents resulting in cell death. Polymyxins are given orally or the topical route, and are active only against gram-negative bacilli, especially *Pseudomonas* spp. The combination of polymyxins with TMP-SMX can be synergistic and can be used for the treatment of serious infections due to *X. maltophilia*, *P. aeruginosa*, *P. cepacia*, and *Serratia* spp.

Bacitracin

Bacitracin, originally isolated from *Bacillus licheniformis*, is a polypeptide antibiotic consisting of peptide-linked amino acids. It is restricted to topical use because of its systemic toxicity, except in the treatment of antibiotic-associated *C. difficile* colitis where it is given orally. If administered orally, it must be used with caution because it is nephrotoxic (toxic to the kidneys). At one time, it was also given orally for severe staphylococcal infections. Bacitracin inhibits dephosphorylation of a lipid pyrophosphate, which is a crucial step in cell wall synthesis, and it disrupts the cytoplasmic membrane. It is mainly used against gram-positive bacteria, especially group A beta-hemolytic streptococci and staphylococci. Bacitracin, neomycin, and polymyxin B are often seen in combination as topical ointment to provide broad-spectrum coverage.

Antimicrobial agents that affect the function of the cytoplasmic membrane

A few antimicrobial agents affect the function of the cytoplasmic membrane of bacteria. The problem with most of these drugs is toxicity occurs not only in the bacterial cytoplasmic membrane, but also in erythrocyte membranes and the membranes of other cells in the human host. Thus, for the most part, these agents are of no clinical use. A few of these antimicrobial agents are used when the mode of application is topical and, occasionally, they are administered by infusion into closed compartments (joints, etc.).

Amphotericin B and nystatin

These antimicrobial agents are also members of the groups discussed earlier, but, as stated, they are chemically quite different from erythromycin, as shown in figure 5-6. Structurally, they have a lipid ring much larger than the 14-member ring of erythromycin, and their modes of action are completely different from that of erythromycin. Neither amphotericin B

(Fungizone, etc.) nor nystatin (Fungicidin, etc.) is an antibacterial agent. Instead, each demonstrates an antifungal activity and is included among the very few systemic antifungal agents available for clinical use; however, these two agents are toxic to the human host and must be used with care. The methyl ester of amphotericin B is currently under study as a possibly less toxic and better-absorbed form of this antibiotic.

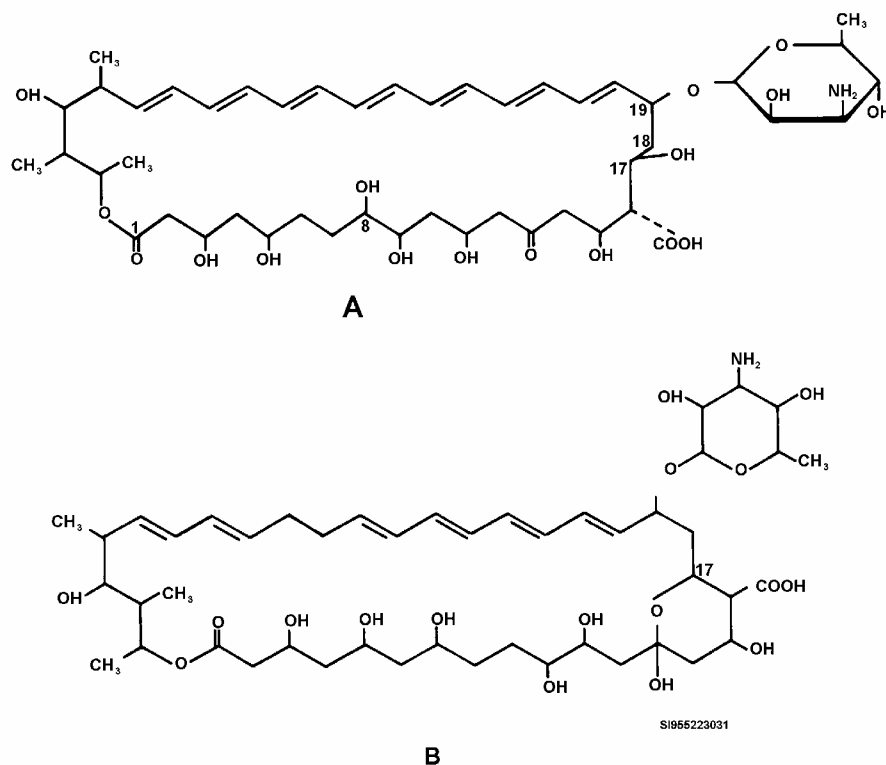


Figure 5-6. Structure of amphotericin B (A) and nystatin (B).

The biologically active antifungal macrolides, such as amphotericin B and nystatin, possess a number of conjugated carbon-carbon double bonds in their structure. These macrolides are, for this reason, quite unstable to both UV light and mild chemical reagents. It is also believed the polyene system (this group of antibiotics is often termed the *polyene macrolides*) is responsible for the known mode of action of these drugs. Those that are active are capable of forming tight complexes with certain sterols (e.g., cholesterol and ergosterol). Cholesterol is an essential component of mammalian cell membranes, and ergosterol plays a similar role in the fungal plasma membrane. Bacteria lack sterols in their plasma membranes, thereby explaining the selective action of these agents against human and fungal cells.

Once the amphotericin B or nystatin molecule has complexed with ergosterol from the fungal membrane, the properties of the membrane are seriously altered. The membranes become “leaky,” and the normal gradients of ions and other metabolites cannot be maintained, leading to the killing of the fungal cell. Because these antimicrobial agents remove cholesterol from the human host cell membranes, there is the possibility of toxicity.

Imidazole antifungal (antiprotozoal) agents

A number of clinically useful antifungal agents possess imidazole rings as partial structures. Of these, miconazole (Monistat, etc.) and ketoconazole (Nizoral, etc.) have received considerable attention. Among the many effects of these imidazole derivatives noted, a leakage of cations across the fungal cell membrane can be related to their antifungal effect. The mechanism of the effect is not known. An important antiprotozoal agent, metronidazole (Flagyl, etc.), also possesses a substituted imidazole ring, and the possibility exists of some similar biologic effect on membrane function.

Isonicotinic acid hydrazide

Isonicotinic acid hydrazide (INH) is one of the most important drugs used in the chemotherapy of tuberculosis. It is usually given in combination with streptomycin and para-aminosalicylate; however, with the availability of rifampin, this particular combination is being changed or modified. Despite the importance of INH, its mode of action is virtually unknown.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

028. Antimicrobial agents that affect bacterial cell-wall synthesis

1. Which organism produces the beta-lactams?
2. How were various types of penicillins obtained from the fermentation of penicillium species?
3. What characteristic of cephalosporin C brought it to the attention of researchers?
4. What is the mode of action of penicillins?
5. What is the organism from which cephalosporins are derived?
6. What are the other beta-lactams?
7. What are the β -lactamase inhibitors?

8. How does clavulanic acid inhibit β -lactamase from staphylococci and many gram-negative bacteria?
9. Which antibiotics are used with clavulanic acid to produce a synergistic effect?
10. Where is vancomycin obtained?
11. What is the mode of action for glycopeptides?
12. Where are polymyxins derived and what is their mode of action?
13. What is the mode of action of bacitracin?
14. What are three antifungal agents?
15. Which drug is used in the treatment of tuberculosis?

5-2. Mechanisms of Antibiotic Resistance

The major factor controlling the use of antimicrobial agents concerns the development of resistant organisms, and, since they are now so widespread, antimicrobial susceptibility tests must be determined for almost all organisms before rational choice of chemotherapy is made.

029. Biochemical mechanisms of resistance to antimicrobial agents

At present, we are in the midst of a “revolution” in our understanding of genetics. According to simple, long-accepted principles, evolution is a logical and relatively slow process. With the rapid recognition from laboratory experiments and clinical studies that new bacterial strains showing resistance to multiple antibiotics (and often to combinations not having common modes of action or structures) can appear suddenly, a new era of genetic understanding began. With prokaryotic cells, such as the gram-positive and gram-negative bacteria that are frequently pathogens, they may yield consequences of all sorts. One new behavioral pattern (phenotype) can include resistance to an antibiotic or other drug to which the parent strain of bacteria previously was susceptible. It is not the purpose of this brief

review to go deeply into the fundamental or applied knowledge concerning the transfer of DNA from one source to another, but a short overview is necessary to help clarify the resistance to antibiotics that often accompanies such transfer.

Mutation or natural selection

A single mutation in the chromosomal DNA of a (bacterial) cell leads, with certainty, to a single biochemical change in the progeny. This phenomenon is referred to as a single point mutation. If resistance to more than one antibiotic is observed because of such a single-point mutation, the antibiotics have a common mode of action at a common target, and they may well all be of the same chemical class (i.e., aminoglycosides, beta-lactams, and macrolides). Using the antibiotics as research tools, a large number of workers began to investigate the mechanisms of rapid acquisition of resistance to multiple antibiotics (drugs in general). This work continues, but what has been learned already is indeed revolutionary. We now realize the genetic information in many cells, especially bacteria and lower forms of eukaryotic cells (yeasts, fungi, etc.), is extremely mobile, and there are several distinct ways by which its composition and function can change rapidly and dramatically.

The fact that resistance to chemotherapeutic drugs occurs has been known at least since the time of Ehrlich. The current view of how resistance appears in a large population of microbial cells exposed to an antimicrobial agent is rather simple. If, in that large population of cells, there are a few genotypically resistant cells (possessing constitutive resistance to the drug in question), the ability of those cells to grow in the presence of the antibiotic leads to a new population of progeny that is mostly of the resistant genotype. The question of how the few genotypically resistant cells arise is related to the process of general microbial mutagenesis. With many agents (e.g., radiation and UV light), a more or less spontaneous genetic change takes place in the chromosomal DNA, or a spontaneous chemical change of the DNA occurs because of any of the chemical or physical forces to which a cell is subjected. These mutational changes take place in either the presence or the absence of an antimicrobial agent, and single-point mutations occur (i.e., in *Escherichia coli* at about the rate of one per 10^5 to 10^7 cells per cell division). If a change leads to resistance to an antimicrobial agent, the resistance appears in either one of two ways—directly or indirectly.

Directly

If the change is specifically related to the drug (i.e., if it increases the amount of an enzyme, such as beta-lactamase, which can hydrolyze penicillin), a high level of resistance is suddenly seen.

Indirectly

If the genetic change is only indirectly related to the biochemical action of the antibiotic (i.e., small increases appear several times with the same microbial population), a gradual development of a much greater resistance to the drug is seen. Both types of changes occur with most of the common antibiotics.

Transformation, transduction, or R-Factor (plasmid) transfer

In contrast to the relatively simple single-point mutation of a bacterial chromosome (in which a single nucleotide base is altered), larger pieces of new or foreign DNA are introduced into a microbial cell in several ways. If these code for enzymes that affect antibiotic sensitivity, profound changes in resistance result.

Transformation

Exposure of a microbial cell to DNA isolated from a different species affords the possibility that some of this DNA will enter the viable cell and be incorporated into its chromosomes. The process operates with relatively low efficiency, and the foreign DNA must come from a strain of microorganism with which it has something in common. This mechanism for changing the antibiotic sensitivity of microbial populations is probably not of great clinical importance, although it has been used by research workers to introduce antibiotic resistance markers into laboratory strains of microorganisms often.

Transduction

Phage particles of both gram-positive and gram-negative cells can enter receptive phage-sensitive cells of related microbial strains. The DNA of the infectious phage can be inserted into the bacterial genome and, once there, it replicates with the bacterial DNA. If the phage DNA codes for proteins that confer drug resistance, this action can be, a mechanism by which the infected cell suddenly acquires resistance to an antimicrobial agent. It is an observed fact that such phage DNA simultaneously carries resistance determinants for more than one antibiotic, and this explains the sudden appearance of resistance to two or more antibiotics, often some unrelated to each other in terms of structure or mode of action.

R-factors

The foreign DNA that enters a recipient cell by direct means (transformation) or via a bacteriophage (transduction) is believed to exert its effect on the cell only after being integrated into its chromosomal DNA. For this to occur, there needs to be a considerable degree of similarity (homology) between the exogenous DNA (at least a substantial part of it) and some region in the chromosome of the recipient. The insertion of the homologous portion of the foreign DNA into the chromosome is more or less efficient, depending on the degree of similarity, and the size of the resultant modified chromosome is not much different from what it was before the exchange took place. As dramatic as the effects of chromosome modification by transformation or transduction are (and these also were considered to be revolutionary when first detected), a far more complex set of mechanisms for the introduction and functioning of foreign DNA in recipient cells is now recognized. Following the outbreak of several epidemics of bacterial dysentery in Japan several years ago, it became known that the pattern of antibiotic resistance in the causative *Shigella* species was very similar to that of nonpathogenic *Escherichia coli* strains present in the same patients. Study of these mixed bacterial populations (*E. coli* and *Shigella*) led to a realization that an “infective” process was occurring in which some of the genetic characteristics of the *E. coli* (in this case, measured as the antibiotic resistance determinants) were being transferred to the *Shigella* strains in a rapid and seemingly direct manner. The mechanism was found to be a direct movement of extrachromosomal DNA from the *E. coli* to the *Shigella* organisms. The transfer occurs in a conjugative mode during which a physical mating of the *E. coli* and the *Shigella* cells occurs. A narrow tube, or *pilus*, connects the two mating cells, and the extrachromosomal DNA passes through the tube from the donor to the recipient. The DNA is termed a plasmid, an episome, or, more often, an R-factor (since in the cases studied, the result of the transfer is acquired resistance to some combination of antibiotics). The ability of two cells of different bacterial genera to participate in this conjugative process is related to their sexuality. The whole matter is quite complicated. The terminology sometimes used is for describing F-factors (fertility factors).

The R-factor-mediated transfer of genetic information between two of ten quite different bacterial strains is now recognized as a general phenomenon and has been studied in a large number of cases beyond those first ones reported in Japan. The properties of plasmids are under intense study at present, and only a few generalizations need be made in this unit. The R-factor DNA is usually quite large (10^6 to 10^8 daltons) and consists of covalently linked closed circles of deoxynucleotide bases. These R-factors do not become incorporated into the chromosomal material of the host, and they reproduce as independent extrachromosomal elements. The time of R-factors reproduction is not related to that of chromosomal replication or other events in the cell cycle of the host bacterium. The number of R-plasmids per bacterial cell also is carefully regulated by some unknown mechanism. It is hard to predict the long-term impact of R-factors, but their study is leading to new and surprising concepts about genetic mobility in microorganisms.

The introduction of foreign DNA sequences into the chromosomes of the host (transformation and transduction) requires the deletion of roughly the same number of nucleotide bases as are introduced, and there must be some degree of homology between the foreign and the native DNA sections. With R-factors, however, it is known their nucleotide-base composition is extremely unstable, and their size is subject to great variation as well. The interaction of foreign DNA with R-factors (and likely the interaction of two or more R-factors) can lead to a profound change in the structure of an R-factor itself, by the addition of the foreign DNA, without much, if any, sequence homology required (except possibly for terminal or initiation base sequences and recognition sites). This process is called “illegitimate recombination” and it can lead to the insertion (or removal) of large portions of DNA from the R-factor. In addition, it is recognized that movement of a portion of the DNA can occur from one structural region of the R-factor to another (transposition). The concept of composite R-factors has developed in which the structure of the DNA is viewed as capable of dissociating into functional subunits, which can then recombine in different ratios or combinations.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

029. Biochemical mechanisms of resistance to antimicrobial agents

1. What is meant by a single point mutation?
2. How can a spontaneous chemical change of the DNA occur?
3. In what two ways can the development of resistance to an antibacterial agent by an organism appear?
4. What criteria must be met in order for transformation to take place?

5. What is one explanation for the sudden appearance of resistance to two or more unrelated antibiotics by an organism?
6. What does the expression of foreign DNA acquired via transformation or transduction require?
7. What must exist if foreign DNA is to be integrated into an organism's chromosomal DNA?
8. What two factors determine the efficiency with which foreign DNA is inserted into the bacterial chromosome?
9. What terms have been used to describe the chromosomal DNA that is passed sexually?
10. What term has been used to describe the ability of two cells of different bacterial genera to participate in the exchange of DNA via pilus?
11. Describe the size and reproduction of R-factors.
12. What is meant by illegitimate recombination?
13. What is meant by transposition?
14. What is the concept of composite R-Factors?

5-3. Antimicrobial Susceptibility Testing

As we noted in Unit 1, antimicrobials were first described as demonstrating effectiveness in managing bacterial infections around 1940. Since then, it was recognized that bacterial infections vary in their innate susceptibility to these agents or can develop resistance. A method to accurately and rapidly determine the specific susceptibility of clinically significant

bacteria was needed, particularly in situations where the susceptibility of the bacteria in question had previously demonstrated variable results to the antimicrobials of choice or in cases where treatment failure was apparent.

The following major events in the development of antimicrobial susceptibility testing outlines the advancements made since the 1940s in developing a suitable *in vitro* test.

- The mid-1940s saw the development of protocols using antimicrobial-impregnated filter paper disk.
- During the 1950s, Dr. Kirby and coworkers investigated the use of individual high-content disks. The drug content and the zone diameter breakpoints for defining susceptibility and resistance were individualized for each antimicrobial.
- Bauer, Kirby, Sherris, and Turck published a standardized procedure in 1966. The Food and Drug Administration (FDA) officially recognized this method, which is the basis for the disk diffusion method used in clinical laboratories today.
- Until the 1970s, most laboratories routinely used the disk diffusion method. However, as the MIC system became commercially available, an increasing number of laboratories adopted it as their method of choice for doing susceptibility testing.

For the most part, the information provided by disk diffusion tests or MIC tests is adequate for the physician to treat the patient. However, when a patient has a serious infection, is immunosuppressed, is infected with very resistant or unusual bacterium, or cannot be treated with the drugs of choice, an additional “special” *in vitro* test can be performed to help establish the adequacy of a specific antimicrobial regimen. These tests are discussed at the close of this section.

030. Disk diffusion testing

The procedure described by Bauer and coworkers forms the basis of the method recommended by the FDA and the Clinical and Laboratory Standards Institute (formerly the NCCLS) for disk diffusion testing. In this test, antimicrobial-impregnated filter paper disks are applied onto an inoculated agar surface. As the disk absorbs water from the agar, the antimicrobial dissolves and diffuses into the agar to form a concentration gradient of the drug. Concurrent with this event is the logarithmic growth of the bacterial population.

Disk diffusion

Initially, there is a greater concentration of antimicrobial near the disk; however, the gradient does change. This change is dependent on several factors, including the size and shape of the antimicrobial molecule, the ionic charge of the antimicrobial molecule, the chemical and physical nature of the agar medium, and the temperature of incubation. Antimicrobial diffusion is usually complete in 6 to 8 hours since most of the drug in the disk is depleted by that time. The zone of inhibition is formed when the gradient concentration of antimicrobial is no longer capable of inhibiting bacterial growth.

Zone diameter breakpoints are used to characterize an isolate as susceptible, moderately susceptible, intermediate susceptible, or resistant. These breakpoints are based on an inverse linear relationship between the zone of inhibition and the log MIC. MIC determinations are discussed shortly. For each antimicrobial, a separate set of breakpoints is determined. Approximately 100 to 150 bacteria, with similar growth characteristics and varying susceptibility to the antimicrobial, are examined. Each isolate, which represents a bacterial species that may respond therapeutically to the antimicrobial in question, is subjected to the

standard disk diffusion test and MIC testing. The optimum concentration of antimicrobial to be incorporated into the disk is determined. A scattergram is constructed by plotting the zone diameter of inhibition against the log MIC for each isolate. Based on therapeutically achievable blood levels obtained with frequently used dosage schedules, MIC cutoffs for defining an organism as susceptible or resistant are established. The regression line is examined to determine the corresponding zone diameter breakpoints that can be used to qualitatively define an organism as susceptible, intermediate susceptible, or resistant. Recently, a moderately susceptible category was added.

As outlined in the following table, susceptible implies an infection caused by the test organism usually responds to the usual doses of that antimicrobial. The moderately susceptible category includes isolates that may be inhibited by concentrations of certain antimicrobials (i.e., beta-lactams using high dosages or at body sites where the drugs concentrate to high levels that occurs in urine). Additionally, for serious enterococcal infections, moderately susceptible suggests the need for large doses of penicillin plus an aminoglycoside. The intermediate category provides a “buffer zone” to prevent major interpretive discrepancies from occurring because of uncontrollable technical factors. These isolates are tested by dilution methodologies for more definitive results, if needed. Resistant isolates are not inhibited by drug concentrations achieved in blood or tissue following administration of standard doses of drug.

Susceptible	This implies an infection caused by the strain of test organism may be appropriately treated with the dosage of antimicrobial agent approved for that type of infection and infecting species, unless otherwise contradictory.
Moderately Susceptible	This implies strains that may be inhibited by achievable concentrations of particular antimicrobial agents (i.e., β -lactams), provided higher dosages are used, or in body sites (i.e., urine) where the drugs are physiologically concentrated. For enterococci, aerococci, and nonenterococcal streptococci, the MIC in the “moderately susceptible” category indicates the need for high-dose penicillin or ampicillin, and for serious infection, such as endocarditis, combined therapy with ampicillin, penicillin, or vancomycin, and an aminoglycoside is suggested to obtain bactericidal activity. Because all but rare nonenterococcal streptococci are highly susceptible to penicillin or ampicillin, a “moderately susceptible” result justifies retesting. Enterococcal urinary infections may be treated with ampicillin or penicillin alone.
Intermediate	This implies a “buffer zone” is provided, which should prevent small, uncontrolled technical factors from producing significant discrepancies in interpretations (i.e., species that should have few or no endpoints in this range, or drugs affected by media variation or with tight pharmacotoxicity margins). For drugs with results falling within the range defined as intermediate, the results may be determined equivocally and, if the organism strain is not fully susceptible to alternative clinical suitable drugs, the test should be repeated. (If this is a disk susceptibility procedure, compare with a dilution test.)
Resistant	This implies test organisms are not inhibited by the usually obtainable systemic concentrations of the agent with normal dosage schedules and/or fall in the range where distinct microbial resistance mechanisms are likely (i.e., β -lactamases), and clinical efficacy has not been dependable in treatment studies.

Performance of the disk diffusion test

The disk diffusion procedure involves several steps that must be rigidly controlled. The first step is inoculum preparation. There are two alternative techniques (direct and indirect) for inoculum preparation for the standard disk diffusion or Kirby-Bauer method. Both require cells be picked from the tops of four to five similar colonies. For the standard inoculum preparation method, the inoculum is grown in a specific optical density. The test organisms

are inoculated into 4- to 5-ml broth (such as trypticase soy broth, brain heart infusion, or Muller-Hinton) and incubated until the turbidity compares to that of a 0.5 McFarland standard, which is approximately 10^8 CFU/ml. This usually requires 2 to 8 hours of incubation. If the turbidity exceeds that of the 0.5 McFarland standard, the suspension is diluted. The direct inoculum preparation technique does not require incubation. Fresh cells are inoculated into broth or saline to obtain turbidity equivalent to the 0.5 McFarland standard.

In the second step, the plate is inoculated. For this, a sterile swab is inserted into the inoculum suspension and the excess liquid is expressed from the swab by pressing it against the inside of the tube above the liquid. The surface of the Mueller-Hinton agar medium is inoculated by swabbing over three separate planes. Disks are applied within 15 minutes of inoculation, with the aid of a dispensing apparatus or manually. They must be pressed firmly against the agar surface to ensure even contact. The plates are inverted and incubated within 15 minutes of the application of the disk, at 35°C for 18 to 24 hours in air, not CO₂. Following incubation, the zone diameters of inhibition are measured using a ruler or calipers by holding the plate against a dark background and illuminating the plate with reflected light. An interpretive guideline is used to determine the relationship of the zone diameter to the organism's susceptibility.

Variables

It is critical to control the technical variables when performing *in vitro* susceptibility tests in order to generate reproducible and meaningful results. Those factors critical to performing the disk diffusion test are many. Mueller-Hinton agar is used for all testing with the exception of some fastidious organisms. The agar depth must be approximately 4.0 mm. This corresponds to pouring 60 to 70 ml into a 150 mm diameter Petri plate. If the agar is too thin, there is faster diffusion of antimicrobial that can result in falsely susceptible results. If it is too thick, falsely resistant results can occur. Agar pH must be 7.2 to 7.4. The activity of some antimicrobials is dramatically altered by a shift in pH. Agar plates are not stored longer than one week unless they are wrapped in tightly sealed packages. Extended storage results in dehydration of the media that affects antimicrobial diffusion.

Mueller-Hinton agar was initially selected because it demonstrates good batch-to-batch reproducibility; is low in sulfonamide, trimethoprim, and tetracycline inhibitors; and allows the satisfactory growth of most pathogens. However, some variation in performance has been noted, particularly with select antimicrobial-organism combinations. The major problem has been the varying concentrations of the divalent cations—calcium and magnesium. These dramatically affect the activity of the aminoglycosides against *P. aeruginosa*. Increasing the concentration of Ca⁺⁺ and Mg⁺⁺ decreases the aminoglycoside activity against *P. aeruginosa*. Since tetracycline tends to chelate with Ca⁺⁺ and Mg⁺⁺, its activity is also reduced by the presence of these cations. Another problem relates to the thymidine content of the media. If the Mueller-Hinton agar is too rich in thymidine, the action of trimethoprim and the sulfonamides is antagonized. This results in the appearance of hazy growth within the zone of inhibition. Some lots of Mueller-Hinton agar have been unsatisfactory for detecting the resistance of staphylococci to penicillinase-resistant penicillins. Due to the heteroresistance of these isolates, *in vitro* susceptibility testing has been a continuing dilemma. It is important to examine the zone of inhibition around the oxacillin, methicillin, and nafcillin disks using transmitted light. Any indication of growth represents resistance.

The inoculum suspension must be accurately standardized. Too light an inoculum can result in false susceptible results and too heavy an inoculum can result in false resistant results. A

time lapse of no longer than 15 minutes should occur between standardization of the inoculum and inoculation of the plate. Tests should be incubated 16 to 18 hours at 35°C. Extended incubation can result in bacterial overgrowth as the antimicrobial deteriorates. If the temperature is too low, organisms grow too slowly; yielding larger zones that can result in false susceptible interpretations. Too high of a temperature can fail to pick up resistance to the penicillinase-resistant penicillins (oxacillin, methicillin, and nafcillin) in staphylococci.

The zones of inhibition must be measured accurately. If resistant colonies appear within a zone of inhibition, they should be tested further. Disks must be stored under the appropriate conditions in order to maintain their potency. For long-term storage, they must be kept in an anhydrous environment at temperatures –20°C or lower. It is appropriate to keep a small working supply at 4°C in a desiccated container for up to 1 week. Containers of disks must be allowed to equilibrate to room temperature for 1 to 2 hours prior to use to minimize the amount of condensation that may be distributed onto the disks. Disks cannot be used beyond their stated expiration date. Disks cannot be crowded on a plate; no more than 12 disks are placed on a 150-mm diameter plate. This prevents overlapping of the effects of the diffusion of the antimicrobials.

The procedures described above are not satisfactory for testing anaerobic bacteria or fastidious aerobic bacteria that require supplemental nutrients and/or incubation conditions for growth. The Clinical and Laboratory Standards Institute has described disk diffusion methods for testing *H. influenzae*, *S. pneumoniae*, *Listeria*, and *N. gonorrhoeae*. Additionally, there are supplemental guidelines for testing streptococci, anaerobes, and other fastidious organisms by the disk diffusion method, which include utilization of blood supplemented Mueller-Hinton agar and zone interpretive criteria specific for this group of bacteria.

NOTE: Consult the updated approved standards from the Clinical and Laboratory Standards Institute for the performance standards of antimicrobial susceptibility testing for disk and dilution methods, the interpretation and reporting of results, and for anaerobic susceptibility testing. These standards will review the new and required procedures.

Antimicrobial susceptibility testing of fastidious aerobic bacteria

Since ampicillin-resistant *H. influenzae* was first recognized in the mid-1970s, the incidence has risen to approximately 20 percent. Most of the ampicillin-resistant *H. influenzae* isolates produce a plasmid-mediated beta-lactamase. Several screening tests have been developed to accurately and rapidly detect beta-lactamase produced by *H. influenzae*, *N. gonorrhoeae*, and staphylococci. Since a small number of nonbeta-lactamase producing ampicillin-resistant *H. influenzae* isolates exist, an alternative method for detecting this resistance is needed. A modification of the standard Clinical and Laboratory Standards Institute disk diffusion protocol for testing *H. influenzae* includes inoculum preparation by the direct preparation technique, use of supplemented Mueller-Hinton agar, and application of specific zone interpretative breakpoints for ampicillin. *Haemophilus influenzae* is tested by dilution methods if growth factors are added to Mueller-Hinton broth. Although uncommon, chloramphenicol-resistant *H. influenzae* have been isolated. This resistance is usually related to the production of chloramphenicol acetyltransferase. A rapid method for detecting this enzyme has been developed.

Additional resistant strains

The first penicillinase (a type of beta-lactamase) producing strain of *N. gonorrhoeae* (PPNG) was reported in 1976. In certain geographic areas, significant numbers of these strains have been isolated. The beta-lactamase test has been useful in detecting most penicillin-resistant *N. gonorrhoeae*; however, there are some resistant strains that are nonbeta-lactamase producers. A disk test using the direct inoculum preparation method with specific media, incubation conditions, and zone diameter interpretive breakpoints is included in the Clinical and Laboratory Standards Institute disk diffusion protocol.

In 1977, an outbreak of multiple-resistant *S. pneumoniae* occurred in South Africa. Strains that are relatively resistant (MICs 0.12 to 1.0 µg/ml) and less commonly resistant (MICs 1.0 µg/ml) to penicillin have also been isolated in the United States. Unlike beta-lactamase production in penicillinase-producing *Neisseria gonorrhoeae* (PPNG) and the majority of ampicillin-resistant *H. influenzae* strains, the phenomenon of penicillin resistance in pneumococci is probably a result of impermeability. The standard 10-unit penicillin disk has been somewhat unreliable for detecting penicillin resistance in pneumococci, although most susceptible strains demonstrate zones 35 mm. Penicillin susceptibility is determined by screening with 1µg oxacillin disks. The inoculum is prepared by the direct inoculum technique, and blood supplemented Mueller-Hinton agar and specific oxacillin zone interpretive breakpoints are used.

Modifications

Although many laboratories use modifications of the standard disk diffusion or dilution tests to perform susceptibility test on other fastidious bacteria, such as *Corynebacteria* spp., well-defined methods with clinically correlated data for testing such fastidious aerobic bacteria are lacking. Susceptibility testing of these isolates and reporting of results must be performed with an understanding of the limitations of the procedures.

The E test

The E test (PDM epsilometer; AB Biodisk NA, Piscataway, NJ) is an *in vitro* quantitative method that combines the disk diffusion and the microdilution methods. An inoculum with a turbidity of a 0.5 McFarland standard is inoculated to a Mueller-Hinton agar plate. Dilutions of the same antimicrobial agent are incorporated into a plastic-coated strip; the strip is then placed on top of the plate. The plate is incubated as required by the test organism. The MIC is read directly from a scale (corresponding to the dilution) on the strip at the point where the ellipse of growth inhibition intercepts the strip. Since the strips can be placed on any enrichment media, which is its greatest advantage, this method may be used for fastidious and anaerobic bacteria.

NOTE: Special procedures for antimicrobial testing for all fastidious aerobic and anaerobic organisms are beyond the scope of this CDC. Please check with the latest edition of civilian references for an in-depth study and special procedures. This includes QC requirements and procedures for detecting antibacterial resistance.

031. Microdilution testing

Microdilution testing is a slight variation of the macrodilution technique that uses much smaller volumes of antimicrobial concentrations in sterile plastic microdilutions trays.

Microdilution procedure

Generally, these trays have approximately 96 wells and accommodate 12 antimicrobials, depending on how many dilutions of each antimicrobial are tested. Trays are filled using devices that automatically fill the wells, and then dilute the antimicrobials. More commonly, the antimicrobial dilutions are prepared in large volumes and placed into a multichanneled dispensing device that simultaneously dispenses a standard volume of each antimicrobial (usually 0.1 ml) into each well. Trays prepared in this manner can be frozen, stored (preferably at -60°C or lower) for up to 6 months, and thawed as needed. The inoculum is standardized and diluted so the final concentration of organisms is approximately 10^4 CFU/well or 10^5 CFU/ml. The equipment available for inoculating microdilution trays vary in the volume they pick up and deliver; the inoculum dilution step must consider this. Disposable multi-pronged plastic inoculators are dipped into the standardized and diluted inoculum suspension and then into the MIC tray to inoculate the antimicrobial dilutions. Semi automated devices that sterilize the prongs, pick up the inoculum, and inoculate the test plate are also available and more practical for laboratories that perform a large number of susceptibility tests. A positive and negative growth control well included on each tray and the inoculum suspension is subcultured for a purity check. After 16 to 20 hours of incubation at 35°C in air, the trays are placed on a reading device and the wells are examined. Instruments that automatically examine the growth in microdilution trays and interpret MIC endpoints have been developed. However, this does not eliminate technologist intervention when unusual results occur.

The reproducibility of a dilution test is generally plus or minus one two-fold dilution. Results obtained from microdilution testing generally agree with macrodilution for gram-positive organisms and are frequently one doubling dilution lower than macrodilution for gram-negative organisms. As with the disk diffusion test, standardization of test methodology is critical for obtaining accurate and reproducible results with dilution test methods. Specifically, inoculum density, incubation time, incubation atmosphere and temperature, and preparation and storage of antimicrobial stock solutions and dilutions must all be controlled. The use of an appropriate medium is of great importance. Mueller-Hinton agar is generally used for agar dilution testing and Mueller-Hinton broth is used for broth dilution testing of nonfastidious aerobic bacteria. The pH of these media should be 7.2–7.4.

The problems with disk diffusion testing of aminoglycosides and *P. aeruginosa* are similar when performing dilution tests. It is usually recommended the medium contain physiologic concentrations of Ca^{++} (50 mg/L) and Mg^{++} (25 mg/L). The agar component of Mueller-Hinton agar contributes concentrations of these cations close to the recommended values; however, Mueller-Hinton broth is essentially devoid of these cations and must be supplemented. As with disk testing, media low in thymidine is optimal for testing sulfonamides and trimethoprim.

Dilution testing, particularly broth dilution testing of penicillinase-resistant penicillins (oxacillin, methicillin, and nafcillin) against staphylococci, has been a significant problem. The detection of this type of resistance is improved by the addition of 2 percent NaCl to cation-supplemented Mueller-Hinton broth when testing oxacillin, methicillin, and nafcillin against staphylococci. Oxacillin (methicillin and nafcillin)-resistant *Staphylococcus aureus* infections have been refractory to cephalosporin therapy even though *in vitro* results often suggest susceptibility. When broth is supplemented with 2 percent NaCl, *in vitro* resistance to cephalosporins usually occurs in these isolates. If salt-supplemented media is not used for testing cephalosporins, it is recommended *S. aureus* resistant to oxacillin, methicillin, and

nafticillin be reported as resistant to cephalosporins regardless of the *in vitro* results. Currently, it is questionable whether this should be followed for coagulase-negative staphylococci as well.

Commercial microdilution MIC systems

In the past few years, several commercial companies have developed microdilution MIC systems. Trays are manufactured in a central facility where the antimicrobial dilutions are incorporated in either a frozen or dried state. The frozen trays are manipulated as described previously. The trays containing dried antimicrobials are rehydrated at the time they are inoculated in the clinical laboratory. These dried trays have increased stability since they can be stored at room temperature and have a longer shelf life than frozen trays; however, the inoculation procedure is somewhat more complicated. Usually, a semi automated inoculation device is available with the dried trays. The commercially available trays include separate panels of antimicrobials for testing gram-positive or gram-negative isolates. Some manufacturers provide panels that contain specific drugs and drug concentrations for testing urinary tract isolates, and other panels for testing anaerobic bacteria. Additionally, some manufacturers supply an expanded spectrum drug panel containing antimicrobials that should be reserved for testing very resistant isolates or isolates contributing to unusual infections. One of the limitations of using commercial MIC panels involves the predetermined selection of antimicrobials and concentrations in the panels. However, commercial MIC panels certainly represent one of the most practical methods for performing MIC tests in smaller clinical laboratories.

Interpretation of MICs

One of the primary advantages of MIC results versus qualitative results is it allows clinicians to correlate the *in vitro* MIC with the concentration of antimicrobial attainable following standard dosages at the specific infection site. Generally, the concentration at the site of the infection must be two to eight times the MIC. Investigators have developed several different formats for reporting and interpreting MIC results. The Clinical and Laboratory Standards Institute suggests four interpretive categories. The final interpretation of the MIC must take into consideration the clinical status of the patient, the pharmacokinetics of the drug, and previous experience treating similar infections.

Quality control for both disk diffusion and dilution testing

As with all QC programs, the goal is to be able to monitor the precision and accuracy of the procedures, dilutions, reagents, interpretations, reporting of results, and performance of the individual who does the test. The Clinical and Laboratory Standards Institute recommends ATCC reference strains to be used in both the disk diffusion and dilution or MIC procedures. Interpretation of zones and/or results criteria is also contained in the Clinical and Laboratory Standards Institute documents and is updated frequently; therefore, you should always refer to the most recent standard. Frequency of testing is performed with each new batch of Mueller-Hinton agar plates, and/or with each new lot number of panel trays or reagents used in MIC procedures. QC should also be performed on a daily basis with the appropriate reference strains. This testing may be reduced if satisfactory performance is documented for 30 consecutive days of testing for each combination of drug and reference strain (see Clinical and Laboratory Standards Institute's documents for criterion and documentation).

032. Beta-lactamase testing

Beta-lactamase production by bacteria and the relationship of specific beta-lactamases to specific beta-lactam antimicrobials are issues of great concern for medical personnel and drug manufacturers. Beta-lactamases represent a group of enzymes that cleave the beta-lactam ring portion of the beta-lactam antimicrobials, and/or bind to the beta-lactam in a specific manner to render it inactive.

Beta-lactamases

Earlier, we learned bacteria resist antibiotics in three general ways. Let's review the area concerned with beta-lactamase. The pathogenic agent may possess some biochemical mechanism (enzyme) that acts to reduce or eliminate the toxic potential of the antibiotic. In a mutant, an increased level of enzymatic activity or even a new mechanism for inactivating the drug can develop. Examples are beta-lactamases that cleave penicillins and cephalosporins to inactive components, acylases that acetylate chloramphenicol to yield inactive derivatives, and enzymes that inactivate aminoglycosides by phosphorylation, adenylation, acetylation, and so forth.

There are several types of beta-lactamases that are simply classified as penicillinase or cephalosporinases, depending on their primary substrate affinity. The beta-lactamases produced by staphylococci are generally penicillinase; whereas, those produced by *Bacteriodes* spp. are cephalosporinases. Hence, the optimal laboratory methods for detecting these utilize penicillin and cephalosporin substrates, respectively. More specifically, beta-lactamases are grouped according to molecular size, amino acid composition, immunologic identity, and inhibition of enzyme activity, isoelectric point, and substrate profile. The different beta-lactam antimicrobials have varying stabilities to the different beta-lactamases. One of the most attractive features of new antimicrobials today is increased stability to degradation by beta-lactamases.

Both gram-positive and gram-negative bacteria produce beta-lactamases. Gram-positive beta-lactamases are inducible and generally require exposure to an inducing agent (usually a beta-lactam) for sizable quantities of enzyme to be produced. Gram-negative beta-lactamases are either inducible or constitutive. Constitutive beta-lactamases are synthesized continuously at the same rate in the presence or absence of an inducing agent. In the laboratory, a common method to enhance the detection of staphylococcal beta-lactamase is to grow the organisms in the presence of very low concentrations of an inducing agent, such as oxacillin or cefoxitin, prior to performing the beta-lactamase test.

Beta-lactamase test

Rapid and simple tests are available to test for beta-lactamase production. These tests are clinically most relevant in testing for ampicillin-resistant *H. influenzae* and penicillin-resistant *N. gonorrhoeae*. If the isolate produces beta-lactamase, by definition, it is resistant to penicillin or ampicillin. However, a small number of isolates in each of these categories is resistant to penicillin and ampicillin by another resistance mechanism. These beta-lactamase negative strains must be tested by conventional susceptibility test to confirm their susceptibility. As described earlier, beta-lactamase testing is useful for testing staphylococci and necessary in certain situations when microdilution test methodologies are used.

Basic methods

The three basic beta-lactamase test methods are the acidometric, iodometric, and chromogenic methods.

Acidometric

The acidometric method utilizes citrate-buffered penicillin G plus phenol red at an alkaline pH. A drop of this solution is added to a heavy suspension of organisms. In the presence of beta-lactamase, penicillin is hydrolyzed and the penicilloic acid formed lowers the pH. This results in a color change of the pH indicator from red (negative) to yellow (positive).

Iodometric

Phosphate-buffered penicillin G, soluble starch, and iodine solution are used in the iodometric method. A solution of iodine and penicillin is added to a heavy suspension of organisms. If penicilloic acid is produced, it reacts with iodine. When starch is added, the iodine is not available to bind with the starch. The result is a colorless reaction (positive). If the organisms do not produce beta-lactamase, penicilloic acid is not formed and the iodine combines with the starch to yield a purple reaction (negative).

Chromogenic

A chromogenic cephalosporin, such as nitrocefin, is the substrate for the chromogenic method. A drop of this is added to a heavy suspension of organisms. If the beta-lactam ring of the cephalosporin molecule is hydrolyzed by beta-lactamase, a color change occurs. The color changes from yellow (negative) to red-brown (positive) or purple (negative) to yellow (positive), depending on the chromogen used. The results of beta-lactamase testing of *H. influenzae* and *N. gonorrhoeae* are obtained rapidly, usually in less than 10 minutes. However, detection of staphylococcal beta-lactamase takes considerably longer, up to one hour. Preinduction can expedite this latter reaction.

Commercial kits

Several commercial kits are available for beta-lactamase testing. When using any of these kits or the methods described, known beta-lactamase positive and negative strains must be tested for QC. Not all methods are appropriate for detecting beta-lactamases produced by all groups of organisms. The tests must, therefore, be performed with an understanding of their limitations.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

030. Disk diffusion testing

1. What factors determine antimicrobial diffusion into the agar surrounding a disk?
2. How many hours are required for complete antimicrobial diffusion from a disk?
3. What are MIC cutoffs for defining an organism as susceptible or resistant based on?
4. What does the term “susceptible” imply when applied to a test organism?

5. What organisms are included in the moderately susceptible category?
6. What purpose does the intermediate category serve?
7. What is the first step in the performance of disk diffusion test?
8. What are the two techniques for inoculum preparation?
9. Which McFarland standard is used to compare the turbidity of the inoculum?
10. What are the incubation requirements?
11. What agar is generally used for all disk diffusion tests?
12. What can result if the agar depth is too thin or too thick?
13. What has been a major problem with using Muller-Hinton agar for disk diffusion testing?
14. What effect does the thymidine content of Mueller-Hinton agar have on disk diffusion testing?
15. How should disks be stored to maintain their quality during long-term storage?
16. What type of resistance is demonstrated by ampicillin-resistant *H. influenzae*?
17. What enzyme is produced by chloramphenicol-resistant *H. influenzae*?

18. What factor is probably responsible for penicillin resistance in *S. pneumoniae*?

031. Microdilution testing

1. The trays used in the microdilution procedure contain how many wells and can generally accommodate how many antimicrobials?
2. At what temperature and for how long can pre-prepared trays be kept?
3. What is subcultured for a purity check?
4. What are the incubation requirements for the trays?
5. The standardization of what specific test methodologies are critical for obtaining accurate and reproducible results with microdilution testing?
6. What must be added to Mueller-Hinton broth when broth dilution testing of penicillinase-resistant penicillins, such as oxacillin, is performed?
7. What is an advantage and a disadvantage of dried microdilution trays?
8. What is one limitation of using commercial MIC panels?
9. What is one of the primary advantages of MIC results versus qualitative results?
10. What consideration must be taken into account before the final interpretation of a MIC can be made?

032. Beta-lactamase testing

1. In what two ways do the beta-lactamases inactivate beta-lactam antimicrobials?
2. What kinds of beta-lactamases exist, and how are they classified?
3. What characteristics are used to specifically group beta-lactamases?
4. In general, what is necessary for sizable quantities of beta-lactamases to be produced by gram-positive organisms?
5. What two categories of gram-negative beta-lactamases exist?
6. How may the detection of staphylococcal beta-lactamases be enhanced?
7. Testing for beta-lactamases in what two organisms is the most clinically relevant testing?
8. What are the three basic beta-lactamases test methods?

Answers to Self-Test Questions**026**

1. A chemical substance, derived from or produced by various species of microorganisms, that is capable in minute concentrations of inhibiting growth.
2. Chemically and in their modes of action.
3. *Bacillus*, *Penicillium*, *Cephalosporium*, *Micromonospora*, and *Streptomyces*.
4. A concept that the pathogen must be destroyed or inhibited without injury to the host.
5. Bacteriostatic.
6. (1) Genetic replication of the cell (transcription).
(2) Expression of genetic information translation.
(3) Assembly or function of cell components (cell walls and membranes).
7. Successful chemotherapy requires that the metabolic process to be attacked in the microorganism be as different as possible from that of the animal host.

8. They are the most toxic to the host and their use must be balanced against the degree of danger to the host's life posed by the microorganism.
9. (1) The antibiotic may be unable to reach the potential target site of its action.
(2) The pathogenic agent may possess some biochemical mechanism (enzyme) that acts to reduce or eliminate the toxic potential of the antibiotic.
(3) The pathogenic agent may have evolved biochemically in such a way that the target site for the antibiotic no longer accommodates the drug and no productive (toxic) interaction occurs.
10. (1) Beta-lactamases that cleave penicillins and cephalosporins to inactive components.
(2) Acylases that acetylate chloramphenicol to yield inactive derivatives.
(3) Enzymes that inactivate aminoglycosides by phosphorylation, adenylation, or acetylation.
11. Human cells possess mitochondria.
12. Mitochondria have many of the characteristics of bacteria and can be damaged by antibiotics.
13. Broad spectrum coverage, decreased potential for development of resistance, decreased dosage and minimize potential toxic side effects, increased bactericidal rates, and treatment of infections that fail to respond to one agent.
14. (1) Synergism.
(2) Antagonism.
(3) Additivity.
15. (1) Beta-lactams cause defects in cell-wall structure, thus allowing increased entry of aminoglycosides and increased inhibition of ribosome-dependent protein synthesis.
(2) Inactivation of beta-lactamase producing enzymes by cloxacillin which potentiates the activity of lactamase-sensitive penicillins.
16. A much higher mortality rate due to the fact that the two agents are antagonistic.

027

1. Probably because the biochemistry of transcription in bacteria is not sufficiently different from that in the human host cells to permit a very favorable therapeutic index.
2. *Ansamacrolides*.
3. *Streptomyces mediterranei*; they were not well absorbed and were not very active.
4. Rifampin, for the treatment of *Mycobacterium tuberculosis*.
5. Rifampin specifically inhibits bacterial RNA polymerase.
6. The drug appears to be relatively impermeable to the mitochondrion.
7. DNA replication.
8. Urine.
9. DNA gyrase.
10. Cinoxacin, norfloxacin, ciprofloxacin, enoxacin, ofloxacin, and lomefloxacin
11. Trimethoprim (TMP) -sulfamethoxazole (SMX).
12. They inhibit folate metabolism which ultimately prevents the synthesis of bacterial DNA.
13. Microcrystalline (Furadantin) and macrocrystalline (Macrocrystalline).
14. Gentamicin, sisomicin, and netilmicin.
15. Streptomycin, neomycin, kanamycin, tobramycin, and paromomycin.
16. The difference in their origin.
17. Streptomycin; resistance in bacteria readily develop, and it is selectively toxic to the eighth cranial nerve.
18. Interference with 30S ribosomal unit function and inhibits protein synthesis.

19. All tetracyclines are amphoteric substances that tend to form insoluble complexes with many anions.
20. Variable and relatively poor absorption of the tetracyclines from the gastrointestinal tract.
21. By binding of the antibiotic to the 30S ribosomal subunit which through a complex process prevents bacterial polypeptide synthesis.
22. The acquisition of plasmids.
23. *Streptomyces*.
24. Macrolides are relatively broad-spectrum against gram-positive and some gram-negative bacteria, mycoplasmas, treponemes, rickettsiae, and chlamydiae.
25. Inhibition of RNA-dependent protein synthesis.
26. *Streptomyces venezuelae*.
27. Chloramphenicol.
28. Blood dyscrasias, aplastic anemia, and depression of bone marrow function.
29. Most bacteria, rickettsiae, and chlamydiae.

028

1. *Penicillium* species.
2. Following supplementation of the medium with certain organic acids.
3. Resistance to inactivation by beta-lactamases.
4. Their ability to inhibit different bacterial enzymes or penicillin-binding proteins (PBP), which are essential for peptidoglycan synthesis.
5. *Cephalosporium acremonium*.
6. Aztreonam and Carbapenem.
7. Clavulanic acid, Sulbactam, and Tazobactam.
8. It acts as a “suicide inhibitor” by forming an irreversible acyl enzyme complex with the β -lactamase.
9. Various penicillins and cephalosporins.
10. *Streptomyces orientales*.
11. Inhibit peptidoglycan synthesis in the bacterial cell wall.
12. *Bacillus polymyxa*; interact with the phospholipids of the bacterial cell membrane which increases cell permeability and disrupts osmotic integrity. This process results in leakage of intracellular constituents resulting in cell death.
13. Inhibits dephosphorylation of a lipid pyrophosphate which is a crucial step in cell wall synthesis and it also disrupts the cytoplasmic membrane.
14. (1) Amphotericin B.
(2) Nystatin.
(3) Imidazole.
15. Isonicotinic acid hydrazide (INH).

029

1. A single mutation in the chromosomal DNA of a (bacterial) cell leads with certainty to a single biochemical change in the progeny.
2. They can occur as a result of any of the chemical or physical forces to which a cell is subject.
3. (1) Directly.
(2) Indirectly.

4. Exposure of a microbial cell to foreign DNA isolated from a different species of bacteria with which it has something in common.
5. Transduction may have occurred.
6. Can only express itself after being integrated into the chromosomal DNA of the recipient cell.
7. A considerable degree of similarity (homology) between the exogenous DNA and some region in the chromosome of the recipient.
8. (1) The degree of similarity (homology).
(2) The size of the resultant modified chromosome.
9. Plasmid, episome, or R-Factor.
10. F-factors.
11. From 10^6 to 10^8 daltons; as independent extrachromosomal elements.
12. The interaction of foreign DNA and interaction between R-factors resulting in profound changes in the structure of the R-factor.
13. Movement of a portion of the DNA from one region of an R-factor to another.
14. The structure of the DNA is viewed as capable of dissociating into functional subunits, which can then recombine in different ratios or combinations.

030

1. The size and shape of the antimicrobial molecule, the ionic charge of the molecule, the chemical and physical nature of the agar medium, and the temperature of incubation.
2. 6 to 8 hours.
3. Therapeutically achievable blood levels obtained with frequently used dosage schedules.
4. An infection caused by the test organism usually responds to usual doses of an antimicrobial.
5. Isolates that may be inhibited by achievable concentrations of certain antimicrobials (for example, beta-lactams) using high dosage or at body sites where the drugs concentrate to high levels.
6. It provides a "buffer zone" to prevent major interpretive discrepancies from occurring as a result of uncontrolled technical factors.
7. Inoculum preparation.
8. The direct and indirect.
9. 0.5.
10. Plates are inverted and incubated at 35°C for 18 to 24 hours in air, not CO₂.
11. Mueller-Hinton.
12. If the agar depth is too thin, there is faster diffusion of antimicrobial that can result in false susceptible results. If it is too thick, false resistant results can occur.
13. Varying concentrations of the divalent cations—calcium and magnesium.
14. If the Mueller-Hinton agar is too rich in thymidine, the action of trimethoprim and the sulfonamides is antagonized.
15. For long term storage, they are kept in an anhydrous environment at temperatures of -20°C or lower.
16. Plasmid-mediated beta-lactamase.
17. Chloramphenicol acetyltransferase.
18. Impermeability of the bacterial cell to penicillin.

031

1. 96 well plates; 12.

2. The trays can be frozen, stored (preferably at -60°C or lower for up to 6 months), and thawed as needed.
3. Inoculum suspension.
4. 16 to 20 hours of incubation at 35°C in air.
5. Inoculum density, incubation time, incubation atmosphere and temperature, and preparation and storage of antimicrobial stock solutions and dilutions must be controlled.
6. Two percent NaCl.
7. Dried trays have increased stability since they are stored at room temperature and have a longer shelf life than frozen trays; however, the inoculation procedure is somewhat more complicated.
8. The selection of the antimicrobials to be tested are predetermined by the manufacturer.
9. They allow clinicians to correlate the *in vitro* MIC with the concentration of antimicrobial attainable following standard dosages at the specific site of infection.
10. The clinical status of the patient, the pharmacokinetics of the drug, and previous experience treating similar infections.

032

1. By cleaving the beta-lactam ring portion of the beta-lactam antimicrobial and/or binding to the beta-lactam in a specific manner to render it inactive.
2. Penicillinases and cephalosporinases; by their substrate affinity.
3. Molecular size, amino acid composition, immunologic identity, inhibition of enzyme activity, isoelectric point, and substrate profile.
4. Exposure to an inducing agent.
5. Inducible and constitutive.
6. By growing the organism in the presence of very low concentrations of an inducing agent, such as oxacillin or ceftiofur, prior to performing the beta-lactamase test.
7. *H. influenzae* and *N. gonorrhoeae*.
8. Acidometric, iodometric, and chromogenic.

Do the Unit Review Exercises (URE) before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter. When you have completed all unit review exercises, transfer your answers to AFIADL Form 34, Field Scoring Answer Sheet.

Do not return your answer sheet to AFIADL.

85. (026) Most antibiotics currently used are *not* derived from which genera of organisms?
- a. Bacillus.
 - b. Penicillium.
 - c. Bacteriodes.
 - d. Streptomyces.

86. (026) The concept that the pathogen must be destroyed or inhibited without injury to the host is called selective
- injury.
 - toxicity.
 - destruction.
 - bacteriostasis.
87. (027) What two drugs work together to inhibit folate metabolism that ultimately prevent the synthesis of bacterial deoxyribonucleic acid (DNA)?
- Amoxicillin and clavulanic acid.
 - Sulfonamides and trimethoprim.
 - Ticarcillin and clavulanic acid.
 - Ampicillin and sulbactam.
88. (027) Erythromycin is one of four closely related antibiotics of the macrolide class that are produced by
- Micromonospora* spp.
 - Bacillus licheniformis*.
 - Streptomyces erythraeus*.
 - Cephalosporium acremonium*.
89. (028) A valuable characteristic of cephalosporin C is
- it inhibits folic acid formation.
 - it's resistant to inactivation by beta-lactamases.
 - it prevents bacteria from attaching to ribosomes.
 - it interferes with protein synthesis at the 30S ribosomal subunit.
90. (028) What is *not* a beta-lactamase inhibitor antibiotic?
- Sulbactam.
 - Tazobactam.
 - Carbapenem.
 - Clavulanic acid.
91. (028) What antibiotic is in the glycopeptide group?
- Penicillin.
 - Bacitracin.
 - Polymyxin.
 - Vancomycin.
92. (028) Antifungal macrolides are
- nontoxic to human cells.
 - not bound by cholesterol.
 - chemically quite similar to erythromycin.
 - quite unstable when exposed to ultraviolet (UV) light and mild detergents.
93. (029) If the change is specifically related to the drug (e.g., if it increases the amount of an enzyme, such as beta-lactamase, which can hydrolyze penicillin), a high level of resistance is suddenly seen. This describes
- transduction.
 - transformation.
 - direct resistance.
 - indirect resistance.

94. (029) A narrow tube or pilus is an integral part of what biochemical mechanism that can dramatically affect an organism's resistance to antibiotics?
- R-factors.
 - Transduction.
 - Transformation.
 - Single-point mutation.
95. (030) "This implies that an infection caused by the strain of test organism may be appropriately treated with the dosage of antimicrobial agent approved for that type of infection and infecting species unless otherwise contradictory" is a definition for
- resistant.
 - susceptible
 - intermediate.
 - moderately susceptible.
96. (031) The media used for microdilution susceptibility testing of nonfastidious aerobic bacteria is
- Mueller-Hinton broth.
 - Trypticase soy broth (TSB).
 - Thioglycolate broth (THIO).
 - Brain heart infusion (BHI) broth.
97. (031) The four categories that outline the interpretation of minimal inhibitory concentration (MIC) results are found in the guidance published by the
- Environmental Protection Agency (EPA).
 - Common Procedural Terminology (CPT).
 - American Type Culture Collection (ATCC).
 - Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards or NCCLS).
98. (031) Quality control (QC), with the appropriate reference strains, should be performed for minimal inhibitory concentrations (MIC)
- daily.
 - weekly.
 - bimonthly.
 - monthly.
99. (032) Beta-lactamase is
- a byproduct of lactose fermentation.
 - an enzyme that cleaves penicillins and cephalosporins.
 - an enzyme generated by penicillin-sensitive organisms.
 - the catalyst that activates the receptor site of the penicillin molecule.
100. (032) A positive result in the iodometric test for beta-lactamase is indicated by the color reaction of
- colorless.
 - yellow.
 - purple.
 - red.

Student Notes

WE NEED YOUR FEEDBACK! When you finish this course, please complete the student survey: <http://www.maxwell.af.mil/au/afiadl/>. Click on Student Info and choose 9502 Survey.

Glossary

Terms

Acetyltransferase—Any of a group of enzymes that catalyze the transfer of an acetyl group from one substance to another.

Actinomyces—A genus of microorganisms of the family Actinomycetaceae, order Actinomycetales, made up of three pathogenic species; called also ray fungus.

Actinomycosis—An infectious disease caused by *Actinomyces israelii* in man and by *A. bovis* in cattle.

Acylase—Any enzyme that catalyzes the hydrolysis of acylated amino acids.-adenosine-a nucleotide, adenine-D-ribose derived from nucleic acid.

Adsorption—The attachment of one substance to the surface of another.

Aerobically—Grown in the presence of molecular oxygen.

Aeromonas—A genus of bacteria of the family Pseudomonadaceae, suborder Pseudomonadineae, order Pseudomonadales, occurring as small rod-shaped cells and usually found in water.

Aerophilic—Requiring air for proper growth.

AFB—Acid fast bacilli.

Agranulocytosis—A symptom complex characterized by marked decrease in the number of granulocytes and by lesions of the throat and other mucous membranes

Alginate—A salt of alginic acid, which is extracted from marine kelp.

Alphanaphthol—A crystalline, antiseptic substance from coal tar.

Aminoglycoside—Any of a group of bacterial antibiotics derived from various species of [*Streptomyces*], which interfere with the function of bacterial ribosomes.

Amphoteric—Having opposite characters; capable of acting either as acid or as a base.

Ampicillin—A semisynthetic, acid-resistant penicillin, used as an antibacterial against gram-negative bacteria, such as *Hemophilus influenzae*.

Anaerobically Growing in the absence of molecular oxygen.

Anaerobiosis—Life only in the absence of molecular oxygen.

Anneal—Separate nuclear strands by heating and gradually cooling which causes complementary strands to attach.

Anorectal—Pertaining to the anus and rectum or to the junction region between the two.

Antigenic—Having the properties of an antigen, capable of eliciting an immune response.

Antimicrobial—Killing microorganisms, or suppressing their multiplication or growth.

Antiphagocytic—Counteracting or opposing phagocytosis.

Antisera—Serum that contains antibodies; it may be obtained from an animal that has been immunized either by injection of antigen into the body.

Arginine—An amino acid produced by the hydrolysis or digestion of proteins.

Ascitic—Effusion and accumulation of serous fluid in the abdominal cavity.

Aseptically—Without infection or septic material.

Asporogenous—Not producing spores.

Asymptomatic Showing or causing no symptoms.

Autolysis—The spontaneous disintegration of tissues or of cells by the action of their own togenous enzymes, such as occurs after death.

Bacillary—Pertaining to rod like forms.

Bacitracin—A polypeptide produced by the growth of a gram-positive, spore forming organism belonging to the lichenifomis group of *Bacillus licheniformis*.

Bacteriogenic—Bacterial in origin.

Bacteriophage —A virus that lyses bacteria.

Bacteriostasis—The inhibition of growth, but not the killing of bacteria

Bacteriostatic—Inhibiting the growth and multiplication of bacteria.

Bacteriuria—The presence of bacteria in urine.

Betalysin—A relatively thermostable lysin for gram-positive bacteria.

Biliary—Pertaining to the bile, and bile ducts.

Biochemistry—The chemistry of living organisms and of vital processes.

Bronchopneumonia—A name given to an inflammation of the lungs which usually begins in the terminal bronchioles. These become clogged with a mucopurulent exudate forming consolidated patches in adjacent lobules.

Bronchoscopic—Pertaining to bronchoscopy or to the bronchoscope and instrument for inspecting the interior of the bronchi.

Buccal—Pertaining to or directed at the cheek.

Capnophilic—Growing best in the presence of carbon dioxide; said of bacteria.

Carotenoid—Any member of a group of red hydrogen-peroxide:hydrogen-peroxide oxidoreductase.

Catalase—A crystalline enzyme that specifically catalyzes the decomposition of hydrogen peroxide and that is found in practically all cells except certain anaerobic bacteria.

Catalyst—Any substance that brings about catalysis; also called accelerant.

Catheter—A tubular, flexible, surgical instrument for withdrawing fluids from (or introducing fluids into) a cavity of the body, especially one for introduction into the bladder through the urethra for the withdrawal of urine.

Cellulitis—Inflammation of cellular tissue; especially purulent inflammation of the loose subcutaneous tissue.

Cephalosporin—Any group of broad-spectrum, penicillinase-resistant antibiotics from *Cephalosporin*, including cephalixin, cephradine, cefadroxil, cefaclor, cefuroxime, axetil, cefprozil, loracarbef, cefixime, and cefpodoxime, which share the nucleus 7-aminocephalosporanic acid.

Cerebrospinal Pertaining to the brain and spinal cord.

Cervical—Pertaining to the neck, or to the neck of any organ or structure.

Cervicitis—Inflammation of the cervix uteri; called also trachelitis.

Chemotherapeutic—Pertaining to chemotherapy.

Chemotherapy—The treatment of disease by chemical agents; first applied to use of chemicals that affect the causative organism unfavorably but do not harm the patient.

Chlamydia—Any member of the genus *Chlamydia*.

Chloramphenicol—An antibiotic substance, originally derived from cultures of *Streptomyces venezuelae*, and later produced synthetically; it occurs as fine, white to grayish or yellowish white, needle-like crystals or elongated plaques, and is used as an antibacterial and antitricketsial.

Cholera—An acute infectious disease caused by *Vibrio cholerae* and characterized by severe diarrhea with extreme fluid and electrolyte depletion, and by vomiting, muscle cramps, and prostration.

Chromatography—Method of chemical analysis in which the solution to be analyzed is poured into a vertical glass tube containing an adsorbent (or stationary phase), the different solutes moving through the stationary phase at different velocities according to their degree of attraction to it, and producing bands of color at different levels of the adsorption column.

Chromogen—Any substance that may give origin to a coloring matter.

Chromogenic—Reducing a pigment or coloring matter.

Chromophore—Any chemical group whose presence gives a decided color to a compound and which unites with certain other groups (auxochromes) to form dyes; called also color radical.

Clot—A semisolidified mass, as of blood or lymph; called also coagulum.

Coagulase—An antigenic substance of bacterial origin, produced chiefly by the staphylococci, that may be causally related to thrombus formation.

Coccobacillus—An oval bacterial cell intermediate between the coccus and bacillus forms.

Coliform—Resembling or being *Escherichia coli*.

Colony—A collection or group of bacteria in culture derived from the increase of an isolated single organism or group of organisms.

Colorimetric—A chemical procedure in a reagent reacts with a particular chemical constituent to produce a measurable color.

Colostomy—To provide with an opening, or mouth. The surgical creation of an opening between the colon and the surface of the body; also used to refer to the opening, or stoma, so created.

Commensa—Living on or within another organism, and deriving benefit without injuring or benefiting the other individual.

Concomitant—Accompanying; accessory; joined with another.

Conductivity—The capacity of a body to conduct a current; when expressed in figures conductivity is the reciprocal of resistance.

Conjugation—A blending or the act of joining together.

Conjunctiva—The delicate membrane that lines the eyelids and covers the exposed surface of the sclera.

Constrictive—Causing constriction or having a tendency to constriction.

Counterstain—A stain applied to render the effects of another stain more discernible.

Cuvette—A glass container, generally possessing well-defined characteristics with regard to dimensions (particularly thickness) and optical properties, and generally used to examine colored and colorless solutions free of turbidity, but also used to examine the light scattering of turbid suspensions.

Cystine—An amino acid, produced by the digestion or acid hydrolysis of proteins.

Cystitis—Inflammation of the urinary bladder hypersensitivity, characterized by a large number of mononuclear leukocytes and eosinophils in the bladder mucosa and musculature, and in the urinary sediment.

Cystostomy—The formation of an opening into the bladder .

Cytomegalovirus—One of a group of highly host-specific herpes viruses that infect man, monkeys, or rodents, with the production of unique large cells bearing intranuclear inclusions.

Cytoplasmic—Pertaining to or contained in cytoplasm.

Dalton—A unit of mass, being one-sixteenth of the mass of the oxygen atom.

Dialysated—The material that passes through the membrane in dialysis.

Diluent—An agent that dilutes or renders less potent or irritant.

Diplococcus—A spherical bacterium occurring predominantly in pairs as a consequence of incomplete separation following cell division in a single plane.

Dysuria—Painful or difficult urination.

Endocarditis—Inflammation of the endocardium; a disease generally associated with rheumatic fever, and sometimes with other acute febrile diseases.

Endocervical—Pertaining to the interior of the cervix uteri.

Endocervix—The mucous membrane lining the canal of the cervix uteri.

Endogenous—Growing from within.

Endospore—A spore produced in the hyphae or cell, as in a spherule of *Coccidioides immitis*.

Endotoxin—A heat-stable toxin present in the bacterial cell but not in cell-free filtrates of cultures of intact bacteria.

Enterococcus—Any streptococcus of the human intestine.

Enterocolitis—Inflammation involving both the small intestine and the colon.

Epididymitis—Inflammation of the epididymis.

Epiglottiditis—Inflammation of the epiglottis.

Episome—In bacterial genetics, any accessory extrachromosomal replicating genetic element that can exist either autonomously or integrated with the chromosome, for example, the F factor.

Erythromycin—A broad spectrum macrolide, produced by a strain of *Streptomyces erythreus* and prepared as yellow, odorless crystals or powder; used as an antimicrobial against gram-positive bacteria.

Etiologic—Pertaining to the cause of a disease.

Eukaryote—An organism whose cells have a true nucleus bounded by a nuclear membrane, and exhibit mitosis.

Exogenous—Growing by additions to the outside; developed or originating outside the organism.

Expectoration –The act of coughing up and spitting out materials from the lungs, bronchi, and trachea.

Extracellular–Outside a cell or cells.

Extrapulmonary–Not connected with the lungs.

Exudate–Material, such as fluid, cells, or cellular debris, which has escaped from blood vessels and has been deposited in tissues or on tissue surfaces, usually as a result of inflammation.

Facultative–Not obligatory; pertaining to or characterized by the ability to adjust to particular circumstances or to assume a particular role.

Filamentous–Composed of long threadlike structures; said of bacterial colonies.

Flagellum–A mobile, whiplike process or stout cilium, especially a coiled filamentous appendage, originating in the cell wall or outer layers of cytoplasm of some rod-shaped bacteria, and serving as an organ of locomotion.

Fluorochrome–A fluorescent compound, as a dye, used to mark protein with a fluorescent label.

Fluoroscopy–Examination by means of a fluoroscope.

Genitourinary–Pertaining to the genital and urinary organs; urogenital; urinosexual.

Genome–The complete set of hereditary factors, as contained in the haploid assortment of chromosomes.

Genotypic–Pertaining to or expressive of the genotype.

Gentamicin–An antibiotic elaborated by the fungi of the genus *Micromonospora*, effective against *Pseudomonas* and certain other gram-negative bacilli.

Gingivae–The gums: the mucous membrane, with the supporting fibrous tissue, which overlies the crowns of unerupted teeth and encircles the necks of those that have erupted.

Glomerulus–Tuft or cluster; used in anatomical nomenclature as a general term to designate such a structure.

Glucoside–Glycoside in which the constituent is glucose.

Hemoflagellate–Any flagellate protozoan parasite of the blood.

Hemolysin–Substance which liberates hemoglobin from red blood corpuscles by interrupting their structural integrity.

Hemolysis–The liberation of hemoglobin.

Hepatotoxicity–The quality or property of exerting a destructive or poisonous effect upon liver cells.

Histoplasmosis –Infection resulting from inhalation or, infrequently, the ingestion of spores of *Histoplasma capsulatum*.

Homologous–Corresponding in structure, position, origin, etc.

Humoral–Pertaining to the humors of the body.

Hypertonic–Biological term denoting a solution which when bathing body cells causes a net flow of water across the semipermeable cell membrane out of the cell.

Hypha–One of the filaments or threads composing the mycelium of a fungus.

Hyphal–Pertaining to hypha.

Iatrogenic—Resulting from the activity of physicians.

Ileostomy—Surgical creation of an opening into the ileum, usually by establishing an ileal stoma on the abdominal wall.

Immunodeficiency—Deficiency in immune response, either in that mediated by humoral antibody or in that mediated by immune lymphoid cells.

Immunoglobulins—Protein of animal origin endowed with known antibody activity.

Immunosuppression—The artificial prevention or diminution of the immune response, as by irradiation or by administration of antimetabolites, antilymphocyte serum, or specific antibody.

Impedance—The opposition to the flow of an alternating current, which is the vector sum of ohmic resistance plus additional resistance.

Impermeable—Not permitting passage, as of fluid.

Indole—Compound, obtained from coal tar and indigo, and produced by the decomposition of tryptophan in the intestines.

Inoculum—The substance used in inoculation.

Intracellular—Situated or occurring within a cell or cells.

Iodophor—Compound consisting of iodine combined with a carrier, such as polyvinylpyrrolidine, used as a preoperative skin disinfectant.

Isoelectric—Showing no variation in an electric potential.

Lactam—Cyclic amide formed from aminocarboxylic acids by the elimination of water.

Lactone—An aromatic liquid prepared by distillation from lactic acid.

Lake—To undergo separation of hemoglobin from the erythrocytes, a phenomenon sometimes occurring in blood.

Lavage—The irrigation or washing out of an organ, such as the stomach or bowel.

Lipochrome—Any one of a group of fat-soluble hydrocarbon pigments, such as carotin, xanthophyll, lutein, chromophane, and the natural coloring material of butter, egg yolk, and yellow corn.

Lipophilic—Having an affinity for fat.

Lipoproteins—Combination of a lipid and protein, possessing the general properties of proteins.

Luciferase—An enzyme, of which there are many forms, that catalyzes the bioluminescent reaction in certain animals capable of luminescence.

Luciferin—heterocyclic phenol which can be reduced and oxidized. It is present in certain animals capable of bioluminescence; when acted upon by luciferase, in the presence of ATP and molecular oxygen, it produces light.

Lymphoid—Resembling or pertaining to lymph or tissue of the lymphatic system.

Lymphoma—General term applied to any neoplastic disorder of the lymphoid tissue, including Hodgkin's disease.

Lymphilization—The creation of a stable preparation of a biological substance (blood plasma, serum, etc.) by rapid freezing and dehydration of the frozen product under high vacuum.

Lyse—Of cause or produce disintegration of a compound, substance, or cell.

Meatus—A general term for an opening or passage; a general term for an opening in the body.

Meningeal—Of or pertaining to the meninges.

Meningitis—Inflammation of the meninges.

Meningoencephalitis—Inflammation of the brain and meninges.

Mesophilic—one of moderate temperature; said of bacteria which develop best at temperatures between 20 and 45 degrees C.

Mesosome—An invagination of the cell membrane occurring in certain bacteria.

Metabolite—Any substance produced by metabolism or by a metabolic process.

Metachromatic—containing differently with the same dye; said of tissues in which different elements take on different colors when a certain dye is applied.

Microaerophilic—growing best in only a small amount of atmospheric oxygen.

Microbiota—The microscopic living organisms of a region; the combined microflora and microfauna of a region.

Microfilaria—The prelarval stage of Filarioidea in the blood of man and tissues of the vector.

Microflora—The plant life, visible only under the microscope, which is present in or characteristic of a special location.

Mitochondria—Small spherical to rod-shaped components (organelles) found in the cytoplasm of cells, enclosed in a double membrane, the inner one having infoldings called cristae. They are the principal sites of the generation of energy (in form of ion gradients and adenosine triphosphate [ATP] synthesis) resulting from the oxidation of foodstuffs, and they contain the enzymes of the Krebs and fatty acid cycles and the respiratory pathway. Mitochondria are organelles with genetic continuity and contain an extranuclear source of DNA.

Monochromatic—Existing in or having only one color.

Monoclonal—Derived from a single cell.

Mordant—Substance capable of intensifying or deepening the reaction of a specimen to a stain.

Mucoid—Any one of a group of mucus-like conjugated proteins of animal origin.

Mucolytic—Destroying or dissolving mucus.

Mucopolysaccharide—Group of polysaccharides which contain hexosamine, which may or may not be combined with protein and which, dispersed in water, form many of the mucins.

Mucoprotein—Compound present in all connective and supporting tissues, containing mucopolysaccharides as prosthetic groups; they are relatively resistant to denaturation.

Mucosa—Mucus membrane.

Mutagenesis—The induction of genetic mutation.

Mutant—A sport or variation which breeds true.

Mycotic—Pertaining to mycosis; caused by fungi.

Nasopharynx—The part of the pharynx which lies above the level of the soft palate.

Nebulization—Conversion into a spray.

Necrosis—Death of tissue, usually as individual cells, groups of cells, or in small localized areas.

Nephropathy—Disease of the kidney.

Nephrostomy–The creation of a permanent fistula leading directly into the pelvis of the kidney.

Neutropenia–A decrease in the number of neutrophilic leukocytes in the blood.

Neutrophil–A granular leukocyte having a nucleus with three to five lobes connected by slender threads of chromatin, and cytoplasm containing fine inconspicuous granules; called also polymorphonuclear, polynuclear, or nertrophilic leukocytes.

Nosocomial–Pertaining to or originating in a hospital, as nosocomial disease.

Nuchal –Pertaining to the nucha, or back of the neck.

Oligonucleotide–A polymer made up of a few (2–10) nucleotides.

Oligopeptides–A structure formed by the linkage of a few amino acids.

Ophthalmia–A severe inflammation of the eye or the conjunctiva, or deeper structures of the eye.

Opsonin–An antibody that renders bacteria and other cells susceptible to phagocytosis.

Oropharynx–That division of the pharynx which lies between the soft palate and the upper edge of the epiglottis.

Osmolality–A property of a solution which depends on the concentration of the solute per unit of solvent.

Osteomyelitis–Inflammation of bone caused by a pyogenic organism.

Ototoxicity–The quality of being poisonous to or of exerting a deleterious effect upon the eighth nerve or upon the organs of hearing and balance.

Oxacillin–A semisynthetic penicillin whose sodium salt is used in the treatment of infections due to penicillin-resistant gram-positive organisms.

Oxidase–Any of a class of (metalloprotein) enzymes that catalyze the reduction of molecular oxygen independently of hydrogen peroxide.

Paracentesis–Surgical puncture of cavity for the aspiration of fluid.

Percutaneous–Performed through the skin, as injection of radiopaque material in radiological examination, or the removal of tissue for biopsy accomplished with a needle.

Pericardium–The fibroserous sac that surrounds the heart and the roots of the great vessels.

Pertussis–Whooping cough.

Phagocyte–Any cell that ingests microorganisms or other cells and foreign particles.

Phenolic–Pertaining to or derived from phenol.

Photometer–A device for measuring the intensity of light.

Phylogeny–The complete developmental history of a race or group of animals.

Pilus–One of the filamentous appendages of the skin, consisting of modified epidermal tissue.

Plasmid–A generic term for all types of intracellular inclusions that can be considered as having genetic functions.

Pleomorphic–Occurring in various distinct forms.

Polychromatic–Exhibiting many colors.

Polymicrobial–Characterized by the presence of several species of microorganisms.

Polymorphonuclear—Having cells of many forms.

Polysaccharide—A carbohydrate which on hydrolysis yields more than 10 monosaccharides.

Potentiator—An agent that enhances another agent so that the combined effect is greater than the sum of the effects of each one alone.

Prokaryote—An organism, for example, that does not have a true nucleus, the nuclear membrane being absent and the nuclear material being either scattered in the cytoplasm of the cell, and that reproduces by cell division.

Prophylaxis—The prevention of disease; preventive treatment.

Prostate—A gland in the male which surrounds the neck of the bladder and the urethra.

Prosthesis—An artificial substitute for a missing body part.

Protease—A general term for a proteolytic enzyme.

Proteose—A secondary protein derivative or a mixture of split products formed by a hydrolytic cleavage of the protein molecule more complete than that which occurs with the primary protein derivatives, but not so complete as that which forms amino-acids.

Psychrophilic—Fond of the cold.

Purulent—Consisting of or containing pus; associated with the formation of or caused by pus.

Pyocyanin—A blue-green antibiotic pigment derived from alpha-hydroxyphenazine by methylation.

Pyogenous—Caused by pus.

Pyuria—The presence of pus in the urine.

Racemase—An enzyme that catalyzes the racemization of an optically active substance, such as lactic acid.

Refraction—The deviation of light in passing obliquely from one medium to another of different density.

Rehydration—The restoration of water or of fluid content to a body or substance which has become dehydrated.

Rhinorrhea—The free discharge of a thin nasal mucus.

Rhodamine—Red fluorescent dye.

Rifampin—Semisynthetic antibacterial, derived from rifamycin SV, used in the treatment of pulmonary tuberculosis and carriers of *Neisseria meningitidis*.

Salmonellosis—Infection with certain species of the genus *Salmonella*, usually caused by the ingestion of food containing the organisms or their products and marked by violent diarrhea attended by cramps and tenesmus and/or paratyphoid fever.

Saprophytes—Any organism, such as a bacterium, living upon dead or decaying matter.

Scattergram—Graph in which the values found in a statistical study are represented by separate, unconnected symbols (dots, crosses, circles, etc.).

Septicemia—Systemic disease associated with the presence and persistence of pathogenic microorganisms in the blood.

Serotype—The type of a microorganism as determined by the kinds and combinations of constituent antigens present in the cell.

Serous—Pertaining to or resembling serum.

Sphincter—A ringlike band of muscle fibers that constricts a passage or closes a natural orifice.

Splenectomy—The surgical removal of the spleen.

Subculture—Culture of bacteria derived from another culture.

Subcutaneous—Beneath the skin.

Substrate—A substance upon which an enzyme acts.

Sucrose—A disaccharide, obtained from sugar cane sugar beet, or other sources.

Supernatant—Situated above or on top of something.

Suprapubic—Situated or performed above the pubic arch.

Supravital—Denoting a staining method in which the dye is added to a medium of cells already removed from the living organism.

Synergy—Correlated action or cooperation on the part of two or more structures or drugs.

Synovia—A transparent alkaline viscid fluid, resembling the white of an egg secreted by the synovial membrane, and contained in joint cavities, bursae, and tendon sheaths; called also synovial fluid.

Tabular—Resembling or shaped like a table.

Thermophilic—Growing best at or having a fondness for high temperatures.

Thionin—A dark purple dye, used as a metachromatic stain in microscopy.

Thrombophlebitis—Inflammation of the veins associated with thrombus formation.

Titration—Determination of a given component in solution by addition of a liquid reagent of known strength until a stoichiometric endpoint is reached.

Trachea—The cartilaginous and membranous tube descending from the larynx and branching into the right and left main bronchi.

Tracheostomy—The surgical creation of an opening into the trachea through the neck.

Transcription—The process by which genetic information contained in DNA produced a complementary sequence of bases in an RNA chain.

Tryptophan—An amino acid, existing in proteins, from which it is set free by tryptic digestion; essential for optimal growth in infants and for nitrogen equilibrium in human adults.

Turbidimetric—A procedure in which the turbidity of a test solution is measured.

Umbilicate—Shaped like or resembling the umbilicus.

Umbonate—Knoblike; button-like; having a button-like raised center.

Urease—A colorless, crystalline globulin first extracted by Takeuchi from soy bean. It is also found in mucous urine passed during inflammation of the bladder. It is an enzyme elaborated by various microorganisms, and is capable of causing the change of urea into benzoic acid and glycolic acid.

Urethritis—Inflammation of the urethra.

Vancomycin—An antibiotic produced by a species of soil bacilli, *Streptomyces orientalis*, which is highly effective against gram-positive bacteria and is especially active against staphylococci.

Viable—Capable of living.

Abbreviations and Acronyms

AIDS	Acquired Immune Deficiency Syndrome
AMS	AutoMicrobic System
ANA	anaerobic agar
AnBAP	anaerobic blood agar plate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BBE	bacteriodes bile esculin
BCYE	buffered charcoal yeast extract
BEA	bile esculin agar
BG	brilliant green
BHI	brain heart infusion
BV	bacterial vaginosis
C	cytosine
CAMPY	campylobacter
CAP	chocolate agar plate
CDC	Centers for Disease Control/Center for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	colony-forming units
CIN	cefsulodin-irgasan-novobiocin
CM	cooked meat/chopped meat
CNA	colistin-nalixidic acid
CPT	common procedural terminology
CSF	cerebrospinal fluid
CTA	cystine trypticase agar
DGI	disseminated gonococcal infection
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
EHEC	Enterohemorrhagic <i>E. coli</i>
EIA	enzyme immunoassay
EMB	eosin methylene blue
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FeS	iron sulfide

FITC	fluorochrome fluorescein isothiocyanate
G	guanine
GI	growth index
GN	gram-negative
H₂S	hydrogen sulfide
HE	hektoen enteric
HEPA	high-efficiency particulate air
HIV	human immunodeficiency virus
HPV	human papillomavirus
INH	isonicotinic acid hydrazide
IV	intravenous
KIA	Kligler's iron agar
KVLB	kanamycin-vancomycin laked blood
LAD	Los Alamos Diagnostics
LE	leukocyte esterase
LED	light-emitting diode
MAC	MacConkey (MAC) agar
MIC	minimal inhibitory concentration
ML	Martin-Lewis
MS	mannitol salt
MTF	medical treatment facility
MTM	modified Thayer-Martin
NAD	nicotinamide adenine dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards (as of January 2005, known as Clinical and Laboratory Standards Institute)
NIH	National Institutes of Health
NYC	New York City
OI	operating instruction
PABA	para-aminobenzoic acid
PBNA	polymyxin B and nalidixic acid
PBP	Penicillin-binding proteins
PCR	polymerase Chain Reaction
PEA	phenylethyl alcohol
PHB	poly- β -hydroxybutyrate

PPE	personal protective equipment
Ppm	parts per million
PPNG	penicillinase-producing <i>Neisseria gonorrhoeae</i>
PRAS	prereduced anaerobically sterilized
psi	pounds per square inch
QC	quality control
R	rough
RBC	red blood cell
RIA	radioimmunoassay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	smooth
SBAP	sheep's blood agar plate
SF	standard form
SMX	sulfamethoxazole
SPS	sodium polyanetholsulfonate
SS	<i>Salmonella</i> and <i>Shigella</i>
SSA	streptococcal selective agar
SSN	Social Security number
STD	sexually transmitted disease
T	thymine
TCBS	thiosulfate citrate bile salts
THIO	thioglycollate
TM	Thayer-Martin
TMP	trimethoprim
TSA	trypticase soy agar
TSB	trypticase soy broth
TSI	triple sugar iron
UCA	Uniform Chart of Accounts
USPS	US Postal Service
UV	ultraviolet
WBC	white blood cell
XLD	xylose lysine desoxycholate

Student Notes

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STUDENT FEEDBACK
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ASSESSMENT SURVEY

STUDENT NAME (Optional): _____

DATE: _____

PURPOSE: This survey is designed to obtain definitive and measurable feedback on the CDC volume you have just completed. It will provide us with your assessment of the quality of the training provided, identify areas where we may need to improve, and, with consolidation of the data, provide an overall assessment of how well we are doing in meeting your needs.

INSTRUCTIONS: Request you respond to the following statements. Please circle the appropriate response according to the following scale:

	1	2	3	4	5	6	7
	Strongly Disagree	Disagree	Somewhat Disagree	Not Applicable	Somewhat Agree	Agree	Strongly Agree
1. The information provided in the CDC provided me with knowledge required to perform on the job.	1	2	3	4	5	6	7
2. The information was presented in a logical sequence and easy to read.	1	2	3	4	5	6	7
3. The CDC volume was							
a. well written,	1	2	3	4	5	6	7
b. provided sufficient details and examples,	1	2	3	4	5	6	7
c. emphasized key information, and,	1	2	3	4	5	6	7
d. the information was easily understood.	1	2	3	4	5	6	7
4. Graphics provided consistently supported the text illustrating and/or clarifying principles, techniques, and procedures.	1	2	3	4	5	6	7
5. The format of the volume made information easy to read and reference or locate information.	1	2	3	4	5	6	7
6. Unit Review Exercises:							
a. Questions were relevant to tasks performed in the workplace and knowledge required to perform on the job.	1	2	3	4	5	6	7
b. Questions and responses could be referenced to the text.	1	2	3	4	5	6	7

Additional write-in comments/recommendations: _____

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