Antimicrobial Susceptibility Testing Protocols

Edited by
Richard Schwalbe
Lynn Steele-Moore
Avery C. Goodwin
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Employees of the Department of Health and Human Services who have contributed to the work are not representing the views and opinions of the U.S. government.
This book is dedicated to Dr. Richard Steven Schwalbe, who passed away September 1, 1998, before our textbook was completed. All of the contributors to Antimicrobial Susceptibility Testing Protocols respectfully and lovingly present our work as a token of our admiration to Rick and in the hope that through our efforts we may carry on his passion and love of susceptibility testing and microbiology.

l’chaim my friend...I miss you.
Preface

Recent reports in the lay press describing bacterial resistance to multiple antibiotics serve to emphasize the importance of accurate susceptibility testing. The clinical microbiology laboratory is often a sentinel for detection of drug-resistant microorganisms. Timely notification of susceptibility results to clinicians can result in initiation or alteration of antimicrobial chemotherapy and thus improve patient care. Standardized protocols require continual scrutiny to detect emerging phenotypic resistance patterns.

The aim of Susceptibility Testing Protocols is twofold: one is to present a comprehensive, up-to-date procedural manual that can be used by a wide variety of laboratory workers. The second objective is to delineate the role of the clinical microbiology laboratory in integrated patient care. Many protocols that are presented are an extrapolation of procedures approved by the Clinical and Laboratory Standards Institute (CLSI). New procedures that are (at present) nonstandardized are also described.

The first section of this manual addresses the basic susceptibility disciplines that are already in place in many clinical microbiology laboratories. These include disk diffusion, macro- and microbroth dilution, agar dilution, and the gradient method. Step-by-step protocols are provided. Emphasis is placed on optimizing procedures for detection of resistant microorganisms. A chapter is devoted to automated susceptibility testing, introducing the systems that are currently available for purchase, including recent laboratory evaluations, and presents an algorithm that can be followed by laboratory workers who are considering purchasing a new automated system.

The second section is devoted to descriptions of susceptibility protocols that may be performed by a subset of laboratories, whether as reference centers or as part of a research protocol. Specialized protocols such as surveillance procedures for detection of antibiotic-resistant bacteria, serum bactericidal assays, time-kill curves, population analysis, and synergy testing are discussed. Emphasis in this section is on clear descriptions of methods leading to reproducible results.

The final section of this manual includes a series of chapters designed to be used as reference sources. Additional chapters focus on antibiotic development and design, use of an antibiogram, and the interactions of the clinical microbiology laboratory with ancillary areas such as hospital pharmacy, infectious disease personnel, and infection control. A table of antibiotic classes and common “bug–drug” susceptibilities are also included.

This manual is directed to personnel engaged in the laboratory disciplines that perform in vitro susceptibility testing, including clinical microbiology, food and agriculture microbiology, pharmaceuticals research, and other applied and basic research environments. It is meant to be used as a bench manual. References are supplied at the end of each chapter to provide additional sources of information to those individuals wishing to pursue a specific topic in greater detail.

Antibacterial Susceptibility Testing Protocols differs from other available sources of information by its scope. Its aim is to combine an updated series of laboratory-based techniques and charts within the context of the role of clinical microbiology in modern medicine.

We would like to acknowledge Debbi Reader Covey for her secretarial assistance and unwaivering support. Most of all, we would like to thank Dr. Richard S. Schwalbe for many, many things and for the great person he was.

Lynn Steele-Moore
Avery C. Goodwin, Ph.D.
Editors

Rick Schwalbe was the director of clinical microbiology at the University of Maryland Medical Systems and the director of clinical microbiology and virology at the Veteran’s Administration Medical Center in Baltimore, Maryland. He was associate professor of pathology at the University of Maryland School of Medicine. At the time of his death he was president of the Maryland ASM. He served on the editorial board of *Antimicrobial Agents and Chemotherapy* and had over 100 publications.

Lynn Steele-Moore was the manager of Dr. William J. Holloway’s Infectious Disease Laboratory at Christiana Care Health Services in Wilmington, Delaware, for 22 years. She currently is employed by the U.S. Food and Drug Administration in Silver Spring, Maryland. Lynn and the late Dr. Richard Schwalbe collaborated on many projects, this text being one of them. Unfortunately, Dr. Schwalbe passed away before the text was completed. However, his contributions can be seen throughout the text with chapters written by many of his friends. We all lovingly dedicate this text to our beloved friend, Rick Schwalbe.

Avery C. Goodwin worked at GlaxoSmithKline on the development of antimicrobial drugs. He is currently employed by the U.S. Food and Drug Administration in Silver Spring, Maryland, as a microbiology reviewer in the Division of Anti-Infective and Ophthalmology Products.
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1 An Overview of the Clinical and Laboratory Standards Institute (CLSI) and Its Impact on Antimicrobial Susceptibility Tests

Arthur L. Barry

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1.1 HISTORICAL PERSPECTIVE

In the middle of the twentieth century, medical technologists and clinical pathologists were expected to maintain expertise in all aspects of the service laboratory; most were poorly trained in diagnostic microbiology. With the widespread use of antimicrobial chemotherapy, the nature of infectious diseases gradually changed and that created new challenges to those concerned with the diagnosis and treatment of infectious diseases. Consequently, clinical laboratories were being asked to perform increasingly sophisticated procedures. To help clinical pathology laboratories meet those new challenges, a number of industries were developed to provide supplies and equipment that laboratorians could no longer make or obtain for themselves. Standardization of methodology was essential for such commercial endeavors to be successful. Performance standards were needed in order for each laboratory to judge the quality of different products. Regulatory agencies were also being asked to monitor the quality of products being sold to diagnostic laboratories. Government agencies were being forced to provide standards for judging the quality of different reagents and equipment; for obvious reasons, laboratory professionals wanted to be involved in writing such standards.

In the mid-1960s a group of interested individuals began discussions that led to the concept of an independent organization that could prepare standards that would be acceptable to everyone using them. This became known as the National Committee for Clinical Laboratory Standards (NCCLS), now called the Clinical and Laboratory Standards Institute (CLSI). The name change (effective January 2005) was felt to be a more accurate representation of the organization. Clinical laboratory standards were to be prepared by committees composed of experts from academia,
government, and industry. Once a standard was written and approved by the CLSI council, it was to be published as a proposed standard, and all interested individuals were asked to make written comments or suggestions. After one year of peer review, the subcommittee was asked to respond to all comments and to make appropriate changes or to explain in writing why some suggestions were not accepted. By this process, each standard would be a true consensus document. After the initial review process, each document would be advanced to the status of an approved standard. When important technical changes are needed, the document should be revised and then go through the consensus review process again. Each document is reviewed every three years and either discontinued or revised. In that way, CLSI standards are living documents that are updated as our understanding of the subject improves.

The CLSI was established in 1967–1968, and a small office was established in Los Angeles, California. A part-time secretary helped to solicit individuals who would volunteer to formulate committees that could address specific issues concerning clinical laboratory medicine. Once the first few standards were published and accepted by clinical laboratories, the CLSI support staff was expanded and was eventually moved to Villanova, PA and later to Wayne, PA. CLSI documents are now accepted throughout the world, and regulatory agents often cite CLSI standards as the accepted state of the art.

1.2 DISK DIFFUSION SUSCEPTIBILITY TESTS

The CLSI began by looking for specific areas that would be most benefited by such consensus documents. In the area of microbiology, the antimicrobial disk diffusion susceptibility test was a natural subject. Just before that time, there was a major effort to standardize antimicrobial susceptibility tests on an international level, through the World Health Organization. Sherris and Ericsson coordinated collaborative studies the results of which were published in 1971 [1]. Their extensive labors helped to standardize broth dilution and agar dilution antimicrobial susceptibility tests; microdilution susceptibility tests were not available at that time. Disk diffusion tests had been carefully standardized for use in the Scandinavian countries by Ericsson [2] and for use in the United States by Bauer et al. [3]. With those two methods, many procedural details were carefully controlled and well-defined. A variety of other methods had been advocated, but there was little effort to control important variables such as the inoculum density, agar medium, incubation conditions, etc. The standardized disk tests of Ericsson [2] and of Bauer et al. [3] both involve measuring the diameter of each zone of inhibition and comparing that to minimum inhibitory concentrations (MICs) obtained by agar or broth dilution susceptibility tests.

In the United States, the majority of clinical microbiologists felt that the time required to standardize inoculum densities and to measure zones of inhibition was too difficult for busy clinical laboratories. The most popular method utilized one or more disks for each agent (high- or low-content disks) and simply reported a strain to be susceptible if there was any zone of inhibition and resistant if it grew up to the disk. That was the method that the U.S. Food and Drug Administration (FDA) approved for inclusion in the package insert that was provided in each package of antibiotic disks [4]. A chaotic situation remained because there was no national effort to bring some degree of standardization to the disk diffusion susceptibility test procedure.

In 1968, I was given the honor to chair a CLSI subcommittee on antimicrobial disk susceptibility tests. After interviewing a number of opinion leaders, those that had the foresight to understand the need for standard methodologies were appointed to the subcommittee and serious discussions of methodologic details pursued. In principle, the method of Bauer et al. [3] was accepted by the subcommittee, but a few minor changes were added. Four years later, a document was prepared and given the designation of M2, the second standard written for the Microbiology Area Committee. That document defined the Kirby-Bauer method and the agar overlay modification of that method [5]. Quality control guidelines were also included even though quality control was unknown in clinical microbiology laboratories at that point in time. The M2 document was forwarded to the
CLSI council for review, but it was not approved until 1975. In the interim, the FDA conducted its own survey and concluded that the methods of Bauer et al. [3] and of Barry, Garcia, and Thrupp [5] were preferable, and the package inserts for antibiotic disks were rewritten [4]. With the changes in the FDA’s recommendations and publication of the M2 document, CLSI standards were widely accepted within the United States and in many other countries. The M2 document has undergone numerous revisions: it is now in its ninth edition [6].

1.3 OTHER CLSI MICROBIOLOGY DOCUMENTS

Once the M2 document was accepted, other subcommittees were established in order to address additional issues concerning antimicrobial susceptibility tests. Table 1.1 describes the standards that have been developed over the years.

H. Frankel and A. Barry cochaired a subcommittee that provided a reference that manufacturers of dehydrated media could use to help standardize Mueller-Hinton agars (CLSI M6). That ongoing subcommittee has successfully improved the performance of Mueller-Hinton agar sold in the United States.

C. Thornsberry chaired a subcommittee to standardize agar and broth dilution tests of aerobic microorganisms [7]. Broth microdilution methods were being popularized at that time, and the subcommittee was able to standardize that procedure before it was widely used and before inappropriate procedures could be well engrained. Consequently, manufacturers of microdilution trays were given specific standards that their product should meet, and there have been very few problems with commercial panels that were subsequently marketed. This document, M7, is now in its seventh edition.

<table>
<thead>
<tr>
<th>CLSI Designation</th>
<th>General Subject Covered</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2</td>
<td>Disk diffusion tests</td>
</tr>
<tr>
<td>M6</td>
<td>Dehydrated Mueller-Hinton agar</td>
</tr>
<tr>
<td>M7</td>
<td>Broth agar dilution tests—aerobes</td>
</tr>
<tr>
<td>M11</td>
<td>Anaerobic dilution tests</td>
</tr>
<tr>
<td>M21</td>
<td>Serum bactericidal test</td>
</tr>
<tr>
<td>M23</td>
<td>Guidelines for new drug reviews</td>
</tr>
<tr>
<td>M24</td>
<td>Mycobacteria and nocardia</td>
</tr>
<tr>
<td>M26</td>
<td>Bactericidal activity</td>
</tr>
<tr>
<td>M27</td>
<td>Antifungal tests of yeasts</td>
</tr>
<tr>
<td>M31</td>
<td>Veterinary drugs and pathogens</td>
</tr>
<tr>
<td>M32</td>
<td>Mueller-Hinton broth</td>
</tr>
<tr>
<td>M33</td>
<td>Antiviral agents</td>
</tr>
<tr>
<td>M37</td>
<td>M23 for veterinary agents</td>
</tr>
<tr>
<td>M38</td>
<td>Antifungal tests of filamentous fungi</td>
</tr>
<tr>
<td>M39</td>
<td>Cumulative susceptibility reports</td>
</tr>
<tr>
<td>M42</td>
<td>Disk diffusion—aquatic animals</td>
</tr>
<tr>
<td>M44</td>
<td>Disk diffusion—yeasts</td>
</tr>
<tr>
<td>M45</td>
<td>Dilution and disk—fastidious bacteria</td>
</tr>
<tr>
<td>M49</td>
<td>Broth dilution—aquatic animals</td>
</tr>
<tr>
<td>M100</td>
<td>Informational supplements for M2 and M7</td>
</tr>
</tbody>
</table>

* Each document is assigned a letter (M for microbiology) and a sequential number; this may be followed by a P (proposed), T (tentative), A (approved) or R (report) and by the number of editions, if more than one. Refer to CLSI website (www.clsi.org) for an updated list each year.
V. Sutter chaired a subcommittee that did the same thing for tests of anaerobic bacteria. Initially, that subcommittee limited their document to agar dilution tests of nonfastidious anaerobes. They defined a reference test that was to be used for evaluating other test procedures and anticipated modifications that would permit testing more fastidious species of anaerobes. That document, M11, has now been expanded and has undergone seven revisions [8].

C. Stratton developed the first draft of two documents; one dealt with methods for measuring the bactericidal activity of antibacterial agents and the other concerned the serum bactericidal tests. J. Jorgensen later brought those documents to the status of approved standards (M21). J. Watts developed two documents that deal with issues that are specific for veterinary practice. G. Woods guided the development of a document that defined procedures for susceptibility tests of Mycobacterium and related species. J. Galgiani and M. Pfaller have chaired a subcommittee that addressed specific reference methods for testing antifungal agents against yeasts (M27) and against filamentous fungi (M38). R. Hodinka chaired a subcommittee that dealt with methods for testing antiviral agents (herpes simplex virus). The reader is referred to Table 1.1 for document numbers.

The subcommittee for disk diffusion susceptibility tests has now been given responsibility for the documents that dealt with aerobic and anaerobic antibacterial dilution tests of human pathogens. J. Jorgensen and M. Ferraro chaired this subcommittee while it was learning to handle its new responsibilities. W. Novick coordinated the preparation of guidelines that defined the type of information that should be provided by drug manufacturers when applying for inclusion in CLSI tables [9]. The original subcommittee was given responsibility for maintenance of four documents (M2, M7, M11, and M23) since these involve evaluation of the same type of data for selecting interpretive criteria and quality control ranges for each new antibacterial agent. In 1964, Bauer et al. [3] provided interpretive criteria for tests of 20 antimicrobial agents, and 42 years later, the 2006 CLSI document describes criteria for over 90 agents. With this increase in the number of antimicrobial agents, the tables became complex and confusing. J. Hindler and J. Swenson have spearheaded a major effort to make the tables significantly more user-friendly, and their efforts resulted in an important improvement. Because new agents may be added to the tables once or twice a year, revision of the documents every three years is not sufficient. In an attempt to maintain an up-to-date document, an informational supplement is published once a year in January [10]. This supplement contains only the most recent version of the tables, without the text of each standard. Consequently, it can be published without the usual delays required by the peer review process. All new entries in the tables are identified and considered tentative for the first year. If written complaints are received in the first year, the subcommittee will reconsider the issue causing concern.

Although the Microbiology Area Committee has concentrated on issues concerning antimicrobial susceptibility tests, other documents have been prepared and are currently available. Subjects that have been addressed are as follows: blood-borne parasitic diseases, intestinal parasites, quality control of commercial media, fetal bovine serum, protection of laboratory workers from infectious agents, Western blot assay for B. burgdorferi, and abbreviated identification of bacteria and yeasts. New documents are in process, including one that addresses PFGE and other methods for bacterial strain typing (see Chapter 15). In all cases, the documents are reviewed on a regular schedule and either withdrawn or revised and updated. Area committees other than microbiology have also been productive in the number of documents that have been published.

1.4 QUALITY CONTROL LIMITS

The most important contribution that was made by these efforts was the selection of quality control (QC) strains and the definition of expected MICs and/or zone diameters for each new antimicrobial agent. The control strains that are currently utilized for different purposes are described in Table 1.2. With these QC strains, laboratories have a way to know whether they are performing the tests appropriately and to monitor the tests that are done on a regular basis. The reader is advised to refer to the most current CLSI documents for an up-to-date list as changes do occur.
Every time a new antimicrobial agent is added to the tables, appropriate multilaboratory collaborative studies must be performed, and the acceptable range of zone diameters or range of MICs is then defined for each QC strain. In general, this range should include at least 95% of all test results, i.e., 1 out of 20 determinations might be just outside of the QC range. If a single determination is out of control by random chance, it should come back into the QC range when repeated. If it remains out of control, some troubleshooting is needed in order to find the problem.

### 1.5 ROLE OF THE U.S. FOOD AND DRUG ADMINISTRATION (FDA)

As directed by Congress, the FDA has legal responsibility for determining safety and efficacy of new anti-infective agents. When a new drug is released for sale in the U.S., the FDA-approved package insert includes specific guidelines for interpretation of *in vitro* tests and quality control limits for tests performed according to CLSI procedures. The CLSI subcommittee also defines interpretive criteria and QC limits for their tests. Because the two groups actually examine slightly different data, it is not surprising that there are occasional discrepancies between the two. Major efforts are being made to avoid unintended differences, but some minor discrepancies are unavoidable.

The devices division of the FDA also certifies commercial products and monitors the reliability of each. The CLSI does not approve or disapprove any commercial product; they only standardize methodology.

The CLSI defines methodologies that can survive the consensus process; they have no regulatory responsibility, but CLSI documents are often cited by agencies that do have such responsibilities.
Many laboratorians feel that they can test only those antimicrobial agents that have interpretive criteria and QC ranges defined in the CLSI tables. Testing and reporting agents that do not have interpretive criteria are the responsibility of the chief microbiologist and such decisions should be made with input from the infectious disease clinicians (see Chapter 17). Clearly, the CLSI has resolved the chaotic situation that existed in the late 1960s, and ongoing activities promise to maintain up-to-date information when it is needed. The area committee for microbiology regularly considers proposals for new projects that may have a substantial impact on the practice of clinical microbiology within the United States.

REFERENCES

Antimicrobial Classifications: 
Drugs for Bugs 

Cassandra B. Calderón and Beulah Perdue Sabundayo 

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2.1 INTRODUCTION

Physicians have used drugs for decades to treat infections. However, chemotherapy as a science began with Paul Ehrlich in the late 1800s. Dr. Ehrlich was a German medical scientist who received the Nobel Prize for Physiology of Medicine in 1908. He realized that like human and animal cells, certain bacteria cells colored with certain dyes while others did not. He postulated that it might be possible to make certain dyes, or chemicals, that would kill bacteria while not harming the host organism. He conducted hundreds to thousands of experiments testing dyes against various microorganisms. It wasn’t until his 606th experimental compound that he discovered a medically useful drug. This compound, later named salvarsan, was arsenic based, and the first treatment for syphilis [1]. In 1889, Vuillemin, a French bacteriologist, suggested using the word antiobiosis, meaning “against life,” to describe the group of drugs that had action against microorganisms [2]. Selman Waksman, an American microbiologist and the discoverer of streptomycin, later changed this term to antibiotic [3]. Many antibiotics have been discovered since then (Table 2.1), but the discovery of penicillin may be one of the most important events in the practice of infectious disease medicine. To date, the U.S. Food and Drug Administration (FDA) has approved 18 antibiotics derived from penicillin and 25 classified as cephalosporins [4,5].

Traditionally, antimicrobial agents have been classified based on their mechanism of action, chemical structure, or spectrum of activity. The primary mode of action is the inhibition of vital steps in the growth or function of the microorganism. These steps include inhibiting bacterial or fungal cell wall synthesis, inhibiting protein synthesis, inhibiting nucleic acid synthesis, or disrupting cell membrane function (Table 2.2) [6].

Antibiotics are often described as “bacteriostatic” or “bactericidal” (Table 2.2). The term bacteriostatic describes a drug that temporarily inhibits the growth of the organism. Once the drug is removed, the organism will resume growth. The term bactericidal describes a drug that causes cell death. For infections that cannot be eradicated by host mechanisms (e.g., endocarditis) or for patients who are immunocompromised (e.g., Acquired Immunodeficiency Syndrome), a bactericidal drug is often required [7].

2.2 ANTIBIOTICS

2.2.1 PENICILLINS

2.2.1.1 Background

Penicillin was first discovered in 1928 by a young Scottish bacteriologist, Alexander Fleming, while studying staphylococcal bacteria at St. Mary’s Hospital in London [8–10]. Unlike his
### TABLE 2.1
Origin of Antibiotic Classes

<table>
<thead>
<tr>
<th>Antibiotic Class</th>
<th>Antibiotic(s)</th>
<th>Origin</th>
<th>Discoverer(s)</th>
<th>Year of Discovery</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prontosil</td>
<td>An azo dye</td>
<td>Gerhard Domagk</td>
<td>1932</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulfanilamide (active component of prontosil)</td>
<td>An azo dye</td>
<td>Trefoul, Nitti, and Bovet</td>
<td>1935</td>
<td></td>
</tr>
<tr>
<td>Penicillins</td>
<td>Penicillin</td>
<td>Penicillium notatum</td>
<td>Alexander Fleming</td>
<td>1928</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Howard Florey and Ernest Chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Therapeutic usefulness recorded in 1940</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Gentamicin</td>
<td>Micromonospora</td>
<td>Weinstein</td>
<td>1963</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Chloramphenicol</td>
<td>Streptomycy venezuelae from soil samples collected in Venezuela</td>
<td>John Ehrlich, Q.R. Bartz, R.M. Smith, D.A. Joslyn, and P.R. Burkholder</td>
<td>Published in 1947</td>
<td></td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Cephalothin</td>
<td>Cephalosporium acriomum from water samples obtained off the Sardinian coast</td>
<td>Giuseppe Brotzu</td>
<td>1948</td>
<td></td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Chlortetracycline</td>
<td>Streptomycy aureofaciens from soil samples collected in Missouri</td>
<td>Benjamin Duggar</td>
<td>1948</td>
<td></td>
</tr>
</tbody>
</table>

associate, who kept a clean and uncluttered laboratory workbench, Fleming was a very untidy person. After returning from a short vacation, Dr. Fleming proceeded to review the petri dishes that he had inoculated. That September day, he noticed something unusual. Some of the culture dishes had been contaminated with a greenish, feathery mold. The contamination wasn’t unusual, but the clearing of staphylococcal colonies around the mold was\[3\]. Alexander Fleming identified the mold as *Penicillium notatum*. He later named his discovery, penicillin, after the mold that produces it\[11\].

Fleming, who may or may not have fully understood the clinical implications of this discovery, was having difficulty extracting the penicillin from the mold in the purity or quantity that would make it useful. He was also having difficulty raising funds to continue his work. Twelve years later, Howard Florey, an Australian pathologist, Ernest Chain, a German biochemist, and others at Oxford University were given funds to study penicillin and its clinical usefulness in treating infections\[3\]. They had performed many tests in tissue cultures and animals but never in human volunteers. On January 17, 1941, a 50-year-old woman with disseminated breast cancer and not long to live, volunteered to undergo toxicity testing. After receiving the injection of penicillin she complained of a musty taste in her mouth and developed a sudden fever and rigor, but no other ill effects\[12\].

It was now time for a therapeutic trial of penicillin. In February of 1941, a 43-year-old Oxford policeman was the first to receive penicillin. He was diagnosed with an overwhelming staphylococcal and streptococcal septicemia including multiple facial and lung abscesses and osteomyelitis of his right humerus. Multiple injections of penicillin were administered intravenously and after 5 days, he was afebrile and eating well. Unfortunately, the supply of penicillin ran out, even though it was recovered in his urine, extracted, purified and readministered. The patient deteriorated and died a month later\[12\].

### TABLE 2.2

*Antibiotic Mechanisms of Action and Description of Activity*

<table>
<thead>
<tr>
<th>Inhibition of Bacterial Cell Wall Synthesis</th>
<th>Inhibition of Nucleic Acid Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins (cidal)*</td>
<td>Fluoroquinolones (cidal)</td>
</tr>
<tr>
<td>Cephalosporins (cidal)</td>
<td>Rifamycins (cidal)</td>
</tr>
<tr>
<td>Carbapenems (cidal)</td>
<td>Sulfonamides (static)</td>
</tr>
<tr>
<td>Monobactams (cidal)</td>
<td>Trimethoprim (static)</td>
</tr>
<tr>
<td>Glycopeptides (cidal)</td>
<td>Cyclic lipopeptides (cidal)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibition of Protein Synthesis</th>
<th>Inhibition of Cell Membrane Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides (cidal)</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>Tetracyclines (static)</td>
<td>Imidazoles (fungistatic)</td>
</tr>
<tr>
<td>Glycylcyclines</td>
<td>Triazoles (fungistatic)</td>
</tr>
<tr>
<td>Chloramphenicol (static)</td>
<td>Echinocandins</td>
</tr>
<tr>
<td>Clindamycin (static)</td>
<td></td>
</tr>
<tr>
<td>Macrolides (static or cidal)</td>
<td></td>
</tr>
<tr>
<td>Ketolides</td>
<td></td>
</tr>
<tr>
<td>Orazolidinones</td>
<td></td>
</tr>
<tr>
<td>Streptogramins (cidal)</td>
<td></td>
</tr>
</tbody>
</table>

* cidal = bactericidal; static = bacteriostatic.

2.2.1.2 Mechanism of Action

All beta-lactam antibiotics, including penicillins and cephalosporins, are inhibitors of bacterial cell wall synthesis. The antibiotic must first bind to the penicillin-binding protein (PBP) located within the cytoplasmic membrane of the cell wall. Once bound, the antibiotic can cause various effects that eventually lead to cell death, but primarily they inhibit the cross-linking of the peptide chains and thereby, prevent the development of normal peptidoglycan structure. All penicillins are bactericidal [11,13].

2.2.2.3 Chemical Structure

There are 56 beta-lactam antibiotics, all of which contain the four-membered beta-lactam ring. The antibacterial activity of these molecules resides in the ring itself, and cleavage of the ring by bacterial beta-lactamases inactivates the compound. All penicillins are derived from the 6-aminopenicillanic acid nucleus, which consists of a five-membered thiazolidine (penam) ring, a four-membered beta-lactam ring, and a side chain. Manipulations in the side chain result in the formation of other penicillins, which differ in their spectrum of activity, beta-lactamase resistance, and pharmacokinetic properties [11,13].

2.2.2.4 Mechanisms of Resistance

Bacteria have three defense mechanisms against beta-lactam antibiotics: destruction of the antibiotic by beta-lactamases, decreased penetration of the antibiotic to reach the PBP, and decreased affinity of the PBP to the antibiotic [13,14]. Some bacteria, such as Staphylococcus species, Haemophilus influenzae, gonococci, and most gram-negative enteric rods produce beta-lactamases (penicillinases). These enzymes can break the beta-lactam ring of the antibiotic, rendering it ineffective [13]. To overcome this resistance, some antibiotics are combined with beta-lactamase inhibitors, such as clavulanic acid (ticarcillin/clavulanate and amoxicillin/clavulanate), sulbactam (ampicillin/sulbactam) or tazobactam (piperacillin/tazobactam), to prevent the destruction of the beta-lactam ring. Other penicillins, such as nafcillin, are resistant to beta-lactamase destruction due to the positioning of their side chain [5].

In order for the penicillins to produce their antibacterial effect, they must first bind to the PBPs. Therefore, anything inhibiting or altering the binding of these antibiotics to their receptors can result in the antibiotic being ineffective. Bacteria differ in their number and types of PBPs. Antibiotics differ in their affinity to bind to the PBP. Also, mutations in the PBPs can cause an organism once sensitive to become resistant [13,14].

2.2.2.5 Classification

The penicillins have been divided into categories based on their spectrum of activity (Table 2.3) [13]. The natural penicillins (penicillin G) were the first agents in the penicillin family to be used clinically to treat infections. Shortly after the introduction of penicillin, the emergence of penicillinase-producing staphylococci caused the natural penicillins to be ineffective for these organisms. This led to the development of the penicillinase-resistant penicillins, also known as the antistaphylococcal penicillins. Methicillin was the first of this group. The addition of a side chain protected the beta-lactam ring by sterically inhibiting the action of the penicillinase. The need for penicillins with extended activity against gram-negative microorganisms prompted further manipulations of the side chains. This led to the development of three new classes of penicillins: the aminopenicillins, carboxypenicillins, and ureidopenicillins [11,13].

2.2.2.6 Antimicrobial Activity and Therapeutic Uses

The natural penicillins have excellent gram-positive activity and until recently were considered the drug of choice for many infections caused by pneumococci, streptococci, meningococci, and
Antimicrobial Classifications

13

non-β-lactamase-producing staphylococci and gonococci. Emerging resistance to these drugs is changing the way these infections are treated [13].

The penicillinase-resistant penicillins, also known, as noted above, as the antistaphylococcal penicillins, have excellent activity against both *Streptococcus* and *Staphylococcus* species (including those strains of penicillinase-producing *Staphylococcus aureus*) but no activity against gram-negative bacteria [4,5,11,13]. They are considered the drugs of choice for the treatment of staphylococcal infections, commonly seen in cellulitis or other skin infections, for example [13].

Aminopenicillins were the first group of penicillin antibiotics to have activity against both gram-positive and gram-negative bacteria. Compared to penicillin G, a natural penicillin, ampicillin has more activity against enterococci, but somewhat less activity against *S. pyogenes, Streptococcus pneumoniae, Neisseria* species, and *Clostridium* species. It also has some activity against gram-negative bacteria such as *Escherichia coli, Proteus mirabilis, Salmonella, Shigella, Listeria*, and non-β-lactamase-producing strains of *H. influenzae* [4,5,11,13]. The emergence of resistant organisms, however, is limiting its use clinically.

Carboxypenicillins have the same antibacterial spectrum of activity as ampicillin, an aminopenicillin, but with greater gram-negative activity. Carbenicillin and ticarcillin are the two drugs in this class. They also have activity against indole-positive *Proteus, Enterobacter, Providencia, Morganella,*

---

**TABLE 2.3 Penicillins Classifications**

<table>
<thead>
<tr>
<th>Classification/Generic Name</th>
<th>Route of Administration</th>
<th>Penicillinase-Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Penicillins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin G</td>
<td>IM, IV, oral</td>
<td>No</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>Oral</td>
<td>No</td>
</tr>
<tr>
<td>Penicillinase-Resistant Penicillins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>Oral</td>
<td>Yes</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>Oral</td>
<td>Yes</td>
</tr>
<tr>
<td>Methicillin</td>
<td>Not available</td>
<td>Yes</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>IM, IV, oral</td>
<td>Yes</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>IM, IV, oral</td>
<td>Yes</td>
</tr>
<tr>
<td>Aminopenicillins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Oral</td>
<td>No</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate</td>
<td>Oral</td>
<td>Yes</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Oral</td>
<td>No</td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>IM, IV</td>
<td>Yes</td>
</tr>
<tr>
<td>Bacampicillin</td>
<td>Oral</td>
<td>No</td>
</tr>
<tr>
<td>Carboxypenicillins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>Oral</td>
<td>No</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>IM, IV</td>
<td>No</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>IM, IV</td>
<td>Yes</td>
</tr>
<tr>
<td>Ureidopenicillins and Piperazine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>IM, IV</td>
<td>No</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>IM, IV</td>
<td>No</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>IM, IV</td>
<td>Yes</td>
</tr>
</tbody>
</table>


* IM = intramuscular; IV = intravenous.
and Pseudomonas aeruginosa. Unlike the aminopenicillin, ampicillin, carbenicillin and ticarcillin are not very active against enterococci [4,5,11,13].

The ureidopenicillins and piperazine penicillins have the broadest spectrum of activity among the penicillins, covering many gram-positive and gram-negative bacteria. The addition of the ureido group to the penicillin structure produces the antibiotics azlocillin and mezlocillin, whereas the addition of the ureido group and a piperazine side chain produces piperacillin. Their coverage is very similar to that of the carboxypenicillins but with enhanced activity against P. aeruginosa [4,5,11,13].

2.2.2.7 Adverse Effect Profile

The major adverse event associated with penicillin use is hypersensitivity reactions, which can range in severity from a mild rash to anaphylactic shock and death. An estimated 1% to 10% of the general population is allergic to penicillins [15]. Although anaphylaxis is more frequent following intravenous therapy, it may also occur with oral use. As with all other orally administered antibiotics, penicillins can cause gastrointestinal disturbances. Symptoms include nausea, vomiting, and diarrhea, and are seen more commonly with ampicillin and amoxicillin than with other penicillins. Hematologic toxicity is rare. Although neutropenia has been documented with all penicillins, it is more common with high doses of penicillin G. Carbenicillin, ticarcillin, and piperacillin have all been associated with thrombocytopenia and/or platelet dysfunction, and are usually reversible upon discontinuation of the medication. Penicillins have caused neurotoxicity, manifested as lethargy, neuromuscular irritability, hallucinations, convulsions, and seizures, when given in large intravenous doses, especially to patients with renal dysfunction. Renal complications, such as interstitial nephritis, are infrequent but are usually associated with high doses of methicillin (Table 2.4) [4,5,13].

2.2.3 CEPHALOSPORINS

2.2.3.1 Background

The success seen with the discovery of penicillin lead to the search for other possible antibiotic-producing microorganisms. In 1948, Giuseppi Brotzu, a professor of bacteriology at the University of Cagliari in Sardinia, was studying water samples from a nearby harbor when he observed a clearing of the water surrounding the sewer outlet and suspected it to contain some antibiotic-producing microorganism. Crude filtrate of this water had antibiotic properties that inhibited the growth of S. aureus in vitro and was successful in treating some patients. The microorganism in the filtrate was later classified as Cephalosporium acremonium, now known as Acremonium chrysogenum [11,16]. Brotzu, unable to complete his work, sent a culture of C. acremonium to Howard Florey at Oxford University. In 1953, after many exhaustive years of study, they discovered the substance produced by the microorganism possessing the antimicrobial properties, known as cephalosporin C [16,17]. This discovery lead to the formation of the class of antibiotics known as cephalosporins, with cephalothin being the first of many [17].

2.2.3.2 Mechanism of Action

Cephalosporins, like other beta-lactam antibiotics, inhibit peptidoglycan cross-linkage and therefore bacterial cell wall synthesis. Like the penicillins, all cephalosporins are bactericidal [5,11,16].

2.2.3.3 Chemical Structure

The 7-aminocephalosporanic acid nucleus of the cephalosporin is very similar to the 6-aminopenicillanic acid nucleus of the penicillin. In addition to the beta-lactam ring, it has a six-membered dihydrothiazine (cephem) ring, and two side chains. The six-membered cephem ring confers a relative
resistance to certain beta-lactamases compared to the penam ring. Unlike the penicillins, manipulation of the cephalosporin’s structure is allowed in two places versus one, resulting in the formation of other antibiotics with different spectrums of activity and pharmacokinetic properties [11,16].

2.2.3.4 Mechanisms of Resistance

Like penicillins, bacterial resistance to cephalosporins can be mediated through three major mechanisms: alterations in PBPs, formation of beta-lactamases (cephalosporinases) that inactivate the drug, or decreased ability of the antibiotics to penetrate the cell wall and reach its PBP [14,16].

2.2.3.5 Classification

Cephalosporins have traditionally been divided into four major groups or “generations” based on their spectrum of activity [4,5,16]. Refer to Table 2.5 for a listing of antibiotics in this class.

2.2.3.6 Antimicrobial Activity and Therapeutic Uses

First-generation cephalosporins are active against the gram-positive cocci, staphylococci, and streptococci, but not enterococci species. Among the gram-negative bacteria, *E. coli*, *Klebsiella pneumoniae*, and *P. mirabilis* are usually susceptible. Even though the first-generation cephalosporins have a broad spectrum of activity and have few side effects, they are rarely the drugs of choice to treat any infection. However, when penicillins are to be avoided, they are commonly prescribed to treat skin and skin-structure infections, streptococcal pharyngitis, and community-acquired pneumonia caused
Antimicrobial Susceptibility Testing Protocols

TABLE 2.5
Cephalosporins Classifications

<table>
<thead>
<tr>
<th>Classification</th>
<th>Generic Name</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Cefazolin</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cephalexin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>Cephapirin</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cephradine</td>
<td>Oral, IM, IV</td>
<td></td>
</tr>
<tr>
<td><strong>Second Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefaclor</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Cefamandole</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>Cefonicid</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cefotetan</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cefprozil</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>IM, IV, oral</td>
<td></td>
</tr>
<tr>
<td>Loracarbef</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td><strong>Third Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefdinir</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Cefixime</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Ceftobuten</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>IM, IV</td>
<td></td>
</tr>
<tr>
<td><strong>Fourth Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefepime</td>
<td>IM, IV</td>
<td></td>
</tr>
</tbody>
</table>


by *S. pneumoniae* [5,16]. Cefazolin remains the drug of choice for prophylaxis in most surgical procedures not involving the bowel [5,16,17].

In general, the second-generation cephalosporins are less active against staphylococci and streptococci compared to the first-generation agents but are more active against selected gram-negative bacilli. *Enterobacter*, *Klebsiella* and indole-positive *Proteus* spp. are usually sensitive, although *P. aeruginosa* is not. Cefoxitin, cefotetan, cefmetazole, and cefamandole all have moderate
activity to *Bacteroides fragilis* and can be useful in mixed infections such as peritonitis or diverticulitis. These agents are also commonly prescribed for surgical prophylaxis, especially intraabdominal procedures [17]. Cefuroxime is active against *H. influenzae, Moraxella catarrhalis,* and *S. pneumoniae* and therefore useful in the treatment of bronchitis and community-acquired pneumonia. First- and second-generation oral cephalosporins, such as cefadroxil, cefaclor, and cefprozil, are commonly prescribed in upper respiratory tract infections, such as otitis media, pharyngitis/tonsillitis, and sinusitis [5,16].

Most third-generation cephalosporins have less staphylococcal and streptococcal activity than the first- or second-generation agents, but possess even better activity against gram-negative bacteria, including *P. aeruginosa* and *Enterobacter* spp. These agents were developed to combat nosocomial, multiresistant, gram-negative bacterial infections. Only cefoperazone and ceftazidime have activity against *P. aeruginosa,* and the latter is commonly prescribed in combination to treat such infections. Ceftazidime is also used in immunocompromised patients to treat fever of unknown etiology. The third- and fourth-generation cephalosporins are the only cephalosporins that penetrate the central nervous system and therefore may be used to treat meningitis, depending on the organism [5,16].

The newest generation of cephalosporins represents an attempt to maintain good activity against gram-positive as well as gram-negative organisms, including *P. aeruginosa,* and is designated as fourth-generation cephalosporins [5]. To date only one antimicrobial agent, cefepime, is considered a fourth-generation cephalosporin.

### 2.2.3.7 Adverse Effect Profile

Oral cephalosporins are generally well tolerated and cause few side effects. Hypersensitivity reactions are the most common side effect and have been reported in less than 5% of patients treated [4]. The most frequently reported side effects to orally administered cephalosporins are nausea, vomiting, and diarrhea. Cephalosporins that contain a methylthiotrazole (MTT) side chain (i.e., cefamandole, cefmetazole, cefoperazone, moxalactam, and cefotetan) have been associated with hypoprothrombinemia and prolonged bleeding [18,19]. These antibiotics are also associated with a disulfiram-like reaction if taken with alcohol or alcohol-containing products (i.e., some cough and cold preparations). This reaction is primarily characterized by rapid onset of flushing, tachycardia, headache, sweating, thirst, nausea, and vomiting (Table 2.6) [18,20].

### 2.2.4 Carbapenems

Imipenem, meropenem, and ertapenem are beta-lactam antibiotics classified as carbapenems. Like all beta-lactam antibiotics, carbapenems inhibit bacterial cell wall synthesis. Their chemical structure is similar to the penicillins, but with a few modifications. The carbapenems have the broadest antimicrobial spectrum of activity of any beta-lactam antibiotics available to date. They have excellent activity against both aerobic and anaerobic gram-positive and gram-negative bacteria. Among the gram-positive organisms, the carbapenems are active against most strains of methicillin-sensitive *S. aureus* (MSSA) and coagulase-negative staphylococci, *Streptococcus* spp., and *E. faecalis*. The two older carbapenems exhibit excellent activity to the majority of gram-negative bacteria, including troublesome nosocomial pathogens such as *P. aeruginosa.* Ertapenem, on the other hand, has excellent activity against most gram-negative pathogens except *P. aeruginosa.* In cases of documented or suspected infections due to *Pseudomonas* spp., ertapenem should not be used. The carbapenems are also active against most strains of clinically significant anaerobes. Due to their broad spectrum of activity, many clinicians think carbapenems should be reserved for the treatment of mixed bacterial infections and the treatment of resistant aerobic gram-negative bacteria that are not susceptible to other beta-lactam antibiotics [5,21]. The most frequent side effects associated with carbapenem administration are phlebitis, nausea, vomiting, diarrhea, and rash [4,5,21]. Seizures occurred in 1%
to 3% of treated patients but occurred more commonly in patients with renal insufficiency or underlying central nervous system disease [21,22].

### 2.2.5 MONOBACTAMS

Aztreonam is a monocyclic beta-lactam antibiotic known as a *monobactam*. Its unique chemical structure is composed solely of the four-membered beta-lactam ring and a side chain. It lacks the five- or six-membered side ring shared by the penicillins and cephalosporins, respectively. Like other beta-lactam antibiotics, aztreonam exerts its bactericidal action by binding to PBPs, disrupting the formation of the peptidoglycan chain and ultimately inhibiting bacterial cell wall synthesis [5,21]. Aztreonam binds primarily to the PBP-3 located on *Enterobacteriaceae, Pseudomonas* spp., and other gram-negative aerobic organisms, but not to the PBPs found on gram-positive bacteria. As a result, aztreonam has a narrow spectrum of activity, limited to gram-negative bacteria [5,11,21]. Aztreonam is well tolerated by most patients and is associated with very few side effects. Patients with a penicillin allergy, as documented through a positive skin test reaction, have received aztreonam with no incidence of anaphylaxis [21,23]. Therefore, due to the lack of cross reactivity, aztreonam is a good alternative for patients who have a serious allergy to penicillin and require treatment. Because it is limited to the treatment of gram-negative infections, it is commonly used in combination with another antimicrobial for empiric therapy [21].
2.2.6 Glycopeptides

2.2.6.1 Background

The discovery of penicillins and cephalosporins was very important for the treatment of many infections, but shortly thereafter, microorganisms began to develop resistance. This resistance resulted in many treatment failures and caused the need for more potent antimicrobials. In 1956, McCormick and associates discovered the organism *Streptomyces orientalis*, now called *Nocardia orientalis*, from soil samples gathered in Indonesia and India. This organism produced an active compound called 05856 that had excellent activity against many gram-positive microorganisms [24]. This compound was later named vancomycin, which was derived from the word *vanishes* [25]. The FDA quickly approved it for clinical use in 1958, because of an increase in penicillin-resistant *S. aureus*. In the 1960s, reports of ototoxicity and nephrotoxicity began to appear in the literature, and with the advent of the less toxic semisynthetic penicillins, vancomycin use began to decline. In the early 1970s, *S. aureus*, now resistant to the semisynthetic penicillin, caused a surge in vancomycin use. Today’s formulations of vancomycin are virtually void of all impurities, as opposed to the earlier formulation, and the incidence of side effects has decreased [26].

Teicoplanin, a glycopeptide structurally related to vancomycin, was derived from *Actinoplanes teichomyceticus*. It is currently not approved for use in the United States but has been used extensively in Europe [27].

2.2.6.2 Mechanism of Action

Vancomycin inhibits cell wall synthesis via a different mechanism than the beta-lactam antibiotics. Its primary mode of action is inhibition of peptidoglycan formation, which is the major structural component of the bacteria cell wall. Vancomycin is bactericidal to most bacteria except enterococci and tolerant strains of staphylococci, which are inhibited but not killed [5,27].

2.2.6.3 Mechanisms of Resistance

Glycopeptide resistance is categorized on the basis of the minimum inhibitory concentration (MIC) of the organism, and inducibility and transferability of resistance to vancomycin and teicoplanin. VanA is the most common type of resistance, and bacterial strains that possess this phenotype are resistant to both vancomycin and teicoplanin. VanB strains have a lower level of resistance to vancomycin and are susceptible to teicoplanin. VanC strains have low-level vancomycin resistance and are susceptible to teicoplanin [5,14,27].

Vancomycin resistance has been transferred to *S. aureus in vitro*, and clinical isolates with intermediate susceptibility have been reported. In May 1996, the first documented infection with vancomycin (glycopeptide) intermediate resistant *S. aureus* (GISA) was diagnosed in a patient in Japan [28]. In July 1997, the first case of GISA was diagnosed in the United States when a patient receiving continuous ambulatory peritoneal dialysis developed peritonitis. In August 1997, the second case of GISA was diagnosed in the United States [29].

2.2.6.4 Antimicrobial Activity and Therapeutic Uses

The antibacterial spectrum and clinical usefulness of vancomycin are limited to the treatment of gram-positive aerobic and anaerobic bacterial infections. Both (MSSA) and methicillin-resistant strains of *S. aureus* (MRSA) and most strains of coagulase-negative staphylococci are highly susceptible to vancomycin. Streptococci, including *viridans* species and penicillin-sensitive and -resistant pneumococci are susceptible. With the increasing incidence of vancomycin-resistant
enterococcus (VRE) seen nationwide, many institutions are reviewing vancomycin prescribing patterns. Recently, the Centers for Disease Control and Prevention (CDC) and the Hospital Infection Control Practices Advisory Committee published guidelines for the appropriate use of vancomycin and situations in which the use of vancomycin should be discouraged (Table 2.7) [30].

Vancomycin is poorly absorbed from the gastrointestinal tract, even when the colon is inflamed, and therefore the oral preparation is used only for the treatment of \textit{C. difficile} colitis [5,27].

### 2.2.6.5 Adverse Effect Profile

The incidence of side effects associated with vancomycin is much less compared with earlier formulations and thought to be due to improved purification techniques. The most frequent adverse effect, the “redman” syndrome, was first described in the 1950s and was associated with a rapid infusion [31]. The syndrome is a nonimmunologic-mediated reaction caused by the release of histamine and is characterized by itching and a flushing of the upper trunk, face, and neck with or without hypotension. This myriad of symptoms may also include chest pain and dyspnea. As a result, it is recommended that vancomycin be infused slowly, over at least an hour in most patients [32]. Otoxicity associated with vancomycin therapy is relatively uncommon. Reports of otoxicity from vancomycin reported in the early 1960s were associated with high serum concentrations ranging from 80 to 100 mg/L; the desired peak is usually 25 to 30 mg/L [27,33]. Some experts have concluded that vancomycin may be ototoxic only when given with other ototoxic agents. There have been many retrospective and prospective studies published in the literature evaluating the incidence of vancomycin-induced nephrotoxicity. Nephrotoxicity associated with vancomycin use has been reported at overall rates of 5% to 17% and as high as 35% when combined with an aminoglycoside [34–36]. This phenomenon is usually reversible, and serum creatinine levels return to baseline following dosage adjustment.

### TABLE 2.7

<table>
<thead>
<tr>
<th>Vancomycin Guidelines for Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appropriate Use of Vancomycin</strong></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Endocarditis prophylaxis as recommended by the American Heart Association</strong></td>
</tr>
<tr>
<td><em><em>Prophylaxis for implantation of prosthetic devices or materials at institutions with high rates of MRSA</em> or MRSE infections</em>*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Situations in Which the Use of Vancomycin Should Be Discouraged</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Routine surgical prophylaxis</strong></td>
</tr>
<tr>
<td><strong>Empiric therapy for febrile neutropenic patients, unless there is evidence of possible gram-positive infection</strong></td>
</tr>
<tr>
<td><strong>Treatment in response to a single blood culture positive for coagulase-negative staphylococci</strong></td>
</tr>
<tr>
<td><strong>Continued empiric use for presumed infection in the absence of positive cultures</strong></td>
</tr>
<tr>
<td><strong>Prophylaxis for infection or colonization of intravascular catheters</strong></td>
</tr>
<tr>
<td><strong>Selective decontamination of the GI tract</strong></td>
</tr>
<tr>
<td><strong>Eradiation of MRSA colonization</strong></td>
</tr>
<tr>
<td><strong>Primary treatment of \textit{C. difficile} colitis</strong></td>
</tr>
<tr>
<td><strong>Routine prophylaxis for very low birth weight infants</strong></td>
</tr>
<tr>
<td><strong>Routine prophylaxis for patients on dialysis</strong></td>
</tr>
<tr>
<td><strong>Treatment chosen for dosing convenience in patients with renal failure</strong></td>
</tr>
<tr>
<td><strong>Topical application or irrigation</strong></td>
</tr>
</tbody>
</table>

* MRSA = methicillin-resistant \textit{S. aureus}; MRSE = methicillin-resistant \textit{S. epidermidis}.
2.2.7 Streptogramins

Quinupristin-dalfopristin was approved in September 1999 by the FDA's accelerated approval process for the treatment of serious or life-threatening infections associated with vancomycin-resistant Enterococcus faecium (VREF) bacteremia. It is the first antibiotic in a new class known as streptogramins to be marketed in the United States. Quinupristin-dalfopristin is a synergistic combination in a 30:70 w/w ratio. This combination has been shown to be bacteriostatic against most strains of E. faecium, including VREF, but has no activity against E. faecalis. It is also active against S. pyogenes and MSSA and therefore also indicated for the treatment of complicated skin and skin-structure infections caused by these microorganisms. The most common side effects are infusion-related reactions (e.g., inflammation, pain, edema) followed by arthralgias, myalgias, and nausea [37].

2.2.8 Cyclic Lipopeptides

2.2.8.1 Background

Daptomycin (Cubicin) is naturally derived from the fermentation process of S. roseosporus. It represents a new class of antibiotics known as cyclic lipopeptides. Daptomycin, formerly LY146032, was first discovered by the Eli Lilly Company in the early 1980s, but concerns about skeletal muscle toxicity lead to the voluntary suspension of clinical trials [38]. Today, it is used at lower doses, which allowed for FDA approval in September 2003.

2.2.8.2 Mechanism of Action

The mechanism of action is not fully understood and is unlike any other antibiotic currently on the market. It inserts its lipid tail into the cytoplasmic membrane of gram-positive bacteria with the aid of calcium. This in turn disrupts the functional integrity of the membrane causing a release of intracellular ions. Cell death occurs as the result of widespread dysfunction, primarily disruption of DNA, RNA, and protein synthesis [38]. Daptomycin is only active against gram-positive bacteria because it is unable to penetrate the outer membrane of gram-negative bacteria.

2.2.8.3 Antimicrobial Activity and Therapeutic Uses

In vitro, daptomycin exhibits rapid concentration-dependent bactericidal activity against a variety of gram-positive pathogens, including MRSA, vancomycin-intermediate susceptible S. aureus, and vancomycin-resistant enterococci, including E. faecalis [39].

Daptomycin was approved by the FDA in September 2003 for the treatment of complicated skin and skin-structure infections caused by susceptible strains of gram-positive organisms, including: S. aureus (including methicillin-resistant strains), S. pyogenes, S. agalactiae, S. dysgalactiae subspecies equisimilis, and Enterococcus faecalis (vancomycin-susceptible strains only) [40]. Daptomycin penetrates poorly into lung tissue and is inactivated by surfactant; therefore, it is not indicated for the treatment of pneumonia.

2.2.8.4 Adverse Effect Profile

Daptomycin is fairly well tolerated when dosed at 4 mg/kg once daily. The most common adverse reactions are gastrointestinal disorders, including constipation, nausea, and diarrhea. But, as discussed previously, earlier studies performed by Eli Lilly showed skeletal muscle toxicity at dosages of 3 or 4 mg/kg twice daily. Two of five subjects receiving the higher dose experienced myalgias and extreme weakness along with elevations in serum creatine phosphokinase levels to >10 times the upper limit of normal [38]. Data suggested these reactions were associated with high trough concentrations and therefore prompted the more recent investigational trials utilizing 4–6 mg/kg once daily. In the more recent Phase 3 complicated skin and skin-structure infections trials, the
incidence of elevated creatine phosphokinase levels occurred in 2.8% of the patients receiving daptomycin compared to 1.8% of the control group. Despite the low incidence of myopathy, the manufacturer recommends that patients receiving daptomycin should be monitored for the development of muscle pain, tenderness, or weakness, particularly of the distal extremities, and creatine phosphokinase levels should be monitored weekly [40].

2.2.9 OXAZOLIDINONES

2.2.9.1 Background

Infections due to *E. faecium* and MRSA have been an increasing problem over the past decade, especially in hospitalized and/or immunocompromised individuals. These organisms are inherently resistant to traditional antibiotics, including vancomycin. More recently, MRSA has made its way into the community setting, and in 1989 the first case of vancomycin-resistant *E. faecium* (VREF) was reported in the United States [41,42]. Linezolid represents a new class of antibiotics known as oxazolidinones and was approved by the FDA in April 2000.

2.2.9.2 Mechanism of Action

Linezolid inhibits bacterial protein synthesis through a mechanism of action different from that of other antibacterial agents; therefore, cross-resistance between linezolid and other classes of antibiotics is unlikely. Linezolid binds to the 50S subunit on the bacterial ribosome and eventually through a variety of steps prevents protein synthesis. Linezolid is considered to be bacteriostatic against enterococci and staphylococci and bactericidal against the majority of streptococci strains.

2.2.9.3 Antimicrobial Activity and Therapeutic Uses

Linezolid is indicated in the treatment of infections caused by VREF, including bacteremia, nosocomial pneumonia caused by methicillin-susceptible or methicillin-resistant *S. aureus* or penicillin-susceptible strains of *S. pneumoniae*, complicated or uncomplicated skin and skin-structure infections caused by *S. pyogenes*, methicillin-susceptible or methicillin-resistant strains of *S. aureus*, and community-acquired pneumonia caused by penicillin-susceptible strains of *S. pneumoniae*.

2.2.9.4 Adverse Effect Profile

The most commonly reported side effects in patients treated with linezolid were diarrhea, headache, nausea, and vomiting [43].

Not associated with its antimicrobial properties, linezolid is a reversible, nonselective inhibitor of monoamine oxidase. Inhibiting this enzyme can result in decreased metabolism of serotonin. Like the antidepressants, patients receiving linezolid should limit or monitor their intake of tyramine-containing food or beverages to prevent the inadvertent rise of serotonin, possibly leading to serotonin syndrome. Such foods include those that undergo protein changes by aging, fermentation, pickling, or smoking to improve flavor, such as aged cheeses, fermented meats, sauerkraut, tap beer, and red wine. Though not seen in clinical trials, linezolid has the potential to interact with serotonergic agents, such as the antidepressants, and selective serotonin surveillance reuptake inhibitors (SSRIs), causing serotonin syndrome. In postmarketing of linezolid, there have been a limited number of case reports of serotonin syndrome when linezolid was used in conjunction with SSRIs.

2.2.10 AMINOGlycosides

2.2.10.1 Background

In 1944, Selman Waksman discovered streptomycin, the first aminoglycoside, from soil samples containing *Streptomyces griseus*. Neomycin, kanamycin, tobramycin, and paromomycin are all naturally
occurring compounds that were isolated from other *Streptomyces* species. Weinstein produced gentamicin in 1963 from *Micromonospora* spp., while amikacin and netilimicin are semisynthetic derivatives. Aminoglycosides derive their names depending on the sources from which they originated: names ending with *mycin* are derived directly or indirectly from *Streptomyces*, whereas those ending in *micin* are from *Micromonospora* spp. [44,45].

### 2.2.10.2 Mechanism of Action

Aminoglycosides are bactericidal against susceptible organisms by irreversibly binding to the bacterial ribosome and inhibiting protein synthesis. In order to achieve this, the aminoglycoside must first penetrate the bacteria cell wall. This process requires active transport and passive diffusion. Passive diffusion of an aminoglycoside can be enhanced by the addition of a cell wall synthesis inhibitor, such as a beta-lactam antibiotic, to the antibiotic regimen. Once inside the nucleus of the cell, the aminoglycoside irreversibly binds to the 30S subunit of the bacterial ribosome, disrupts protein synthesis, and eventually causes cell death through leakage of essential bacterial constituents [5,44,45].

### 2.2.10.3 Mechanisms of Resistance

Three primary mechanisms can result in resistance to the aminoglycosides. First, alterations in active transport or passive diffusion make the drug unable to penetrate the bacterial cell wall and bind to the ribosome. Second, chromosomal mutations result in the drug being unable to bind to the receptor on the 30S subunit of the bacterial ribosome. Third, the microorganism can produce enzymes to inactivate the aminoglycoside [5,14,44,45].

### 2.2.10.4 Antimicrobial Activity and Therapeutic Uses

Gentamicin, tobramycin, and amikacin have traditionally been used as empiric therapy in the treatment of febrile neutropenic patients and those with severe nosocomial infections because of their excellent activity against enteric gram-negative bacilli. Gentamicin is usually considered the aminoglycoside of choice in most institutions because of its low cost, although in institutions with higher rates of resistance, tobramycin or amikacin may be prescribed more frequently. To decrease the likelihood of resistance formation, aminoglycosides are commonly used in combination for the treatment of serious gram-negative infections, especially those caused by *P. aeruginosa*. Even though aminoglycosides have some limited activity against gram-positive bacteria, they are never used alone for this purpose. They are commonly used in combination with a cell-wall active agent such as a beta-lactam or vancomycin to treat enterococcal, streptococcal, and severe staphylococcal infections [5,44,45]. Aminoglycosides, particularly streptomycin, are used in the treatment of tuberculosis. The CDC recommends streptomycin be added to the drug regimen for the treatment of presumed multidrug-resistant tuberculosis unless the likelihood of resistance to isoniazid or rifampin is low [46]. It is also prescribed when a patient has a contraindication to use or toxicity to another agent.

### 2.2.10.5 Dosing

Aminoglycosides have traditionally been administered as an intravenous infusion in divided daily doses every 8–12 h, depending on renal function. Currently, once-daily dosing of aminoglycosides is more frequent. The rationale for this dosing regimen is to enhance the bactericidal activity of aminoglycosides by maximizing the peak concentration/MIC ratio for the infecting organism. By administering an aminoglycoside as a large single daily dose, a clinician can take advantage of its concentration-dependent bacterial killing, time-dependent toxicity, and a prolonged postantibiotic effect. Data from animal models and clinical trials suggest that once-daily dosing is safe and effective, with no increase in adverse reactions and possibly a decrease in nephrotoxicity and ototoxicity compared with conventional dosing [47].
2.2.10.6 Adverse Effect Profile

All aminoglycosides can cause ototoxicity and nephrotoxicity. Ototoxicity is manifested by both auditory (cochlear damage) and vestibular symptoms. Auditory toxicity is irreversible and characterized by hearing loss, primarily of high frequency tones (those not used in conversation). Tinnitus, nausea, vomiting, and loss of balance may occur and are usually signs of vestibular damage [44,45]. Aminoglycosides have also been associated with the development of nephrotoxicity. The exact mechanism is not clear, and the incidence varies greatly. Nephrotoxicity induced by aminoglycoside use is usually mild and reversible. It occurs more frequently in critically ill patients, in the elderly, in dehydrated patients, in patients receiving other nephrotoxic drugs, and in those with elevated trough concentrations and prolonged therapy. Other risk factors associated with nephrotoxicity include preexisting renal dysfunction, shock, liver dysfunction, and obesity [48]. Serum peak and trough concentrations are commonly monitored for more accurate dosage adjustments and individualized patient dosing. All aminoglycosides may produce neuromuscular blockade. However, this is a rare complication, usually resulting from very rapid intravenous administration. Extra care should be taken when administering this drug to patients who have myasthenia gravis or are receiving neuromuscular-blocking agents (Table 2.8) [49]. Investigators from the CDC have reported an endotoxin-like reaction associated with the administration of intravenous gentamicin. Within 3 h. of receiving a dose, 20 patients at a major medical center in California developed severe shaking chills often accompanied by fever, tachycardia, and/or a decrease in systolic blood pressure. The investigators believe this reaction is associated with an endotoxin present in a particular brand of gentamicin. They explained that with traditional dosing, the endotoxin present in the gentamicin solution is administered in two or three divided doses over 24 h, whereas once-daily dosing delivers larger amounts of endotoxin over a shorter period of time, usually 1 hour. Studies are currently in process to determine the extent of these reactions and to identify their etiology [50].

<table>
<thead>
<tr>
<th>TABLE 2.8 Adverse Effects Associated with Aminoglycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypersensitivity</strong></td>
</tr>
<tr>
<td>Anaphylaxis</td>
</tr>
<tr>
<td>Rash</td>
</tr>
<tr>
<td>Urticaria</td>
</tr>
<tr>
<td><strong>Gastrointestinal</strong></td>
</tr>
<tr>
<td>Nausea and vomiting</td>
</tr>
<tr>
<td>Diarrhea</td>
</tr>
<tr>
<td><strong>Hematologic</strong></td>
</tr>
<tr>
<td>Anemia</td>
</tr>
<tr>
<td>Eosinophilia</td>
</tr>
<tr>
<td>Leukopenia</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
</tr>
</tbody>
</table>

2.2.11 TETRACYCLINES

2.2.11.1 Background

Benjamin Duggar discovered the first tetracycline, chlortetracycline (Aureomycin®), in 1948 while studying soil samples for antimicrobial properties. These soil samples, collected in Missouri, contained Streptomyces aureofaciens. Duggar named the organism and the antibiotic after the Latin word for gold (aurum) because of the golden yellow pigment it produced [51]. The second compound, oxytetracycline (Terramycin®), was isolated from Streptomyces rimosus in 1950. These two compounds were so similar in structure and clinical importance that they collectively called them tetracyclines (tetra, Latin for four, and cyclos, Latin for rings) [2]. Tetracycline was produced as the result of the catalytic dehalogenation of chlortetracycline in 1953 [52].

2.2.11.2 Mechanism of Action

Tetracyclines reversibly bind to the 30S ribosomal subunit, thereby inhibiting protein synthesis, which is responsible for their bacteriostatic action [5,52,53].

2.2.11.3 Mechanisms of Resistance

Once a microorganism develops resistance to one tetracycline, it generally confers resistance to the entire class of drugs. Resistance occurs more commonly because of chromosomal mutations in the outer membrane of the organisms resulting in a decreased penetration of tetracycline into the cell. Resistance can also occur due to an energy-dependent pumping of the drug out of the cell. Another mechanism, although poorly elucidated, is the biologic or chemical inactivation of tetracyclines by resistant bacteria. Bacteria can produce proteins that interact with the ribosome so that protein synthesis may continue despite the presence of tetracycline within the cell [5,14,52,53].

2.2.11.4 Classification

Tetracyclines are classified based upon their duration of action (Table 2.9) [5].

2.2.11.5 Antimicrobial Activity and Therapeutic Uses

Because of their broad spectrum, tetracyclines are used for a wide variety of infections. They are particularly useful in the treatment of Rocky Mountain spotted fever and are considered the drug of choice for the treatment of early Lyme disease [54]. Tetracyclines are very effective in the treatment of many sexually transmitted diseases. The CDC along with the Public Heath Service recommend doxycycline as the drug of choice for the treatment of lymphogranuloma venereum; nongonococcal urethritis; uncomplicated urethral, endocervical, or rectal C. trachomatis infections; pelvic inflammatory disease (with cefoxitin or cefotetan); and epididymitis (with ceftriaxone). Doxycycline may also be used as an alternative for nonpregnant patients with syphilis who report an allergy to penicillin [55]. They are often prescribed as alternative therapy when the drug of choice in not feasible (e.g., allergy to the beta-lactams, sulfas, or quinolones).

2.2.11.6 Adverse Effect Profile

The most common side effect associated with tetracyclines is gastrointestinal related and include epigastric burning, abdominal discomfort, nausea, vomiting, and anorexia. Food may alleviate these symptoms but may also decrease the absorption by up to 50%. Doxycycline may be taken with food with no alterations in absorption. Tetracyclines can cause alterations in the normal bowel flora, causing large bulky stools or diarrhea [56]. Photosensitivity reactions can range from a red rash to blisters on sun-exposed areas and are most common with demeclocycline but may occur with any of the
Antimicrobial Susceptibility Testing Protocols

2.2.12 GLYCyclines

2.2.12.1 Background

Emerging antimicrobial resistance has created the need for the development of new antimicrobials that are less likely to be affected by the commonly known mechanisms of resistance formation. Tigecycline is a new broad-spectrum antimicrobial agent that represents the first in its class of semisynthetic glycylcyclines. This drug was specifically designed by Wyeth to overcome the two most common mechanisms of resistance to tetracyclines: efflux pumps and ribosomal protection proteins. By adding a bulky side chain at position 9 of the minocycline molecule, tigecycline is less affected by resistance mutation and has improved activity and tolerability.

2.2.12.2 Mechanism of Action

Like tetracycline, tigecycline also binds to the 30S subunit of the bacterial ribosome to inhibit protein synthesis. The glycylcycline derivatives bind approximately five times more effectively than tetracycline.

2.2.12.3 Antimicrobial Activity and Therapeutic Uses

Tigecycline has activity in vitro against many gram-positive organisms including MRSA, glycopeptide-intermediately resistant *S. aureus* (GISA), penicillin-resistant *S. pneumoniae*, and van-

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**TABLE 2.9**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Generic Name</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short Acting</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline HCl</td>
<td>Oral, IM, IV</td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline HCl</td>
<td>Oral, IM</td>
<td></td>
</tr>
<tr>
<td><strong>Intermediate Acting</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demeclocycline</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td><strong>Long Acting</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline hyclate</td>
<td>Oral, IV</td>
<td></td>
</tr>
<tr>
<td>Doxycycline calcium</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Doxycycline monohydrate</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Minocycline</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Minocycline HCl</td>
<td>Oral</td>
<td></td>
</tr>
</tbody>
</table>


* IM = intramuscular; IV = intravenous.
comycin-resistant enterococci (VRE). It is also active against many gram-negative organisms including Acinetobacter baumanii, Stenotrophomonas maltophilia, most Enterobacteriaceae, including some strains producing extended-spectrum B-lactamases (ESBLs), and against many atypical bacteria and anaerobes, including B. fragilis and Clostridium perfringes and C. difficile. It does not have clinically significant activity against P. aeruginosa or Proteus species [58]. Tigecycline is currently FDA approved for the treatment of patients with complicated skin and skin-structure infections and complicated intraabdominal infections caused by susceptible strains.

2.2.12.4 Adverse Effect Profile

Nausea and vomiting appear to be dosed related and are the most common adverse effects associated with tigecycline, occurring in 30% and 20%, respectively. These symptoms usually occur in the first 1 to 2 days of treatment and are more common in women than men. Tigecycline is structurally similar to tetracycline and may cause similar adverse effects, including photosensitivity and discoloration of the teeth if used during tooth development [59].

2.2.13 MACROLIDES

2.2.13.1 Background

In 1952, McGuire and colleagues discovered erythromycin, the prototype and first antibiotic in the class of macrolides. It was isolated from the metabolic products of Streptomyces erythus, originally collected from soil samples obtained in the Philippine archipelago [53]. To date, the FDA has approved azithromycin, clarithromycin, dirithromycin, erythromycin (including many salt forms), and troleandomycin (Table 2.11) [4,5]. A 12–16 membered lactone ring characterizes the chemical structure of macrolides. Azithromycin is a semisynthetic compound that contains a 15-membered

**TABLE 2.10**
Adverse Effects Associated with Tetracyclines

<table>
<thead>
<tr>
<th>Hypersensitivity Reactions</th>
<th>Teeth and Bones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urticaria</td>
<td>Yellow to gray-brown discoloration</td>
</tr>
<tr>
<td>Morbilliform rashes</td>
<td>Hypoplasia of the enamel</td>
</tr>
<tr>
<td>Exfoliative dermatitis</td>
<td>Bone growth retardation</td>
</tr>
<tr>
<td>Anaphylaxis</td>
<td></td>
</tr>
<tr>
<td>Stevens-Johnson syndrome</td>
<td></td>
</tr>
<tr>
<td><strong>Gastrointestinal</strong></td>
<td><strong>Photosensitivity and Hyperpigmentation</strong></td>
</tr>
<tr>
<td>Epigastric/abdominal pain</td>
<td>Red rash to blisters</td>
</tr>
<tr>
<td>Nausea and vomiting</td>
<td>Pigmentation of nails, skin, and sclera</td>
</tr>
<tr>
<td>Anorexia</td>
<td>Discoloration of gums</td>
</tr>
<tr>
<td><strong>Hepatic</strong></td>
<td><strong>Miscellaneous</strong></td>
</tr>
<tr>
<td>Intrahepatic cholestatis</td>
<td>Vertigo</td>
</tr>
<tr>
<td>Jaundice</td>
<td>Systemic lupus erythematous-like syndrome</td>
</tr>
</tbody>
</table>

structure and is considered the prototype for a new macrolide structure termed azalides; the ketolides, discussed below, have a 14-membered lactone ring.

### 2.2.13.2 Mechanism of Action

Macrolides penetrate the cell wall of susceptible gram-positive and -negative microorganisms and reversibly bind to the 50S ribosomal subunit, inhibiting RNA-dependent protein synthesis. They are primarily bacteriostatic in nature but can be bactericidal, depending on the microorganism and drug concentration [5,53,60].

### 2.2.13.3 Antimicrobial Activity and Therapeutic Uses

Macrolides are highly active against many gram-positive organisms and have good activity against some gram-negative organisms, especially those commonly isolated from the respiratory tract. For this reason, they are considered the preferred agent for the treatment of outpatient community-acquired pneumonia, and erythromycin is considered the drug of choice when *Legionella* is suspected [61]. Macrolides are also prescribed for acute bacterial exacerbations of chronic bronchitis. Upper respiratory tract infections, including pharyngitis and tonsillitis, acute maxillary sinusitis, and otitis media, are commonly caused by *S. pyogenes*, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* to which macrolides have good activity. They also have good activity against those organisms most commonly implicated in skin and skin-structure infections: methicillin-sensitive *S. aureus*, *S. pyogenes*, and other *Streptococcus* species. *N. gonorrhoeae*, *C. trachomatis*, *Mycoplasma* species, and *Ureaplasma urealyticum*, to which macrolides have good activity, commonly cause sexually transmitted diseases. Erythromycin is considered the drug of choice for treating chlamydial infections in pregnancy, whereas azithromycin is commonly prescribed as a one-time dose for the treatment of *C. trachomatis* in patients in whom compliance is an issue [55]. Clarithromycin combined with omeprazole and/or metronidazole is prescribed for the treatment of *H. pylori*-associated duodenal ulcer disease. The United States Public Health Service recommends a macrolide, usually clarithromycin or azithromycin, be used in combination with other medications for the

### TABLE 2.11

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Route of Administration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>Oral, IV*</td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Dithromycin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Erythromycin base</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Erythromycin estolate</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Erythromycin stearate</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Erythromycin ethylsuccinate</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Erythromycin lactobionate</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Erythromycin gluceptate</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>Oral</td>
<td></td>
</tr>
</tbody>
</table>


* IV = intravenous.
treatment of *Mycobacterium avium* complex infections. These two macrolides have also been studied as alternative agents for the prevention of *M. avium* complex infections [62].

### 2.2.13.4 Adverse Effect Profile

The majority of side effects associated with the macrolides are mild and transient, and as a class they are generally well tolerated. The most common complaints involve the gastrointestinal tract and include diarrhea, nausea and vomiting, and abdominal pain. Patients receiving clarithromycin may complain of an abnormal or metallic aftertaste. Hepatotoxicity is a very rare but serious side effect associated primarily with the estolate salt of erythromycin [4,5,53,60].

### 2.2.14 Ketolides

Telithromycin is the first member in a new class of antibiotics known as the *ketolides* approved for use in the United States. This compound, derived from erythromycin A, is very similar in structure to the macrolides but has a ketone bond in the 3-position of the erythronolide ring, instead of the sugar cladinose. Like the macrolides, ketolides inhibit protein synthesis by reversibly binding to the 50S ribosomal subunit of the bacterial ribosome. Their spectrum of activity is similar to the macrolides, except they provide a broader coverage against macrolide-resistant streptococci. They are being developed for respiratory indications, including community-acquired pneumonia, acute bacterial exacerbations of chronic bronchitis, sinusitis, and pharyngitis, especially when resistant *S. pneumoniae* strains may be a concern [63]. The most commonly occurring adverse effects associated with the use of telithromycin were gastrointestinal in nature, including nausea, vomiting, and diarrhea, occurring in 8% to 10% of patients. Visual disturbances (blurred vision, diplopia, and accommodation difficulties) occurred in 0.6% of patients. These reactions more commonly occurred after the first or second dose of the medication and persisted for several hours. Liver abnormalities are currently under review with this medication after a recent paper published in the *Annals of Internal Medicine* described three patients who experienced serious liver toxicity following the administration of telithromycin. On June 29, 2006, the FDA notified healthcare professionals and patients that it had completed its safety review of telithromycin. They concluded that additional warnings about the risk of liver toxicity are required and instructed the manufacturer to revise their package insert.

### 2.2.15 Fluoroquinolones

#### 2.2.15.1 Background

The fluoroquinolones can be classified into four generations based on their antimicrobial activity. Nalidixic acid, a first-generation fluoroquinolone discovered in 1962, has a narrow gram-negative coverage and limited tissue distribution. Even though it has a very limited place in therapy, it served as the prototype for many other fluoroquinolones [64]. In the 1980s, it was discovered that manipulating the basic structure of nalidixic acid led to agents with a broader spectrum of antibacterial activity, better tissue distribution, and improved pharmacokinetics. These “second-generation” fluoroquinolones include ciprofloxacin, enoxacin, levofloxacin, lomefloxacin, norfloxacin, ofloxacin, and temafloxacin. Temafloxacin was voluntarily withdrawn from the market in 1992 by Abbott Laboratories, because of severe adverse effects, including six deaths, hemolytic anemia, renal and hepatic dysfunction, and hypoglycemia associated with its use in the first three months of marketing [65]. The “third-generation” fluoroquinolones include gatifloxacin, sparfloxacin, moxifloxacin, and grepafloxacin. These agents retain expanded gram-negative and atypical pathogen coverage, as compared to the second generation, but also have improved gram-positive pathogen coverage. On October 27, 1999, grepafloxacin was voluntarily withdrawn from the
market because the pharmaceutical company, GlaxoSmithKline, concluded that the therapeutic benefit was outweighed by the potential cardiac risks, especially given the availability of alternative treatments [66]. The fourth generations of fluoroquinolones have improved gram-positive coverage, maintain gram-negative coverage, and gain anaerobic coverage. This generation includes trovafloxacin, which was approved by the FDA in December 1997, and gemifloxacin, approved April 4, 2003. In June 1999, the FDA issued a public health advisory to all health care professionals that described serious liver injury associated with the use of trovafloxacin. The drug is currently available for use, but the FDA and Pfizer Pharmaceuticals strongly advise it be reserved for patients who meet specific criteria [67]. Since then, two additional fluoroquinolones have been approved by the FDA, moxifloxacin and gatifloxacin (Table 2.12) [4,5]. In May 2006, the FDA in cooperation with Bristol-Myers Squibb (gatifloxacin) mailed a letter to all health care professionals describing serious, sometimes fatal, cases of both hypoglycemia and hyperglycemia. This was prompted by a case-control study published in the New England Journal of Medicine that found that the use of gatifloxacin among outpatients was associated with an increased risk of both hypoglycemia and hyperglycemia as found among inpatients [68].

### 2.2.15.2 Mechanism of Action

The mechanism of action of the fluoroquinolones has not been clearly defined. However, it is clear that they inhibit bacterial DNA synthesis by inhibiting the enzyme DNA gyrase. This enzyme is

### TABLE 2.12

**Fluoroquinolones Classifications**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Generic Name</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td><strong>Second Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Oral, IV(^a)</td>
<td></td>
</tr>
<tr>
<td>Enoxacin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>Oral, IV</td>
<td></td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>Oral, IV</td>
<td></td>
</tr>
<tr>
<td><strong>Third Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>Oral, IV</td>
<td></td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>Oral</td>
<td></td>
</tr>
<tr>
<td><strong>Fourth Generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trovafloxacin (alatrovafloxacin)</td>
<td>Oral, IV</td>
<td></td>
</tr>
<tr>
<td>Gemifloxacin</td>
<td>Oral</td>
<td></td>
</tr>
</tbody>
</table>


\(^a\) IV = intravenous.
essential in bacterial replication and is responsible for breaking the superhelical twist of DNA, allowing the incorporation of new DNA and resealing the break [5,69,70].

2.2.15.3 Antimicrobial Activity and Therapeutic Uses

There are currently 12 fluoroquinolones on the market. These agents are considered bactericidal and have a wide range of activity against gram-negative and some gram-positive organisms. Trovafloxacin is the only quinoline with FDA approved labeling for use against anaerobes [4,5]. Among the gram-negative organisms, quinolones have excellent activity against the Enterobacteriaceae and H. influenzae, N. gonorrhoeae, N. meningitidis, and M. catarrhalis. Compared with the other quinolones, ciprofloxacin is more active against pseudomonads and most gram-negative organisms. They also exhibit good, but variable, activity against gram-positive organisms, including methicillin-susceptible S. aureus and S. epidermidis, but less activity against Streptococcus and Enterococcus species [4,5,69,70].

Norfloxacin is effective for infections involving the genitourinary and gastrointestinal tracts. It is poorly absorbed and therefore achieves minimal serum levels, and the MICs for most bacteria outside of these two sites preclude it for use in systemic infections. The other fluoroquinolones have been studied extensively as empiric treatment of patients with community-acquired pneumonia. According to recent guidelines from the Infectious Disease Society of America, respiratory fluoroquinolones such as levofloxacin, moxifloxacin, gatifloxacin, or gemifloxacin or a beta-lactam antibiotic with an advanced macrolide (azithromycin or clarithromycin) are considered preferred agents for most patients treated empirically for community-acquired pneumonia [61]. Quinolones have also been used for the treatment of nosocomially acquired pneumonia, bronchitis, sinusitis, and exacerbations of cystic fibrosis.

2.2.15.4 Adverse Effect Profile

The quinolones are generally well tolerated, although the frequency of adverse effects may increase with higher dosage. The most common side effects are gastrointestinal; primarily nausea and diarrhea; however, vomiting, epigastric burning, abdominal cramps, anorexia, and flatulence have been reported. Adverse reactions involving the central nervous system are the second most commonly encountered reaction and occur in 1% to 3% of the patients taking quinolones [70]. Symptoms include tremulousness, headache, dizziness, anxiety, nightmares, and insomnia. As a result of the cartilage damage seen in young animals, the fluoroquinolones are currently not recommended for use in children, pregnant women, or nursing mothers. Moderate to severe phototoxic reactions have occurred in patients exposed to direct or indirect sunlight or artificial ultraviolet light (sunlamps) while taking quinolones. The incidence of this reaction is higher with lomefloxacin and fleroxacin compared with the other quinolones [71]. As mentioned earlier reports of hepatotoxicity prompted Pfizer Laboratories to mail “Dear Prescriber Letters,” emphasizing the proper use of trovafloxacin for patients with serious infections in which therapy was initially started in the hospital or nursing home [72]. Additional scientific evaluation and analyses to clarify this are ongoing. Other less common adverse effects include cardiovascular, musculoskeletal, renal, hematological, ocular, and respiratory effects.

2.2.16 SULFONAMIDES AND TRIMETHOPRIM

2.2.16.1 Background

In 1932, after many exhaustive experiments, Domagk tested compound K1-730, a red azo dye, against a streptococcus growing in the test tube and in laboratory mice [73]. The compound had no effect against the streptococcus in the test tube, but when given to the infected mice, they were protected. This compound was named Prontosil rubrum. In 1935, Trefouel and colleagues manipulated Prontosil,
breaking the azo bond, and tested the by-products for antibacterial activity. One chemical that was produced was sulfanilamide, which was found to be as potent against streptococcus as Prontosil. They concluded that sulfanilamide was the active component of Prontosil and responsible for the antibacterial effect. Ironically, Gelmo had described sulfanilamide in 1908 while working on his doctoral thesis. He had no idea of its antibacterial properties but was working on the synthesis of dyes (Table 2.1) [69].

Included in the class of sulfonamides are sulfisoxazole, sulfamethoxazole, sulfadiazine, sulfadoxine, sulfasalazine, and sulfapyridine [4,5].

2.2.16.2 Mechanism of Action

The sulfonamides and trimethoprim inhibit the growth of susceptible microorganisms by preventing the formation of nucleic acids, and therefore cell growth. Either drug when used alone is bacteriostatic, but when combined they are bactericidal for susceptible organisms [69,74]. A combination of trimethoprim and a sulfonamide results in a sequential blockade in the folate synthesis pathway in bacteria. Specifically, sulfamethoxazole inhibits the enzyme responsible for the incorporation of para-aminobenzoic acid (PABA) into the precursor of folic acid (dihydrofolic acid), therefore blocking folic acid production in bacteria. Trimethoprim is a potent inhibitor of the enzyme dihydrofolate reductase and interferes with the conversion of folic acid to folinic acid (tetrahydrofolinic acid). Folinic acid is necessary in the production of purines, the backbone of both bacterial and mammalian DNA, and therefore inhibition of such results in cell death. Fortunately, mammalian cells can utilize folic acid obtained from the diet, bacteria cells cannot and therefore trimethoprim has little effect on mammalian cells [5,69,74].

2.2.16.3 Antimicrobial Activity and Therapeutic Uses

Trimethoprim-sulfamethoxazole has a wide range of activity against both gram-positive and gram-negative organisms, including *H. influenzae*, *S. pneumoniae*, *S. aureus*, *Salmonella* spp., *Shigella* spp., *Nocardia* spp., *Listeria* spp., *E. coli*, most *Enterobacteriaceae*, and *Pseudomonas* species other than *P. aeruginosa*. It is indicated for a variety of infections involving the genitourinary, gastrointestinal, and respiratory tracts. It is commonly prescribed for urinary tract infections and upper respiratory tract infections (i.e., otitis media, sinusitis, and bronchitis). It is considered the drug of choice for the treatment and prophylaxis of *Pneumocystis carinii* pneumonia and *Toxoplasmosis gondii* [5,69,74].

Sulfadiazine and sulfisoxazole have been shown effective in the treatment of nocardia infections. Sulfasalazine is indicated in the management of mild-to-moderate ulcerative colitis. Topical sulfonamide can be found in a variety of preparations including acne creams, antibacterial eye drops, vaginal creams, and burn creams [74].

2.2.16.4 Adverse Effect Profile

Trimethoprim-sulfamethoxazole is well tolerated by most people. Rashes occur in about 3% of patients who use this product and are usually mild. Severe skin reactions such as Stevens-Johnson syndrome have occurred but are rare. More frequent but less serious side effects include nausea, vomiting, and diarrhea [4,5,74].

2.3 ANTIMYCOBACTERIAL AGENTS

2.3.1 Isoniazid (INH)

Isoniazid, introduced in 1952, is the most active drug for the treatment of tuberculosis and has some activity against the other mycobacterium species. The mechanism of action is not fully
understood but is thought to be through inhibition of the mycobacterial cell wall synthesis. It is bactericidal against *M. tuberculosis* when actively growing, but bacteriostatic if the organism is not replicating [75,76]. Peripheral neuropathy, most likely caused by interference with the metabolism of pyridoxine, is associated with isoniazid. Pyridoxine, vitamin B-6, is commonly given to patients to prevent peripheral neuropathy. Isoniazid has also been associated with minor asymptomatic elevations in liver function tests that usually resolve with continued treatment. Though fulminate hepatotoxicity is rare, it may occur at any time, but more likely 4–8 weeks after treatment has begun (Table 2.13) [4,5].

2.3.2 RIFAMPIN, RIFABUTIN, AND RIFAPENTINE

The rifamycins are a class of antibiotics produced by *Streptomyces mediterranei*. First described in the 1960s, there are now three rifamycins currently approved for clinical use in the United States. Rifampin, rifabutin, and, more recently, rifapentine (a long-acting rifamycin) are used primarily for the treatment of mycobacterial infections but are also active against some gram-positive and gram-negative organisms. They inhibit nucleic acid synthesis by binding to DNA-dependent RNA polymerase [5,75,76]. The most common adverse effect is gastrointestinal upset. The rifamycins are excreted in the urine, tears, sweat as well as other body fluids and can cause a red–orange discoloration. Patients should be warned of the discoloration of body fluids and advised of the possibility of discoloration of soft contact lenses. Other reactions include rash, hepatitis, myalgias, arthralgias, and rarely thrombocytopenia and neutropenia. Uveitis is associated with rifabutin and appears to be dose-dependent. Symptoms include acute onset of ocular pain, blurry vision, photophobia, and diminished visual acuity. The patient should be advised to report these symptoms to their health care provider immediately, at which time rifabutin should be discontinued (Table 2.13) [4,5]. Rifabutin, rifapentine, and, to a lesser extent, rifampin are inducers of hepatic microsomal enzymes and therefore have the potential for drug interactions with other medications metabolized through the liver. They may increase the clearance of zidovudine, dapsone, methadone, coumadin, glucocorticoids, estrogens, oral hypoglycemic agents, quinidine,

<table>
<thead>
<tr>
<th>Medication</th>
<th>Usual Adult Dosage</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>300 mg po qd</td>
<td>Peripheral neuropathy, hepatitis, skin rash, arthralgia, drowsiness, fever</td>
</tr>
<tr>
<td>Rifampin</td>
<td>600 mg po qd</td>
<td>Hepatitis, rash, thrombocytopenia, flu-like syndrome, orange-red discoloration</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>30 mg/kg po qd</td>
<td>Hepatitis, fever, skin rash, arthralgia, hyperuricemia, abdominal distress</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>15–25 mg/kg po qd</td>
<td>Optic neuritis, skin rash, abdominal distress</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>15 mg/kg IM/IV qd</td>
<td>Hearing loss, ataxia, nystagmus, nephrotoxicity</td>
</tr>
<tr>
<td>Amikacin</td>
<td>15 mg/kg IV qd</td>
<td>Hearing loss</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>400 mg po q12h</td>
<td>Abdominal distress, headache, anxiety</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>750 mg po q12h</td>
<td>Abdominal distress, headache, anxiety</td>
</tr>
</tbody>
</table>


* Dosages are for adults weighing 60 kg and with normal renal function.

* po = by mouth; IM = intramuscular; IV = intravenous; qd = daily; q12h = every 12 h.
verapamil, theophylline, anticonvulsants, cyclosporin, protease inhibitors, and nonnucleoside reverse transcriptase inhibitors [4,5].

2.3.3 **Pyrazinamide (PZA)**

Pyrazinamide, a synthetic analog of nicotinamide, is bactericidal for *M. tuberculosis*. The most common side effects are nausea and vomiting. The most important adverse reaction to pyrazinamide is liver damage. Hyperuricemia occurs frequently, but acute gout is uncommon. Skin rash and gastrointestinal intolerance are also seen (Table 2.13) [4,5,75,76].

2.3.4 **Ethambutol (ETH)**

Ethambutol was discovered in 1961 and, at usual doses, is generally considered bacteriostatic against *M. tuberculosis*. It may have bactericidal effects when given at higher doses, but its precise mechanism of action is unknown. Its spectrum of activity is limited to mycobacteria. It has no activity against gram-positive or gram-negative organisms or fungi [5,75,76]. The drug is usually well tolerated with a low frequency of side effects. At higher dosage, retrobulbar neuritis is the most serious side effect of ethambutol. Symptoms include blurred vision, decreased visual acuity, and red–green color blindness (Table 2.13) [4,5].

2.3.5 **Antimicrobial Activity and Therapeutic Uses**

Tuberculosis must be treated for a long duration as compared with most other infectious diseases; if not, the infection may reoccur, and the patient may develop drug-resistant mycobacteria. Due to the high rates of resistant tuberculosis, it is recommended that all patients receive at least an initial four-drug regimen treatment of isoniazid, rifampin, pyrazinamide, and ethambutol which can later be pared down to a three-drug regimen. (Table 2.14) [46].

The treatment of tuberculosis in persons infected with HIV is complicated by many factors. The risk of drug-resistant tuberculosis is higher among persons with known HIV infection and therefore requires multiple antituberculosis drugs. In addition the concomitant use of rifamycins and protease inhibitors results in potentially harmful drug interactions.

Preventive therapy reduces the risk of developing clinically active tuberculosis in infected persons. Isoniazid is the only antituberculosis drug shown to be effective for preventive therapy. Depending on skin test results, age, and risk categories, it is usually given for 6–12 months in adults and 9 months in children. At least six months of preventive therapy with isoniazid is recommended for adults and children who have abnormal chest radiographs consistent with a past infection of tuberculosis. A 12-month regimen of rifampin alone or in combination with PZA or ethambutol is recommended for people exposed to a patient with INH-resistant *M. tuberculosis* or if the patient has failed treatment. If the index case has documented multidrug resistant tuberculosis, combination therapy with PZA and ofloxacin is recommended [32]. For HIV-infected adults, a nine-month regimen of isoniazid can be administered daily or a two-month regimen of a rifamycin (rifabutin or rifampin) and pyrazinamide can be administered daily. Rifabutin is recommended for patients receiving a protease inhibitor, whereas rifampin is recommended for patients not receiving protease inhibitors. The concurrent administration of rifampin and a protease inhibitor is not recommended [77].

Various treatment regimens for the treatment of disseminated *M. avium* complex (DMAC) infections have been studied. The ideal regimen remains unclear. Azithromycin or clarithromycin, ethambutol, rifabutin, clofazimine, ciprofloxacin, and amikacin are the agents used, usually in combination, for the treatment of DMAC. Rifabutin, clarithromycin, and azithromycin have all been used as prophylactic agents. The U.S. Public Health Service recommends a macrolide, usually azithromycin, clarithromycin, generally in conjunction with ethambutol, clofazimine, ciprofloxacin, or rifabutin, for those patients previously treated for DMAC infections. Prophylaxis should be
### TABLE 2.14
Drug Regimens for Culture-Positive Pulmonary Tuberculosis Caused by Drug-Susceptible Organisms

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Initial Phase</th>
<th>Continuation Phase</th>
<th>Range of total doses (minimal duration)</th>
<th>Rating(^a) (evidence)(^b)</th>
<th>HIV-</th>
<th>HIV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INH, RIF, PZA, EMB</td>
<td>Seven days per week for 56 doses (8 wk) or 5 d/wk for 40 doses (8 wk)(^c)</td>
<td>INH, RIF</td>
<td>Seven days per week for 125 doses (18 wk) or 5 d/wk for 90 doses (18 wk)(^d)</td>
<td>182–130 (25 wk)</td>
<td>A((</td>
</tr>
<tr>
<td>2</td>
<td>INH, RIF, PZA, EMB</td>
<td>Seven days per week for 14 doses (w wk), than twice weekly for 12 doses (8 wk) or 5 d/wk for 10 doses (2 wk), than twice weekly for 12 doses (wk)</td>
<td>INH, RIF</td>
<td>Twice weekly for 36 doses (18 wk)</td>
<td>62–58 (26 wk)</td>
<td>A(</td>
</tr>
<tr>
<td>3</td>
<td>INH, RIF, PZA, EMB</td>
<td>Three times weekly for 24 doses (8 wk)</td>
<td>INH, RIF</td>
<td>Three times weekly for 54 doses (18 wk)</td>
<td>78 (26 wk)</td>
<td>B((</td>
</tr>
<tr>
<td>4</td>
<td>INH, RIF, PZA, EMB</td>
<td>Seven days per week for 58 doses (8 wk) or 5 d/wk for 40 doses (8 wk)</td>
<td>INH, RIF</td>
<td>Seven days per week for 217 doses (31 wk) or 5 d/wk for 156 doses (31 wk)</td>
<td>273–195 (38 wk)</td>
<td>C((</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** EMB = Ethambutol; INH = Isoniazid; PZA = pyrazinamide; RIF = rifampin; RPT = rifperistine.

\(^a\) Definitions of evidence ratings: A = preferred; B = acceptable alternative; C = other when A and B cannot be given; E = should never be given.

\(^b\) Definition of evidence ratings: \(|) = randomized clinical trial; || = data from clinical trials that were not randomized or were conducted in other populations; ||| = expert opinion.

\(^c\) When DOT is used, drugs may be given 5 days/week and the necessary number of doses adjusted accordingly. Although there are no studies that compare five with seven daily doses, extensive experience indicates this would be an effective practice.

\(^d\) Patients with cavitation on initial chest radiograph and positive cultures at completion of 2 months of therapy should receive a 7-month (31 week; either 217 doses [daily] or 62 doses [twice weekly]) continuation phase.

\(^e\) Five-day-a-week administration is always given by DOT. Rating for 5 day/week regimens is A(\(|)\).

\(^f\) Not recommended for HIV-infected patients with CD\(_4\)\(^e\) cell counts <100 cells/µl.

\(^g\) Options 1c and 2b should be used only in HIV-negative patients who have negative sputum smears at the time of completion of 2 months of therapy and who do not have cavitation on initial chest radiograph (see text). For patients started on this regimen and found to have a positive culture from the 2-month specimen, treatment should be extended an extra 3 months.

considered for all HIV-infected persons without previous history of DMAC infection who have a CD4+ count of <75 cells/mm³, although some experts would wait until the CD4+ count is <50 cells/mm³ [62].

2.4 ANTIVIRAL AGENTS

2.4.1 ANTIRETROVIRAL AGENTS (FUSION INHIBITORS, NUCLEOSIDE AND NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS, NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS, AND PROTEASE INHIBITORS)

2.4.1.1 Mechanisms of Action

Targets in the viral replicative cycle that are potentially susceptible to antiviral intervention include entry of the virus into the cell, reverse transcriptase, integrase, protease, and viral maturation and release [78].

Fusion inhibitors directly interfere with host and cellular membrane fusion, thereby preventing entry of HIV-1 into the cell. The HIV-1 envelope contains surface glycoproteins (gp120) and transmembrane glycoproteins (gp41). The initial attachment between virus and cell is the coupling of the viral gp120 subunit to the CD4 cell, resulting in a conformational change that exposes the gp41 fusion peptide and pulls the cell and viral membranes close, allowing fusion and viral entry. Fusion inhibitors bind to a region of gp41, inhibiting apposition and therefore fusion and viral entry [79,80].

Nucleoside reverse transcriptase inhibitors (NRTIs) must be converted to the active triphosphate derivative via a three step process, whereas nucleotide RTIs only require a two step process. The active intracellular moiety causes a 3´ modification, resulting in a missing 3´-hydroxyl group and thereby not allowing the addition of any nucleotides. The ensuing chain termination ultimately stops HIV replication [78].

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are highly specific, direct, noncompetitive inhibitors of HIV-1 reverse transcriptase. These agents do not require cellular metabolism to be active, are not incorporated into the elongating strand of DNA, and do not cause chain termination [78].

HIV encodes for the protease enzyme that is essential for viral infectivity and the processing of core proteins. The protease cleaves the polyprotein precursors of viral proteins, creating mature virion particles. Protease inhibitors (PIs) compete for the active cleavage site on the protease enzyme, inhibiting the formation of mature core proteins, resulting in the release of noninfectious virion from the host cell [78].

2.4.1.2 Mechanisms of Resistance

HIV resistance to the available antiretroviral agents is inevitable. Highly specific mutations can be transmitted with primary infection and will evolve over time in a patient, particularly in the setting of incomplete viral suppression. There is considerable cross-resistance within the classes of antiretroviral drugs, but cross-resistance between the classes does not occur. The accumulation of mutations results in the expression of amino acid substitutions in the pol gene that encodes for reverse transcriptase and in the viral protease or the gp41 envelope gene associated with resistance to entry inhibitors. Coexpression of multiple and variable substitutions at these positions may occur and are generally associated with higher levels of resistance [81,82].

2.4.1.3 Classification

The antiviral agents used for the treatment of HIV infection are divided into classes based on their mechanism of action (Table 2.15).
2.4.1.4 Antiretroviral Activity and Therapeutic Uses

All drugs in this category are effective against HIV type 1 (HIV-1) [5,6]. In addition, all of the NRTIs and PIs possess activity against HIV type 2 (HIV-2). A select few of these agents also possess activity against hepatitis B virus (HBV), such as lamivudine, emtricitabine, and tenofovir [82,83].

### TABLE 2.15
**Antiretroviral Drugs Classifications**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Generic Name (Brand Name)</th>
<th>Route of Administration</th>
<th>Common Adult Dosagesa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleoside(tide) Reverse Transcriptase Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zidovudine (Retrovir)</td>
<td>IV, b Oral</td>
<td>300 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Didanosine (Videx)</td>
<td>Oral</td>
<td>Tablets: 200 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Didanosine EC (Videx EC)</td>
<td>Powder: 250 mg twice a day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC capsules: 400 mg once a day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zalcitabine (Hivid)</td>
<td>Oral</td>
<td>0.75 mg three times a day</td>
<td></td>
</tr>
<tr>
<td>Stavudine (Zerit)</td>
<td>Oral</td>
<td>40 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Lamivudine (Epivir)</td>
<td>Oral</td>
<td>300 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Abacavir (Ziagen)</td>
<td>Oral</td>
<td>600 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Emtricitabine (Emtriva)</td>
<td>Oral</td>
<td>200 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Tenofovir (Viread)</td>
<td>Oral</td>
<td>300 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Zidovudine/lamivudine (Combivir)</td>
<td>Oral</td>
<td>300 mg/150 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Zidovudine/lamivudine/Abacavir (Trizivir)</td>
<td>Oral</td>
<td>300 mg/150 mg/300 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Lamivudine/abacavir (Epzicom)</td>
<td>Oral</td>
<td>300 mg/600 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Emtricitabine/tenofovir (Truvada)</td>
<td>Oral</td>
<td>200 mg/300 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Tenofovir/tenofovir (Atripla)</td>
<td>Oral</td>
<td>600 mg/200 mg/300 mg once a day</td>
<td></td>
</tr>
<tr>
<td><strong>Nonnucleoside Reverse Transcriptase Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nevirapine (Viramune)</td>
<td>Oral</td>
<td>200 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Delavirdine (Rescriptor)</td>
<td>Oral</td>
<td>400 mg three times a day</td>
<td></td>
</tr>
<tr>
<td>Efavirenz (Sustiva)</td>
<td>Oral</td>
<td>600 mg once a day</td>
<td></td>
</tr>
<tr>
<td><strong>Protease Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saquinavir (Invirase, Fortovase)</td>
<td>Oral</td>
<td>Invirase: 1000 mg twice a day with ritonavir 100 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Ritonavir (Norvir)</td>
<td>Oral</td>
<td>600 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Indinavir (Crixivan)</td>
<td>Oral</td>
<td>800 mg every 8 h</td>
<td></td>
</tr>
<tr>
<td>Nelfinavir (Viracept)</td>
<td>Oral</td>
<td>1250 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Amprenavir (Agenerase)</td>
<td>Oral</td>
<td>1200 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Lopinavir/ritonavir (Kaletra)</td>
<td>Oral</td>
<td>400 mg/100 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Atazanavir (Reyataz)</td>
<td>Oral</td>
<td>400 mg once a day</td>
<td></td>
</tr>
<tr>
<td>Fosamprenavir (Lexiva)</td>
<td>Oral</td>
<td>1400 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Tipranavir (Aptivus)</td>
<td>Oral</td>
<td>500 mg twice a day with 200 mg of ritonavir twice a day</td>
<td></td>
</tr>
<tr>
<td>Darunavir (Prezista)</td>
<td>Oral</td>
<td>600 mg twice a day with 100 mg of ritonavir twice a day</td>
<td></td>
</tr>
<tr>
<td><strong>Fusion Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enfuvirtide (Fuzeon)</td>
<td>Subcutaneous</td>
<td>90 mg twice a day</td>
<td></td>
</tr>
</tbody>
</table>


*a Dosages are for adults weighing 60 kg, with normal renal and hepatic function and without ritonavir boosting except those that are only FDA approved as such.

*b IV = intravenous; EC = enteric coated.
Zidovudine, an NRTI, was the first drug approved by the FDA (1987) for the treatment of HIV and was the only drug available until 1991. Since then, six other nucleoside(tide) reverse transcriptase inhibitors have been added to this class [82,83].

The second class of drugs developed and approved by the FDA, the PIs, was introduced in 1995. There are now ten PIs available for the treatment of HIV infection. These agents, when combined with two NRTIs, revolutionized anti-HIV therapy. Triple combinations of these agents led to what is called “highly active antiretroviral therapy” or “HAART” [82,83].

In 1996, the FDA approved nevirapine, the first drug in the third class of antiviral drugs used for the treatment of HIV. Since then, two other agents have joined the class of NNRTIs, bringing the total to three [82,83]. Triple combinations of an NNRTI with two NRTIs are also referred to as HAART.

The fourth class of anti-HIV drugs introduced to the market was the fusion inhibitors, entering the market in 2003. Enfuvirtide is the only drug from this class that is currently available and can only be given by subcutaneous injection. As such, its use has been limited to salvage therapy when other options are limited [82,83].

Today, there are 22 antiviral agents in four different classifications used for the treatment of HIV infection. Earlier studies demonstrated better clinical utility due to delaying resistance when two NRTIs were combined together for treatment [7]. Current guidelines for the use of these agents are reviewed and updated routinely as new agents and data become available. Currently, recommendations for therapy consist of regimens that combine at least three agents, with the preferred regimens being two NRTIs with either lopinavir/ritonavir (a boosted PI) or efavirenz (NNRTI). Further discussion on the treatment of HIV infection can be found elsewhere [83].

Zidovudine has been shown to be effective in reducing the risk of HIV transmission from mother to infant by about two-thirds [8]. In the trial of the Pediatric AIDS Clinical Trial Group (PACTG) 076, mothers who received zidovudine during the second and third trimester of pregnancy and intravenous zidovudine during delivery, in addition to the infant who received 6 weeks of zidovudine immediately after birth, had a transmission rate of approximately 8%. Those who did not receive this regimen had a transmission rate of about 28% [85]. As a result, it became standard practice to recommend zidovudine treatment to HIV-infected women beyond the first trimester of pregnancy and to the woman’s infant for six weeks after birth [86]. Current recommendations have changed to include the use of HAART in all pregnant women. Pregnant women should be evaluated for HAART in much the same way that all other HIV-infected patients are evaluated. The specific regimen that is finally chosen depends upon the risks to the mother and to the fetus [86].

Occupational exposure to HIV may occur in the health care workplace. Introduction of postexposure prophylaxis can greatly reduce the risk of HIV transmission. Current recommendations may involve two to three drugs, two from the NRTIs and potentially one from the PIs (the third drug). The recommendation depends upon the status of the source patient, the severity of the exposure, and the potential for transmission. Therapy should be started within the first 24 h after an exposure to ensure the greatest possibility of preventing transmission and should be continued for 4 weeks [87].

2.4.1.5 Adverse Effect Profile

All the NRTIs share the possibility of lactic acidosis with hepatic steatosis, a rare, but potentially life-threatening toxicity. Most of the NRTIs can cause gastrointestinal complaints like nausea and vomiting and some may cause peripheral neuropathy (didanosine and stavudine). Bone marrow suppression is a concern when using zidovudine, although the frequency is relatively uncommon without the simultaneous use of other potentially bone marrow toxic drugs. Abacavir has the potential to cause a hypersensitivity reaction, resulting in discontinuation of the drug for resolution and does not allow for rechallenges because this may result in serious and sometimes fatal reactions. The NNRTIs as well as the PIs tipranavir, fosamprenavir, and amprenavir are known to cause rash. The NNRTIs and the PIs are also associated with increases in hepatic transaminase levels. The PIs are also known to cause fat redistribution and lipid abnormalities. The PIs are reported to cause hyperglycemia, sometimes leading
to the development of diabetes mellitus. The PIs have been associated with gastrointestinal discomfort (nausea, vomiting, and diarrhea). Indinavir is associated with nephrolithiasis; therefore, patients are cautioned to drink at least 1.5 L of fluid, preferably water, per day to decrease this risk (Table 2.16). The most common complaint with enfuvirtide is injection site reactions, occurring in 98% of patients on this drug. Rare cases of pneumonia have also been reported with its use [82,83].

<table>
<thead>
<tr>
<th>TABLE 2.16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adverse Effects Associated with Antiretroviral Drugs</strong></td>
</tr>
</tbody>
</table>

**Nucleoside and Nucleoside Reverse Transcriptase Inhibitors**

<table>
<thead>
<tr>
<th>Gastrointestinal</th>
<th>Neurologic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>Peripheral neuropathy (didanosine, stavudine)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Headache</td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hematologic (Primarily Zidovudine)</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemia</td>
<td>Lactic acidosis with hepatic steatosis</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>Dyslipidemias (stavudine, zidovudine)</td>
</tr>
</tbody>
</table>

**Nonnucleoside Reverse Transcriptase Inhibitors**

<table>
<thead>
<tr>
<th>Hypersensitivity Reactions</th>
<th>Central Nervous System (Primarily Efavirenz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rash</td>
<td>Headaches</td>
</tr>
<tr>
<td>Hepatic</td>
<td>Dizziness</td>
</tr>
<tr>
<td>Increased transaminase levels</td>
<td>Somnolence and insomnia</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>Abnormal dreams</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Confusion</td>
</tr>
<tr>
<td>False-positive cannabinoid test (efavirenz)</td>
<td>Abnormal normal and impaired concentration</td>
</tr>
<tr>
<td>Teratogenic (efavirenz)</td>
<td>Amnesia</td>
</tr>
<tr>
<td>Dyslipidemias</td>
<td>Hallucinations</td>
</tr>
</tbody>
</table>

**Protease Inhibitors**

<table>
<thead>
<tr>
<th>Gastrointestinal</th>
<th>Metabolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Fat redistribution</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Dyslipidemias</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td></td>
</tr>
<tr>
<td>Dyspepsia</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Renal</th>
<th>Hematologic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrolithiasis (indinavir)</td>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td><strong>Hepatic</strong></td>
<td>Bleeding episodes in patients with hemophilia</td>
</tr>
<tr>
<td>Increased indirect bilirubinemia</td>
<td>Miscellaneous</td>
</tr>
<tr>
<td>Elevated transaminase enzymes</td>
<td>Headache</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>Asthenia</td>
</tr>
</tbody>
</table>

2.4.2 ANTIVIRAL DRUGS

2.4.2.1 Mechanisms of Action

The drugs approved for the treatment of viral infections, other than those for HIV, work by interfering with viral messenger RNA, inhibiting viral DNA synthesis, blocking viral M2 protein ion channels thereby inhibiting viral uncoating and hence viral proliferation, or inducing cellular enzymes that interfere with the synthesis of viral proteins (Table 2.17). Many of these agents must undergo intracellular phosphorylation to their active component in order to be effective [78,88].

2.4.2.2 Mechanisms of Resistance

Viral resistance results from mutations in the viral DNA polymerase gene or by qualitative changes in viral thymidine kinase. Changes in viral RNA segments account for resistance to amantadine and rimantadine. There is cross-resistance between acyclovir, famciclovir, and ganciclovir, and complete cross-resistance between amantadine and rimantadine. Ribavirin shares no cross-resistance with any other drugs in this class. As with the drugs used for the treatment of HIV infection, combination therapy decreases the rate at which resistant viruses emerge during therapy and could potentially lead to decreased dosages and, therefore, decreased risk of adverse events [73].

2.4.2.3 Classification

These agents are classified based on their chemical structure. The chemical structures can be grouped into classes consisting of nucleoside analogs, 10-carbon-ring amines, a nucleotide analog, a pyrophosphate analog, a recombinant protein produced in bacteria, neuraminidase inhibitors, and monoclonal antibodies (Table 2.17) [4,5,88].

2.4.2.4 Antiviral Effects and Therapeutic Uses

The antiviral drugs are effective against a wide variety of viruses (Table 2.18). These agents have been used for the treatment and prevention of many viral infections in both immunocompetent and immunocompromised patients. Some common uses include mucosal, cutaneous, and systemic herpes infections; genital herpes; varicella zoster (shingles) infections; chicken pox; cytomegalovirus (CMV) infections; influenza A; respiratory syncytia virus (RSV); and Kaposi’s sarcoma (HHV8) [88,89].

2.4.2.5 Adverse Effect Profile

The most common adverse effects associated with these agents are gastrointestinal in origin. Patients may complain of nausea, vomiting, and/or diarrhea. Headache is also a common complaint. Other potential adverse effects are listed in Table 2.19 [4,5,88,89].

2.5 ANTIFUNGAL AGENTS

2.5.1 BACKGROUND

Recent advances in antimicrobial therapy, trauma resuscitation, and supportive care in the ICU have decreased mortality but increased the risk of fungal superinfections. Candidemia is the most deadly bloodstream infection, with a mortality rate of approximately 40%. Patients at risk for candidemia and systemic candidiasis include patients with a compromised immune system, have received stem-
<table>
<thead>
<tr>
<th>Classification</th>
<th>Generic Name</th>
<th>Mechanism of Action</th>
<th>Route of Administration</th>
<th>Common Adult Dosagesa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleoside Analogues</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acyclovir</td>
<td>DNA chain terminator</td>
<td>Oral, Topical</td>
<td>IV: 5–10 mg/kg every 8 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral: 200–800 mg every 4–12 hours</td>
<td></td>
</tr>
<tr>
<td>Adefovir</td>
<td>DNA chain terminator</td>
<td>Oral</td>
<td>10 mg daily</td>
<td></td>
</tr>
<tr>
<td>Entecavir</td>
<td>DNA chain terminator</td>
<td>Oral</td>
<td>0.5 mg daily</td>
<td></td>
</tr>
<tr>
<td>Famciclovir</td>
<td>Inhibits DNA synthesis (Prodrug of penciclovir)</td>
<td>Oral</td>
<td>125–500 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>Inhibits DNA synthesis</td>
<td>Oral</td>
<td>IV: 5 mg/kg twice a day</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral: 1 gram three times a day</td>
<td></td>
</tr>
<tr>
<td>Penciclovir</td>
<td>Inhibits DNA synthesis</td>
<td>Oral</td>
<td>1% cream every 2 hours</td>
<td></td>
</tr>
<tr>
<td>Ribavirin</td>
<td>Inhibits mRNA</td>
<td>Inhalation, oral</td>
<td>Inh: 6 grams delivered over 18 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral: 500–600 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Valacyclovir</td>
<td>DNA chain terminator (Prodrug of acyclovir)</td>
<td>Oral</td>
<td>500–1000 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Valganciclovir</td>
<td>Inhibits DNA synthesis</td>
<td>Oral</td>
<td>900 mg twice a day for 21 days then 900 mg daily</td>
<td></td>
</tr>
<tr>
<td><strong>Nucleotide Analogue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cidofovir</td>
<td>Inhibits DNA synthesis</td>
<td>Oral</td>
<td>5 mg/kg every 2 weeks</td>
<td></td>
</tr>
<tr>
<td><strong>Pyrophosphate Analogue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foscarnet</td>
<td>Inhibits DNA synthesis</td>
<td>Oral</td>
<td>40–60 mg/kg 2–3 times per day</td>
<td></td>
</tr>
<tr>
<td><strong>10-Carbon-Ring Amines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amantadine</td>
<td>Inhibits viral uncoating</td>
<td>Oral</td>
<td>100 mg twice a day</td>
<td></td>
</tr>
<tr>
<td>Rimantadine</td>
<td>Inhibits viral uncoating</td>
<td>Oral</td>
<td>200 mg once a day</td>
<td></td>
</tr>
<tr>
<td><strong>Recombinant Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interferon alfa</td>
<td>Inhibits protein synthesis</td>
<td>Oral</td>
<td>5 million units daily or 10 million units three times weekly</td>
<td></td>
</tr>
<tr>
<td>Peginterferon alfa-2a</td>
<td>Inhibits protein synthesis</td>
<td>Oral</td>
<td>180 mcg every week with Ribavirin</td>
<td></td>
</tr>
<tr>
<td>Peginterferon alfa-2B</td>
<td>Inhibits protein synthesis</td>
<td>Oral</td>
<td>1.5 mcg/kg every week with Ribavirin</td>
<td></td>
</tr>
<tr>
<td><strong>Neuraminidase Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>Inhibits influenza virus neuraminidase</td>
<td>Oral</td>
<td>75 mg twice a day for 5 days</td>
<td></td>
</tr>
<tr>
<td>Zanamivir</td>
<td>Inhibits influenza virus neuraminidase</td>
<td>Inhalation</td>
<td>10 mg twice a day for 5 days</td>
<td></td>
</tr>
<tr>
<td><strong>Monoclonal Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palivizumab</td>
<td>Monoclonal antibody</td>
<td>Oral</td>
<td>50–100 mg once a month throughout RSV season</td>
<td></td>
</tr>
</tbody>
</table>


*a* Dosages are for adults weighing 60 kg with normal renal function.

*b* IV = intravenous; IM = intramuscular; sub-Q = subcutaneous.
cell transplants, are receiving chemotherapy, are critically ill in the ICU, have catheters, have recently had surgery, and who are on prolonged antibiotics. To date, the various amphotericin-based products and the azole antifungals have been the mainstay of therapy, but the troublesome side effects and resistance formation have increased the need for improved therapeutic options. More recently a new class of antifungals, the echinocandins, has been FDA approved, including: caspofungin, micafungin, and anidulafungin (Eraxis). The echinocandins were actually first discovered in 1974, as a naturally occurring product derived from *Aspergillus nidulans*. The first echinocandin,
cilofugin (Lilly), never made it to the marketplace because early clinical trials were halted due to nephrotoxicity. It was later determined that the nephrotoxicity was related to the vehicle, polyethylene glycol, and not the medication itself.

2.5.2 MECHANISMS OF ACTION

The antifungal agents are grouped according to mechanism of action. The macrolide antifungal agents work by binding to ergosterol in fungal cell membranes, leading to weakening of the membranes and then leakage of the cellular components. This eventually leads to death of the fungal cell. The azole antifungal agents (triazoles and imidazoles) exert their effect by inhibiting the formation of sterols, which alters fungal cellular membranes. When membrane structure is disrupted, there is increased membrane permeability and therefore leakage of essential cell contents and eventually death. The echinocandins specifically target or inhibit the formation of 1,3-β-D-glucan, a key component in the fungal cell wall. Of the miscellaneous agents, flucytosine is taken up by the fungal cell and then it inhibits protein synthesis. Griseofulvin acts by disrupting the cell cycle and possibly by formation of defective DNA, which cannot replicate. Nystatin and terbinafine exert their effect by interfering with the formation of fungal sterols, much like the azole antifungal agents [4,5,90].

2.5.3 MECHANISMS OF RESISTANCE

Mutants with decreased susceptibility (resistance) to the macrolide antifungal agents have been acquired in vitro; however, the clinical relevance of such resistance is unknown. Detection of antifungal resistance is difficult. It is unclear if clinical failure is related to fungal resistance or failure on the part of the host because these agents are typically used in immunocompromised patients. Some fungal strains have demonstrated increased MICs to the azole antifungal agents and generally share this potential for resistance with other agents within the class (cross-resistance) [5,90].

TABLE 2.19
Adverse Effects Associated with Antiviral Drugs (not used for HIV infection)

<table>
<thead>
<tr>
<th>Gastrointestinal</th>
<th>Central Nervous System (Amantadine, Rimantadine, Interferon Alfa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea and vomiting</td>
<td>Headaches</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Dizziness</td>
</tr>
<tr>
<td>Hypersensitivity Reactions</td>
<td>Insomnia</td>
</tr>
<tr>
<td>Rash</td>
<td>Confusion</td>
</tr>
<tr>
<td>Worsening of respiratory symptoms (ribavirin)</td>
<td>Suicide attempts</td>
</tr>
<tr>
<td>Conjunctivitis (ribavirin)</td>
<td></td>
</tr>
<tr>
<td>Renal</td>
<td>Hematologic</td>
</tr>
<tr>
<td>Increased BUN*</td>
<td>Anemia</td>
</tr>
<tr>
<td>Increased serum creatinine</td>
<td>Neutropenia</td>
</tr>
<tr>
<td>Urine protein</td>
<td>Miscellaneous</td>
</tr>
<tr>
<td>Electrolyte imbalances (foscarnet)</td>
<td>Phlebitis</td>
</tr>
<tr>
<td></td>
<td>Fever</td>
</tr>
<tr>
<td></td>
<td>Hypotension</td>
</tr>
</tbody>
</table>


* BUN = blood urea nitrogen.
2.5.4 CLASSIFICATION

The antifungal agents are classified based on mechanism of action (Table 2.20) [4,5].

### TABLE 2.20
Antifungal Drug Classifications

<table>
<thead>
<tr>
<th>Classification</th>
<th>Polyene Macrolides</th>
<th>Azoles (Imidazoles, Triazoles, and Echinocandins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generic Name</td>
<td>Route of Administration</td>
<td>Common Dosages&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Polyene Macrolides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B deoxycholate (Ampho B)</td>
<td>IV,&lt;sup&gt;b&lt;/sup&gt; oral, topical</td>
<td>0.25–1 mg/kg/day</td>
</tr>
<tr>
<td>Amphotericin B complex (ABLC)</td>
<td>IV</td>
<td>5 mg/kg/day</td>
</tr>
<tr>
<td>Amphotericin B dispersion (ABCD)</td>
<td>IV</td>
<td>3–6 mg/kg/day</td>
</tr>
<tr>
<td>Amphotericin B liposomal</td>
<td>IV</td>
<td>3–5 mg/kg/day</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Oral, topical</td>
<td>100,000–600,000 units two–four times a day</td>
</tr>
<tr>
<td><strong>Imidazoles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butoconazole</td>
<td>Topical</td>
<td>2% cream at bedtime</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>Oral, topical</td>
<td>Oral: 10 mg five times a day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Topical: 100 mg/500mg/5 grams at bedtime</td>
</tr>
<tr>
<td>Econazole</td>
<td>Topical</td>
<td>1% cream twice a day</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Oral, topical</td>
<td>400 mg oral/2% topical once a day</td>
</tr>
<tr>
<td>Miconazole</td>
<td>IV, topical</td>
<td>IV: 200–3600 mg divided three times a day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Topical: 100 mg/200 mg/2% cream at bedtime</td>
</tr>
<tr>
<td>Oxiconazole</td>
<td>Topical</td>
<td>1% cream at bedtime</td>
</tr>
<tr>
<td>Sulconazole</td>
<td>Topical</td>
<td>1% solution once a day</td>
</tr>
<tr>
<td><strong>Triazoles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>IV, oral</td>
<td>100–400 mg once a day</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Oral, topical</td>
<td>200 mg once a day</td>
</tr>
<tr>
<td>Terconazole</td>
<td>Topical</td>
<td>80 mg/0.4% cream at bedtime</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>IV, oral</td>
<td>4–6 mg/kg twice a day</td>
</tr>
<tr>
<td><strong>Echinocandins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>IV</td>
<td>50–100 mg once a day</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>IV</td>
<td>50 mg once a day</td>
</tr>
<tr>
<td>Micafungin</td>
<td>IV</td>
<td>50–150 mg once a day</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciclopirox olamine</td>
<td>Topical</td>
<td>1% cream/lotion twice a day</td>
</tr>
<tr>
<td>Fluconazole olamine</td>
<td>Oral</td>
<td>100–150 mg/kg divided four times a day</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>Topical</td>
<td>500 mg–1 gram per day</td>
</tr>
<tr>
<td>Haloprogin</td>
<td>Topical</td>
<td>1% cream or solution twice a day</td>
</tr>
<tr>
<td>Naftifine</td>
<td>Topical</td>
<td>1% cream twice a day</td>
</tr>
<tr>
<td>Terbinfine</td>
<td>Oral</td>
<td>250 mg per day</td>
</tr>
<tr>
<td>Tolnaftate</td>
<td>Topical</td>
<td>1% twice a day</td>
</tr>
</tbody>
</table>


<sup>a</sup> Dosages are for adults weighing 60 kg and having normal renal function.

<sup>b</sup> IV = intravenous.
The antifungal agents are effective against a wide variety of infections caused by pathogenic fungi (Table 2.21). They are often used for the treatment of invasive, as well as localized fungal infections in immunocompetent and immunocompromised patients such as those with cancers and AIDS (Table 2.22) [90].

### TABLE 2.21
**Antifungal Activity**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Antifungal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyene Macrolides</strong> (Activity Based on Studies with Amphotericin B Deoxycholate)</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B deoxycholate</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Aspergillus species, and mucormycoses caused by Mucor, Phizopus, Absidia, Entomophthora, and Basidiobolus</td>
</tr>
<tr>
<td>Amphotericin B complex</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Amphotericin B dispersion</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
</tbody>
</table>

**Imidazoles**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antifungal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butoconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Aspergillus species, and mucormycoses caused by Mucor, Phizopus, Absidia, Entomophthora, and Basidiobolus</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Econazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Miconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Oxiconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Sulconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
</tbody>
</table>

**Triazoles**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antifungal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Terconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
</tbody>
</table>

**Echinocandins**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antifungal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anidulafungin</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Micafungin</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
</tbody>
</table>

**Miscellaneous**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antifungal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciclopirox olamine</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Phialophora species, Trichophyton species, Epidermophyton species, Microsporum species, Malassezia furfur, Cryptococcus neoformans</td>
</tr>
</tbody>
</table>

(continued)
Some of these agents have been used together or are proposed for use together. The combination of the amphotericins and the azoles is thought to be potentially antagonistic because the azoles inhibit the product to which the amphotericins bind and exert their effect (ergosterol). Further clinical trials are needed to determine if this effect does indeed happen. Synergism between flucytosine and amphotericin has been reported. As a result, these two agents have often been used together against *Cryptococcus neoformans* [5,90].

The lipid formulations of amphotericin were developed in hopes of decreasing some of the adverse effects of amphotericin B deoxycholate. By incorporating the amphotericin into a lipid product, there is reported to be less uptake by the kidneys and yet greater uptake into other areas such as the reticuloendothelial system (RES). More drug, therefore, may be delivered to the site of infection while decreasing the harmful effects on the kidneys [90,91].
2.5.6 Adverse Effect Profile (Table 2.23)

The amphotericin products are known to cause flu-like symptoms, such as fever, chills, malaise, and generalized pain. These agents may also cause nephrotoxicity resulting in increased BUN (blood urea nitrogen) and creatinine concentrations. Electrolyte abnormalities, particularly hypokalemia and hypomagnesemia, are reported as well [4,5,91]. While many of these effects are less common with the lipid products, there is still potential for these effects, particularly with prolonged use, which is often necessary to adequately treat fungal infections [91].

The azoles and other miscellaneous antifungal agents can be divided into topical and systemic drugs when discussing adverse effects. Overall, these agents are well tolerated at usual doses. Using the topical agents may result in localized reactions, such as local irritation, sensitization, itching, burning, erythema, and rashes. Topical agents used for genital fungal infections may also cause dyspareunia and/or vulvovaginal burning [4,5,91].

The most common side effect associated with the systemic (oral) use of the azole drugs is gastrointestinal distress. Symptoms include nausea, vomiting, abdominal pain, and diarrhea. Some patients may experience transient increases in liver transaminases. Flucytosine may cause gastrointestinal distress and has been associated with neutropenia and thrombocytopenia in some patients [4,5,91].

---

**TABLE 2.23**

Adverse Effects Associated with Antifungal Drugs

<table>
<thead>
<tr>
<th>Polyene Macrolides</th>
<th>Hepatic</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurologic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td>Chills</td>
</tr>
<tr>
<td>Headache</td>
<td></td>
<td>Back pain</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td></td>
<td>Nausea</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td></td>
<td>Rigors</td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
<td>Malaise</td>
</tr>
<tr>
<td>Nausea</td>
<td></td>
<td>Generalized pain</td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td>Anemia</td>
</tr>
<tr>
<td>Cardiac</td>
<td></td>
<td>Epistaxis</td>
</tr>
<tr>
<td>Tachycardia</td>
<td></td>
<td>Anorexia</td>
</tr>
<tr>
<td>Chest pain</td>
<td></td>
<td>Hypotension</td>
</tr>
<tr>
<td>Renal</td>
<td></td>
<td>Hyperspnea</td>
</tr>
<tr>
<td>Increased serum creatinine concentration</td>
<td></td>
<td>Edema</td>
</tr>
<tr>
<td>Increase in BUN</td>
<td></td>
<td>Cough</td>
</tr>
<tr>
<td>Hypokalemia</td>
<td></td>
<td>Insomnia</td>
</tr>
<tr>
<td>Hypomagnesemia</td>
<td></td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyperglycemia</td>
</tr>
</tbody>
</table>

**Topical Azoles (Imidazoles and Triazoles) and Miscellaneous Agents**

<table>
<thead>
<tr>
<th>Topical Azoles (Imidazoles and Triazoles) and Miscellaneous Agents</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Local irritation</td>
<td>Erythema</td>
</tr>
<tr>
<td>Sensitization</td>
<td>Rash</td>
</tr>
<tr>
<td>Pruritis</td>
<td>Folliculitis</td>
</tr>
<tr>
<td>Burning</td>
<td>Pain</td>
</tr>
<tr>
<td>Allergic contact dermatitis</td>
<td>Dryness</td>
</tr>
</tbody>
</table>

*(continued)*
TABLE 2.23
Adverse Effects Associated with Antifungal Drugs (continued)

Systemic Azoles (Imidazoles and Triazoles)

<table>
<thead>
<tr>
<th>Hepatic</th>
<th>Gastrointestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased liver enzymes (AST, ALT, alkaline phosphatase)</td>
<td>Nausea</td>
</tr>
<tr>
<td>Bilirubinemia</td>
<td>Vomiting</td>
</tr>
<tr>
<td>Dermatologic</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Rash</td>
<td>Abdominal pain</td>
</tr>
<tr>
<td>Pruritus</td>
<td>Flatulence</td>
</tr>
<tr>
<td>Urticaria</td>
<td>Dyspepsia</td>
</tr>
<tr>
<td>Stevens-Johnson Syndrome (rare)</td>
<td></td>
</tr>
</tbody>
</table>

Endocrine

<table>
<thead>
<tr>
<th>Dermatologic</th>
<th>Gastrointestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gynecomastia</td>
<td>Nausea</td>
</tr>
<tr>
<td>Decreased libido</td>
<td>Vomiting</td>
</tr>
<tr>
<td>Impotence</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Adrenal insufficiency</td>
<td>Abdominal pain</td>
</tr>
<tr>
<td></td>
<td>Flatulence</td>
</tr>
<tr>
<td></td>
<td>Dyspepsia</td>
</tr>
</tbody>
</table>

Echinocandins

<table>
<thead>
<tr>
<th>Injection site reaction</th>
<th>Possible Histamine-Mediated Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phlebitis</td>
<td>Rash</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Pruritus</td>
</tr>
<tr>
<td>Nausea</td>
<td>Facial swelling</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Vasodilation</td>
</tr>
</tbody>
</table>

Miscellaneous Systemic (Flucytosine and Griseofulvin)

<table>
<thead>
<tr>
<th>Central Nervous System</th>
<th>Gastrointestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>Nausea</td>
</tr>
<tr>
<td>Dizziness</td>
<td>Vomiting</td>
</tr>
<tr>
<td>Insomnia</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Dermatologic</td>
<td>Abdominal pain</td>
</tr>
<tr>
<td>Urticaria</td>
<td>Flatulence</td>
</tr>
<tr>
<td>Rash</td>
<td></td>
</tr>
<tr>
<td>Photosensitivity</td>
<td></td>
</tr>
<tr>
<td>Hematologic</td>
<td></td>
</tr>
<tr>
<td>Hepatic</td>
<td></td>
</tr>
<tr>
<td>Increased liver enzymes (AST, ALT, alkaline phosphatase)</td>
<td>Anemia</td>
</tr>
</tbody>
</table>


AST = aspartate aminotransferase; ALT = alanine aminotransferase; BUN = blood urea nitrogen.

REFERENCES

55. Centers for Disease Control and Prevention. 1998 Guidelines for treatment of sexually transmitted
57. Frost, P., Weinstein, G.D., and Gomez, E.C. Phototoxic potential of minocycline and doxycycline,
58. Bouchillon, S.K., Hoban, D.J., Johnson, B.M. et al. In vitro activity of tigecycline against 3989 gram-
negative and gram-positive clinical isolates from the United States Tigecycline Evaluation and Sur-
59. Smith, K.L., McCabe, S.M., and Aeschlimann, J.R. Tigecycline a novel glycylcyclines antibiotic,
60. Steigbigel, N.H. Macrolides and clindamycin, in Principles and Practices of Infectious Diseases,
Practice Guidelines for the Management of Community-Acquired Pneumonia in Immunocompetent
62. USPHS/IDSA Prevention of Opportunistic Infections Working Group. USPHS/IDSA guidelines on
opportunistic infections in infected persons with human immunodeficiency virus: Disease-specific
64. Lesher, G.Y., Froelich, E.J., and Gruetton, D. 1,8-Napthyridine derivative. A new class of chemother-
67. Public Health Advisory Food and Drug Administration. Trovan, FDA/Center for Drug Evaluation and
68. Park-Wyllie, L.Y., Juurlink, D.N., Kopp, A. et al. Outpatient gatifloxacin therapy and dysglycemia in
69. Mandell, G.L. and Sande, M.A. Sulfonamides, trimethoprim-sulfamethoxazole, quinolones, and agents
for urinary tract infections, in Goodman and Gilman’s The Pharmacological Basis of Therapeutics,
1047–1064.
70. Hooper, D.C. Quinolones, in Principles and Practices of Infectious Diseases, Mandell, G.L., Bennett,
73. Otten, H. Domagk and the development of the sulphonamides, J. Antimicrob. Chemother., 17, 689–696,
1966.
Diseases, Mandell, G.L., Bennett, J.E., and Dolin, R., Eds., Churchill Livingstone, New York, 1995,
354–364.
Diseases, Mandell, G.L., Bennett, J.E., and Dolin, R., Eds., Churchill Livingstone, New York, 1995,
389–401.
76. Mandell, G.L. and Sande, M.A. Drugs used in the chemotherapy of tuberculosis and leprosy, in
Goodman and Gilman’s The Pharmacological Basis of Therapeutics, Goodman Gilman, A., Rall,
77. American Thoracic Society, Centers for Disease Control and Prevention, and Infectious Diseases
Society of America. Treatment of tuberculosis, Morbidity and Mortality Weekly Report, 52(RR11),
3 Disk Diffusion Test and Gradient Methodologies

Audrey Wanger

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3.1 INTRODUCTION

3.1.2 HISTORICAL PERSPECTIVE

The earliest methods for assessing the effects of antibiotics on bacteria involved the use of ditches cut into agar plates. Antibiotic solution poured into the ditch was allowed to diffuse out to inhibit the growth of the bacteria streaked perpendicular to the ditch. In 1943, Foster and Woodruff used paper strips impregnated with antibiotics as an alternative source of diffusion [1]. The agar diffusion principles used in the bioassay of penicillin G in plasma were further used to develop the disk diffusion test [2]. Antibiotic reservoirs that initially consisted of antibiotic solutions in agar wells or cylinders were soon replaced by paper disks impregnated with antibiotics.
The results of early diffusion tests were arbitrarily interpreted: the presence of an inhibition zone suggested susceptibility and no zone, resistance. It became obvious at an early stage that many test parameters influenced the inhibition zone size irrespective of the susceptibility of the test organism. Qualitative results were also considered a disadvantage, and multiple disks with a high and a low antibiotic content were then introduced [3]. Categorical interpretations were derived based on the following: zones around both disks were indicative of susceptibility, a zone around the high potency disk only was indicative of intermediate susceptibility, and no zones around either disk was indicative of resistance.

3.1.3 First Attempts at Standardization

By virtue of its simplicity, the disk diffusion test was rapidly and widely used by many laboratories. However, the lack of standardized procedures gave erroneous and nonreproducible results. In 1954, Ericsson published the first article describing a standardized technique called the paper disk method (PDM) for sensitivity testing of bacteria [4]. This technique became the PDM disk diffusion product line manufactured by AB BIODISK, Solna, Sweden, in the mid-1950s. The use of strictly calibrated antibiotic disks (PDM_ASD) together with a specially formulated antibiotic susceptibility test medium (PDM ASM) with controlled levels of thymine, thymidine, and various cations, and the use of a well-defined procedure with prediffusion and lighter inoculum, formed the backbone of this method [5].

Rigorous control of test reagents (PDM disks and media) on a batch to batch basis and the use of a well-defined test procedure made it possible to standardize all components of the PDM method. The zone diameter, an unknown or dependent variable, was then calibrated against reference agar dilution minimum inhibitor concentration (MIC) values (known or independent variable) using regression analysis based on the assumption that there was a linear correlation between zone sizes and MIC values (Figure 3.1).

Information on drug levels, the site of infection, and MIC distributions of biological populations of bacteria were used to select the MIC cutoffs or break points for the PDM method to predict whether bacteria with a certain zone-MIC correlate would respond to therapy with standard dosages. It is surprising that many of the susceptibility-testing principles used today were meticulously defined 50 years ago.

3.1.4 International Collaborative Study

In 1961, Ericsson and Sherris in collaboration with the World Health Organization undertook an initiative to define and standardize susceptibility testing on a global level. Experts from 16 countries
stretching from the United States across to Japan contributed to this, which, in its time, was a monumental effort to obtain global consensus on the standardization of susceptibility testing with MIC and disk diffusion methods. The findings and recommendations of this project were published in the *Acta Pathologica et Microbiologica Scandinavica* and titled “Antibiotic sensitivity testing, report from an International Collaborative Study (ICS)” [5]. This comprehensive reference, which has been cited over 5,000 times in the peer reviewed literature, was taken one step further by the Clinical Laboratory Standards Institute (CLSI) formerly NCCLS in the United States to develop specific clinical laboratory standards for antimicrobial susceptibility testing. In the meantime, the Food and Drug Administration (FDA) began its efforts to define the quality specifications of antibiotic disk reagents based on the efforts and findings of Ericsson et al. and to regulate their use for clinical testing [6].

### 3.1.5 Theory of Inhibition Zones

Despite its simplicity, the disk diffusion test is based on sophisticated physicochemical principles governing the dynamics of diffusion of antibiotics simultaneous to bacterial growth in an agar system. Cooper and Woodman [6] applied the formula for diffusion of neutral particles in gases to the diffusion of antibiotics through agar gels as follows:

\[
X^2 = 4DT \cdot 2.3 (\log M_o/M)
\]

where
- \(X\) = zone radius
- \(D\) = diffusion coefficient
- \(T\) = critical time for zone demarcation
- \(M_o\) = antibiotic concentration at the reservoir
- \(M\) = critical concentration of antibiotic inhibiting the organism

The critical time \(T\) can be further related to the growth characteristics of the test organism and the inoculum factor as follows:

\[
T = L + n \log N/N_o
\]

where
- \(L\) = lag period
- \(n\) = generation time
- \(N\) = critical mass of cells formed at \(T\)
- \(N_o\) = inoculum density used in the test

Thus, it is clear that many drug- and organism-related variables directly influence the zone size. To obtain meaningful results, as many test variables as possible need to be strictly standardized such that the rate-limiting step influencing the zone size is proportional to the critical concentration of the drug (\(M\)) inhibiting the organism relative to its susceptibility or resistance.

### 3.1.6 The Race Between Diffusion and Growth

When an antibiotic disk comes in contact with an inoculated agar surface, the “drug–bug” race begins. Antibiotic molecules diffuse out from the disk into the agar, creating a dynamically changing gradient of antibiotic concentrations, while the test organism starts to divide and growth progresses toward the critical mass. The zone edge is formed at the critical time where the concentration of antibiotic that is just able to inhibit the organism reaches an overwhelming cell mass. At this point, the density of cells is sufficiently high such that the antibiotic in the immediate vicinity can be
absorbed, thus maintaining concentrations at subinhibitory levels and enabling the test organism to
grow. The critical times of most rapidly growing aerobic and facultative anaerobic bacteria vary
between 3 and 6 h and should not be confused with the incubation period needed to achieve visible
growth as seen by the naked eye.

Besides molecular properties of the drug such as molecular weight and size, ionic charge, and
aqueous solubility, the diffusion coefficient of the drug is also influenced by the viscosity and depth
of the agar as well as the assay temperature and incubation conditions. Besides characteristics
intrinsic to the organism, growth is affected by the nutritive capacity of the test medium, the density
and growth phase of the inoculum, and the incubation temperature [7,8].

3.1.7 National Variants of Disk Tests

In an attempt to “tame” the disk diffusion test and provide national guidelines, expert groups in several
countries began to develop local variants of the disk test. Bauer et al. [9] defined the American version,
as we know it today, based on the use of an inoculum density of approximately $10^8 \text{CFU/mL}$ to give
semiconfluent growth using Mueller-Hinton agar (MHA) as the test medium. To overcome certain
disadvantages of MHA, as observed in the past, e.g., poorer support of growth of gram-positive
organisms, higher levels and lot-to-lot variable levels of sulfonamide antagonists, and, in general,
significant batch-to-batch reproducibility, European versions opted for the use of other formulations
such as PDM ASM (AB BIODISK, Solna, Sweden) and IsoSensitest (Oxoid, Basingstoke, UK). A
100-fold lighter inoculum ($10^6$) (or 1:100 dilution of a 0.5 McFarland turbidity) was also chosen to
achieve the so-called ICS inoculum of “dense but not confluent growth.” This provided an optical
check, and small variations of the lighter inoculum size had less effect on zone sizes than did the
heavier inoculum. In the PDM method, Ericsson further implemented a prediffusion period of between
30 and 60 min prior to incubation to allow the gradient to stabilize before growth is initiated.

In the United Kingdom, the Stokes method [10] was introduced in an effort to control the
influence of testing variables on the zone size and to improve the reliability of the interpretation
of the test result. This technique compares the zone size of the test organism to a few control
organisms with known susceptibilities. The test strain is concentrically inoculated in a section
adjacent to the control organism on the same agar surface by rota-plating. Different antibiotic disks
are then applied at the junction of the test and control. When the zone of the test strain for a certain
antibiotic is equivalent to or larger than that of the susceptible control, the result is interpreted as
a susceptible category and vice versa for a resistant result. Although the Stokes method is based
on a rational assumption, the lack of suitable control strains to represent susceptibility categorization
for an increasing number of antibiotics and control strains belonging to different species and with
growth rates comparable to those of a wide variety of clinical pathogens have limited its validity
and usefulness. Currently, laboratories in the United Kingdom and other former Commonwealth
countries are replacing this method with a standardized disk test as described by the BSAC (British

3.1.8 Rational Use of Antibiotics

Results of in vitro susceptibility tests can at best be used as an educated guess to predict the
therapeutic outcome of standard antibiotic dosage regimens in normal patients. Many factors besides
the in vitro result affect the potential clinical efficacy of a particular antibiotic. These include the
pharmacokinetic (PK) properties of the drug such as its absorption, distribution metabolism, elim-
ination, and protein binding and thus the resulting bioavailable drug levels obtained in different
fluids and tissues. The pharmacodynamics (PD) or drug effects on the bacteria, such as inhibition
of growth (bacteriostatic), killing kinetics of the organism (bactericidal), and postantibiotic effects
will equally affect organism eradication and clinical outcome. Host factors, such as the inflammatory
response, suppression of normal immune defense mechanisms, site of the infection, and presence
of foreign bodies or abscess formation are important factors that will also influence treatment outcome. Thus, susceptibility test results of qualitative methods like the disk diffusion test must be correlated to quantitative reference MIC values in micrograms per milliliter (µg/ml) that are in turn used in different PK/PD models to help optimize antibiotic selection, fine-tune dosage regimens, and minimize resistance selection. Again, these seemingly sophisticated principles were well described by Ericsson in a Ph.D. thesis entitled, “Rational Use of Antibiotics in Hospitals” [12].

The main advantages of disk diffusion testing are the simplicity of use, no requirement for expensive equipment, flexible choice of antibiotics for testing, and cost-effectiveness. The main disadvantage, besides not providing quantitative information, is the amount of technologist time required for setting up the test and for manual reading of zone sizes and consultation with interpretive standards prior to interpretation of results. In many clinical situations, disk diffusion testing and a qualitative interpretation of susceptibility is an acceptable option. In the treatment of most “healthy” patients with, for example, uncomplicated urinary tract infection, the choice of the test method is not critical. In these cases, disk diffusion, break-point agar, or broth dilution systems are equally satisfactory for predicting patient outcome [13].

In critical infections such as endocarditis, meningitis, or osteomyelitis, or in the immunocompromised host, accurate quantitative determination of the exact MIC value is crucial for therapy guidance. In these situations, an MIC value of 0.016 µg/mL has significantly different therapeutic implications than does an MIC of 1 µg/mL in influencing the antibiotic choice and dose relative to factors such as body weight, site of infection, potential drug toxicity, and drug levels [13]. Clinical laboratories usually implement multiple susceptibility test systems and base their choice of test method on patient type, source of specimen, organism species, and expected problems of resistance detection. The need for quantitative methods was emphasized over 30 years ago. Ericsson et al. [5] stated “The basic point of reference should be the MIC determined under reproducible conditions,” and the ICS report [5] encouraged “the development and evaluation of new techniques for measuring sensitivity,” not just arbitrarily grouping isolates into categories.

3.1.9 Antibiotic Gradient Plate

As early as the 1950s, Bryson and Szybalski [14] recognized the limitations of diffusion methods due to the undefined and unstable gradients generated. For research studies on antibiotic resistance, the selection of bacterial populations with different levels of resistance required a calibrated quantitative gradient. They developed the “wedge plate” method of creating a gradient in an agar plate that would be stable long enough to select out resistant populations of bacteria at defined concentrations of antibiotics in a reproducible manner. A layer of plain agar is poured into a petri dish, preferably square or rectangular in shape, and the agar is allowed to solidify as a slope by placing the plate at an angle. A second layer of agar containing a defined amount of antibiotic is poured on top of the slope and the “sandwich” agar composite is allowed to solidify on a horizontal surface. The drug diffuses vertically downward creating a gradient of antibiotic concentrations across the wedge length of the plate. The concentration gradient is calibrated by calculating the drug concentration in total volume of agar at different sections along the wedge.

3.1.10 Spiral Gradient Plate

The spiral gradient technique [15] uses a diluter-dispenser pump to deposit a continuously diluted bacterial suspension onto an agar surface using a spiral plater. The method was initially developed for the enumeration of bacteria for research and industrial purposes. It was further adapted for dispensing a continuously diluted antibiotic solution as closely spaced concentric rings on an agar surface. After a period of equilibration, an antibiotic gradient is generated with a high concentration in the middle and decreasing progressively toward the rim of the agar plate. In a way, it resembles a “huge” disk diffusion gradient with a concentration maximum in the middle of a circle. After
growth of the organism, which is streaked from the center outward like the spokes in a wheel, the
distance from the center to the point of inhibition is measured and the inhibiting concentration of
the antibiotic can be calculated. Although automated, the technique still requires preparation and
quality control of fresh antibiotic solutions, and the stability of the gradients generated is maintained
for a relatively short period of time. The technique is useful for research but not for daily use in a
clinical laboratory.

3.1.11 ETEST

An innovative gradient technique that combined the principles of both the disk diffusion and agar
dilution was developed in Sweden and first presented to the scientific community at the Interscience
Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meeting in 1988 [16]. A pre-
formed and predefined gradient of antibiotic concentrations is immobilized in a dry format onto
the surface of a plastic strip (Figure 3.2).

The continuous concentration gradient is calibrated across a corresponding MIC range covering
15 twofold dilutions. When applied to the surface of an inoculated agar plate, the antibiotic on the
Etest strip is instantaneously transferred to the agar in the form of a stable and continuous gradient
directly beneath and in the immediate vicinity of the strip (Figure 3.3).

The stability of the gradient is maintained for up to 18 to 20 hours, which covers the critical
times of a wide range of pathogens, from rapid growing aerobic bacteria to slow growing fastidious
organisms, including anaerobes and fungi. The stable gradient also provides inoculum tolerance
where a 100-fold variation in CFU/mL has minimal effect on the MIC results of homogeneously
susceptible strains. This minimizes the inoculum-related day-to-day variability seen in routine
susceptibility testing [17]. More importantly, the stable gradient also allows the use of a mac-
romethod with heavier inoculum to optimize the detection of low-level resistance, heteroresistance,
and resistant subpopulations.

Etest has been approved by the U.S. FDA since 1991 and is currently cleared for clinical testing
of aerobes, anaerobes, pneumococci, streptococci, haemophilus, and gonococci against a wide
variety of antibiotics, and for Candida spp. against several antifungal agents. Further, it has been
extensively validated with over 3,000 international scientific references, more than 1,000 of which

FIGURE 3.2 Configuration of the Etest strip with the antibiotic scale on one side and the predefined gradient
on the reverse side.
have been published in peer-reviewed journals (Etest reference list). These investigations have documented Etest performance to be equivalent to reference MIC methods with a high degree of reproducibility in most laboratory settings.

### 3.2 PROCEDURE — DISK DIFFUSION

#### 3.2.1 INOCULUM AND INOCULATION

A major source of error in disk diffusion testing is the inoculum variation [18]. In the CLSI (former NCCLS) procedure [19], the inoculum density must be standardized to a final concentration of $1–2 \times 10^8$ CFU/mL. This can be accomplished either by the growth method or direct colony suspension. For the growth method, a loop is used to touch the top of three to five colonies of the same morphological type from an agar plate culture. This is suspended in 4–5 mL of a suitable broth, for example, tryptic soy broth, and incubated at 35°C until it achieves or exceeds the turbidity of a 0.5 McFarland standard (usually 26 h). The turbidity of the actively growing cells when adjusted to the 0.5 McFarland standard using sterile saline will provide an inoculum of approximately $12 \times 10^8$ CFU/mL (as initially determined using *E. coli* ATCC 25922 as a reference). The inoculum adjustment can be done visually using a barium sulfate turbidity standard or with a photometric device. Barium sulfate standards must be verified monthly and vigorously agitated before use. Commercially available latex standards (Remel, Lenexa, KS, and Hardy Diagnostics, Santa Monica, CA) with a long shelf life are a practical alternative. The latex suspension should be mixed by inverting gently and not on a vortex mixer.

A more practical alternative is to prepare the inoculum by suspending isolated colonies from an 18–24-h agar plate in broth or saline to a turbidity matching a 0.5 McFarland standard. This is the recommended method for fastidious organisms and for testing staphylococci for the detection of oxacillin resistance. The Prompt (BD Diagnostic Systems, Sparks, MD) inoculum preparation
Antimicrobial Susceptibility Testing Protocols

system can also be used to optimize workflow since the organisms are supposedly maintained at the same density for 6 h. However, the system is not suitable for mucoid strains or fastidious organisms since the amount of cell mass absorbed onto the tip of the sampling wand may be insufficient to give the appropriate inoculum density in terms of CFU per milliliter. Furthermore, the inoculum solution contains Tween, a surfactant that can have potential effects on certain organisms, and the diffusion of antibiotics from the disks. Colony counts should be regularly performed to validate that the inoculum density is correct.

The inoculum suspension should be used within 15 min of preparation, and this is particularly important for fastidious organisms that lose their viability rapidly. A sterile cotton swab (not too thin or too tightly spun) is dipped into the suspension and pressed firmly on the inside of the tube to remove excess liquid. The dried surface of the appropriate agar plate is inoculated by streaking the entire surface and repeating it twice, rotating the plate 60° degrees each time to obtain an even distribution of the inoculum. The inoculated plate is allowed to dry with the lid left ajar for no more than 15 min.

3.2.2 Application of Disks

Once the agar plate is completely dry, the different antibiotic disks are applied either manually or with a dispensing apparatus. In general, no more than 12 disks should be placed on a 150-mm agar plate or 5 disks on a 90-mm plate (less for very susceptible organisms, see Figure 3.4).

Optimally, disks should be positioned at a distance of 30 mm apart and no closer than 24 mm apart when measured center to center to minimize overlap of inhibition zones. Most dispenser devices are self-tamping (disks are tapped or pressed onto the agar surface). If applied manually, the disk must be pressed down to make immediate and complete contact with the agar surface. Once in contact with the agar, the disk cannot be moved because of instantaneous diffusion of antibiotics from the disk to the agar.

3.2.3 Incubation and Reading

Agar plates are incubated in an inverted position (agar side up) under conditions appropriate for the test organism (Table 3.1). Plates should not be stacked more than five in a pile to ensure that the plate in the middle reaches incubator temperature within the same time frame as the others.

FIGURE 3.4 Kirby-Bauer disk diffusion result for a very susceptible strain of Staphylococcus species.
After 16–18 h of incubation for rapidly growing aerobic bacteria, or longer where appropriate for fastidious organisms or specific resistance detection conditions, the agar plate is examined to determine if a semiconfluent and even lawn of growth has been obtained before reading the plate. If individual colonies are seen, the inoculum is too light, and the test should be repeated since zone sizes would be falsely larger. The same holds true for excessively heavy inoculum where zone edges may be very hazy and the zone sizes falsely small.

If the lawn of growth is satisfactory, the zone diameter is read to the nearest millimeter using a ruler or sliding calipers. For MHA (without blood supplements), zone diameters are read from the back of the plate. For blood-containing agar, zone diameters are read from the surface of the agar. The zone margin is identified as the area where no obvious visible growth is seen by the naked eye unless otherwise specified; faint growth or microcolonies detectable only with a magnifying glass or by tilting the plate should be ignored. Although selection of the end point should logically be related to the mode of action of the drug, for example, bactericidal drugs to be read at 100% or complete inhibition of all growth and bacteriostatic drugs at 80% inhibition, such specific recommendations are not provided by the CLSI. The following are highlighted in the CLSI document [19]:

- Discrete colonies in an otherwise clear zone should be subcultured, reidentified, and retested. With *Proteus* spp., the swarming growth within a discernable zone should be ignored.
- For streptococci, the zone of growth inhibition should be measured and not the hemolysis seen on blood agar plates.
- With trimethoprim and sulphonamides, trailing of growth at the zone edge should be disregarded and the most obvious margin as seen by the naked eye should be read.
- Transmitted light should be used to examine oxacillin and vancomycin zones for light growth or microcolonies, both of which are indicative of resistance.

Some examples of different zone edges can be seen in Figure 3.5.

Reading of zone sizes can be automated using a camera-based image analysis systems such as the BIOMIC (Giles Scientific, New York, NY), Aura (Oxoid, Basingstoke, UK), Protozone (Microbiology International, Frederick, MD), and SirScan (i2a, Paris, France). The agar plate is placed into the reader, which is connected to a computer system. The system then reads the different zone

### Table 3.1: Disk Diffusion Testing Conditions

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Temp.</th>
<th>Atmosphere</th>
<th>Duration (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae</td>
<td>MHA</td>
<td>35°C</td>
<td>Ambient</td>
<td>16–18</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>MHA</td>
<td>35°C</td>
<td>Ambient</td>
<td>16–18</td>
</tr>
<tr>
<td><em>Acinetobacter</em> spp.</td>
<td>MHA</td>
<td>35°C</td>
<td>Ambient</td>
<td>16–18</td>
</tr>
<tr>
<td><em>Staphylococcus</em> spp.</td>
<td>MHA</td>
<td>35°C</td>
<td>Ambient</td>
<td>16–18 (oxacillin)</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>MHA</td>
<td>35°C</td>
<td>Ambient</td>
<td>16–18 (vancomycin)</td>
</tr>
<tr>
<td><em>Haemophilus</em> spp.</td>
<td>HTM</td>
<td>35°C</td>
<td>5% CO₂</td>
<td>16–18</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td>GC</td>
<td>35°C</td>
<td>5% CO₂</td>
<td>20–24</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp. other than S. pneumoniae</td>
<td>MHB</td>
<td>35°C</td>
<td>5% CO₂</td>
<td>20–24</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>MHB</td>
<td>35°C</td>
<td>5% CO₂</td>
<td>20–24</td>
</tr>
</tbody>
</table>

* MHA = Mueller-Hinton agar, HTM = *Haemophilus* test medium, GC = gonococcal agar, MHB = Mueller-Hinton + 5% sheep blood agar.
sizes directly from the agar plate and converts the result to a susceptibility category interpretation. Although zone sizes are automatically read, the user can intervene and adjust the results. The BIOMIC system also claims that zone sizes can be converted to MIC equivalents using a database of regression analyses based on the assumption of a linear correlation between the zone size and MIC value. Although a few studies have shown acceptable correlations between zone extrapolated MIC equivalents provided by BIOMIC when compared with reference methods [20,21], accuracy limitations must be recognized. The large standard errors (Sy.x, i.e., variation of y/zone size along each x/MIC value) associated with such linear regression analysis assumptions, combined with the zone size reproducibility of ±3 mm at best, implies that the extrapolated MIC may have an inherent error range of 3 to 4 dilutions. An extrapolated MIC of 4 µg/mL may mean a range of 1 to 16 µg/mL and may potentially incur errors that can vary from minor ones to serious major errors (false resistance) to very major errors (false susceptibility). The use of such MIC extrapolates as substitutes for quantitatively determined real MIC values is questionable especially for critical isolates and for bacterial populations that cluster around interpretive MIC break points.

3.2.4 INTERPRETATION

Zone diameters are converted into different susceptibility categories using the zone/MIC interpretive criteria from the most recent annually published CLSI M100 S series documents for disk diffusion using the appropriate tables for the organism being tested. Organisms are categorized as susceptible, intermediate, or resistant to the antibiotics tested. These categories are defined as follows:

- **Susceptible** — an infection caused by the organism will most probably respond to treatment with a standard dosage of the antibiotic as indicated for use for that infection.
- **Intermediate** — these results are usually regarded as indicative of nonuseful therapeutic options similar to the resistant category for treatment purposes. It also serves as a buffer zone to help prevent major categorical errors caused by slight changes in the zone sizes due to the influence of technical variables. However, infections at sites where the antibiotic is likely to be highly concentrated, such as β-lactams in the urinary tract, may respond to therapy.
- **Resistant** — the infection is not likely to respond to therapy.

Disk diffusion was initially developed for susceptibility testing of rapidly growing aerobic bacteria, including enterococci, staphylococci, Enterobacteriaceae, and *P. aeruginosa*. Modifications have been described by the CLSI for certain fastidious organisms including: *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, and *Streptococcus* spp. The inoculum for fastidious organisms should be prepared using the direct inoculation method. In general, these organisms are highly susceptible to most antibiotics tested and therefore, no more than 9 disks should be placed on a large 150-mm agar plate compared to the 12 disks that can be used when testing rapidly growing aerobic organisms. Table 3.1 provides a description of media, inoculum, and incubation conditions recommended.
3.2.5 Special Tests

In certain clinical situations, it may be appropriate to use disk diffusion to screen for certain resistance mechanisms. A single antibiotic can also be used as a group representative to predict susceptibility to agents belonging to the same class. The most commonly used disk screen is the oxacillin 1-µg disk to predict susceptibility of *S. pneumoniae* to β-lactams. All nonsusceptible results by the disk screen must be confirmed by a MIC method. MIC testing of *S. pneumoniae* is recommended as the primary method for sterile body site isolates.

Disks with a higher concentration of either gentamicin or streptomycin can be used to screen enterococci for high-level resistance to aminoglycosides (HLAR) to predict potential synergy or lack thereof between aminoglycosides and penicillin. Enterococci are generally resistant when tested with a standard disk content for aminoglycosides (30-µg disk). However, susceptibility to high-level gentamicin (120 µg) or streptomycin (300 µg) disks predicts potential synergy between that aminoglycoside and penicillin that may contribute to bactericidal effects with drug combination therapy, an important consideration for serious infections.

Two or more of the following disks — cefpodoxime, ceftazidime, aztreonam, cefotaxime, and/or ceftriaxone — can be used to screen strains of *E. coli*, *Klebsiella pneumoniae*, and *K. oxytoca* for the presence of extended spectrum β-lactamase (ESBL). Modified zone size criteria are provided by the CLSI for ESBL screening purposes. Disk diffusion can also be used to confirm the presence of ESBLs by using cefotaxime and ceftazidime disks with and without clavulanic acid. The combination disks initially had to be prepared by hand but are now commercially available (BD Diagnostics, Sparks MD; and Remel, Lenexa, KS). The test principle is based on the fact that most ESBLs will be inhibited by the β-lactamase inhibitor clavulanic acid, hence restoring susceptibility to the cephalosporin. Thus, an increase in zone size in the presence of the inhibitor compared with the cephalosporin alone by 5 mm or more is indicative of an ESBL-producing strain (Figure 3.6). If confirmed, the ESBL-producing strain should be considered and reported to be resistant to all β-lactam antibiotics.

Inducible resistance to clindamycin in gram-positive organisms can be detected by placing a clindamycin and an erythromycin disk 15–20 mm apart and observing for a flattening of the clindamycin zone on the side closest to the erythromycin disk (Figure 3.7) [22]. Detection of a flattened zone correlates with the presence of the *erm* gene and resistance to clindamycin even though it may appear susceptible to clindamycin by routine testing methods.

All organisms not mentioned above are not currently included in the CLSI recommendations for disk diffusion susceptibility testing and should be tested by a MIC method since either the procedure is not applicable or no interpretive criteria have been developed to date. The CLSI guidelines for the use of MHA for aerobic bacteria, unless otherwise specified, and the inoculum described should be strictly followed to achieve acceptable results.

![FIGURE 3.6 Detection of ESBLs using ceftazidime and cefotaxime disks plus or minus clavulanic acid.](image)
The most recent CLSI disk diffusion document, Table 1 and 1A [19], provide suggested groupings of antibiotics that could be tested as primary, secondary, and supplemental panels for aerobes and fastidious organisms, respectively. Disk diffusion testing with a group representative concept can be exemplified by the use of an erythromycin disk to predict susceptibility to all other macrolide antibiotics, including azithromycin and clarithromycin. Other antibiotic–organism combinations can also be tested, although caution should be taken to avoid combinations specifically contraindicated. The most common contraindications are listed in Table 3.2. If all these disk diffusion testing provisions have been met and problems still occur with the test, for example, quality control results are outside given specifications, the source of possible errors must be investigated and corrective action taken (Table 3.3) [23].

3.2.5.1 Etest

Etest is based on the use of a stable predefined gradient of antibiotic to generate precise MIC values. The gradient immobilized on the plastic strip is placed on an inoculated agar surface to transfer the antibiotic to the agar whereby a stable and continuous gradient is established under and alongside the strip. The stability of this gradient is maintained for up to 20 h. Etest differs from disk diffusion in that it is a preformed and stable gradient applicable for use with a wide range of aerobic and anaerobic organisms, both fastidious and nonfastidious, regardless of varying growth rates and critical times and testing procedures while disk diffusion is only suitable for rapidly growing aerobic bacteria. The key advantages of Etest are that it comprises a stable gradient, is an agar-based method, and can also be used in a macromethod format to optimize detection of antibiotic resistance. Furthermore, molecular properties of the antibiotic do not affect the performance of Etest since the
gradient is preformed and not dependent on diffusion. Thus, large molecules such as vancomycin and lipophilic compounds such as amphotericin that comprise a limitation for disk diffusion testing can be reliably tested with the Etest predefined gradient.

The procedure for inoculum preparation and inoculation of the agar plates is the same as that described for disk diffusion. The plate can also be efficiently streaked using a rota-plater e.g., Retro C80 (AB BIODISK) to obtain an even lawn of growth. After the plate is streaked, it is very important that the agar surface be allowed to dry completely before applying the strips. Etest strips can be applied to the agar surface by using either forceps, a manual single strip applicator, Nema C88, a vacuum-controlled pen, or Simplex C76, or an automatic multiple strip dispenser (Figure 3.8). All applicator devices are available from AB BIODISK.

Up to six strips can be optimally positioned on a large agar plate (150 mm) or one or two on a small agar plate (90 mm). Strips should be applied in an equidistant spoke-wheel pattern with the highest concentration positioned toward the edge of the plate.

The side of the strip carrying the antibiotic gradient must be placed in contact with the agar surface (MIC scale facing upward). Templates provided by AB BIODISK can be used to facilitate the optimal positioning of strips (Figure 3.9).

Because of the immediate release of the antibiotic, the strip cannot be moved once it is in contact with the agar surface. However, if the strip fell with the MIC scale side facing down, it can be picked up, turned over and reapplied correctly. The plate is incubated in an inverted position (agar side up) under conditions appropriate for the organism. Incubation conditions for most organisms are the same as for disk diffusion (Table 3.1). Information on appropriate inoculum, media, and incubation conditions for different organism groups are provided in the product inserts and the Etest Media, Inoculum and Incubation Guide provided by AB BIODISK.

**TABLE 3.3**

<table>
<thead>
<tr>
<th>Result</th>
<th>Possible Cause(s)</th>
<th>Suggested Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zones too small</td>
<td>Inoculum too heavy</td>
<td>Use McFarland standard or calibrator to carefully measure inoculum density and perform colony counts</td>
</tr>
<tr>
<td></td>
<td>Agar too thick</td>
<td>Measure agar depth carefully</td>
</tr>
<tr>
<td></td>
<td>Disk expired or inactive</td>
<td>Use new lot of disks or unopened cartridge</td>
</tr>
<tr>
<td></td>
<td>Inoculated plates left too long prior to application of disks</td>
<td>Apply disks within 15 minutes</td>
</tr>
<tr>
<td></td>
<td>Wrong medium for organism</td>
<td>Follow NCCLS guidelines for appropriate choice of media, perform quality control</td>
</tr>
<tr>
<td>Zones too large</td>
<td>Inoculum too light</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Agar too thin</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Poor growth (too fastidious, wrong media, not fresh isolate)</td>
<td>Same as above</td>
</tr>
<tr>
<td>Single disk out of control</td>
<td>Improper storage of disks</td>
<td>Maintain majority of disk stock at −20°C, only keep maximum of 1 week supply at 4°C (be cautious with β-lactams, clavulanic acid containing disks, and imipenem)</td>
</tr>
<tr>
<td></td>
<td>Media pH too low or too high</td>
<td>Particularly effects tetracycline macrolides and clindamycin (CO₂ incubation can decrease pH)</td>
</tr>
<tr>
<td></td>
<td>Cation concentration too low</td>
<td>Especially effects aminoglycosides and <em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>Transcription or reading error</td>
<td>Reread or reset the test</td>
</tr>
<tr>
<td>Colonies within zone</td>
<td>Mixed population</td>
<td>Reisolate or Gram stain the colonies</td>
</tr>
<tr>
<td>Deformation of zone</td>
<td>Disks too close to each other</td>
<td>Place fewer disks on plate (especially with very susceptible organisms)</td>
</tr>
</tbody>
</table>

In Table 3.3, the possible cause(s) and suggested resolution for various results of disk diffusion troubleshooting guide are listed. The guide is useful for troubleshooting the disk diffusion test.
Antimicrobial Susceptibility Testing Protocols

technical guides and organism-specific application sheets are also available to facilitate the use of Etest. These guides can be downloaded from the AB BIODISK Web site (www.abbiodisk.com) or requested from AB BIODISK directly or the local Etest distributor.

After incubation, a parabolic shaped inhibition zone centered alongside the test strip is seen. The MIC is read at the point where the growth/inhibition margin of the organism intersects the edge of the calibrated strip (Figure 3.10).

MIC endpoints for most antibiotic–organism combinations can be easily read visually. A magnifying glass and/or tilting the plate can be helpful for visualizing microcolonies and hazes or
other colonies within the inhibition ellipse when reading the end points of bactericidal drugs and/or when detecting different resistances such as heteroresistance to oxacillin in *S. aureus*.

Specific guidelines are used for reading different types of Etest results depending on the mode of action of the antibiotic or antifungal agent. End points for bacteriostatic drugs are read at 80% inhibition and bactericidal drugs at 100% inhibition. Comprehensive guidelines for reading of an array of end points are provided in Etest reading guides that contain color pictures of a wide range and variety of results for different organisms, drugs, and resistance mechanisms. Incubation of capnophilic organisms such as pneumococci, streptococci, gonococci, and *Haemophilus* spp. is done in 5% CO₂. The resultant acidification and decrease in pH can affect the activity of certain antibiotics such as macrolides. The MIC values of azithromycin and clarithromycin may be increased by one to three dilutions under CO₂ conditions compared with ambient incubation, and MIC interpretive criteria and quality control specifications adjusted for CO₂ according to the manufacturer’s instructions should be used whenever appropriate.

Because of the continuous concentration gradient, Etest MIC values can fall in between conventional twofold dilution values. The MIC should be reported as the exact value obtained, for example. 0.38 µg/ml; however, it should be rounded up to the next higher 2-fold dilution value, that is, 0.5 µg/mL (Figure 3.11) before using CLSI interpretive criteria for susceptibility categorization.

A major advantage of Etest is that it can be used as a macromethod because it is "inoculum tolerant" to maximize the detection of resistance mechanisms that may be heterogeneous and expressed at varying levels. A common example of heterogeneous resistance is oxacillin resistance in staphylococci. Laboratories may choose to confirm equivocal oxacillin results from their routine systems using an alternative MIC method such as Etest. Inducible β-lactamases in gram-negative organisms such as *Enterobacter*, *Serratia*, and *Citrobacter* species can also be difficult to detect in standard methods. Resistance to cephalosporins may be falsely reported as susceptible in automated systems, which use a lower inoculum, or falsely resistant if the organism produces excessive filaments. Equally, labile compounds such as imipenem may give unexpected resistant results, in both based methods due to freeze–thaw effects.

Certain antibiotics can be problematic to test with the disk diffusion method because of specific physicochemical properties of the molecule. Glycopeptides such as vancomycin have a high molecular weight (1450) and diffuse very slowly in agar. The limited diffusion and poorly resolved concentration gradient around a vancomycin disk result in only a few millimeter difference in zone size for separating susceptible and resistant strains, thus, results may not be reliable. Because of this limitation, the Centers for Disease Control and Prevention recommends that an MIC method like Etest be used for vancomycin testing with staphylococci for detection of reduced susceptibility

![Image](image.png)

**FIGURE 3.11** Interpretation of Etest half dilution MICs using penicillin MICs for *Streptococcus pneumoniae* as an example.
to vancomycin [24]. Although standardized MIC methods can detect strains with higher level resistance to vancomycin (MICs $\geq 8 \mu g/mL$), strains with lower MIC values (4–8 $\mu g/mL$) (intermediate and/or heterogeneous resistant strains) will be better detected using a richer medium like brain–heart infusion agar, a higher inoculum, and extended incubation as described for the Etest macromethod for vancomycin intermediate $S. aureus$ (VISA) and hetero-VISA (h-VISA) detection [25, 26]. Hiramatsu [27] first reported that a clinical strain of $S. aureus$ (Mu3) heteroresistance to vancomycin (h-VISA) was a precursor strain to the more stable resistant variant Mu50 (VISA), which was associated with clinical failure. More evidence has been generated since then to suggest that the heteroresistant phenotype can be clinically significant in different therapeutic situations. Several studies have demonstrated the limitations of disk diffusion and automated systems for testing of $S. aureus$ against vancomycin [28]. Walsh et al. [29] also demonstrated the limitations of most standardized MIC and screening methods for the detection of vancomycin resistance in staphylococci. A modification of the Etest procedure using an inoculum density equivalent to a McFarland 2.0 and brain–heart infusion agar achieved a sensitivity and specificity of 98% when compared with population analysis profiling as the reference method [26]. CLSI also recommends that all enterococci found to have intermediate susceptibility to vancomycin by disk diffusion testing be confirmed with an MIC method. Using a combination of on-scale MIC values to vancomycin and teicoplanin, vancomycin-resistant enterococci (VRE) can also be differentiated into the various phenotypes ($vanA$, $vanB$, and $vanC$).

Unique Etest gradient formats are also available for detecting specific resistance mechanisms such as extended spectrum-lactamases (ESBLs) and metallo-β-lactamases (MBLs). Etest ESBL strips comprise double-sided gradients of either cefotaxime or ceftazidime across seven dilutions on half of the strip and a gradient of the same drug overlaid with a constant concentration of clavulanic acid on the other half. A decrease in MIC of at least three twofold dilutions in the presence of clavulanic acid compared with the parent cephalosporin alone or the presence of a “phantom” or deformed zone along the strip confirms the presence of an ESBL (Figure 3.12 and Figure 3.13).

![FIGURE 3.12 Positive ESBL result (top, cefotaxime, cefotaxime/clavulanic acid) and negative ESBL result (bottom, ceftazidime, ceftazidime/clavulanic acid) and overall ESBL positive confirmation.](image-url)
The Etest MBL strip will be an important tool for tracking the emergence of MBLs in gram-negative aerobic bacteria and also in anaerobes [30]. The MBL strip based on the same configuration as the ESBL strip uses imipenem gradients with and without a zinc chelator EDTA (Figure 3.14). Since MBL enzymes require zinc as an activity cofactor, removing zinc will reduce the activity of the enzyme and restore imipenem activity. Similarly, a decrease in imipenem MIC by at least three dilutions or a deformation of the ellipse or a phantom zone is indicative of a MBL.

Because of the stability of the concentration gradient, Etest can also be used for testing very slow growing organisms, fastidious organisms, and a wide variety of clinically significant organisms that belong to many species. Clinical situations often warrant susceptibility testing of rarely encountered, unusual, and/or opportunistic pathogens for which no CLSI guidelines are available. Scientific references can be used to guide the choice of appropriate media, incubation conditions, and antibiotics to test, and MIC values can be reported to the clinician without providing any interpretations. The electronic version of the Etest reference list that can be provided by AB BIODISK is a useful source of information for these unusual applications. Interaction with the physician is essential to ascertain the necessity for susceptibility testing and which agents to test, and to aid in
the understanding of the MIC value itself. Etest can also be applied to susceptibility testing of yeast, molds and mycobacteria. Although mold and microbacteria applications are not cleared for clinical use in the United States by the FDA, equivalence to CLSI and other reference methods has been demonstrated [31,32,34]. The use of a wide concentration range MIC method (15 dilutions) in an agar-based system that provides good growth of most organisms would be advantageous for obtaining quantitative MIC information in difficult clinical situations.

Clinical laboratories must differentiate between CLSI recommendations of reference methods in contrast to the regulatory role of the FDA, which clears the clinical use of commercial test systems. Each commercial manufacturer has to demonstrate that the performance of their product is substantially equivalent to the CLSI reference method, based on comprehensive testing criteria as required by the FDA. CLSI does not “approve” or endorse any commercial test system, and this is clearly outlined in their documents. However, the CLSI also states that alternative methods like the “antibiotic gradient” may be used in place of CLSI reference methods if shown to be substantially equivalent. Additional research applications of Etest include antibiotic combination testing [35], determination of minimum bactericidal concentrations (MBC), kill curve assays, and postantibiotic effect measurements [36].

3.3 QUALITY CONTROL

The correct use and accuracy of any susceptibility test system is dependent on good quality control (QC) practices. Quality of the reagents, media, and testing procedures, and personnel competency must be assessed on a regular basis. It is important that QC data are retained in such a way that deviation trends can be quickly and easily observed. Patient data cannot be released if results for QC strains are out of specification. QC testing should be performed in the same manner and by the same personnel who are testing patient isolates.

3.3.1 QUALITY CONTROL STRAINS: USE AND STORAGE

The CLSI provides guidelines for the selection of QC strains and frequency with which QC should be performed for both disk diffusion and MIC testing [19]. Guidelines for MIC testing should be used for QC of Etest. QC strains chosen for MIC testing should be such that the expected MIC value for the strain falls in the middle of the concentration range for that antibiotic. QC specifications for each Etest reagent are also provided in the product package insert. QC strains can be purchased in a dehydrated form either as Culti-loops (Remel), pellets, or swabs (KWIK-STIK, Hardy Diagnostics, Santa Maria, CA), or lyophilized in multiple unit containers (LYFO-DISK, MicroBioLogics, St. Cloud, MN). After processing according to the manufacturer’s instructions, organisms should be maintained on an agar slant, stored at 2–8°C, and can be subcultured weekly for up to 1 month, after which a new QC stock organism should be used. QC organisms can be stored for prolonged periods of time at −70°C in broth (such as brain–heart infusion or brucella) or skim milk. Strains should be subcultured at least twice after the initial rehydration or removal from frozen stocks before being used for QC testing. Testing of QC strains should be performed either on each day of clinical testing for infrequently performed tests or weekly after qualifying an initial 30-day QC specification. CLSI provides flow diagrams for frequency of QC testing, troubleshooting, and corrective action for out-of-control results.

3.3.2 REAGENT HANDLING AND STORAGE

Antibiotic disks are available from several manufacturers (Becton Dickinson, Remel, and Hardy Diagnostics). They should be strictly handled and stored according to the manufacturer’s instructions. Short-term (within one week) disks can be stored at 4°C in disk dispensers containing desiccant for day-to-day use. Long-term storage of unopened cartridges should be at −20°C, and
it is preferable to store all β-lactam antibiotics at −20°C. Packages should be allowed to reach room temperature prior to opening (BBL package insert). Etest is manufactured by AB BIODISK (Solna, Sweden) and is marketed and sold in the United States through their subsidiary in Piscataway, NJ. Etest reagents are provided in packages of 100 strips or 30 for some items. Unopened packages should be stored at −20°C until their expiry date, and the shelf life of most Etest products is longer than 3 years. Prior to use, the package is removed from the freezer and allowed to reach room temperature before opening the package (approximately 30 min if stored at −20°C and longer if stored at −70°C). Once opened, the remaining unused strips from the package must be stored in a storage tube or other airtight container with desiccant. Laboratories may find it convenient to store the Etest strips in an applicator tray with antibiotic selections for specific organism applications. The tray with Etest strips can be stored in a plastic container with desiccant such as a Tupperware box, and can be placed directly in the freezer (Figure 3.15). Strips from opened packages, if appropriately stored at −20°C, have the same expiration date as the unopened package.

3.4 CONCLUSIONS

Disk diffusion is a relatively “inexpensive,” easy to use, flexible agar-based method that provides qualitative results for rapidly growing aerobic bacteria. However, disk diffusion is based on the use of a relatively unstable antibiotic concentration gradient generated from a point source diffusion center, that is, the disk. Many in vitro variables such as prediffusion, inoculum density, and growth phase, and variations in the agar plate, for example, depth, can directly influence zone sizes and therefore give unreliable results. Larger zones will be obtained with slow growing, fastidious organisms compared with rapidly growing aerobic bacteria directly as a consequence of a longer critical time and not necessarily a lower level of susceptibility. High molecular weight compounds such as vancomycin and polymyxins will not diffuse well in agar and are therefore difficult or inappropriate to test by disk diffusion.

Disk testing is an indirect qualitative method that has to be calibrated against reference MIC methods to generate clinically meaningful zone size–MIC-based interpretive criteria. Like most break-point susceptibility testing methods and broth microdilution systems with limited dilution ranges, the disk method provides categorical results and not quantitative on-scale MIC values. Automated systems, which frequently report results as greater than or less than a numerical value,
are just as qualitative as disk diffusion. Disk diffusion is suitable for testing rapidly growing aerobic bacteria isolated from nonsterile body sites. Use of an MIC method and preferably with a wide concentration range would be more appropriate to obtain exact MIC values for isolates from critical patients and critical specimens.

Etest complements disk diffusion testing and other methods currently used for routine susceptibility testing. Since Etest is based on the use of a stable preformed gradient, it can be used for testing a wide range of fastidious and/or slow growing organisms. This is reflected in the FDA clearance of Etest for both gram-positive and gram-negative aerobic bacteria, including gram-negative nonfermenters, anaerobic bacteria, pneumococci, streptococci, gonococci and Haemophilus; Etest is the only system cleared for gonococci. It has also been cleared for antifungal testing of Candida spp. As a macromethod that is inoculum tolerant, it is suitable for detection of low-level, inducible, or heterogeneous mechanisms of resistance such as penicillin resistance in pneumococci, inducible β-lactamases in gram-negative aerobes, and vancomycin or oxacillin heteroresistance in staphylococci. Treatment of serious life-threatening infections and management of critical patients often require targeted therapy where the choice and dosage have to be optimized using the MIC and PK/PD data. For these situations, Etest is a valuable therapeutic tool that can provide an exact and accurate MIC value for PK/PD-targeted selection of both the antibiotic and the dosage regimen, that is, fine-tuning of therapy for critically ill patients as described in the study by Mohr, Wanger, and Rex [37].

REFERENCES


4 Macro- and Microdilution Methods of Antimicrobial Susceptibility Testing

Sadaf Qaiyumi

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4.1 INTRODUCTION

Sensitivity and resistance patterns of microorganisms are constantly changing, and many microorganisms have developed resistance even to the newest antibiotics. Since susceptibility patterns are unpredictable, it is necessary to test isolated pathogens against appropriate antimicrobial agents. The clinical microbiology laboratory plays an important role in assisting physicians in determining an appropriate treatment for an infectious disease. Therefore, once the organism has been isolated and identified from a patient’s specimen, it is necessary to determine an antimicrobial susceptibility profile of the isolated organism. With this information, the clinician can choose the appropriate antibiotic to be used in treating the patient.

The lowest concentration of an antibiotic that will inhibit the growth of the organism being tested is known as the minimal inhibitory concentration (MIC). The MIC may assist a physician in deciding the concentration of the antibiotic needed to inhibit the pathogen. The MIC test can be performed on agar or on liquid medium. The traditional method of determining the MIC is with the broth dilution technique, where serial dilutions of antibiotic are incorporated into the broth media in either the wells of microtiter plates or in culture tubes. The concentration of antibiotic used may vary with the antimicrobial, organism identification, and site of infection. Each tube or well contains a different concentration of the antimicrobial agent and is inoculated with a fixed amount of the organism being tested. After appropriate incubation, the lowest concentration showing
no visible growth is considered as the MIC. A complete guide to this protocol can be found in the Clinical Laboratory Standards Institute (CLSI) guidelines document M7-A7 [1]. This is a quantitative test, the result of which is expressed in micrograms per milliliter (μg/mL).

In testing for antimicrobial susceptibility, the laboratory must maintain a high level of accuracy and reproducibility in the testing procedures. Because of varying conditions, such as, inoculum size, composition of the culture media, incubation time, temperature, pH, etc., a dilution test may not provide the same end point each time [2]. Generally, the acceptable reproducibility of the test is within a two-fold dilution of the actual end point. There must be a good correlation between the result and the clinical response of the patient. Although, at times, an antimicrobial agent that shows poor in vitro activity against a pathogen is used in a patient with good results; the opposite effect may also occur.

4.2 MACROBROTH DILUTION METHOD

The macrobroth dilution method was among the first to be developed and still serves as a reference method. This assay is generally performed in glass test tubes containing a broth volume of >1 mL. Two test tubes containing broth without antimicrobial agent added should be included in each test. One of these tubes should be inoculated with the test organism; the other should be left uninoculated and will serve as a check for media sterility.

4.2.1 PREPARATION OF ANTIBIOTIC SOLUTION

The antibiotic stock solutions should be made to a final concentration of 10 mg/mL or 10 times the highest concentration to be tested and then diluted to an appropriate concentration in broth. It is very important to follow the manufacturer’s guidelines when preparing stock solutions of antimicrobial agents. Sterile water is generally used in the preparation of the antibiotic solutions. There are some antibiotics that have limited solubility; therefore, a stock solution of lower concentration should be prepared for such antibiotics. Other antimicrobials require solvents other than water. If such is the case, use a minimal amount of the recommended solvent and make further dilutions with water. For example, rifampin, erythromycin, or chloromphenicol are insoluble in water, but they dissolve readily in 95% ethanol. Therefore, a minimum volume of ethanol should be added dropwise to the antibiotic, until the powder dissolves; the final volume is then brought up in water.

Antibiotic powder obtained from the manufacturer is not always 100% pure. Therefore, prior to making a stock solution, it is important to ensure the potency of each antibiotic. The following formula may be used to determine the amount of antimicrobial needed for the desired volume:

\[ \text{Weight (mg)} = \frac{\text{volume (mL)} \times \text{desired concentration (μg/mL)}}{\text{antibiotic potency (μg/mg)}}. \]

Therefore, for 3 mL of 10 mg/mL solution of drug X with 99.4% potency (994 μg/mg)

\[ X = \frac{3 \times 10,000}{994}. \]

Weigh out 30.2 mg and dissolve it in 3 mL of sterile distilled water.

Another way to achieve the required concentration is to weigh out the desired amount (or slightly more) of antimicrobial powder and then determine the exact volume of the diluent by using the following formula. It is recommended to weigh 10 mg of the powder or more to improve the accuracy.

\[ \text{Volume (mL)} = \frac{\text{weight (mg)} \times \text{potency (μg/mg)}}{\text{concentration (μg/mg)}}. \]
Weigh out drug $X$ (e.g., 10 mg)
Drug purity is $994 \, \mu g/mL$
Desired concentration is $10,000 \, \mu g/mL$

\[
\text{Volume} = \frac{10 \times 994}{10,000}.
\]

Dissolve 10 mg of drug $X$ in 0.994 mL of sterile distilled water to get 10 mg/mL of drug $X$.

The stock solutions can be further diluted in broth (e.g., Mueller-Hinton) to the highest concentration needed to test the antibiotic by following the formula $M_1V_1 = M_2V_2$. Suppose a working solution of $256 \, \mu g/mL$ is desired in $5 \, mL$. Then

\[
M_1V_1 = M_2V_2.
\]

\[
10,000 \, \mu g/mL \times V_1 \, mL = 256 \, \mu g/mL \times 5 \, mL.
\]

Therefore, $0.128 \, mL (128 \, \mu l)$ of the 10,000 $\mu g/mL$ stock solution should be suspended in 4.872 mL of broth.

Small volumes of the stock solution can be stored at $-70^\circ C$ in freezer vials, without the loss of any activity. Solutions may be stored for up to 6 months unless otherwise indicated in the manufacturer’s package insert. Frozen stocks should be thawed the day of use, and any left over is generally discarded.

**4.2.2 Mueller-Hinton Broth**

Mueller-Hinton Broth Cation adjusted is a specially formulated medium for susceptibility testing. It is recommended by the CLSI for testing most commonly encountered aerobic bacteria found in food and clinical samples. The medium demonstrates fairly good batch-to-batch reproducibility for susceptibility testing; it is low in sulfonamide, trimethoprim and tetracycline inhibitors, and yields satisfactory growth of most pathogens.

The amount of divalent cations should be standardized in the Mueller-Hinton broth. The susceptibility testing of antibiotics like aminoglycosides and tetracyclines is greatly affected without the supplementation of these ions. Cation-adjusted Mueller-Hinton broth can be easily purchased from various commercial sources. If the broth does not contain the appropriate concentration of $Ca^{2+}$ and $Mg^{2+}$ (20–25 mg of $Ca^{2+}$/L and 10–12.5 mg of $Mg^{2+}$/L), then an adjustment is recommended. For the supplementation of cations in Mueller-Hinton broth, a 10 mg/mL stock solution of CaCl$_2$ and MgCl$_2$ is prepared. Magnesium stock solution is prepared by dissolving 8.36 g MgCl$_2$·6H$_2$O in 100 mL of deionized water, and calcium stock solution is prepared by adding 3.68 g of CaCl$_2$ to 100 mL of deionized water. The stock solutions should be filtered, sterilized, and chilled before addition to the broth.

Before the addition of cations, it is important to know the cation contents of the Mueller-Hinton broth because unnecessary supplementation may cause false results. If the Mueller-Hinton broth contains no cations, then 2 mL of calcium stock solution and 1 mL of magnesium stock solution can be added to 1 L of cooled Mueller-Hinton broth. The pH of each batch of Mueller-Hinton broth should be checked and must lie between 7.2 and 7.4. Before use, each batch of media should be checked for its ability to support the growth of the most relevant control strains.

**4.2.3 Procedure**

Label sterile capped 13 × 100 mm test tubes 1 through 11:
Antimicrobial Susceptibility Testing Protocols

a) Pipette 0.5 mL of Mueller-Hinton broth into tubes 2–11.
b) Pipette 0.5 mL of antibiotic solution into tubes 1 and 2.
c) Transfer 0.5 mL from tube 2 to tube 3 and continue through tube 9. Be certain to changepipettes between tubes to prevent carryover of antibiotic.
d) Discard 0.5 mL from tube 9. The tenth tube, which serves as a control, receives noantibiotic.
e) Select 4–5 isolated colonies from an overnight culture and dilute in broth to a turbiditycomparable to that of a 0.5 McFarland turbidity standard (approximately $1 \times 10^8$ CFU/mL).This suspension is further diluted 1:100 ($10^6$ CFU/mL) with sterile water, 0.85% saline,or broth. Within 15 min of the preparation of this inoculum add 0.5 mL of bacterial broth suspension to each tube except the eleventh (last) tube, which is the broth control tube. The final inoculum concentration of $10^5$ CFU/mL is achieved. Note that the final concentration of the antibiotic is now one half of the original concentration in each tube. If there is unavoidable delay in inoculation, the inoculum can be stored at 4°C for up to2 h. Streak a 10 μl loopful of suspension from the growth control tube onto a sheep blood agar plate (or other appropriate medium) and incubate overnight to check the purityof the test isolate and correct inoculum density. The presence of approximately 50colonies would indicate an inoculum density of $5 \times 10^5$ CFU/mL.

4.2.4 Incubation

All assay tubes should be incubated overnight in an ambient air incubator at 35–37°C. Duringincubation, care should be taken to ensure adequate humidity. With methicillin-resistant staphylococci and vancomycin-resistant enterococci, a 24-h incubation period is recommended.

4.2.5 Control Strain

When testing a patient isolate, the corresponding ATCC quality control (QC) organism should be testedconcurrently. To consider a test result valid, the MIC of the QC organism must fall within the acceptablelimits for quality control strains stated in the CLSI guidelines the M100-S16 document [3].

4.2.6 Result Interpretation

The lowest concentration of the antimicrobial agent that will inhibit the growth of the microorganismbeing tested as detected by lack of visual turbidity, matching with a negative control included with the test, is known as MIC. The MIC of each antimicrobial agent is recorded in microgram per milliliter (mg/mL). Before reading and recording the results obtained with clinical isolates, it isrecommended that the tubes be shaken gently before performing a reading. The tubes containing the QC strain should be checked to ensure that their values are within the acceptable range for thatantibiotic. In addition, the antibiotic-free growth control tube should be examined for the viabilityof the test isolate. This is needed to ensure that the culture conditions, such as media, temperature,etc., were suitable for the growth of the organism. The broth control tube included in the assaycontains everything except the inoculum of test strains. This control tube should remain clear (no growth). Growth in this tube is an indicator of contamination of one of the components of the test system. In that case, the assay is invalid and should be repeated.

When using an antibiotic such as sulfonamide or trimethoprim, care should be taken in deter-mining the end point because a trailing effect may be observed as a result of drug carryover with the inoculum. Therefore, the MICs of these antimicrobial agents should be interpreted as the endpoint at which an obvious 80% or more reduction of growth occurs when compared with the growth control tube. A trailing effect is also possible with bacteriostatic drugs such as chloromphenicol and tetracycline.
4.3 MBC DETERMINATION

The minimal bactericidal concentration (MBC) is defined as the lowest concentration of an antibiotic killing the majority (99.9%) of a bacterial inoculum. Since MIC is the ability of inhibitory status, it is possible that if the antibiotic is removed, the microorganism will begin to grow again. In some infections (i.e., evclocaditis), it is necessary to actually kill the microorganism. To determine the ability of the antibiotic to kill the microorganism, a growth test can be performed. This test is referred to as the MBC.

4.3.1 METHOD

Following a broth dilution MIC test, from each tube that shows no growth, remove 50 μL of suspension and spread it onto sheep blood agar plates or other appropriate medium. Incubate the plates overnight at 37°C. The number of colonies growing from each of the test tubes is counted and the number of colonies corresponding to a thousand-fold reduction (as compared to the colony count of the start inoculum) is recorded as the MBC (99.9% killing).

4.3.1.1 Microbroth Dilution Method

The broth dilution method is an adaptation of the broth dilution method using small volumes for routine testing. It utilizes microtiter plastic plates containing 96 wells. The advantage of the system is that it utilizes small volumes of reagents and allows a large number of bacteria to be tested relatively quickly.

4.3.2 WELL VOLUMES

With the exception of those wells acting as drug-free controls, each well should receive either 100 μL or 50 μL of a two-fold dilution series of the antibiotic solution. Each well, except those acting as sterility controls, should then receive either 100 μL or 50 μL of bacterial suspension (containing 10^5 CFU). Note that this protocol results in a final concentration of the antimicrobial agents in the assay wells being twofold lower than in the aliquots added to the wells. Each plate should contain a column of wells that contain no antimicrobial agent (A12–H12). A plate should also include one row containing the full dilution series inoculated with a relevant control strain (A1–A11) and one column inoculated with the sterile 0.9% saline used to prepare the bacterial suspensions (H1–H11).

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Automated Systems: An Overview

Sheryl Stuckey

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5.1 INTRODUCTION

One of the most significant tests performed by the clinical microbiology laboratory for the management of infectious disease is antimicrobial susceptibility testing. Pathogenic organisms that are resistant to frontline antimicrobial agents are constantly emerging, and laboratory technicians and clinicians are more concerned than ever with the resistance patterns of these organisms.

Antimicrobial resistance and the potential of these organisms to acquire resistance dominate the infectious disease arena. In this age of diagnosis-related groups, local medical review policies, managed care health plans (HMOs, IPOs, PPOs, etc.), and budgetary constraints, clinical microbiologists
are faced with the challenge of identifying etiological agents quickly, efficiently, accurately, and at a relatively low cost.

Today, a myriad of automated susceptibility testing instruments are incorporated into many institutions. These instruments offer greater sensitivity and speed than manual methods, and aid in the interpretation of antimicrobial susceptibility test endpoints. To alleviate ambiguity, it is important that automated instruments be standardized at regular intervals.

5.2 CONSIDERATIONS IN CHOOSING AN AUTOMATED SYSTEM

Several issues must be considered in determining the appropriate automated instrument for antimicrobial testing:

5.2.1 LABORATORY TEST UTILIZATION

What level and volume of antimicrobial testing do you currently perform, and what is the result turnaround time required by the clinicians you serve? Depending on your staffing level, case mix, and organism antibiogram, if you are doing less than 10 tests per day, the cost of automation may not be warranted. Are your manual results within the acceptable range for accuracy, and do they correspond with patient treatment outcomes? If so, do you need to change?

5.2.2 CASE MIX AND ORGANISM ISOLATION RATES

Is your institution’s case mix primarily acute or chronic? If it is acute, do you serve a large percentage of patients for whom infection can be rapidly fatal? Are you isolating significant numbers of multidrug-resistant or difficult to treat pathogens? If you are in an acute setting with potentially high morbidity, limited infection control isolation rooms, or significant numbers of potentially communicable pathogens, the cost savings to the patient and the institution on the treatment side may outweigh the expense of automating the laboratory. However if, on the other hand, you are primarily performing urine cultures and the predominant isolate is a multidrug-susceptible *Escherichia coli*, do you need automation?

5.2.3 ANTIMICROBIAL UTILIZATION AND PRACTICE GUIDELINES

What are the primary antibiotics used by the institution? Consultation with local infectious disease physicians and clinical pharmacy personnel is essential.

Are there clinician practice guidelines in place that require the use of particular agents and/or that forgo the performance of specimen culture? Such guidelines can be a determining factor in whether you need automation because of their impact on test volume.

5.2.3.1 Methodology

Based on the predominant classes of your isolates, will the instrument methodology be compatible with your needs and current reporting options, or will it require extensive training and education of laboratory personnel and clinicians? If you are currently using Kirby-Bauer methodology, you must consider the time and energy involved in learning new methodologies such as broth microdilution. If you are already using broth microdilution, do you require automation of the inoculation process, the reader process, or both? Will automation be labor saving or labor intensive?

5.2.3.2 Instrument Ease of Use

How difficult is the instrument to use? Are frequent calibrations or maintenance checks required? Are the steps to process or troubleshoot tests straightforward? If you must spend significant amounts
of time preparing the instrument for processing tests or if downtime of the instrument is frequent and troubleshooting cumbersome, the automation process may cause delays in patient treatment.

5.2.3.3 Instrument Performance

If you test an *E. coli* that is known to be susceptible to a given level of ampicillin, will the instrument accurately interpret the reading, and if not, with what frequency will it fail? What level of failure is acceptable to you, and what level will be acceptable to your risk management department?

5.2.3.4 Instrument Breadth of Line

Can you test all of your common isolates on the instrument? Can you test all of the formulary antibiotics using the automated method? How many times will you have to perform additional testing because of test accuracy problems associated with certain drug versus bug combinations?

5.2.3.5 Instrument Disposables

If cost is a factor, both the supplies and time of automated testing must be taken into account. In addition, the type and number of disposables involved in doing each test should be considered. Each disposable involves some time and expense in its handling, and this can increase labor costs. If reagents are used, you must also consider potential chemical hazards and disposal costs.

5.2.3.6 Instrument Turnaround Time to Result

Once the automated antimicrobial system is inoculated, how quickly do you need or can you get the result? Do you want your results rapidly? If so, what is your definition of rapid? Will speed or accuracy have any measurable effect on patient outcome?

5.2.3.7 Personnel and Regulatory Considerations

Regulatory issues can impact your decision to acquire automation. What are the licensure requirements? Depending on the process you are automating, what is the regulatory complexity of your testing? Given the previous considerations of methodology and ease of use, what level of personnel proficiency and education is required for your testing?

5.3 EVALUATING AN AUTOMATED SYSTEM

Now that you have decided to invest in automated antimicrobial resistance testing, how do you go about evaluating which test system is right for you?

5.3.1 Search for References

Clinical laboratories may choose among several automated instruments to assist in their routine work. However, before a purchase is made, it is important to obtain written references (journal articles, monographs, research data, etc.) and a list of long-term current and former users. The company representative can assist you with this, but it is important to conduct your own research. You should focus your search on the needs of your particular institution.

5.3.2 Choice of Test Isolates

The key to evaluating your choice of instrumentation is knowing your institution’s antibiograms. An antibiogram is the overall antimicrobial susceptibility profile of a bacterial isolate to a battery of antimicrobial agents. Furthermore, it may be useful to take notes of isolation trends that may
adversely affect the mix of isolates and their antimicrobial resistance patterns. Below is a list of the types of organisms that should be included in your evaluation:

- Common predominant wild type organisms (those naturally occurring in the environment) found in your institution (include the range of possible antimicrobial resistance levels for each antibiotic of interest)
- Infrequently isolated wild type organisms seen in your institution
- Clinically relevant organisms appropriate for the class of antibiotics of interest (including $\geq 35$ known resistant isolates for each antibiotic tested)
- Organisms that the evaluation instrument and any competing instrument under consideration have been noted to have difficulties with interpretation, ease or speed in their interpretation
- Quality control organism required for the type of testing
- Quality control organisms required by the manufacturer

Finally, care should be taken to avoid wild type isolates from the same source unless there is a significant change in the resistance level beyond the acceptable interpretative standard deviation of the method employed.

5.3.3 Preparing Isolates for Evaluation Testing

Whenever possible, it is best to prepare evaluation organism sets; this is particularly important when evaluating more than one instrument under consideration so as to remove the possibility of test organism bias. The fairest comparative evaluation of two instruments is to challenge both with the same test isolate. However, this may be difficult to do if careful planning (with regard to the timing of instrument evaluations) is not done prior to the commencement of the evaluation process. Test isolates may be prepared in the following manner:

- Perform antimicrobial resistance testing on the isolate using the current system employed by your laboratory; record and maintain the results for future use.
- Prepare an 18–24-h broth culture of the isolate in appropriate broth media.
- After incubation, subculture the broth to a second broth media and reincubate for at least 18–24 h.
- After incubation, subculture the broth to the appropriate solid agar media and incubate at the organism's optimal temperature and atmosphere for at least 18–24 h.
- After incubation, use well isolated colonies to prepare a stock culture of the organism using the method employed by your laboratory.

5.3.4 Performing the Evaluation

You should obtain the assistance of the manufacturer’s personnel (i.e., the clinical applications or technical specialist) for guidance on the use of the automated instrument. Quality control organisms should be used to determine the performance characteristics of the automated instrument and to demonstrate the reproducibility of results. All quality control activities should be recorded.

In order to have a sufficient variety and number of isolates to detect the performance errors of the automated system, it is suggested that you plan to test isolates equivalent in number to at least 5–10% of your normal annual workload or at least a minimum of 100 isolates exclusive of the required quality control organisms.

Isolates from stock cultures should be prepared for testing in accordance with the guidelines of the laboratory (frozen isolates should be subcultured at least twice before testing). Unless the manufacturer’s media or organisms preparation requirement dictate otherwise, the same culture and/or inoculum should be used for concurrent testing with the current antimicrobial resistance test method (reference method).
and the automated method being evaluated (test method). The manufacturer’s instructions should be strictly adhered to during the performance of testing. Upon the completion of testing, the results should be recorded using the system employed by the laboratory for the current test system and that of the manufacturer for the evaluation instrument, and should be maintained for review.

5.3.5 EVALUATION OF RESULTS

Now that you have completed your testing, you must carefully review the results in the context of those factors you considered important in choosing an automated antimicrobial testing system. You can use the test system to challenge data if there are no organisms between the previous reference system results and the current reference system results. It is important to compare your previously maintained reference system results with the current reference system result and look for significant changes that may have resulted from long-term storage and/or repeated subculture of the organism. The test and the reference system must be in categorical agreement (test and reference method achieve the same result) for a given organism versus antibiotic challenge. The degree and frequency of the following errors must be determined by using the challenge data. (When the quality control testing appears to have failed, then the degree and frequency of the following errors must be determined by using the challenge data):

Very major error (test system susceptible/reference system resistant): false susceptible result
Major error (test system resistant/reference system susceptible): false resistant result
Minor error (test system susceptible or resistant/reference system intermediate)

This review often is the most labor intensive and tiresome of the evaluation process. However, it can be streamlined with a carefully planned evaluation tool that affords easy review and detection of errors and categorical agreement. Figure 5.1 and Figure 5.2 are examples of a simplified evaluation tool.

If your laboratory is accredited or licensed by a body that employs the Clinical Laboratory Standards Institute (CLSI) criteria for evaluation [1], be advised that the CLSI minimum levels of acceptable interpretative error in susceptibility testing are quite restrictive. Very major errors should not exceed 1.5%, major errors should not exceed 3.0%, and overall categorical agreement should equal or exceed 90% for each organism/antibiotic challenge [2]. The following are formulas for calculating these evaluation indices [12]:

\[
\text{Very major error rate} = \frac{\# \text{ of very major errors}}{\text{total # of resistant strains}} \times 100
\]

\[
\text{Major error rate} = \frac{\# \text{ of major errors}}{\text{total # of susceptible strains}} \times 100
\]

\[
\text{Minor error rate} = \left( \frac{\text{total # of tests}}{\# \text{ of very major errors}} - \# \text{ of major errors} - \# \text{ with categorical agreement} \right) \times \frac{100}{\text{total # of tests}}
\]

\[
\% \text{ categorical agreement} = \frac{\# \text{ of tests with categorical agreement}}{\text{total number of tests}} \times 100
\]
ANTIBIOTIC CHALLENGE: Ampicillin

<table>
<thead>
<tr>
<th>ISOLATE</th>
<th>REFERENCE SYSTEM</th>
<th>TEST SYSTEM</th>
<th>VERY MAJOR</th>
<th>MAJOR</th>
<th>MINOR</th>
<th>CATEGORICAL AGREEMENT</th>
</tr>
</thead>
<tbody>
<tr>
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<td>S</td>
<td>X</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E. coli 2</td>
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<td>X</td>
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<td></td>
</tr>
<tr>
<td>E. coli 3</td>
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<td>R</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli 4</td>
<td>S</td>
<td>I</td>
<td></td>
<td>X</td>
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<tr>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
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<tr>
<td>E. coli 10</td>
<td>R</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

**FIGURE 5.1** Antibiotic challenge: ampicillin.

**FIGURE 5.2** Antibiotic challenge

For example, using the chart in Figure 5.2, the rates would be as follows:

\[
\text{Very major error rate} = \frac{1 \text{ very major error}}{4 \text{ resistant strains}} \times 100 = 25\%
\]

\[
\text{Major error rate} = \frac{1 \text{ major error}}{3 \text{ susceptible strains}} \times 100 = 33\%
\]
It is important to keep in mind that some organisms may fail to achieve sufficient growth for interpretation or may fail to grow at all in the automated test system. The number of these organisms should not exceed 10% of the total challenges [4]. Also, antibiotics that lack an intermediate category interpretation can have a one-dilution difference result in a false determination of a very major error or major error. In these cases, there should be $\geq 90\%$ agreement within one dilution between the reference system and test system being evaluated [5].

If upon completion of the review of results you find that the performance of the instrument fails to meet the minimum requirements for acceptable testing, then it is important to check your methodology. It is also important to review the technique of the personnel performing the evaluation, calculations, etc., to ensure that no errors have been made.

5.4 THE AUTOMATED SYSTEM ZODIAC

There are three currently available automated instruments capable of generating rapid susceptibility test results: the Dade Microscan system (Dade Behring, West Sacramento, California), the bioMerieux Vitek system (bioMerieux Vitek, Inc., Hazelwood, Missouri), and the Becton Dickinson Phoenix (Becton Dickinson Microbiology Systems, Cockeysville, Maryland). These devices help to facilitate data recording and long-term data storage.

5.4.1 Dade Microscan

The Dade Microscan system [6–8] consists of a large, self-contained incubator and reader that affords the user the possibility of semiautomated (reader only AutoScan-4) and fully automated (incubator/reader Walkaway 40 and Walkaway 96) instrumentation with rapid and/or conventional testing. The system allows for identification and antimicrobial resistance testing, and results may be obtained in as little as 2 to 7 h.

Conventional testing is performed using colorimetric and turbidity readings. Halogen tungsten light passes through one of six colorimetric interference filters specific for the optimal light source for the substrate being read and is focused on a photometer providing a reading. The antimicrobial resistance test reading is determined by detection of changes in the turbidity of each dilution well of the antibiotic. The information is then transferred to a data management computer that determines a biotype, accesses the appropriate database, and produces a list of probable organisms and, based on this identification, the interpretation of the antibiogram for the isolate.

The rapid-testing system utilizes fluorogenic substrates and fluorometric indicators. For identification testing, the presence of preformed enzymes results in changes in the pH of the substrate, causing a decrease or increase in fluorescence that is detected by the instrument. In the case of antimicrobial resistance testing, the substrate is bound to a fluorescent molecule that in the presence of a specific metabolic enzyme will be cleaved, releasing the fluorescence that is detected by the instrument. Identification and interpretation of the antibiogram then proceeds as for conventional testing.

\[
\text{Minor error rate} = \left( \frac{10 \text{ total tests}}{10 \text{ total tests}} - 1 \text{ very major error} - 1 \text{ major error} - 5 \text{ with categorical agreement} \right) \times 100 = 30\%
\]

\[
\% \text{ categorical agreement} = \frac{5 \text{ tests with categorical agreement}}{10 \text{ total tests performed}} \times 100 = 50\%.
\]
Both the AutoScan-4 and Walkaway 40/96 systems come with data management computer programs that can be used to interpret, store, and report data. In addition, the system includes an expert antibiotic resistance pattern alert system that can be customized to meet the needs of the specific end user, and an antibiotic therapy tracking interface for the pharmacy. These systems also may be interfaced with many current laboratory information systems.

5.4.2 **BioMerieux Vitek**

5.4.2.1 **Vitek Classic**

The Vitek “Classic” [9–11] was originally developed for use by the National Aeronautics and Space Administration (NASA) in its space exploration efforts of the 1970s. The original Vitek was highly automated and relatively compact. It was subsequently introduced into the diagnostic microbiology laboratory arena.

Vitek has a modular format consisting of a filler-sealer module, a reader incubator with a capacity for up to 240 test cards, and a computer control module. The system utilizes separate individual small plastic reagent cards containing a variety of substrate or antibiotic wells for identification testing or antimicrobial resistance testing.

Cards are inoculated, sealed, and placed in the reader-incubator. After baseline readings are taken, light emitting diodes and phototransistor detectors scan the cards; a photometer detects changes in light transmission. These readings are transferred to the computer control module, which determines the organism’s biotype, identification, and antibiogram.

Like the Microscan instruments, the Vitek system comes with a data management computer for the interpretation, storage, and reporting of data, and can be readily interfaced with several laboratory information systems. In addition, this system pioneered the “expert” system for on-line identification and susceptibility result validation and antimicrobial resistance evaluation. The system uses an inference engine, which, given the probability of certain antimicrobial resistance patterns occurring under certain circumstances, flags anomalies for the user with suggestions to verify or validate the result. This has proven to be a useful tool in some clinical microbiology laboratory settings.

5.4.2.2 **Vitek 2**

The Vitek 2 [9,13,14] uses fluorogenic technology that allows the instrument to take an optical reading of each test well on the card every 15 min. Each test card is bar coded to avoid the possibility of the clerical errors and unintelligible handwriting that plagued the original Vitek. The Vitek 2 autofills, autoseals, autoincubates, and even autodisposes the test cards upon completion of testing.

5.4.2.3 **Vitek 2 Compact**

Vitek 2 Compact [9,13,14] was recently introduced by bioMerieux. The system utilizes chromogenic substrates with an “advanced colorimetry” system. The Vitek 2 Compact also makes use of an advanced system that includes an evaluation of the quality of the test. In addition, the system allows connection to bioMerieux’s Stellara wireless patient management system that affords integration of laboratory, pharmacy, and other systems to aid the physician in making clinical and therapeutic interventions all from a handheld personal digital assistant (PDA).

5.4.3 **Becton Dickinson Phoenix**

The Becton Dickinson Phoenix [15–17], with the exception of manual test card inoculation, is a fully automated, fully integrated identification and susceptibility testing system. The Phoenix uses both chromogenic and fluorogenic substrates in the same test card. Using standardized inoculums,
panels are manually inoculated and placed in the Phoenix instrument for incubation and reading. Panels are read every 20 min until the testing is completed. Like the other systems, test data is stored in a data management computer, and an “expert” system for reviewing results is included. However, the data management system for the Phoenix is designed to be integrated with other Becton Dickinson microbiology system instrumentation (i.e., blood culture instruments) to provide a total microbiology data management program.

REFERENCES

6 Agar Dilution Susceptibility Testing

Ann Hanlon, Merwyn Taylor, and James D. Dick

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6.1 INTRODUCTION

The agar dilution susceptibility-testing method is utilized for the determination of the minimal inhibitory concentration (MIC) of an antimicrobial agent required to inhibit the growth of a microorganism. As with broth dilution susceptibility tests, the agar dilution method provides a quantitative result in the form of an MIC, in contrast to disk diffusion susceptibility tests that result in an indirect measure of susceptibility and provide a qualitative interpretative result. The agar dilution method is the most well-established method for determining antimicrobial susceptibility and is commonly used as the standard or reference method for evaluation of new antimicrobial agents and susceptibility test methods [1–10]. In addition, it offers the convenience of simultaneously testing a number of isolates (32–36), being able to detect mixed cultures or heterogeneous populations, and flexibility in antibiotic selection and concentration range to be tested.
6.2 SIGNIFICANCE

Selection of an appropriate antimicrobial agent should be considered in the context of the microorganism, the antibiotic, and the host [11]. Factors that are involved in this triad include (a) the in vitro susceptibility of the infecting organism, (b) the relative susceptibility of the infecting organism compared to other members of the same species, (c) the pharmacokinetic/pharmacodynamic properties of the antibiotic, such as its toxicity, distribution, adsorption, excretion, protein binding, half-life, and postantibiotic effect, (d) prior clinical experience of the efficacy of the antibiotic in similar clinical situations, and (e) the nature of the pathophysiology underlying the infection in the host and its potential effect on chemotherapy and the immune status of the host. Among these considerations, the concentration of antibiotics required for in vitro inhibition or killing can be measured in the clinical laboratory. It is the responsibility of the laboratory to provide the clinician with accurate and appropriate in vitro susceptibility information, which, in conjunction with the previously described pharmacologic and host information, can be used to formulate the optimal therapeutic approach for the patients. In addition to direct application in guiding antimicrobial therapy on a patient-by-patient basis, in vitro susceptibility information should be capable of and utilized to detect new emerging mechanisms of resistance as well as changing patterns of resistance in a particular location, i.e., ward, institution, or among a particular group of microorganisms.

6.3 AGAR DILUTION PROCEDURE

6.3.1 PREPARATION OF AGAR DILUTION PLATES

6.3.1.1 Media Selection

The basal media used for testing is determined on the basis of the organisms and in some cases the antibiotic to be tested.\(^1\)

- Enterobacteriaceae, nonfermentative gram-negative bacilli, staphylococci, enterococci, nonfastidious bacteria: Mueller-Hinton agar
- Oxacillin/methicillin/nafcillin testing of staphylococci: Mueller-Hinton agar supplemented with 2% (w/v) NaCl
- Streptococci: Mueller-Hinton agar supplemented with 5% (v/v) defibrinated sheep blood (for testing of a sulfonamide, lysed horse blood should be used)
- Neisseria gonorrhoeae: GC base with 1% defined growth supplement (NCCLS, Table 2F-M7-A5, M100-S16)
- Haemophilus: Haemophilus test medium (HTM) (NCCLS, Table 2E-M7-A5, M100-S12)

6.3.1.2 Antibiotic Concentration Range

Choosing the appropriate concentration to test is based on two parameters:

1. Interpretive break points set by NCCLS for both the antibiotic and the organism being tested
2. Quality control organism being utilized

The number of concentrations being tested will vary by the individual laboratory. In addition, it is essential that the interpretive break points be included as well as concentrations that allow for adequate quality control (QC) testing.

\(^1\) See NCCLS Table 7-M7-A5; M100-S16 for defined growth supplement and for further information on dilution testing and screening tests for organisms not listed here.
6.3.1.3 Antimicrobial Powders

Pharmaceutical companies will usually supply on request standard antimicrobial reference powder for antibiotics that they market. Some off-patent antibiotics can be obtained from commercial chemical companies.

6.3.1.4 Weighing Antimicrobial Powders

6.3.1.4.1 Potency

Most antibiotics being used for agar dilution are not 100% pure. It is important to know the assay potency of the lot of antibiotic being utilized. Using this information, a standardized solution can be formulated. This information may be found on the vial of the reference powder or may be included in the information enclosed with the drug. The following formula should be used:

\[
\text{Weight (mg)} = \frac{\text{volume (mL) \times concentration (μg/mL)}}{\text{assay potency (μg/mg)}}.
\]

**Example**

To prepare 10 mL of a stock solution containing 1,000 μg/mL of a particular antibiotic with a potency of 869 μg/mg, perform the following calculation:

\[
\text{Weight (mg)} = \frac{10 \text{ mL} \times 1000 \text{ μg/mL}}{869 \text{ μg/mg}}.
\]

Weight = 11.5 mg

6.3.1.4.2 Weighing

Antimicrobial powders should be weighed on an analytical balance that has been calibrated with National Institute of Standards and Technology weights or other approved reference weights.

6.3.1.5 Preparing Concentrations for Testing

A. Prepare a stock solution of 10,000 μg/mL or 1,000 μg/mL for each antibiotic being tested.
   - Water can be used as a solvent for most antibiotics.
   - Some antibiotics require solvents other than water. It is important to use the minimal amount of solvent needed to solubilize the drug and to use water or another appropriate buffer to make the final stock concentration and dilutions. See the Appendix for a list of buffers.
   - Stock solutions can be stored at 60°C or below for 6 months or more without significant loss of activity with the exception of imipenem and ticarcillin/clavulanate, which can only be stored for 1 month. Quality control should always be performed to verify that no significant deterioration has occurred.

B. Using the stock solution and Table 6.1, the dilution scheme for each antibiotic can be achieved. The reader is also referred to the latest M100 NCCLS document for a scheme for preparing dilutions used in agar dilution.

---

1 To make a 10,000 μg/mL solution, you can weigh out the same amount of antibiotic and add solvent to a volume of 1.0 mL, or weigh out 10 times as much antibiotic and add solvent to a volume of 10 mL.
TABLE 6.1
Antibiotic Dilution Scheme

<table>
<thead>
<tr>
<th>Volume of Stock</th>
<th>To Be Added to 1 Liter of Agar (mL)</th>
<th>Final Concentration in Agar (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 μg/mL</td>
<td>25.6</td>
<td>256</td>
</tr>
<tr>
<td>12.8</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>6.4</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>3.2</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>1.6</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>1,000 μg/mL</td>
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</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

If you have concentrations of 128 μg/mL and/or 256 μg/mL in your dilution scheme in addition to lower concentrations, always make a 10,000 μg/mL solution and dilute it to a 1,000 μg/mL solution. This way your quality control will be testing your entire dilution scheme.

### 6.3.1.6 Pouring the Plates

1. Label square or round petri plates for each concentration of antibiotic being tested. Label in such a way that the label can be used to orient the plate, i.e., put the label in the bottom right corner of the square plates or on the bottom edge of round plates.
2. Prepare 1 L of Mueller-Hinton agar or other base media for each concentration of antibiotic being tested per manufacturer’s instructions. Before autoclaving the media, add an anti-bubble additive such as Pourite (Compliance Technology, Inc., San Francisco, CA).
3. Cool the media in a water bath to between 45°C and 50°C.
4. Add any supplements to the media, including sheep or horse blood.
5. Take the pH of each batch of media. It should be between 7.2 and 7.4 at room temperature. This should be done once the agar is solidified. This is essential since the potency of some antibiotics will change with the hydrogen ion concentration.
6. Add antibiotic to the liquid agar media.
7. Swirl the flask to mix thoroughly.
8. Pour into the petri plates on a level surface to a depth of 3–4 mm (approximately 40–50 mL/plate).
9. Allow the plates to solidify at room temperature.
10. Store the plates at 2°C–8°C for up to two weeks for routine susceptibility testing (with the exception of imipenem and clavulanate plates, which should be prepared and used within seven days) but no more than five days for reference work. These times are for benchmark purposes.
11. Different antibiotics can be more or less stable, and therefore quality control must be performed on each day of use.

### 6.3.1.7 Control Plates

In addition to preparing antibiotic dilution plates, it is important to also prepare control plates. These plates consist of only the agar-based media with no antibiotic added. Optimally, a control
plate should be made at the beginning of each agar dilution run and at the end to evaluate an organism’s ability to grow on the base media, as well as any contamination and antibiotic carryover.

6.3.1.7.1 Testing Isolates Using Agar Dilution Plates

6.3.1.7.1.1 Initial Preparation

1. Remove all concentrations of agar dilution plates and control plates from the refrigerator to allow the plates to come to room temperature and the agar surface to dry. This can be achieved by slightly opening the lids of each plate and allowing them to remain on the bench for 1–2 h.

2. The number of plates of each concentration of antibiotic you will need to test is dependent on the number of isolates being tested and the type of petri plate being used:
   - Square plates — 36 isolates, including the QC
   - Round plates — 32 isolates, including the QC

3. Preparing a grid
   Each patient isolate and quality control organism needs to be accounted for when setting up an agar dilution run.

   A square plate setup is numbered as follows:

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>19</td>
<td>20</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>24</td>
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<tr>
<td>25</td>
<td>26</td>
<td>27</td>
<td>28</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>31</td>
<td>32</td>
<td>33</td>
<td>34</td>
<td>35</td>
<td>36</td>
</tr>
</tbody>
</table>

   A round plate setup is numbered as follows:

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>23</td>
<td>24</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>29</td>
<td>30</td>
<td>31</td>
<td>32</td>
</tr>
</tbody>
</table>

4. When the plates are dry, close them and arrange the plates so that they are marked as to their correct orientation.

6.3.1.7.1.2 Preparing the Inoculum

1. Growth method
   a. Inoculate 1 mL of Mueller-Hinton broth with a portion of three to five colonies of the organism to be tested. The colonies should be morphologically the same.
   b. Incubate at 35°C for 2–6 h. The growth should reach a turbidity that is equivalent to or greater than a 0.5 McFarland standard.
   c. Adjust the turbidity of the culture using sterile saline to achieve a turbidity equivalent to a 0.5 McFarland standard. This will contain approximately $1.5 \times 10^8$ CFU/mL.

2. Direct colony suspension method
   a. This method should be use when testing the following organisms:
      - Haemophilus spp.
      - N. gonorrhoeae
      - Steptococci
Antimicrobial Susceptibility Testing Protocols

Staphylococci when looking for potential methicillin or oxacillin resistance
b. Isolated colonies for testing should come from 18- to 24-h agar plates.
   The organism should be grown on nonselective media such as blood agar.
c. A suspension is made using a portion of three to five colonies that are morphologically
   similar. Adjust the suspension so that it is equivalent to a 0.5 McFarland standard.

3. Dilution inoculum
   a. Dilute the adjusted suspensions 1:10 in sterile saline. This will give you an inoculum
      concentration of $10^7$ CFU/mL.
   b. Use the diluted suspensions within 15 min of preparation.

6.3.1.7.1.3 Inoculation Agar Dilution Plates

1. Use of a replicator device at this point is helpful. Replicators consist of a block of wells
   where the organisms go and a transferring head containing metal pins. These pins are
   usually 3 mm in diameter and transfer 1–2 μl of inoculum on the agar plate creating a 5–8-
   mm spot. This gives a final inoculum amount on the agar surface of $10^4$ CFU/mL.
2. Arrange all tubes containing the diluted suspensions in a rack that corresponds to the
   grid prepared earlier.
3. Fill the corresponding well in the replicator block half full of the diluted suspension
   using a sterile transfer pipette. Leave a small portion of the suspension in the pipette and
   use this to streak an agar plate to be used as a purity check. This plate will also provide
   fresh isolated colonies from which further testing or repeat testing can be performed.
4. Using the replicator, inoculate the growth control plate.
5. Inoculate each set of antibiotics starting with the lowest concentration.
6. Inoculate a second control plate at the end.

6.3.1.7.1.4 Incubation

1. Allow the plates to sit at room temperature until the inoculum spots have dried, approx-
   imately 10 min.
2. Invert the plates and incubate as follows:

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Incubation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfastidious bacteria</td>
<td>16–20 h, 35°C, non-CO₂</td>
</tr>
<tr>
<td>Streptococci</td>
<td>20–24 h, 35°C, CO₂, if necessary for growth</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>20–24 h, 35°C, 5%–7% CO₂</td>
</tr>
<tr>
<td>Oxacillin, methicillin, and vancomycin testing of staphylococci</td>
<td>24 h, 33–35°C (do not exceed 35°C), non-CO₂</td>
</tr>
<tr>
<td>Vancomycin testing of enterococci</td>
<td>24 h, 35°C, non-CO₂</td>
</tr>
</tbody>
</table>

6.3.1.7.1.5 Determining End Points

1. After the appropriate incubation, check both control plates for growth and any obvious
   contamination. Any organisms either not growing on the control plate or contaminated
   should not be read for MICs.
2. Check the purity plates for contamination or mixed cultures. Any plate with more than
   one morphology of an organism should not be read for MICs.
3. Antibiotic plates should be read for end points on a dark nonreflecting surface.
4. All concentrations of the particular antibiotic being read should be laid out in front of
   you with the lowest concentration to your left.
5. The MIC is read as the first antibiotic concentration that inhibits the growth of the
   organism completely. A faint haze caused by the inoculum or a single colony should not
   be read as growth.
6. Purity should be questioned in the following situations:
   - Greater than one colony on any plate past an obvious endpoint
   - Higher concentrations have growth when lower concentrations have no growth
7. All procedures from 1 through 6 should be repeated by another independent reader.
8. The two readings should be compared to each other, and any discrepancies should be resolved.

6.3.1.7.1.6 Quality Control
Quality control of the agar dilution method of susceptibility testing is important to ensure that all the steps of the procedure result in accurate MICs being reported. In addition to those procedures previously described, the inclusion of four to six control strains on every run adds an additional level of assurance to the susceptibility data.

Quality control isolates should consist of organisms that have MICs that fall into the range of concentrations being tested for each antibiotic. Reference American Type Culture Collection (ATCC) stains that are recommended by NCCLS are as follows:

- Enterococcus faecalis ATCC 29212
- Enterococcus faecalis ATCC 51299
- Escherichia coli ATCC 25922
- Escherichia coli ATCC 35218
- Haemophilus influenzae ATCC 49247
- Klebsiella pneumoniae ATCC 700603
- Neisseria gonorrhoeae ATCC 49226
- Pseudomonas aeruginosa ATCC 27853

Additional control isolates, particularly for gram-positives and nonfastidious gram-negatives, can be selected for inclusion as control organisms. These isolates should be selected on the basis of their reproducibility and as controls for antibiotics that are not controlled by the NCCLS-recommended strains. It is important to assess the accuracy of the standardization of the test inoculum to ensure consistency throughout all the testing. Plate counts should be obtained from a random well in the replicator device on a periodic basis by a conventional pour or spread agar quantitative technique.

6.4 DATA PROCESSING AND ANALYSIS
The rapidly expanding spectrum of antimicrobial agents, microorganisms, and resistance determinants has resulted in the need for computerized processing and analysis of data generated through both identification- and susceptibility-testing systems in large clinical laboratory settings. Automated data analysis permits the integration and evaluation of both species and antibiotic profiles, which are the two essential components for determination of specific resistance determinants not apparent using direct interpretation of a single antibiotic test result. Nonintegrated systems require manual integration of identification and susceptibility data, data evaluation, and decision making, which can result in a significant burden for the bench microbiologist. Many of the automated systems have incorporated “expert systems” into their software to solve this problem; however, laboratories using manual or combinations of automated and manual technologies are still faced with the burden of manually evaluating speciation and susceptibility information prior to final reporting of culture results. The following is a data-processing system used in our laboratory for integration and evaluation of identification- and susceptibility-profile information. The system was designed to accept semiautomated identification and susceptibility data from an agar-based system but is applicable for totally automated, manual–automated, and manual systems that interact with a computerized laboratory information system.
6.4.1 DATA-PROCESSING METHODOLOGY — AGAR DILUTION

The agar dilution computer system was designed to support the data entry and processing required to identify and report a microbiology laboratory’s results using the agar dilution method. The computer system is composed of three primary components illustrated in Figure 6.1.

These components are the graphical user interface (GUI), data processing unit, and database unit. The GUI provides a framework for technologists to interact with the system. The processing unit accepts data from the GUI, checks it for validity, and applies interpretation rules to it. The database unit stores all the data. The three components are connected to the same network, and it is the shared network connectivity that allows the component to send information to other components.

The first component of the agar dilution computer system is the GUI. The interface is composed of several dialog boxes that technologists use to enter data. The interfaces are used to communicate with various modules contained in the processing unit. Figure 6.2 illustrates the main dialog boxes that are used.

The data entry process begins with the creation of a “RUN.” The interface presents the technologists with a $6 \times 6$ grid to enter the isolate identifiers that will be used in the current run. A $6 \times 6$ grid was selected as the interface of choice because it reflects the positioning of specimen samples on the dishes and because it is intuitive. The isolate numbers in the $6 \times 6$ grid correspond to the positions of the specimen samples. Once the information has been entered, it is transferred to the processing unit. Among other things, the processing unit checks the isolate numbers for validity and transfers the isolate numbers to the database unit where the “RUN” is registered.

After the RUN is created, technologists can enter the biochemical identification reactions for the isolates that have been recorded for that run. The dialog box for the biochemical data entry is similar to that of the RUN setup dialog box. This dialog box also consists of a $6 \times 6$ grid, the name of the biochemical substrates tested, and within each cell, drop-down boxes that contain options for that cell. The values entered into the cells are registered with the isolates that have been entered into the corresponding cells of the run and ensure that the technologists enter valid values. Furthermore, by using a dialog box, technologists can enter data in any order and make corrections to data prior to submitting the final results to the processing unit.

Double data entry is used for quality control when registering biochemical results for isolates. When the second technologist enters data, the technologist will be presented with values from the first collection that differ from those just entered. The technologist can override or accept the previous value. To support this feature, the database unit has to be designed to appropriately manage the first and second batch of values, and the processing unit must compare these values when the second technologist enters any data.

The second data entry interface is the susceptibility-reading data entry dialog box. Technologists use this dialog box to enter MIC readings. The susceptibility data entry dialog box is similar to that of the biochemical data entry dialog box. One noticeable difference is that the pull-down selections for the cell differ with the antibiotics that are being processed. The interface communicates with the processing unit to retrieve the allowable options for the cells. This feature ensures that the technologists will only enter allowable values and reduces errors in the system. As in the first interface, double data entry is used for quality control.
The third major interface is the individual specimen results entry dialog box. Technologists use this interface to enter data for isolates of the same specimen. This interface is used to report the identification of organisms that are not identified using the agar method. Using this interface, technologists can enter the organisms that have been identified, the quantity of colonies, the biochemicals used, and the MIC readings. This interface contains shortcuts that reduce the amount of typing required by the technologists. For example, technologists can enter a short three to four letter mnemonic for an organism, and the interface will replace the mnemonic with the organism’s name when the organism button is pressed. Since there are over 1,200 organisms that can be entered into the organism text box, this shortcut avoids searching a long list and saves time. If the organism button is pressed and the organism text field does not contain the mnemonic, the technologist is presented with a selection list. This feature is also used for entering quantities, results, and methods.

Technologists enter MIC values in a text box after pressing the “+” button in either the “SCREEN” or “REPORT” list. In the event that an MIC reading was entered in the SCREEN list but should have been entered in the REPORT list, the interface allows the technologist to move entries from one list to another. This feature makes it easy to recover from mistakes and saves time for the technologists. Removing entries from either list identification is quite intuitive. Technologists simply highlight the entry and press the “=” button. This portion of the interface uses icons that reflect the actions the technologists perform and adds to the intuitive feel of the interface.

The goal of the GUI is to present the technologists with a series of menus and intuitive, easy to use dialog boxes that can be used to navigate through the agar dilution computer system and to enter data. Selection lists are used to minimize errors in data entry. The GUI gets data for the selection lists from the database by sending requests to the processing unit. The processing unit
extracts data from the database and passes it along to the GUI. Shortcuts provide efficient options for data entry. Several dialog boxes were designed similarly to ensure consistent interaction with the computer system where appropriate.

6.4.2 Processing Unit

The processing unit is the component of the computer system that interacts with the GUI. It is an application server that waits for data from the user interface. Therefore, the user interface and the processing unit are individual computer processes that work together. This component has several modules designed specifically for the individual dialog boxes in the GUI. Two of the more important modules are illustrated in Figure 6.2. These modules are the organism identification module and the antibiotic susceptibility screening module.

The organism identification module is invoked after the second collection of biochemical reactions has been entered for the runs. The computer system maintains a list of biochemical patterns and corresponding organisms that are identified by the patterns. This module retrieves the biochemical patterns from the database and compares them to those that have been supplied for individual isolates. If the biochemical results for an individual isolate match those in the biochemical patterns list, the corresponding organism is registered with the isolate.

To improve the efficiency of the search, the identification module keeps the patterns indexed on the first three biochemicals. By searching the first three components of a pattern, the module can quickly narrow down the patterns that may potentially match the technologist's inputs. This feature saves a considerable amount of time since patterns that do not match the biochemical results entered by the technologist can be safely avoided without checking them in their entirety.

The antibiotic screening module is used to determine which antibiotic results should be reported to clinicians and which results should be saved but screened. This module is invoked after the antibiotic susceptibility data has been entered either for isolates that are included in a RUN or for specimens using the specimen result entry dialog. The screening rules are based on the body sources of the specimens, the identified organism, and the antimicrobial agents. This screening information is maintained in the database. One interesting feature of these rules is that they can contain references to classes of organisms as opposed to individual organisms. The database maintains a hierarchy of organisms that can be accessed to resolve individual organisms to their higher level classes. Figure 6.3 contains a sample hierarchy that is used in the system.

Other modules include the organism taxonomy management module and the extensible markup language (XML) parsing module. The organism taxonomy management module provides access to organisms and their subclasses. This information is used in screening rules, and it makes the rule-screening system extremely flexible. Most messages in the computer system are XML-like messages. The XML parsing module is used to transform XML-like messages to structures containing data that can be processed by other modules in the processing unit. The XML-like syntax is a communications standard used by the GUI and the processing unit that allows for the seamless integration of the two units.
6.4.3 DATABASE

The database is an integral part of the agar dilution computer system. It stores the information that is needed to identify organisms, screen antibiotics, supply the user interface with allowable values for selection lists, and manage the data generated by the technologist and the processing modules. Consequently, the database had to be designed to efficiently support these functions. A relational database architecture is used in which data are stored in tables. When necessary, tables may reference one another to link data from one table to that of another.

Figure 6.4 illustrates the subset of the database schema that is used to store the isolate and susceptibility data. The isolate table contains data that is pertinent to the isolate and does not reference the susceptibility data. This is a valid approach because isolates can exist without susceptibility data. The susceptibility table contains information that is pertinent to the MIC values. Since susceptibility readings typically correspond to isolates, the susceptibility table cross-references the isolate table to create a link between isolates and their susceptibility readings.

The biochemical patterns are expressed as XML-like streams. Each pattern is associated with an organism and one or more comments that are to be reported with the organism identification. A typical pattern is expressed as

```
<PATTERN> <PARTIAL ES="+" NA="+" ED="-"/> <FULL NO="+" SC="+" LC="+" MN="+" ST="+" AB="+" SS="-"/> <PATTERN>
```

The biochemical pattern is partitioned into two components, the partial component and the full component. The partial component is a prefix that is used to index patterns that begin with the same sequence. The full component denotes the remainder of the pattern that is different from other patterns with the same prefix. Both patterns contain mnemonics for the biochemicals that are matched. This information is loaded, parsed, and indexed by the organism identification module.

The antibiotic screening rules are also expressed as XML-like streams. A typical screening rule is expressed as

```
<BODYSOURCE BLOOD/> <ORGANISM ENFM/> <ANTCON> <GT VAN (Vancomycin) = "64" /> </ANTCON>
```

The preconditions of this rule are (a) the body source of the specimen has to be “BLOOD,” (b) the identified organism should be ENFM (Enterococcus faecium) or any of its descendants, and (c) the MIC for “VAN” should equal to 64. If the preconditions are all satisfied, the actions for this rule are applied. The actions for a rule are typically expressed as

```
<REPORT TET P-T/> <SCREEN SUL VAN/>
```

In this example, the MIC for TET (tetracycline) will be reported while the MICs for SUL (sulfasoxizole) and VAN will be suppressed from the final report.

![Isolate and susceptibility tables.](image)
XML tags are used in the biochemical patterns and the antibiotic screening rules to instruct the processing modules on how to construct patterns and rules, respectively. Embedding XML tags in relational tables simplifies the process of amending existing patterns and rules, and provides a flexible framework for adding patterns and rules that are structured differently. This overcomes one of the shortcomings of relational database technology, i.e., changing the structure of the existing data. By adding new tags and embedding tags in different structures, new patterns and rules can be added to the database while avoiding the major task of restructuring the database schema. This design decision makes the agar dilution computer system very flexible.

The database contains an organism taxonomy that was created to allow the antibiotic screening rules to reference classes of organisms. Organism classes can be resolved to individual organisms by traversing the taxonomy. The database contains a table that lists the parent–child relationships that exist among organism classes and organisms. The organism taxonomy table contains three columns, the “super” category, the “sub” category, and the relationship type. For example, PC Aerobe, STAP, and ISA appear in the database and state that STAP is a subclass of PC Aerobe. The processing unit uses the information to build a taxonomy of organisms and provides services that allow the various modules to probe the taxonomy.

Creating a computer system to support agar dilution requires that one carefully consider the design of the user interface, the processing modules, and the storage of data. Ideally, the three components should work together. However, they should not be tightly coupled since a tight coupling among the components creates a less flexible overall system. The user interface should be easy to use and powerful enough to allow the technologists to complete their tasks. The processing unit should contain all the modules that are necessary to complete its tasks and be efficient enough to handle large workloads. The database unit should provide access to data that is presented to users in the GUI, such as selection lists, as well as store the results of all processing modules. Using these design principles should ensure that the computer systems will be flexible and easy to upgrade.

### 6.5 SUMMARY

The agar dilution antimicrobial testing method is a well-established, standardized technique that is frequently used as a reference for evaluating the accuracy of other susceptibility test methods. The method provides a quantitative result in the form of an MIC to which interpretive criteria can be applied, i.e., susceptible (S), intermediate (I), or resistant (R). MIC data can be utilized directly in conjunction with pharmacokinetic and pharmacodynamic parameters of the antimicrobial agent in optimizing therapy on a case-by-case basis. The method also provides for the simultaneous testing of many isolates (32–36), including control isolates, for each antibiotic and dilution tested. In contrast to broth methods, agar dilution is more amenable to the visual detection of contamination or microbial heterogeneity at the time of testing. Perhaps the greatest advantage of agar dilution susceptibility testing is its flexibility. Since antibiotic-containing plates are most commonly prepared by the user, antibiotics and the concentrations tested are at the laboratory’s discretion rather than that of the manufacturer. This permits the user to better correlate their particular antibiotic test battery with those antibiotics being used, i.e., hospital formulary, at their institution. This has the potential for cost savings by not testing unnecessary or nonformulary drugs, which might be included in a commercial antibiotic panel. In addition, since the user is the manufacturer, antibiotic-testing changes can be implemented in a timely manner such as at the time of FDA approval of a new antibiotic. Finally, although the method is labor-intensive, significant cost savings can be realized if it is implemented by a well-trained staff when compared to the cost of commercial susceptibility test systems. The major disadvantages of the agar dilution system are associated with the labor-intensive tasks of preparing and conducting quality control of the antibiotic-containing plates. Additionally, in the absence of a computerized interface for data analysis, the manual evaluation and collating of susceptibility results, particularly when a large number of antibiotics are tested, and of identification information can be time-consuming.
APPENDIX — ANTIBIOTIC BUFFERS

1. Saturated sodium bicarbonate: Add sodium bicarbonate to approximately 5 mL of water until no more will go into solution.
2. 0.025 M \(\text{KH}_2\text{PO}_4\) buffer, pH 5.5: Dissolve 0.34 g \(\text{KH}_2\text{PO}_4\) in 100 mL \(\text{H}_2\text{O}\) using a volumetric flask. Adjust to pH 5.5 with approximately 0.07 mL 1 N \(\text{NaOH}\).
3. 1 mol/L \(\text{NaOH}\): 4 g \(\text{NaOH}\) + 100 mL \(\text{H}_2\text{O}\).
4. 0.1 mol/L phosphate buffer, pH 7.0: Prepare the following two solutions in separate 100-mL volumetric flasks:
   A. 2.76 g \(\text{NaH}_2\text{PO}_4\) + 100 mL \(\text{H}_2\text{O}\)
   B. 2.84 g \(\text{Na}_2\text{HPO}_4\) + 100 mL \(\text{H}_2\text{O}\)
   In another volumetric flask, add 19.5 mL of solution A and 30.5 mL of solution B. Bring to 100 mL with \(\text{H}_2\text{O}\).
5. 0.01 mol/L phosphate buffer, pH 7: Prepare the following two solutions in separate 100-mL volumetric flasks:
   0.272 g \(\text{KH}_2\text{PO}_4\) + 100 mL \(\text{H}_2\text{O}\)
   0.35 g \(\text{K}_2\text{HPO}_4\) + 100 mL \(\text{H}_2\text{O}\)
   In another volumetric flask, add 19.5 mL of solution A and 30.5 mL of solution B. Bring to 100 mL with \(\text{H}_2\text{O}\). The pH should be 7.0.
6. 0.1 mol/L \(\text{KH}_2\text{PO}_4\), pH 4.5: Dissolve 1.362 g of \(\text{KH}_2\text{PO}_4\) in 100 mL \(\text{H}_2\text{O}\) using a volumetric flask.

REFERENCES

Susceptibility-Testing Protocols for Antibiograms and Preventive Surveillance: A Continuum of Data Collection, Analysis, and Presentation

John G. Thomas and Nancy T. Rector

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7.1 INTRODUCTION AND BACKGROUND

7.1.1 ORIGIN

The origin of antibiograms is reflected in the growing emergence of bacterial resistance (Figure 7.1), i.e.,

- Limited resistance development in the 1950s and 1960s
- Penicillin, then methicillin (1970) to *Staphylococcus aureus*
- Resistant *Mycobacterium tuberculosis*
- WHO (World Health Organization) addressed susceptibility testing via the standardization of disk diffusion, i.e., Kirby-Bauer (1973)

![Figure 7.1](image-url)  
**FIGURE 7.1** Correlation of antibiotic use and developing resistance.
7.1.2 History and Review of Antibiograms

- Automation (1970s) required documentation
- Need for new antimicrobials required tracking
- Development of parallel drugs in the same class but with supposedly better activity
- Center for Disease Control (CDC) computer tracking (“Dare-to-Compare”) provided free to interested laboratories for data collection and comparison (1970s)
- Differences by geography and institutional type became apparent

7.1.3 Definitions

There are several types of antibiograms, and the following two are most common:

- Cumulative single-dimension antibiogram
- Focused antibiogram

A cumulative single-dimension antibiogram measures the cumulative sensitivity/resistance against a group of antibiotics; one must carefully define exclusive/inclusive criteria for the organisms as well as concentrations of each antibiotic evaluated. One also needs to use consistent interpretations, usually utilizing a consensus oversight organization such as the Clinical and Laboratory Standards Institute (CLSI) formerly NCCLS (National Committee for Clinical Laboratory Standards). Significant differences do occur between countries, because Japan, United Kingdom, Europe, and the United States use different criteria.

The second method, a focused antibiogram, uses a selected subset of information to unmask and eliminate low-frequency data that would be hidden in a standard cumulative antibiogram.

7.2 Relevance of Antibiograms

7.2.1 Why Are Antibiograms Important?

The pattern of resistance or sensitivity in a hospital or indeed any environment is the negative imprint of the use of antibiotics in that environment

Hans M. Ericsson, 1977, Professor of Microbiology

Table 7.1 tracks this important observation in real terms, while Table 7.2 describes the obvious association with the overuse of antibiotics. Table 7.3 highlights resistance development or loss of susceptibility for intensive care unit (ICU) isolates. Tables 7.4 and 7.5 relate resistance to cost, which in today’s economic environment is critical. Table 7.5 emphasizes the importance of “Burden of Disease,” generally and specifically, for ventilator associated pneumonia (VAP).

“Preventive surveillance” is another way of defining the benefits of an antibiogram. The word antibiogram implies a “static” process, while preventive surveillance constitutes active participation and denotes involvement, which is what microbiologists need to do.

7.2.2 How Can Antibiograms Be Used in Patient Care?

- Help in empiric antibiotic selection
- Alert to developing resistance (trends)
- Track outbreaks/epidemiology
- Guide hospital formulary decisions (pharmacy)
- Satisfy regulatory agencies
TABLE 7.1
Concentration (µg/mL) of Anti-Infective Agents That Would Inhibit or Kill Bacteria at Their Discovery and in 1999

<table>
<thead>
<tr>
<th>Anti-Infective</th>
<th>Bacteria</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At Discovery</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>S. pneumonia</td>
<td>0.008</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>S. aureus</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>K. pneumonia</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>N. gonorrhea</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>S. typhi</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>H. influenza</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>S. typhi</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>S. pyroxenes</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>MRSA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>M. tuberculosis</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

<sup>a</sup> MRSA = Methcillin Resistant <i>Staphylococcus aureus</i>.

TABLE 7.2
Use of Antibiotics in Hospitals

>30% of all patients receive antibiotics
60% of all antibiotics are administered in the absence of documentation of infection
13%–15% of administered antibiotics are incorrect
70% of antibiotics chosen are the more expensive variety, i.e., an advanced generation antibiotic, when the cheaper antibiotic could be used.
19%–35% of a pharmacy’s budget deals with antibiotic use
20% of antibiotics used have complications in their use, e.g., renal toxicity or nosocomial infections

TABLE 7.3
Comparison of Nosocomial Resistance Rates in ICUs by Selected Phenotypes

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin-resistant &lt;i&gt;Enterococcus&lt;/i&gt;</td>
<td>25.9</td>
<td>58</td>
<td>47</td>
<td>28.5</td>
<td>12</td>
</tr>
<tr>
<td>MRSA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54</td>
<td>865</td>
<td>43</td>
<td>59.5</td>
<td>11</td>
</tr>
<tr>
<td>Methicillin-resistant CNS</td>
<td>86.7</td>
<td>789</td>
<td>2</td>
<td>89.1</td>
<td>1</td>
</tr>
<tr>
<td>Cef3-resistant &lt;i&gt;E. coli&lt;/i&gt;</td>
<td>3.2</td>
<td>316</td>
<td>23</td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>Cef3-resistant &lt;i&gt;K. pneumoniae&lt;/i&gt;</td>
<td>8.9</td>
<td>248</td>
<td>–1</td>
<td>20.6</td>
<td>47</td>
</tr>
<tr>
<td>Imipenem-resistant &lt;i&gt;P. aeruginosa&lt;/i&gt;</td>
<td>18.5</td>
<td>298</td>
<td>35</td>
<td>21.1</td>
<td>15</td>
</tr>
<tr>
<td>Quinolone-resistant &lt;i&gt;P. aeruginosa&lt;/i&gt;</td>
<td>23</td>
<td>480</td>
<td>49</td>
<td>29.5</td>
<td>9</td>
</tr>
<tr>
<td>Cef3-resistant &lt;i&gt;P. aeruginosa&lt;/i&gt;</td>
<td>20</td>
<td>490</td>
<td>&lt;1</td>
<td>31.9</td>
<td>20</td>
</tr>
<tr>
<td>Cef3-resistant Enterobacter spp.</td>
<td>36.4</td>
<td>335</td>
<td>3</td>
<td>31.1</td>
<td>–6</td>
</tr>
</tbody>
</table>


<sup>a</sup> MRSA = Methcillin Resistant Staphylococcus aurea; CNS = Coagulase Negative Staphylococcus; Cef3 = 3rd Generation Ceflsporin.
**TABLE 7.4**

Economic Burden of Infectious Diseases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of non-AIDS STDs</td>
<td>$5.0 billion</td>
</tr>
<tr>
<td>Ear infections</td>
<td>$1.2 billion</td>
</tr>
<tr>
<td>Intestinal infections (direct costs and lost productivity)</td>
<td>$30.0 billion</td>
</tr>
<tr>
<td>Nosocomial infections</td>
<td>$4.5 billion</td>
</tr>
<tr>
<td>Antimicrobial resistance</td>
<td>$4.0 billion</td>
</tr>
</tbody>
</table>

Infectious diseases contribute significantly to economic losses and lost productivity in the United States.

**TABLE 7.5**

Burden of Three Selected Infectious Diseases in the USA

<table>
<thead>
<tr>
<th>Hospitalizations</th>
<th>Mortality Rate</th>
<th>Mortality</th>
<th>$ per Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe sepsis</td>
<td>660,000</td>
<td>23%</td>
<td>150,000</td>
</tr>
<tr>
<td>Community acquired</td>
<td>395,000</td>
<td>13%</td>
<td>50,000</td>
</tr>
<tr>
<td>Hospital acquired</td>
<td>265,000</td>
<td>38%</td>
<td>100,000</td>
</tr>
<tr>
<td>Adequate initial Rx (70%)</td>
<td>185,000</td>
<td>28%</td>
<td>51,000</td>
</tr>
<tr>
<td>Inadequate initial Rx (30%)</td>
<td>80,000</td>
<td>62%</td>
<td>49,000</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>1,300,000</td>
<td>9%</td>
<td>115,000</td>
</tr>
<tr>
<td>Community acquired</td>
<td>1,000,000</td>
<td>2.4%</td>
<td>24,000</td>
</tr>
<tr>
<td>Hospital acquired</td>
<td>300,000</td>
<td>30%</td>
<td>90,000</td>
</tr>
<tr>
<td>Ventilator associated pneumonia</td>
<td>135,000</td>
<td>45%</td>
<td>61,000</td>
</tr>
<tr>
<td>Adequate initial Rx (56–75%)</td>
<td>75,000–100,000</td>
<td>10–20%</td>
<td>30%–50%</td>
</tr>
<tr>
<td>Inadequate initial Rx (25–44%)</td>
<td>35,000–60,000</td>
<td>40–60%</td>
<td></td>
</tr>
</tbody>
</table>

*Note: In an average 500-bed hospital, studies indicate that patients with acquired infections will receive inadequate initial antimicrobial therapy XXX per year:
  * About 40 with hospital acquired sepsis
  * Between 20–30 with ventilator associated pneumonia

---

<table>
<thead>
<tr>
<th>Reference</th>
</tr>
</thead>
</table>
7.2.3 **Who Uses an Antibiogram?**

Health care providers, including:

- Physicians
- Intensivists in intensive care units
- Infection control practitioners
- Pharmacists
- Microbiologists
- Administrators

7.3 **Procedure**

7.3.1 **Overview of Antibiogram Development**

The process of antibiogram development is a continual interaction of three analytical phases: pre-analytic, analytic, and post-analytic (Figure 7.2). To begin the process, the best starting point is pre-analytic. The first step is data design (I) followed by actual data collection (II). The process then moves into data compilation, using varied types of statistical tools to best exhibit the desired results (III). The post-analytic phase involves the different chart types and presentation methods that best suit the institution and the internal customers (IV). The transition phase between pre-analytic and post-analytic is comparative analysis that brings to light any changes needed to be addressed in the data designing phase for the next cycle of antibiogram design and development.

7.3.2 **Preanalytical—Data Designing**

(See checklist for Implementing an Antibiogram, Table 7.6)

7.3.2.1 Data Designing

The antibiogram must fit the surveillance needs of the institution in order to have real value. Several criteria must be identified in order to achieve this goal.

![Strategy for Development](image)

**FIGURE 7.2** Overview of designing, implementing, and evaluating an antibiogram.
### TABLE 7.6
Checklist for Implementing an Antibiogram

**A. Pre-analytical**
1. Identify who the members are of the current P&T/Infection Control committee:
   - __Infection control
   - __Pharmacy
   - __Microbiology
   - __Administration
   - __Medical doctor to represent institution specialties
2. __Set team goals
3. __Perform a team review
4. __Assess the current antibiogram
5. __Determine frequency of the antibiogram development
6. __What is the frame necessary to completely exhibit trending?
7. What are the desired demographics?
   - __Patient identifier
   - __Patient location
   - __Specimen identifier
   - __Specimen source/type
   - __Organism subset such as Methcillin Resistant *Staphylococcus aureus* (MRSA)/Methcillin Susceptible *Staphylococcus aureus* (MSSA)
   - __Patient age
   - __Institution
   - __Diagnosis
   - __Clinical specialty
   - __Other ________________________
8. __Off-site locations such as geriatric (nursing home)
9. Choose data type
   - __AST method/result
   - __Formulary list requirements
   - __Drug class
   - __Individual drugs formulary
   - __Antimicrobial agents
   - __Organism identification list

**B. Analytical**
10. __Needs of internal customers
11. Where is the information?
   - __The Hospital Information System (HIS)
   - __The Laboratory Information System (LIS)
   - __The Instrument Information System (Microbiology ID/AST system)
   - __The Hospital Pharmacy Information System (HPS)
   - __An electronic database
12. __Determine the available fields in the institution’s computer

**C. Post-analytical**
13. __Patient management (template)
14. __Hospital management (template)
15. __Determine presentation types (tables and figures)
   - __Chart type
   - __Bar graph
   - __Pie chart
   - __Percent
16. __Report type
   - __Traditional
   - __Nontraditional

---

P&T = Pharmacy and Therapeutics; AST = Antimicrobial and Susceptible Testing; ID/AST = Identification and Antimicrobial and Susceptible Testing.
7.3.2.1.1 Define the Internal Customer
This aspect is dependant on institution type, size, and specialty. Identify who the members are of the current Pharmacy and Therapeutics (P&T) and Infection Control committee. A minimum of a core team is required to successfully cover all areas of involvement. These core team members include infection control, pharmacy, microbiology, and administration. Additional members such as a medical doctor, who represents the institution specialties, can be added if necessary.

7.3.2.1.2 Set Goals
The next set of criteria to be addressed is the team goals. The following points should be considered:

- Surveillance for institution resistance trends: The results of this would fall under the responsibility of the institution team administrative representative.
- Surveillance of formulary functionality within the institution: The pharmacist team member would utilize the antibiogram to evaluate the efficacy and cost-effective nature of the available institution therapies.
- Surveillance of emerging pathogen specifics: The infectious disease physician would participate in the development of the antibiogram to track the incidence of pathogen activity.

7.3.2.1.3 Assess the Team’s Needs
Perform a team review and assess the current antibiogram if it exists or discuss the following points with the team for antibiogram development.

Determine frequency of the antibiogram development. Is it a rolling report? What is the time frame necessary to completely exhibit trending? The minimum time frame to best exhibit a trend is 6 months. If data is not available for 6 months, begin assessing data at 3 months.

7.3.2.1.4 Identify the Desired Report Demographics
The primary demographics required in an antibiogram must include:

- Unique patient identification number
- Healthcare facility for institutions with multiple laboratory locations
- Patient location. Inpatient vs. outpatient; community vs. noncommunity. (These terms may be used interchangeably.)

The primary specimen information required in an antibiogram must include:

- Specimen unique identifier
- Specimen source/type. Blood vs. non-blood; urine vs. non-urine. Delineation of specimen source into larger groups is necessary to separate appropriate therapy methods (urinary antimicrobics vs. non-urinary antimicrobics) on the antibiogram chart.

Other institution information:

- Clinical services:
  - ICU (Medical ICU/Surgical ICU)
  - Medical or surgical unit (acute care)
  - Pediatric
  - BMTU (bone marrow transplant unit)
  - ED (emergency department)

Organism subsets such as Methcillin Resistant Staphylococcus Aureus (MRSA)/Methcillin Susceptible Staphylococcus Aureus (MSSA).
Secondary demographics that can be of value in final data assessments are: patient age or gender, institution service such as oncology or the area of specialty treatment within the institution, diagnosis types such as cystic fibrosis, and off-site locations such as geriatric (nursing home).

7.3.2.1.5 Data Type
The choice of data to be exhibited should reflect the needs of the institution.

*Antimicrobial susceptibility testing (AST) method and result.* Ask the team the following question: Does the medical staff utilize minimum inhibitory concentration (MIC) results? If so, then analysis of MIC results should be displayed on the antibiogram as MIC<sub>50</sub> or MIC<sub>90</sub>. If MIC results are not utilized, then use the percent susceptible (S), intermediate (I), or resistant (R) on the antibiogram.

* Routinely calculate the percent susceptible, excluding intermediate.* Exceptions include:
  * combining percent S and I for *Streptococcus pneumoniae*
  * penicillin and ESBLs (extended spectrum β-lactamase)
  * penicillin and viridians group streptococci

*Please note:* some institutions have chosen to group antibiotic effectiveness by selected categories including: “1st choice,” “alternative,” “growing resistance,” and “don’t use.”

7.3.2.1.6 Formulary List Requirements
The next step is to discuss the following points with the team to assure the pharmaceutical relevance of the antibiogram:

  * Drug class: The antibiogram may be divided into drug classes over a more broad range of antimicrobials if the formulary is not strictly observed by the prescribing physicians
  * Individual: Select drugs in use at the institution can be listed.
  * Only those antimicrobial agents on formulary should be listed if enforcement of the formulary is required.
  * This list may or may not include restricted antimicrobials.
  * Do not include combinations with no clinical indication/utility.

7.3.2.1.7 Organism Identification List
This is the last set of data to be discussed within the team.

  * Only the most common isolates (15–25) should be listed. These isolates may be different for selected specimens or patient service areas.
  * Do not include isolates with very low numbers (<10 isolates).
  * For *S. aureus*, provide routine analysis, but include an analysis of oxacillin-resistant subset.

7.3.2.2 Data to Avoid

  * Eliminate duplicates by including the “first isolate” for each patient. Duplicate results can skew the data and should be avoided. Parameters should be agreed upon within the team as to what constitutes a “duplicate.” A specific time frame and associated specimen type defines a duplicate culture. For example, two blood cultures with the same organism isolate and susceptibility pattern from the same patient within three days would constitute a duplicate culture.
  * Inappropriate organism and antimicrobial agent combinations such as *E. coli* and erythromycin should be avoided.
  * Known innate resistance patterns such as *P. aeruginosa* and ampicillin, *Enterococcus* spp. and cephalosporins, and *P. mirabilis* and nitrofurantoin should not be part of the antibiogram.
• Organism and antibiotic combinations with no resistance mechanisms described to date such as Vancomycin-resistant *Staphylococcus* spp. or Ceftriaxone-resistant *Haemophilus* spp.
• Isolates from surveillance cultures.

### 7.3.2.3 Summary of Pre-Analytical Stage

The team members have been chosen and their antibiogram needs explored. With these needs in mind, the design of the antibiogram content is complete. The next stage is the data analysis or *analytic* phase.

### 7.3.3 Analytic — Data Collection and Storage

(See checklist for Implementing an Antibiogram, Table 7.6, Part B.)

#### 7.3.3.1 Data Collection Sources

There are several possible sources that depend upon the institution structure and complexity. Larger sized institutions will have more data mining opportunities. A higher level of computerization within the institution will also ease the data mining process. In any case, the data can be retrieved using manual techniques or computerization.

Manual data retrieval will require a larger time obligation, as all laboratory and microbiology records will need to be handled manually. Automated data retrieval will depend greatly on the available computer systems within the institution.

The first step is to look at the needs of the internal customers (team members) and decide what information is necessary to accomplish the team goals. The second step is locating the information? In an institution with a computerized information system, the first place to look for the data is the available reports that may be generated from within that system.

The hospital information system (HIS) will generally have patient history and demographics. This system also will have current and historical laboratory patient reports available for data mining either on a manual or electronic basis. Often the HIS has an interface with the laboratory information system (LIS).

The LIS will provide current laboratory testing information through a direct link from the testing instruments. Most LISs are interfaced to the individual instruments that provide actual testing results. In some cases, where the electronic interface is not present, the result and demographic data are entered manually by the technologist performing the test.

The instrument information system (microbiology ID/AST system) will produce printed patient reports (include patient demographic information) or laboratory reports (include the lab result with a patient identifier) or electronically stored reports with accessible data and an available data tracking system. The LIS is the primary generator of patient reports and the instrument data manager usually produces the lab report. It’s these lab reports that show the microbiologist more detailed analysis of the patient’s actual results, biochemical reactions and MIC/Interpretations that assist the microbiologist in determining the accuracy of the result. The patient result is an LIS stripped down version of this showing the physician only the filtered results that are relevant to the patient. ID/AST instrument manufacturers provide software that will generate reports that can be customized to meet the antibiogram needs:

The BD EpiCenter™ Microbiology Data Management System supports the BD Phoenix™ AST/ID system with two expert systems, “pro-active” alerting for microbiologists and other key staff, and extensive epidemiology reporting functionality. Phoenix™ provides both resistance marker confirmation and true doubling MIC dilutions that employs a proprietary redox indicator. Phoenix™ delivers this data to the EpiCenter every 20 minutes. Data are analyzed through the two expert systems resident in EpiCenter. The first of these
systems BDXpert™ analyzes data using a “best practice” class CLIS rule set that can be customized by the user. These data are then further refined using the BD EpiCenter EpiCARE™ system. This patent pending “Clinical Applications Rules Editor” provides microbiologists with software that can further expertise the data using specific factors unique to the patient population. These data are linked to the software’s “pro-active” alerting systems that can alert the microbiologists, physicians, pharmacists, or infection control staff of sentinel events defined by their institution.

EpiCenter also provides a true multi-user environment enabling the lab, infection control and pharmacy to simultaneously and securely use the software’s extensive epidemiology library and reporting tools. The system’s library and built-in report design tools empowers users to actively mine data to analyze outbreaks and emerging resistance trends, create customized presentations and then share this information electronically with their colleagues. These tools also provide the ability to routinely monitor events and then use EpiCARE to set up sentinel event alerts. EpiCenter also simultaneously supports BD Bactec™ blood culture systems integrating the microbiology department for improved efficiency. The system integrates seamlessly with the existing LIS eliminating duplicate data entry.

The bioMeriéux VITEK instrument software is configured to detect emerging resistance. The VITEK 2 brings the Advanced Expert System® (AES), which assigns a specific phenotype to each organism tested. This second generation expert system assigns a phenotype to each isolate based on the identification of the organism and the MIC distribution of the antibiotics tested against that organism. The database is comprised of over 2,000 phenotypes and 20,000 MIC distributions. The AES matches the phenotype with the resistance mechanisms known to be associated with that phenotype. This allows for biologic and therapeutic corrections to be made to the microbiology report. AES also allows for the detection of rare phenotypes and phenotypes yet to be described, obviously important in monitoring and reporting emerging antibiotic resistance. The AES is kept current with software upgrades that allow for the incorporation of new phenotypes and resistance mechanisms and the expected distribution of MICs can be modified, as new information becomes available, to insure the detection of known resistance mechanisms if the MIC changes over time. Unlike other expert systems, AES validates all results by assigning them to a known phenotype. This allows users to configure the VITEK 2 as they deem appropriate; for example, a laboratory may choose to “auto-release” those results that AES has found to be fully consistent with a known phenotype. This insures results are released to clinicians in the shortest possible time frame.

VITEK 1 and VITEK 2 therefore detect resistance, including emerging resistance, using several mechanisms. Increased range of dilutions allows for the detection of creeping resistance. Software is used for the generation of antibiograms and MIC trending reports. Expert systems, including the second generation Advanced Expert System, validate results in addition to assigning a phenotype with associated resistance mechanisms to each organism isolated in the laboratory.

The Hospital Pharmacy Information System (HPS) tracks patient demographics, laboratory results and current antimicrobial therapies.

An Electronic Database such as TSN (The Surveillance Network) has accumulated susceptibility and identification statistics from individual institutions and globally to produce resistance statistics. The TSN Database–USA, initiated in 1994 by Focus Technologies, Inc. (formerly MRL, Herndon, Virginia), is a queriable real-time database that electronically assimilates antimicrobial susceptibility testing data and patient demographic data from a network of 270 accredited hospitals in the United States. A laboratory’s inclusion in TSN is based on factors such as hospital bed size, patient population, geographic location, and antimicrobial susceptibility testing methods employed. Susceptibility testing
of patient isolates is conducted on-site by each participating laboratory as a part of their routine diagnostic testing.\textsuperscript{21}

7.3.3.2 What Information?

Specific Infection Control team report parameter needs are delineated in available database fields located in the LIS or ID/AST instrument software. Once team needs are well in hand, the best place to discover data availability is in the LIS or HIS. The available information fields will restrict or enhance data accessibility. The field contains an information packet that identifies a data group or searchable statistic. See the sample with identified fields below.

<table>
<thead>
<tr>
<th>Collection Date</th>
<th>Specimen Number</th>
<th>Source Organism</th>
<th>Organism Code</th>
<th>Antibiotic Tested</th>
<th>Susceptibility Code</th>
<th>Result (µg/mL)</th>
<th>Interpretation</th>
</tr>
</thead>
</table>

This table contains the information accessible to the technologist for data mining.\textsuperscript{1} Coding is utilized to prevent transcription inconsistencies with data entered by various individuals.\textsuperscript{2}

Next, assess the field information availability. The fields of information may be available but not currently defined or in use. The institution information services contact will address the implementation of needed fields or packets of information to provide team required statistics. An example of this follows: the LIS has a field for patient location, but that particular field is turned off or not in use. The LIS manager may be able to work with the team needs to make that information accessible.

7.3.4 POST-ANALYTIC — RETRIEVING DATA, COMPILATION, AND PRESENTATION

(See checklist for Implementing an Antibiogram, Table 7.6, Part C.)

7.3.4.1 Antibiogram Type

Laboratories historically have created a yearly single dimension cumulative antibiogram for the institution. In the mid-1980's, patient types and specialization expanded, thus it became apparent that institutions needed to unmask potentially infrequent but significant resistant phenotypes and subset the population. Thus, focused antibiograms started to emerge with the number of subsets reflective of the specialization, size and educational commitment of the institutions. This culminated in the organization of two distinct types of focused antibiograms: a) those that deal with the patient management and selection of antimicrobial therapy and b) those that deal with hospital pharmacy and infection control management, where issues of the epidemiology and the evaluation and tracking of selected resistant phenotypes unique to that institution are followed. In describing the options available, a template is created that allows an institution to design and generate, in advance, those sort-parameters for patient management or hospital management to routinely track the antibiotic signatures in their institution.

7.3.4.1.1 Patient Management (Templates)

- Specialty organisms: The “other” Microbes, Mycobacteria, Yeast/Moulds
- Hospital Pharmacy/Infection Control management by source, service, and by Profession, i.e. (School of Dentistry)
- Microbial Phenotypic tracking
- Hospital epidemiology
- Traditional bug/drug format Total vs. Focused and non—traditional format
- Specific Example/Recommendations
7.3.4.2 Presentation

Hospital susceptibility/resistance data can be maximized by the method of presentation. Historically, these were tables that listed the focused antibiotic and organism data. Today, aided with software programs, one can maximize the targeted organism antibiotic combinations and measure selected changes visually by expressing data in a variety of manners. Hence, some data is best presented as a bar graph, some as a pie chart and some as percentages, recognizing the statistical validity of the data.

Recently, advances have also been made so that individuals may present both a graphic presentation with an overall signature and the data that corresponds to that in the same table.

7.3.4.2.1 Chart Types

- Bar graph
- Pie chart
- Tabular
- Scientific Examples/Recommendations by organism

7.3.4.2.2 Chart Presentation

As greater susceptibility/resistance data is available and our access to software analysis becomes more convenient, the options grow in presenting these data. Traditional antibiograms including focused, demonstrate time honored formats. However, non-traditional antibiograms are emerging, including susceptibility profiles based on patient disease/DRG, antibiotics organized by mode of action rather than by class, and combinations of bugs/drugs that represent the seven most frequent combinations in the institutions and their total median resistance plotted over years, i.e. tracking the “institution profile.”

- Traditional% (Table 7.7)
- Non-traditional (Table 7.8) — Composite of X-microbiology, Y-pharmacy, and Z-costs as a guide. These are usually syndrom focused.

---

### TABLE 7.7

**Traditional Data Compilation, Analysis, and Presentation**

| A. | Single Dimension Antibiogram                  |
|    | (Percent resistance or susceptibility): Tracking or preventative surveillance |
| B. | Hospital or community location               |
|    | IP/OP/hospital location                       |
| C. | Specimen source                              |
|    | Urine versus non-urine or blood only         |
| D. | MICₜ₅/MIC₉₀ (selected isolates)               |
| E. | Floating 6-month “window”                    |
| F. | Date of admission                            |
|    | <3 days — community acquired                 |
|    | ≥4 days — Nosocomial                         |
| G. | Organism                                     |
|    | *S. pneumoniae*                              |
|    | MRSA/MRSE                                    |
|    | VRE                                         |
|    | ESBL                                        |
|    | VISA/VRSA                                    |
|    | SA: Clindamycin/Erythromycin                 |
Antimicrobial Susceptibility Testing Protocols

7.3.4.3 Levels of Monitoring

West Virginia University (WVU) provides six levels of monitoring susceptibility programs as described below, usually on a yearly basis. Hospitals of varying size and diversity may need to tailor their needs (Figure 7.3). As described earlier, the primary purpose of antibiograms is five-fold:

1. Provide guidance in selecting empiric therapy.
3. Complement epidemiologic investigations.
4. Satisfy regulatory agencies.
5. Guide hospital formulary discussions.

7.3.4.3.1 Level I

A generalized antibiogram illustrates the composite antibiotic susceptibility signature of the entire in-patient population. This does not separate organism-antibiotic combinations by age, hospital service and/or clinics. This establishes a base-line database and allows for generalized trend analysis over a period of time. This is the more traditional manner of summarizing susceptibility data.
7.3.4.3.2 Level 2

Recently, focused antibiograms have become the method of choice for measuring small changes in susceptibility patterns that may be masked in the “Generalized Antibiogram.” In the Focused Antibiogram, selected age groups, clinical presentations, i.e. cystic fibrosis and hospital locations such as ICUs, are separated from the generalized data and “focused” upon for selected bug-drug combinations. It has been found that these reveal hidden trends that may not be evident in the Generalized Format; of particular focus are the ICUs, where concentrated use of cephalosporins is encountered.

7.3.4.3.3 Level 3

Fungal (yeast) susceptibility data is tracked in two ways: 1) MIC distribution for the 6 most common yeast and 2) a yeast Histogram.

7.3.4.3.3.1 Anaerobic Bacteria, Mycobacteria, Virus and Unusual, Fastidious Organisms Susceptibility Profiles

For anaerobic bacteria established national susceptibility profiles have been generated. The CDC does not recommend routine susceptibility testing; in mixed anaerobic infections or for single anaerobic isolates, published guidelines should be used in selecting antimicrobial therapy. However, due to growing resistance, some laboratories assay anaerobes as a “batch,” usually once a month.

7.3.4.3.4 Level 4

7.3.4.3.4.1 Miscellaneous Susceptibility Data

The microbiology laboratory sends significant clinical isolates to national reference laboratories for susceptibility testing not routinely performed. These results are collated annually and are available via consultation with the Medical Director. These include mycobacteria, virus, and unusual or fastidious isolates.

7.3.4.3.5 Level 5

7.3.4.3.5.1 Predictable (Established) Susceptibility Patterns; i.e., “Preventative Surveillance”

Stable bug-drug patterns over five years can be used to eliminate routine susceptibility testing, as an example, outpatient urines.

7.3.4.3.6 Level 6

Lastly, by computer analysis, all sensitivities are compared daily to the established patterns or predictable norms of previous isolates via the antibiograms over the past three years. Unusual patterns are highlighted and/or discussed with the attending physician as appropriate on a case/case basis.
7.3.4.4 Examples of Data Compilation

See Figure 7.4.

7.3.4.4.1 Comparative National Data: Surveillance

The use of electronic surveillance and national databases has increased dramatically over the last ten years. There presently are several national databases that allow the participating institutions to download their data on a daily basis and to analyze it in real time. Depending on the national database and its sophistication there is also the potential of searching that database by a variety of parameters in real time. A number of these are described. In addition to the analysis, the real time availability allows for integration of this database into the workflow of both the laboratory and patient management in clinical rounds. This is highlighted (Figure 7.4) describing the partnering of microbiology data, pharmacy data and clinical needs during rounding with medical staff. Further, this database allows one to establish on a routine basis analysis of information provided by the source. Hence, templates can be created and information requested on a routine timely basis as defined by the user.

7.3.4.4.2 Generalized — Electronic, Real Time-International, National, Regional

TSN — Personalized — Multiple Sort Parameters
(Computer Screen/Selection)

7.3.4.4.3 Focused — Retrospective

In contrast to national electronic databases, information can be obtained via participation in national surveys. There are a number of these and they have unique features, recognizing they are static and that their implementation or changing of antibiotic therapy is done based on a yearly summation, not on a daily basis as is the national electronic database. These are dependent on users and their participation in these studies.

- ISS — Merck: ICU gram-negative rods
- SMART — University of Iowa: BSI
- TRUST — Ortho McNeil: URI

FIGURE 7.4 Example of data compilation (traditional).
7.3.4.5 **Electronic Data Collection Devices**

Real time use of electronically acquired antibiotic data has been generally tethered to a desktop computer. Recently, with the advance of PDRs (Personal Data Resource), information is available dependent upon network connection/availability. This clearly will have tremendous expansion potential and in the future may present the most realistic mode of acquiring national data, analyzing it and having a return of these data to the user in a real time scenario.

### 7.4 IMPLEMENTATION/INFORMATION MANAGEMENT/UNLOCKING DATA/MICROECONOMICS

Data is power. As the availability and utilization of susceptibility data grows, its uses, in heretofore-unrecognized scenarios, also grows. This is particularly true in the economic environment that we are presently involved with. It is necessary to routinely run susceptibility patterns on outpatient urine cultures for organisms that have established and stable susceptibility patterns over five years. Can one use a database management that allows for tracking of patterns and predictions based on the stability of those patterns over time? The answer would appear to be yes, given that there is some way of sampling a significant portion to routinely monitor the potential of changes without doing all patients at all times. Antibiograms and the information they provide is an empowerment for the microbiologist. The information held in the antibiograms is unfathomable. Yet its application and its daily use have been very restricted. This should not continue. With the increasing use of electronic surveillance and the availability of immediate recall, antibiograms will be a means by which a laboratory can further prove its value, and to document benefit to patient care via shortening of length of stay and reduced hospitalization costs, i.e. outcomes (Figure 7.5).

Recognizing the importance of Cumulative Antibiograms for Clinical Microbiology, the CLSI in May of 2002, approved guidelines for the tabulation of susceptibility data and provided a second edition in 2006 (Analysis and Prevention of Cumulative Susceptibility Test Data; Approved Guidelines-2nd Edition). This guideline is a consensus document and clearly has as a focus, standardization for hospital reporting. It has both “pros” and “cons” depending upon the institution’s previous attempts. One of the major issues discussed during the document development was whether “resistance” or “susceptibility” should be reported. Ultimately, history prevailed, recognizing that most physicians and users have long been accustomed to the term “susceptible”. The CLSI standard has

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**FIGURE 7.5** Summary of antibiogram utility and focus.
several points that need to be considered when producing a Cumulative Antibiogram. The report should be prepared annually. It should establish Susceptible (S) isolates only, not including Intermediate (I) isolates. It should include results from the first isolate of a given species encountered by a patient. It should exclude surveillance isolates, and it should report results for all drugs tested that are appropriate for the species, however, one should not report supplemental drugs that are selectively tested on resistant isolates only. It also clearly defines how frequently repeat isolates from the same patient should be tabulated. Most importantly, a statistical analysis section was added, which focused on the number of isolates needed to define a standard deviation. This was emphasized in “tables,” which demonstrated, based on the number of isolates, the range that in fact was represented by that susceptibility result of the institution (see Figure 7.5).

Unfortunately a large proportion of hospitals do not have the capacity to sort by all of these parameters. It is important to note that in our experience, the “first isolates” susceptibilities versus the “total isolates” susceptibilities, including removing duplicated isolates, results in a difference of about 5%.

To facilitate the improved utilization of this incredible data, we have included several options that will guide the user in correctly implementing the steps necessary for accurate and timely antibiotic data (Table 7.6).

7.4.1 Tier I

- Focused Antibiogram: Organism Prevalence ICU
- Focused Antibiogram: % Susceptible ICU
- Relative Resistance
- Relative Resistance Prevalence by Resistance Type
- Trending: Over/Under Analysis

The most frequently used antibiograms are cumulative summaries (usually a year) and have been discussed in detail up to this point. However, with increased resistance of selected patient types and the diversification of hospital organization with “cohorting” by age, disease type and subspecialties, total cumulative antibiograms have limited application. Hence, a number of newer applications of antibiotic data are presented here, focusing particularly on those institutions that are larger than 400-bed and/or teaching and university centers.

The first is a “focused” antibiogram. Antibiotic resistance is sorted by selected parameters, generally the hospital service area, age of the patient, organism, and/or specimen source, i.e. bloods, urines, and respiratory. Focused antibiograms can be tailored to the most prevalent organisms within that service area, and have the advantage of highlighting low frequency resistant phenotypes which may be unrecognized in a standard cumulative antibiogram. Two examples presented here are focused antibiograms for the ICU, which list first, the prevalence within that subset and secondly, the susceptibility of those selected organisms to antimicrobials that would be most useful in dealing with infections in those areas.

The second emerging modification is that of the “Relative Resistant” antibiogram. “Relative Resistance” is based on selected phenotypes and rather than establishing the percent of that individual phenotype, tabulates the percent of that resistant phenotype against the other selected resistant phenotypes (i.e. non-susceptible isolated only); hence, the percentage plot represents a true indication of the frequency of that resistance phenotype within the population tested. This clearly unmasks the most prevalent resistance and can be subset according to hospital location, organism type, age, etc.

Table 7.9 describes Relative Resistance based on eleven important phenotypes and underscores the importance of the frequency of the individual isolate relative to the frequency of all key non-susceptible isolates. Figure 7.6 plots the relative resistance as a visual presentation.
Another way of demonstrating the change in relative resistance is to plot the selected non-susceptible isolates phenotypes by year (Figure 7.7) and combine this by an “over/under” change as shown in Figure 7.8. Here, a comparison demonstrates that methicillin-resistant CNS decreased in the time period evaluated, whereas gentamicin resistance and imipenem non-susceptible/resistance increased significantly. This idea of trending using the “over/under” analysis is key for selected areas, particularly where cyclical use of antimicrobials may be apparent, as in the ICU; the antibiogram data is focused on a particular service area, with a particular antibiotic, and a particular organism.

**TABLE 7.9**
Relative Resistance

<table>
<thead>
<tr>
<th>Rank</th>
<th>Resistance Type</th>
<th>Total N</th>
<th>N Res</th>
<th>%R</th>
<th>Relative resistance %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MRCNS</td>
<td>4,928</td>
<td>3,597</td>
<td>73.0%</td>
<td>62.4</td>
</tr>
<tr>
<td>2</td>
<td>MRSA</td>
<td>2,915</td>
<td>1,031</td>
<td>35.4%</td>
<td>17.9</td>
</tr>
<tr>
<td>3</td>
<td>CIPRO Res P. aeruginosa</td>
<td>1,348</td>
<td>372</td>
<td>27.6%</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>GENT Res P. aeruginosa</td>
<td>1,739</td>
<td>263</td>
<td>15.1%</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>VAN Res E. faecium</td>
<td>273</td>
<td>164</td>
<td>60.1%</td>
<td>2.8</td>
</tr>
<tr>
<td>6</td>
<td>IMP Res P. aeruginosa</td>
<td>1,339</td>
<td>152</td>
<td>11.4%</td>
<td>2.6</td>
</tr>
<tr>
<td>7</td>
<td>CIPRO Res E. coli</td>
<td>6,825</td>
<td>71</td>
<td>1.0%</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>PEN Res S. pneumoniae</td>
<td>243</td>
<td>67</td>
<td>27.6%</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>CAZ Res K. pneumoniae</td>
<td>475</td>
<td>43</td>
<td>9.1%</td>
<td>0.7</td>
</tr>
<tr>
<td>10</td>
<td>CRO Res E. coli</td>
<td>4,916</td>
<td>2</td>
<td>0.0%</td>
<td>0.0</td>
</tr>
<tr>
<td>11</td>
<td>LEVO Res S. pneumoniae</td>
<td>3</td>
<td>0</td>
<td>0.0%</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The Relative Resistance is calculated as the % of each resistant phenotype as part of all non-susceptible isolates only. It is a true indicator of the impact of that isolate on the patient population.

**FIGURE 7.6** Relative resistance prevalence.
7.4.2 Tier II

- Cumulative Presentation (Figure 7.9)
- Cumulative MIC
- Cumulative Resistance by Mechanism of Resistance
- Cumulative Antibiogram: Cross Resistance

Here the cumulative presentation over a selected period of time, usually a year, may be sorted by a selected characteristic of the antibiotic, particularly emphasizing its mechanisms of resistance. Three examples are shown. First, Cumulative Distribution of MICs tabulates the percent of susceptibility associated with an increasing concentration of the anti-infective. Generally, the organism in the upper left hand corner is more efficacious and is less efficacious as one moves from the upper left to the lower right in the graph.
Cumulative “Mechanism of Resistance” is a unique and uncommon presentation: it does however have unique links. The mechanism of resistance is generally defined by the organisms themselves, so that antibiotics with similar mechanisms via selected phenotypes are graphed together (Figure 7.10). This is often referred to as non-susceptible isolate distribution, because the organisms that are plotted are non-susceptible or resistant isolates only.
A unique amplification of mechanisms of resistance is shown in Figure 7.11, which describes the mechanism of resistance, but compared by years. Here, selected non-susceptible isolates are grouped by mechanism of resistance, and changes over time are clearly highlighted.

Cumulative antibiograms separated by cross-resistance are shown in Figure 7.12. The focus of this antibiogram is multidrug cross-resistance and determines that if a singular phenotype or a
particular antibiotic combination is associated with a simultaneous increase in resistance for another antibiotic.

Shown are the data for ceftazidime MICs, which when measured with other antibiotics, remain stable only for imipenem; all other antibiotics show increasing MIC with higher ceftazidime MICs.

7.4.3 TIER III

- Pharmaconomics (X, Y, Z)
- CAG: Comprehensive Antimicrobial Guide, Integration of % Susceptible, Empiric Selection, and Economics (X, Y, Z)
- Syndrome Approach (X, Y, Z)

Tier 3 represents the amalgamation of the various components involved in patient care and burden of disease. It is an attempt to integrate data from microbiology (represented by X), pharmacy (represented by Y), and cost (represented by Z). One way of integrating data and linking these three features is shown in Figure 7.13, the PharmacoEconomic Form. In cascade fashion, one addresses a susceptibility breakpoint quotient (SBPQ), a serum-inhibitory quotient (SIQ), and finally a cost code, recognizing a 24-hour cost divided by the SIQ. By using the E test, one may illustrate for comparative strips, the antibiotic MIC breakpoint and the zone of inhibition (MIC).

Data are shown prominently in the Comprehensive Antimicrobial Guide shown in Figure 7.14. Here antimicrobials are ranked in efficacy (1–3), based on the features of microbiology susceptibility data, pharmacologics of the antimicrobial, and the cost per 24 hours by the P&T Committee. It avoids the problems of antibiotic percentage comparisons, given that an antibiogram with cumulative percentages only, represents one feature of the three parts. Number 1 is the preferred drug of the institution, number 2 is good coverage, and number 3 is alternative with R representing significant resistance emerging (5-10% change/year).

Figure 7.15 is another attempt at integrating the three features of X, Y and Z, often called the Syndrome Antibiogram. Here, antimicrobials are ranked as either 1) being effective or 2) less effective, but focusing on organisms associated with syndromes, with a simple cost of therapy dollars per day from pharmacy. This is particularly useful when physicians select empiric therapy based on clinical presentation matched to the frequency with which organisms appear within the institution. This can be tailored to the individual hospital, given the geographic differences are noted and reflective also of the type of institutional, its size and patient capabilities. This is a composite presentation, which is simple, but highly effective.

7.4.4 TIER IV

- Other Antibiogram Examples
  - Fungal/Yeast
  - Yeast MICs
  - Dental
- Methods of Distribution (www. etc) - at least 6 methods:
  - Card (Traditional)
  - Electronic (Intra or Internet)
  - PDA (Hand held device)
  - Booklet
  - Path Lab Manual
  - Hard copy always in Front of Patient Chart.

This outlines Antibiograms for organisms or services other than the traditional microbiology laboratory. Here, the focus has recently been on yeast susceptibilities, particularly Candida albicans
Antimicrobial Susceptibility Testing Protocols

There are now choices that were not available several years ago. In recognizing that most laboratories list C. albicans as one of the five most frequent blood culture isolates in blood cultures, its prevalence is clearly significant. Given that there are several FDA approved commercial antifungal assays, a fungal Antibiogram is important.

FIGURE 7.16 shows a Fungal Antibiogram for four antifungals: amphotericin B, fluconazole, 5FC and itraconazole. Endpoints for other and newer antifungals are still controversial and are as yet

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<table>
<thead>
<tr>
<th>Code</th>
<th>X - MICROBIOLOGY</th>
<th>Y - PHARMACY</th>
<th>Z - COST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt</td>
<td></td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1.</td>
<td>Organism:</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>2.</td>
<td>Source:</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>3.</td>
<td>Ant Infective:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>NCLSI &quot;S&quot; Breakpoint</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>MIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>S, I, R, Category</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>MBEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>MBEC/MIC RATIO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Susceptibility Breakpoint Quotient (S-BP/MIC)</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>10.</td>
<td>Serum Level (C_{max}) / MIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>S / Q (Quotient)</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>12.</td>
<td>Cost/24 HR Quotient (Life Treating) WUH ($)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>AWP ($)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Cost / SIG Quotient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Ranking</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 7.13 Pharmacoeconomics. The integration of three important factors when selecting antibiotics for hospital formulary: microbiology (X), pharmacy (Y), and costs (Z).

and the other Candida species, given that a number of antifungals are now available. There are now choices that were not available several years ago. In recognizing that most laboratories list C. albicans as one of the five most frequent blood culture isolates in blood cultures, its prevalence is clearly significant. Given that there are several FDA approved commercial antifungal assays, a fungal Antibiogram is important.
FIGURE 7.14 Susceptibility-based antibiotic guide. Implementation of the X-Y-Z factors into a useable resource. On this CAG (Comprehensive Antibiotic Guide) card (not shown) are listed cost comparisons, empiric selection by source/bug/symptoms segregated by adult and pediatric concentrations.
Antimicrobial Susceptibility Testing Protocols

unrecognized, but clearly for the laboratory, measuring an MIC and tracking it is equally as important, given the cost of the various antifungals available. Between the first generation antifungals and the more recent third generation, a cost difference over a hundred fold is not unusual.

The next two figures (Figures 7.17 and 7.18) list Candida MIC data. A cumulative plot of the MICs for C. albicans and C. galbrata is highlighted, recognizing the considerable concern about the development of resistance and/or intrinsic resistance in question.

Figure 7.19 shows the importance of fungal susceptibility, highlighting an algorithm and the use of PNA-FISH technology to rapidly recognize and detect C. albicans in blood cultures.
Here based on the antibiograms for yeast, an algorithm has been described which incorporates two parameters: rapid molecular methodology and previous history of resistance within the institution.

### Fungal Antibiogram Interpretation

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total Tested</th>
<th>mpB</th>
<th>Fluc</th>
<th>5FC</th>
<th>Itra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>285</td>
<td>287</td>
<td>282</td>
<td>278</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
<td>283</td>
<td>277</td>
<td>278</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Sus %</td>
<td>99%</td>
<td>97%</td>
<td>99%</td>
<td>92%</td>
<td></td>
</tr>
<tr>
<td>S-DD%</td>
<td>0%</td>
<td>2%</td>
<td>1%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Res %</td>
<td>1%</td>
<td>2%</td>
<td>0%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sus %</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>S-DD%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Res %</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
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<td>10%</td>
<td>98%</td>
<td>2%</td>
<td></td>
</tr>
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<td>S-DD%</td>
<td>0%</td>
<td>72%</td>
<td>0%</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>Res %</td>
<td>0%</td>
<td>18%</td>
<td>2%</td>
<td>85%</td>
<td></td>
</tr>
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<td>Candida krusei</td>
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<td>6</td>
<td>6</td>
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</tr>
<tr>
<td>Sus n</td>
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<td>1</td>
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<td>Sus %</td>
<td>100%</td>
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<td>83%</td>
<td>17%</td>
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<td>S-DD%</td>
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<td>17%</td>
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<tr>
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<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sus %</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>S-DD%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Res %</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
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<td>59</td>
<td>59</td>
<td>58</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
<td>59</td>
<td>56</td>
<td>58</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Sus %</td>
<td>100%</td>
<td>95%</td>
<td>100%</td>
<td>53%</td>
<td></td>
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<tr>
<td>S-DD%</td>
<td>0%</td>
<td>5%</td>
<td>0%</td>
<td>46%</td>
<td></td>
</tr>
<tr>
<td>Res %</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
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<td></td>
</tr>
<tr>
<td>Candida species</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sus %</td>
<td>100%</td>
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<td>100%</td>
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<tr>
<td>S-DD%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
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</tr>
<tr>
<td>Res %</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
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<td>35</td>
<td>16</td>
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<tr>
<td>Sus %</td>
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<td>91%</td>
<td>81%</td>
<td>37%</td>
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<tr>
<td>S-DD%</td>
<td>0%</td>
<td>9%</td>
<td>0%</td>
<td>58%</td>
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</tr>
<tr>
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<td>0%</td>
<td>0%</td>
<td>19%</td>
<td>5%</td>
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<tr>
<td>Cryptococcus neoformans</td>
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<td>13</td>
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<td>13</td>
<td></td>
</tr>
<tr>
<td>Sus n</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Sus %</td>
<td>100%</td>
<td>100%</td>
<td>92%</td>
<td>100%</td>
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</tr>
<tr>
<td>S-DD%</td>
<td>0%</td>
<td>0%</td>
<td>8%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Res %</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>

**INTERPRETATION**

<table>
<thead>
<tr>
<th>ANTIFUNGAL</th>
<th>MIC</th>
<th>S</th>
<th>SDD*</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>μ/ml</td>
<td>≤2</td>
<td>None</td>
<td>&gt;2</td>
</tr>
<tr>
<td>5-FC</td>
<td>μ/ml</td>
<td>≤4</td>
<td>8-16</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>μ/ml</td>
<td>≤8</td>
<td>16-32</td>
<td>≥64</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>μ/ml</td>
<td>≤0.125</td>
<td>0.25-0.5</td>
<td>≥1</td>
</tr>
</tbody>
</table>

**SSD* - Susceptible, but Dose Dependent**

**FIGURE 7.16** Fungal antibiogram.
**FIGURE 7.17** *Candida albicans* MIC data. Distribution of resistance by MIC showing essentially no resistance for all antifungals.

**FIGURE 7.18** *Candida glabrata* MIC data. Distribution of resistance by MIC showing essentially no resistance for all antifungals, except significant resistance for fluconazole, itraconazole, and ketoconazole.
Figure 7.20 lists a cumulative antibiogram for isolates recovered in the Oralfacial Microbiological laboratory (Dental) at West Virginia University Hospitals (WVUH). There is growing awareness of the oral-systemic link and of the need for persistent infections to be eradicated by selective therapy. Here the organisms listed are frequent periopathogens, with antibiotics used by the dental community, evaluated using the E-test MIC. The method of Relative Resistance has been utilized for dental isolates and has recently shown that the most frequent resistant phenotype, is ciprofloxacin-resistant gram-negative rods.
Antimicrobial Susceptibility Testing Protocols

7.5 LIMITATIONS OF DATA, DATA ANALYSIS, AND DATA PRESENTATION

7.5.1 ART vs. AST

The emergence and dissemination of resistance has made it imperative for laboratories to use in vitro tests that can detect resistance. The concept of Antimicrobial Resistance Testing (ART) should replace Antimicrobial Susceptibility Testing (AST). ART requires flexibility in testing systems and current AST methods are often handicapped. This is due to an over emphasis on standardization, not accounting for the diversity and complexity of resistance mechanisms.

7.5.1.1 Limitations of AST

Methods were originally developed when antibiotic resistance was low. Techniques such as disk diffusion, microdilution and rapid automated systems are suited for testing rapidly growing bacteria. They were designed for convenience, rapid turnover of clinical samples and simplification of data handling. Some of the underlying principles of these systems accounts for their limited ability to detect resistance. Because of the unstable antibiotic gradient in a disk test, zone sizes are directly influenced by inoculums and growth rates regardless of susceptibility. This makes the test only useful for screening the susceptibility of rapidly growing aerobes. Microdilution, restricted by volume and limiting nutritive capacity, tends to give false susceptible results. Rapid methods may not detect resistance because many mechanisms are not expressed within the 4-6 hour incubation period used. When using a small inoculum, a necessity in both manual and automated micro-methods, the odds of capturing resistant subpopulations are minimal.
7.5.2 Compilation and Statistical Tools

7.5.2.1 Estimating Resistance

Resistance is measured as a proportion. It is the proportion of organisms that survive a given dosage of a particular antibiotic in a specific place at a certain point in time. We will refer to that proportion as $\pi$. Neurobiology laboratories routinely test bacteria in order to establish drug specific resistances and the changes in those resistances over time.

If, in a certain laboratory, five patients are infected with XXXXX in a given month, and if one of those organisms proves resistant to a particular drug then a $1/5 = 20\%$ resistance is reported. Of course, we know that if a larger sample (hypothetically, all XXXXX bacteria) were tested, the proportion of resistant organisms would not be exactly 20%. Another way to view it is to say that value, $p=20\%$, is just an estimate of the actual resistant proportion and that there some amount of uncertainty in the estimate. By way of notation, the sample proportion is indicated by $p$, in contrast with the true proportion, $\pi$.

One way in which uncertainty is expressed is through the standard error of the estimate. Roughly speaking, the standard error of an estimate is the average amount by which the estimate misses the true value (parameter) it is intended to estimate. So, if the standard error is reported to be 4%, that indicates that on average the estimate is “off” by 4%. The standard error is calculated as:

$$SE = p \frac{(1 - p)}{n}$$

where $n$ is the sample size.

Another way in which uncertainty is expressed is accomplished by reporting a confidence interval (CI) for the true resistant proportion. A confidence interval is a statement that may take one of three different forms. The first is called a two-sided confidence interval. It is a statement like “we are $95\%$ sure that the interval from 2.7% to 6.8% contains the true resistance”. Alternatively, we may use one-sided confidence intervals that lead to statements like “we are $95\%$ sure that resistance is less than 6.6%” or, “we are $95\%$ sure that resistance is at least 3%”. The differences in the statements stem from a desire to communicate to the audience the highest likely value of resistance, the lowest likely value of resistance, or a specific range of likely values.

The calculation of confidence intervals is relatively simple. Two-sided intervals are formed from $p + z \left(\frac{p(1-p)}{n}\right)$ while one-sided confidence intervals are formed from the same formula using only the “$+$” sign. Note that the parenthesized term is just the standard error of the estimate. The confidence probability depends on the value of $z$. While any confidence probability may be used, $95\%$ confidence intervals are almost universally reported. For a $95\%$ two-sided confidence interval, the appropriate value of $z$ is 1.96. For a one-sided interval in which in upper bound is given, $z = 1.645$. For a one-sided interval in which a lower bound is to be given, $z = -1.645$. For example, if 100 XXXXXX organisms were tested and 10 of them proved resistant to a particular antibiotic at a certain dosage then, $p=10/100=10\%$. The standard error is given by $0.1(0.9)/100 = 0.03$ so a two-sided $95\%$ confidence interval for $\pi$ is given by the interval with lower bound 0.10-1.96 (0.03) or about 0.04. The upper bound is $10 + 1.96 (0.3) = 1.6$. And, we may say we are $95\%$ sure that the true resistance is between 4% and 16%.

When sample sizes are such that $np$ is less than 5, the formula for confidence intervals displayed above may give values that are less accurate than we would like. For circumstances in which $np < 5$ “exact” confidence interval in the form of either graphs or tables may be obtained from various sources.

Of particular interest is the question of placing bounds on a resistance when no resistant organisms have yet been found. In such cases $p = 0 / n$. The estimated resistance is zero but it is quite clear that we cannot assert that no X organisms are resistant. In cases where $n$ is small, the problem is particularly important since, for $n = 5$, if $10\%$ of the organisms are resistant we have a
59% chance that none of our tested organisms will exhibit resistance. In this case we are interested in reporting an upper bound on the proportion of resistant organisms and the sample sizes are often, though not always, small. Suppose that after 100 tests, no organisms have been found to be resistant to a certain dosage of a given antibiotic. We would like to place an upper bound on the proportion of resistant organisms consistent with our experience. One rule of thumb is known as “three-over-n”. It approximates the upper bound of a 95% confidence interval by $3/n$. In this case $3/n$ is equal to 0.03, a value that is in good agreement with the exact limit of (approximately) 0.0298 which may be gotten in a much more complex way. A slightly more accurate approximation than $3/n$ may be gotten from $3/(n+1.5)$, which, in this case, yields 0.02955665. The difference in the two approximations is only important for very small sample sizes. The difference in the two approximations is less than $1$ of one percent so long as $n > 30$.

7.5.2.2 Verification

It is very important to vary antibiotic susceptibility data. One means is to evaluate known bug-drug patterns and antibiotics within a class.

7.5.2.2.1 Data Verification/QA Monitor; Compare these antibiotic patterns

Comparison:

- Gentamicin vs. Tobromycin vs. Amikacin
- Sulfa vs. trimethoprine/sulfamethoxazole (TMP.SMX)
- Oxacillin vs. Cephalosporin

Also, one needs to verify one algorithm a month, as well as check and review the computer print-out.

- Verify algorithms
- Check/Review
- Computer Printout, Monthly.

7.5.2.2.2 Influence of Small Numbers/Data Bases

Also, the laboratory needs to establish criteria for:

- Selected organisms tested, only
- Reflexive testing
- Duplicates
- Low numbers
- Inaccurate Data

7.6 CONCLUSION


The initial document took a significant first step in standardizing laboratory prepared antibiotic data; it places significant emphasis on verification and validation. It was very specific, however, and has unique key elements: first isolate only selected sub-sets, and >10 isolate criteria. This is one more supporting document for the importance of a well-constructed antibiogram. It is our
means of survival and promotion of our values. The current document contains updates to term definitions to include replacing the term “antibiogram” with “cumulative antimicrobial susceptibility test data summary.” Updates also include the following areas: Antimicrobial susceptibility test results, Data inclusion/exclusion, Calculations, Reports, Data Review/Quality Assurance and Statistical significance of changes in %S.

7.7 ACKNOWLEDGEMENTS

Ron Masters, MRL/TSN (Focus Technologies, Herndon, VA)
Jerry Hobbs, Associate Professor, West Virginia University, Morgantown, WV
Lindsay Nakaishi, Research Assistant, West Virginia University, Morgantown, WV

7.8 REFERENCES

TRADITIONAL


WEB BASED

18. For a discussion of TSN and MRL, see http://www.mrlworld.com/mrlworld/index.html

AVAILABLE TEMPLATES

20. Templates for creating microbiology antibiogram reports and pharmacy antibiotic cost charts were available from Roche Laboratories, Inc., 2001.
8 Anaerobe Antimicrobial Susceptibility Testing

Darcie E. Roe-Carpenter

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8.1 INTRODUCTION

It has been well established for many years that anaerobic bacteria can cause medically significant infections [1]. The earliest medical writers have described the distinctive clinical features of tetanus, gas gangrene, and other anaerobic infections. With the discovery of antibiotics and the introduction of agents such as penicillin for prophylaxis and treatment, the incidence of anaerobic infections decreased. Historically, anaerobic infections were treated with antibiotics empirically, so there was little need for routine susceptibility testing. However, with the emergence of the “superbugs” that are resistant to many antimicrobial agents, it is becoming harder to predict the susceptibility of many bacterial species. Although antibiotic resistance in anaerobic bacteria has been increasing, physicians could still choose antibiotics for anaerobic infections empirically from surveillance studies reported in the literature or from data obtained in their own institutions [2]. It is presently much more difficult to predict anaerobe susceptibility patterns based on the literature. An example of this is a report of the antibiograms of anaerobic bacteria from several different hospitals within one metropolitan area. The results of this study showed that there were dramatically different antibiograms between the hospitals and that resistance was based on the use of specific agents at that institution [3].

8.2 RELEVANCE

The current Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS), recommendations for anaerobe susceptibility testing (M11-A7) are as follows [4]. Susceptibility testing should be done to determine patterns of susceptibility or resistance for new agents. Periodic testing in individual hospitals should be done to monitor susceptibility patterns. Testing of anaerobic bacteria from a clinical sample should be done when a pathogen is recovered that is known to be resistant, when therapeutic failure has occurred with the current therapy, when appropriate therapy is critical to patient outcome, with severe infections, when long-term therapy is necessary, or when not enough information is available to make an empirical judgment about the therapy [3,4].

The first guidelines for antibiotic susceptibility testing in anaerobic bacteria were in the CLSI M11-A document, which was a proposed standard in 1979 and became an approved standard in 1985. In the M11-A document, the only procedure described is the modification of the classic agar dilution test for anaerobic bacteria [5]. Wilkins-Chalgren agar was the medium recommended for agar dilution testing in this document. Since the publication of the M11-A document, additional methods have been added: limited agar dilution, Wadsworth agar dilution, broth microdilution procedure, broth macrodilution procedure, and disk elution [6]. It was later demonstrated that the results obtained with the disk elution procedure did not correlate with the agar dilution method, and this procedure was removed from the M11 document [7]. Before the creation of the M11-A4 document, the anaerobe working group did several multicenter studies [8]. The purpose of these studies was to evaluate the best medium for susceptibility testing of a wide range of anaerobic bacteria using the agar dilution procedure. The results of these studies indicated that brucella agar, supplemented with 5% laked sheep blood, vitamin K₁, and hemin, better supports the growth of more fastidious anaerobic species compared with Wilkins-Chalgren agar. Therefore, the CLSI anaerobe working group combined the agar dilution procedure with the Wadsworth agar dilution
procedure to create the new procedure that was first described in the M11-A4 document [8]. For broth microdilution, the M11-A4 document had five media formulations that could be used. In addition, these broths could be further supplemented to support the growth of fastidious anaerobic species. The CLSI anaerobe working group completed three studies to optimize the media for the microbroth dilution method, and these results were incorporated into the M11-A6 document [9]. This document lists only one media, brucella broth supplemented with lysed horse blood, hemin, and vitamin K₁, to be used with only Bacteroides fragilis group isolates. The CLSI anaerobe working group has made no recommendations for the microbroth dilution method with organisms outside of the B. fragilis group. Even though the agar dilution method is the reference method, it is usually performed in research and large clinical laboratories whereas microbroth and the Etest are the methods frequently used by typical clinical laboratories. In 2006, the CLSI anaerobe working group completed a quality control study to add ATCC 700057 Clostridium difficile to the approved list of quality control organisms for the agar dilution method. The quality control ranges for this new organism are listed in M11-A7 [4].

8.3 PROCEDURE

8.3.1 AGAR DILUTION — CLSI REFERENCE METHOD

8.3.1.1 Introduction

Agar dilution is the reference method that has been established by the CLSI for susceptibility testing of anaerobic bacteria. This procedure involves making a series of agar plates, each containing a specific concentration of an antimicrobial agent. With the use of a replicating device, up to 36 different isolates can be tested at one time. However, this procedure is very labor intensive and does not lend itself well to daily susceptibility testing by the typical clinical laboratory. This procedure is usually used for periodic testing to monitor resistance in anaerobic bacteria at a facility or for testing a new antimicrobial agent to determine its activity against a wide range of species. This is the gold standard to which all other methods are compared. If any other method can prove its equivalency to this method, it is acceptable for use in the clinical lab. It is not the function of the CLSI to validate every susceptibility method available, so there will always be other methods available that CLSI does not describe or discuss.

8.3.1.2 Materials

1. Media.
   a. Antibiotic sources.
      1) United States Pharmacopeia (USP).
      2) Sigma Chemical Company.
      3) The antimicrobial agent manufacturer.
         • Many antimicrobial agents can be obtained in small amounts from the drug manufacturer at no charge by request through the drug company’s customer service department or equivalent department. (Consult the Physicians Desk Reference [PDR] text or your local pharmaceutical representatives for telephone numbers.)
   b. Brucella agar.
      1) Brucella agar is the only medium that should be used for the susceptibility testing of anaerobic bacteria. The media should be made according to the manufacturer’s instructions (Becton Dickinson, Remel, Accumedia).
      2) The medium should be supplemented with 1 mL/L of hemin stock solution (5 mg/mL) and 1 mL/L of working vitamin K₁ stock solution (1 mg/mL) before autoclaving or dispensing.
c. Sterile distilled water.
d. Laked sheep blood.
   1) Sterile whole defibrinated sheep blood can be purchased from several manufacturers (Remel, PML, Hemostat, Hardy Diagnostics).
   • Sterile laked blood can be purchased from Hardy Diagnostics, PML, and Hemostat.
   2) To prepare laked blood in the laboratory from whole blood, completely freeze the whole sheep blood at –20°C overnight or longer. When the blood is thawed, the red blood cells will lyse and the blood is “laked.” The thaw can be done rapidly in a 35°C–37°C water bath or incubator. The thaw can also be done slowly at 2°C–8°C overnight.
   3) Laked blood can be stored in the freezer at –20°C for up to 1 year.
      • Once thawed, laked blood has a 2½-week shelf life at 2°C–8°C and should be labeled with a new expiration date when thawed.
e. Hemin stock solution (5 mg/mL).
   1) Weigh out 0.5 g of hemin (Sigma # H2250).
   2) Dissolve the hemin powder in 10 mL of 1 mol/L NaOH (ACS certified).
   3) Make sure that the hemin powder is completely dissolved.
      • If the water is added to the hemin solution before it is completely dissolved, the hemin will remain unsuspended.
      • If the hemin will not go into solution in NaOH, the solution can be heated to help dissolve the hemin. *This does not work if it is done after the addition of water.*
   4) Bring the volume of the solution to 100 mL with distilled water.
   5) Make sure that the solution is in a brown bottle or wrapped in foil to protect it from light. Sterilize the hemin solution at 121°C for 15 min.
   6) Cool to room temperature and store at 4°C–8°C.
   7) This solution has a 1-month shelf life and should be labeled with an expiration date when made.
   8) Add 1 mL of the hemin stock solution (5 mg/mL) to 1 L of medium.
f. Vitamin K₁ solution (10 mg/mL).
   1) Stock solution (10 mg/mL).
      a) Add 0.2 mL of vitamin K₁ solution (3-phytylmenadione, Sigma # V3501) to 20 mL of 95% ethanol.
      b) Mix the ethanol with the vitamin K₁ solution.
      c) Place in a sterile dark bottle and store at 4°C–8°C.
      d) The solution has a 2-year shelf life and should be labeled with an expiration date when made.
         • This solution has not been sterilized; therefore, sterile technique should be used when making this solution.
         • Always inspect the solution for possible contamination before use.
         • Vitamin K₁ can be autoclaved; however, other forms of vitamin K cannot.
   2) Working solution (1 mg/mL).
      a) Add 1 mL of stock vitamin K₁ solution (10 mg/mL) to 9 mL of sterile distilled water.
      b) Place in a sterile dark bottle and store at 4°C–8°C.
      c) This solution has a 1-month shelf life and should be labeled with an expiration date when made.
      d) Add 1 mL of the *working* vitamin K₁ stock solution (1 mg/mL) to 1 L of medium.
         • This solution has not been sterilized; therefore, sterile technique should be used when preparing this solution.
         • Always inspect the solution for contamination before use.
         • Any medium can be autoclaved after the addition of the vitamin K₁.
g. Brucella broth.

1) The media should be made according to the manufacturer’s instructions (Becton Dickinson, Remel, Accumedia).

2) The media should be supplemented with 1 mL/L of hemin stock solution (5 mg/mL) and 1 mL/L of working vitamin K₁ stock solution (1 mg/mL) before autoclaving or dispensing of the media.

3) Dispense into 1–5 mL volumes (depending on how many plates are to be inoculated) if using the direct colony suspension method for preparing inoculum suspensions and 5 mL volumes if using the growth inoculum procedure.

h. Sterile phosphate buffers at various pHs (Table 8.1).

2. Supplies.

a. Test tubes.

- Any standard laboratory sterile screw cap test tube that holds at least 10 mL can be used for making antibiotic dilutions.

- Glass tubes with screw caps are needed for making the agar deeps. These test tubes need to be autoclavable and have dimensions greater than 16 × 150 mm (having at least a 22 mL capacity).

- It is not recommended to reuse the tubes that were used to make antibiotic dilutions. It is very labor intensive and difficult to clean the glass tubes to ensure that there is no carryover of antibiotics between runs. In addition, it has been shown that some antibiotics (i.e., quinolones) are very efficient at sticking to glass surfaces even after proper cleaning in an automated dishwasher (industrial strength).

b. Petri dishes.

- The 32-prong replicator fits a 100 × 15 mm round petri dish.

- The 36-prong replicator fits a 100 × 15 mm square petri dish.

- A standard 100 × 15 mm round petri dish has an internal measurement of 87 mm and is 10 mm high. Petri dish manufacturers have started making space saver dishes that are now currently being used by media manufacturers. These space saver petri

---

**TABLE 8.1**

**Antimicrobial Agent Solvents**

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin, ticarcillin, clavulanic acid</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Phosphate buffer, pH 8.0, 0.1 mol/L</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
</tr>
<tr>
<td>Cefotetan⁴</td>
<td>DMSO⁴</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>95% ethanol</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>Metronidazole, opt 80 and tinidazole</td>
<td>DMSO</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>Nizoxanide and tizoxanide</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>Imipenem and Ertapenem</td>
<td>Phosphate buffer, pH 7.2, 0.01 mol/L</td>
<td>Same as solvent</td>
</tr>
<tr>
<td>Rifaximin</td>
<td>Methanol</td>
<td>Phosphate buffer, pH 7.4, 0.1 mol/L,+ 0.45% SDS</td>
</tr>
<tr>
<td>Tilmicosin and tylosin</td>
<td>95% ethanol</td>
<td>Sterile distilled water</td>
</tr>
</tbody>
</table>

If antibiotic is not listed in the table, the solvent and diluents are sterile distilled water.

⁴ All other cephalosporins and cephamycins should be dissolved in phosphate buffer, pH 6.0, 0.1 mol/L, and all further dilutions should be done with sterile distilled water.

⁵ DMSO (dimethyl sulfoxide) is potentially toxic. Consult the material safety data sheets (MSDS) available from the product manufacturer before using this material; SDS = sodium dodecyl sulfate.
dishes have an internal dimension of 85 mm and a height of 8 mm. The loss of 2 mm is enough that it makes inoculation of these petri dishes more difficult. The corner isolates in the replicator are inoculated at the extreme edge of the plate and can result in growth that is more difficult to interpret.

c. Pipettes (1-, 5-, 10-, and 25-mL sterile serological pipettes).
d. Pipetting aid for serological pipettes.
e. 0.5 McFarland standard.

1) Procedure for making McFarland standards [10].

a) Combine the appropriate amount of BaCl$_2$ and H$_2$SO$_4$.
   • For the 0.5 McFarland standard, use 0.5 mL of 0.048 mol/L BaCl$_2$ (1.175% w/v BaCl$_2$·2H$_2$O) and 99.5 mL of 0.18 mol/L H$_2$SO$_4$ (1% v/v).
   • For the 1 McFarland standard, use 1 mL of 0.048 mol/L BaCl$_2$ (1.175% w/v BaCl$_2$·2 H$_2$O) and 99 mL of 0.18 mol/L H$_2$SO$_4$ (1% v/v).

b) Mix the solutions thoroughly.

c) Using the same size screw-cap tubes that are to be used for the preparation of bacterial inoculum suspensions, aliquot 4–6 mL into each tube.

d) Seal the caps with tape or Parafilm.

e) Store these tubes in the dark at room temperature when not in use.

f) Mix the standard solution well prior to each use.
   • Manufactured McFarland standards are available from PML and Hardy Diagnostics.

2) Validation of McFarland standard turbidity.

a) Absorbency.
   i. Read the optical density of the standard on a spectrophotometer with a 1-cm light path using a matched set of cuvettes.
   ii. The absorbency should be read at 625 nm.
      • 0.5 McFarland standard should have a reading of 0.08 to 0.10 $\lambda$.
      • 1 McFarland standard should have a reading of 0.16 to 0.20 $\lambda$.

b) Colony count.
   • The density of a standard can be validated by performing a spread plate colony count on a suspension of $E. coli$ ATCC 25923 equivalent to the turbidity of your standard.
      • A 0.5 McFarland standard is considered equivalent to 1–2 $\times$ 10$^8$ organisms/mL.
      • A 1 McFarland standard is considered equivalent to 3 $\times$ 10$^8$ organisms/mL.

   • The density of the McFarland standard being used should be verified monthly by one of these two methods.

3) Other methods.

a) Manufactured latex standards.
   • Latex standards have the advantage of a 2-year shelf life and are not light sensitive.
   • Latex standards are available from Remel (Lenexa, KS).
   • Like all standards, the density of latex standards should be verified monthly.

b) Turbidity meters.
   • Available from Vitek, MicroScan, and Anaerobe Systems.
   • Need to be calibrated with turbidity standards.
   • The frequency of calibration depends on the manufacturer.

f. Sterile swabs.

3. Equipment.

a. Autoclave.

b. Water bath 48°C–50°C.

c. Steers replicator (Figure 8.1).
• Using a Steer’s replicator [11].
  1) Transfer 0.6 mL of each isolate’s standardized inoculum to the appropriate individual well of the sterile seed plate.
  2) Move the slide plate to the extreme left, placing the seed plate directly under the inoculating head.
  3) Place the appropriate agar dilution petri dish without a lid in the left-hand position on the slide plate.
  4) Push down on the piston followed by a controlled release; this results in the movement of the inoculating rods in and out of the seed plate.
    • If the release of the inoculating head is not done in a controlled manner, the inoculum may splatter, resulting in cross-contamination of the isolates.
  5) Move the slide plate to the extreme right, placing the inoculating rods directly over the petri dish.
  6) Gently depress the plunger until the inoculating rods rest on the surface of the medium.
    • This will deliver 1–2 μl of the inoculum on the medium per rod.
  7) Controlled release of the pressure results in the inoculating rods rising from the surface of the medium.
    • If the release is not controlled, inoculum splattering on the agar plate will occur.
• The current manufacturer of Steers replicators is CMI-Promex (Pedricktown, NJ). They are available in stainless steel and aluminum, and with 32 prongs for a round plate and 36 prongs for a square plate. The 32-prong pattern offers the advantage of using standard petri dishes. The 36-prong pattern uses a square plate, which is often not a standard order item for most laboratories.

![32-prong pattern]

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20</td>
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<tr>
<td>23</td>
<td>24</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>29</td>
<td>30</td>
<td>31</td>
<td>32</td>
</tr>
</tbody>
</table>
• Steers replicator maintenance.
  a) The replicator should be decontaminated after every use by autoclaving, then
     cleaned using water, and then reautoclaved to sterilize for the next use.
  b) Soap or any type of disinfectant should not be used to clean the replicator.
     Residues and the sticking of these agents can affect the results the next time
     the replicator is used. Only water and scrubbing should be used to clean this
     instrument.
  c) Repeated autoclaving of the aluminum replicators will result in pitting, which
     will affect the accuracy of the unit over time. However, the aluminum replicators
     can be treated, by the process of hard anodizing performed by a metal machinist,
     to prevent pitting.

d. Incubator.
  • The incubator should be set for 35°C–37°C.
  • Either the incubator is in an anaerobic chamber or is a non-CO₂ incubator for use
    with anaerobic jars.
e. Anaerobic chamber or anaerobic jars.
  • The following are manufacturers of anaerobic systems: Coy, EM, Forma, Micro-
    biology International, Mitsubishi, Oxoid, Plas-Labs, Sheldon Manufacturing, Tou-
    can Technologies.

8.3.1.3 Quality Control

1. Quality control strains:
   • Bacteroides fragilis ATCC 25285.
   • Bacteroides thetaiotaomicron ATCC 29741.
   • Eubacterium lentum ATCC 43055.
   • Clostridium difficile ATCC 700057.

2. Frequency of quality control testing.
   • At least two of the above isolates should be tested with every agar dilution run.
   • E. lentum is the hardest of the four quality control isolates to grow on agar medium
     and produces the lightest growth pattern, which makes MIC determination more
     difficult. C. difficile was added as a quality control organism to provide an alternative
     gram-positive organism to E. lentum for agar dilution.
   • All quality control strains should be maintained following procedures described by
     CLSI [12].

3. Evaluating results.
   • The MIC values for all the quality control organisms tested in one run must be within
     acceptable ranges for the antimicrobial being tested for the results to be considered
     valid. These ranges are listed in the current CLSI M11 document and in Table 8.2.
   • It is important to keep in mind the function of these quality control organisms, which
     is to check that the antibiotic concentrations were made properly, meaning the amount
of antibiotic in the agar plate really matches what you think is there. These quality control organisms do not test the quality of the media or the presence of an anaerobic environment.

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Bacteroides fragilis ATCC 25285</th>
<th>Bacteroides thetaiotaomicron ATCC 29741</th>
<th>Clostridium difficile ATCC 700057</th>
<th>Eubacterium lentum ATCC 43055</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/clavulanic acid (2:1)</td>
<td>0.25/0.125–1/0.5</td>
<td>0.5/0.25–2/1</td>
<td>0.25/0.125–1/0.5</td>
<td>—</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>16–64</td>
<td>16–64</td>
<td>1–4</td>
<td>—</td>
</tr>
<tr>
<td>Ampicillin/sulbactam (2:1)</td>
<td>0.5/0.25–2/1</td>
<td>0.5/0.25–2/1</td>
<td>0.5/0.25–4/2</td>
<td>0.25/0.125–2/1</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>8–32</td>
<td>32–128</td>
<td>—</td>
<td>4–16</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>32–128</td>
<td>32–128</td>
<td>—</td>
<td>32–128</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>8–32</td>
<td>16–64</td>
<td>—</td>
<td>64–256</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>4–16</td>
<td>32–128</td>
<td>—</td>
<td>32–128</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4–16</td>
<td>8–32</td>
<td>—</td>
<td>4–16</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>—</td>
<td>4–16</td>
<td>—</td>
<td>16–64</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>32–128</td>
<td>64–256</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2–8</td>
<td>4–16</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Clinafloxacine</td>
<td>0.03–0.125</td>
<td>0.06–0.5</td>
<td>—</td>
<td>0.03–0.125</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.5–2</td>
<td>2–8</td>
<td>2–8</td>
<td>0.06–0.25</td>
</tr>
<tr>
<td>Doripenem</td>
<td>—</td>
<td>—</td>
<td>0.5–4</td>
<td>—</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>0.06–0.25</td>
<td>0.25–1</td>
<td>—</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Garenoxacin</td>
<td>0.06–0.5</td>
<td>0.25–1</td>
<td>0.5–2</td>
<td>1–4</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.03–0.125</td>
<td>0.06–0.25</td>
<td>—</td>
<td>0.125–0.5</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2–8</td>
<td>2–8</td>
<td>1–4</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.03–0.25</td>
<td>0.125–0.5</td>
<td>0.5–4</td>
<td>0.125–1</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.25–1</td>
<td>0.5–2</td>
<td>0.125–0.5</td>
<td>—</td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>16–64</td>
<td>8–32</td>
<td>—</td>
<td>8–32</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.12–0.5</td>
<td>1–4</td>
<td>1–4</td>
<td>0.125–0.5</td>
</tr>
<tr>
<td>Nitazoxanide</td>
<td>—</td>
<td>—</td>
<td>0.06–0.5</td>
<td>—</td>
</tr>
<tr>
<td>Opt 80</td>
<td>—</td>
<td>—</td>
<td>0.06–0.25</td>
<td>—</td>
</tr>
<tr>
<td>Penicillin</td>
<td>8–32 (16–64)*</td>
<td>8–32 (16–64)*</td>
<td>1–4</td>
<td>—</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>2–8</td>
<td>8–32</td>
<td>4–16</td>
<td>8–32</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>0.125/4–0.5/4</td>
<td>4/4–16/4</td>
<td>4/4–16/4</td>
<td>4/4–16/4</td>
</tr>
<tr>
<td>Ramoplanin</td>
<td>—</td>
<td>—</td>
<td>0.125–0.5</td>
<td>—</td>
</tr>
<tr>
<td>Rifaximin</td>
<td>—</td>
<td>—</td>
<td>0.004–0.016</td>
<td>—</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.125–0.5</td>
<td>8–32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>16–64</td>
<td>16–64</td>
<td>16–64</td>
<td>16–64</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>—</td>
<td>0.5/2–2/2</td>
<td>16/2–64/2</td>
<td>16/2–64/2</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>—</td>
<td>—</td>
<td>0.125–1</td>
<td>—</td>
</tr>
<tr>
<td>Tinidazole</td>
<td>—</td>
<td>—</td>
<td>0.125–0.5</td>
<td>—</td>
</tr>
<tr>
<td>Tizoxanide</td>
<td>—</td>
<td>—</td>
<td>0.06–0.5</td>
<td>—</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td>0.06–0.5</td>
<td>0.25–1</td>
<td>—</td>
<td>0.25–1</td>
</tr>
<tr>
<td>Tylosin</td>
<td>—</td>
<td>—</td>
<td>0.125–0.5</td>
<td>—</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>—</td>
<td>—</td>
<td>0.5–2</td>
<td>—</td>
</tr>
</tbody>
</table>


* Penicillin units in parentheses are in units/mL.
8.3.1.4 Procedure

1. Plan.

It is important to completely plan out the entire procedure because it involves many steps over several days. Agar dilution is a very complicated multistep procedure. It is important to have everything ready ahead of time to complete an agar dilution run in a timely manner. It is also important to understand all the steps in the procedure before starting. This procedure can be performed by one person; however, it is significantly more manageable with two people.

- Determine the number of isolates to be tested, the number of antibiotics to be tested, and the range of concentrations for each antibiotic to be tested.
- The antibiotic range testing should include at least two dilutions below and two dilutions above the break-point values for that antibiotic. The break points are listed in the current CLSI M11 document and in Table 8.3.

### TABLE 8.3
Interpretive Criteria (μg/mL) (break points for agar dilution and broth microdilution)

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Activity</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/clavulanic acid (2:1)</td>
<td>C</td>
<td>4/2</td>
<td>8/4</td>
<td>16/8</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>C</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ampicillin/sulbactam (2:1)</td>
<td>C</td>
<td>8/4</td>
<td>16/8</td>
<td>32/16</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>C</td>
<td>16</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>C</td>
<td>16</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>C</td>
<td>16</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>C</td>
<td>16</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>C</td>
<td>16</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>C</td>
<td>32</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>S</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>S</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>C</td>
<td>4</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>C</td>
<td>4</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Meropenem</td>
<td>C</td>
<td>4</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>C</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>C</td>
<td>32</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Penicillin</td>
<td>C</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>C</td>
<td>32</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>C</td>
<td>32/4</td>
<td>64/4</td>
<td>128/4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>C</td>
<td>4</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>S</td>
<td>32</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>C</td>
<td>32/2</td>
<td>64/2</td>
<td>128/2</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td>C</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

Values were taken from the CLSI M11-A7, 2007 document.

* bactericidal = C, bacteriostatic = S.
2. Agar dilution plates.
   Prepare the appropriate number of brucella agar deeps. These deeps can be made in
   advance and stored in the refrigerator for up to 1 month at 2°C–4°C or 1 week at room
temperature.
   a. Based on the number of runs to be done and the antibiotics to be tested, calculate the
   number of agar deeps needed (# runs × number of antibiotic dilutions + controls =
   # of agar deeps needed).
   b. Calculate the total amount of agar needed (# of agar deeps × 17 mL = total volume
   needed).
   c. Weigh out the appropriate amount of brucella agar base following the manufacturer’s
   instructions.
   d. Add the appropriate amount of powder and distilled water to a bottle or flask and mix.
   e. Add 1 mL/L of hemin stock solution and 1 mL/L of vitamin K1 working solution to
   the liquid medium.
   f. Bring the medium to a full rolling boil for 10 min to ensure that the agar is completely
   dissolved. The medium will be a consistent color and somewhat translucent; if the
   media is not homogeneous, boil longer.
   • If the agar is not completely dissolved in the liquid, it will result in agar deeps
   with different concentrations of agar.
   g. Move hot medium to a 50°C water bath and allow the medium to cool to at least 60°C
   before dispensing the hot medium.
   h. While leaving the boiled medium in the 50°C water bath, pipette 17 mL ± 0.2 mL of
   liquid medium into glass or polypropylene test tubes (25.5 mL ± 0.2 mL for square
   petri dishes).
   • The test tubes do not have to be sterile.
   i. Once all of the medium has been dispensed, cap all of the tubes.
   • The caps do not need to be sterile and should not be put on tightly.
   j. As soon as the tubes have been dispensed, they should be autoclaved at 121°C for 15
   min with a slow exhaust.
   k. After autoclaving, allow the tubes to cool and solidify. Once cooled, the tubes should
   be tightly capped and then can be stored at 4°C for 1 month or at room temperature
   for 1 week. Tubes should be placed in a 50°C water bath if they are going to be used
   that day.
   l. If the agar deeps are allowed to solidify, they need to be remelted before use. It is
   important that the media inside the tube reaches a full boil to ensure that all of the
   agar is completely melted. The agar deeps are melted by autoclaving at 121°C with
   a slow exhaust for 3 min or by boiling in a water bath, a steamer, or in a microwave
   for 3–5 min (time is dependent on the diameter of the tube and the strength of the
   microwave). Once the medium has reached a full boil, the tubes are transferred to a
   50°C water bath and allowed to cool to 50°C before use.
   m. Once the media has cooled to 48°C–50°C, it is ready for the addition of laked sheep
   blood and antibiotic solutions.

   a. All antibiotic powders should be stored according to their manufacturer’s instructions.
   b. All antimicrobial agents are assayed by the manufacturer to determine the units of
   activity. This difference must be taken into account when making stock solutions.
Antimicrobial Susceptibility Testing Protocols

\[
\text{Weight (mg)} = \frac{\text{volume (ml)} \times \text{desired concentration (mg/ml)}}{\text{antibiotic potency (mg/mg)}}
\]

c. When possible, more than 100 mg of antimicrobial powder should be weighed out at one time.
d. All stock solutions should be made at the same concentration to minimize confusion. Stock solutions of 2,560 μg/mL work well for most standard susceptibility ranges.
e. The antimicrobial powder should be dissolved in water or other appropriate diluent in a sterile test tube (see Table 8.1). Once the antibiotic is in solution, all further dilutions should be made in water unless otherwise indicated (see Table 8.1).
f. Stock solutions should be aliquoted into volumes necessary for a typical run (approximately 2.5 mL). They should be stored in polypropylene or polyethylene sterile test tubes and frozen at −60°C or lower. Stock solutions should not be stored in frost-free freezers.
g. The stock solutions should be accurately labeled with the antibiotic’s name and concentration, preparation date, and an expiration date. The expiration date should be 6 months after the date the stock solution was made or the manufacturer’s expiration date for the powder, whichever is earlier.
h. Stock solutions should be discarded at the end of each day and should never be refrozen.
i. Because of the high concentration of antibiotic in the stock solution, sterilizing the solution is not necessary.
j. If using low concentrations of antibiotic or if there is a need to sterilize the antibiotic stock solution, it should be done using a membrane.
  • Paper, asbestos, or sintered filter should not be used since they may bind the antimicrobial agent.
  • If the stock solution is filter sterilized, the absence of adsorption must be documented by appropriate assay procedures. This procedure is not recommended.

   a. All test tubes should be labeled with the antibiotic’s name and appropriate concentration.
   b. Add appropriate amounts of sterile water or proper diluent to each tube (see Figure 8.2).
   c. Add the appropriate amount of the stock solution to the first antibiotic dilution tube.
   d. Continue with the serial dilutions (Figure 8.2).

5. Plate preparation.
   a. Label all the plates with the antibiotic’s name, concentration, and run number if appropriate.
   b. Stack plates in an organized fashion starting with the lowest concentration to the highest at the top of the stack.
   c. Remove the melted agar deep from the 50°C water bath, and wipe off the excess water on the outside of the test tube.
   d. Add 1 mL of laked blood to the melted agar deep (1.5 mL for square petri dishes).
   e. Add 2 mL of antibiotic dilution to the melted agar deep (3 mL for square petri dishes).
   To prepare control plates, add 2 mL of sterile water in place of the antibiotic solution (3 mL for square petri dishes).
   f. Mix by gentle inversion of the test tube three times; turning the tube upside down and back to the upright position counts as one inversion. Try to avoid making any bubbles during this process, and do not shake tubes violently.
   g. Pour the entire volume of media into the appropriate prelabeled sterile petri dish. Rotate petri dish gently to evenly distribute the agar and move any bubbles to the edge. If bubbles still remain in the center of the petri dish, a sterile needle or sterile loop can be used to pop the bubble or move it to the edge. If the bubble is large in size, it is best to try and pop the bubble. This process needs to be done before the agar solidifies.
**FIGURE 8.2** Tube dilution scheme.

<table>
<thead>
<tr>
<th>Stock</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic Con (µg/ml)</td>
<td>2560</td>
<td>1280</td>
<td>640</td>
<td>320</td>
<td>160</td>
<td>80</td>
<td>40</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Source</td>
<td>Stock</td>
<td>Stock</td>
<td>Stock</td>
<td>Stock</td>
<td>Stock</td>
<td>Stock</td>
<td>Stock</td>
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</tr>
<tr>
<td>Source Volume</td>
<td>2 ml</td>
<td>1 ml</td>
<td>1 ml</td>
<td>2 ml</td>
<td>1 ml</td>
<td>1 ml</td>
<td>2 ml</td>
<td>1 ml</td>
<td>1 ml</td>
<td>2 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Diluent Volume</td>
<td>2 ml</td>
<td>3 ml</td>
<td>7 ml</td>
<td>2 ml</td>
<td>3 ml</td>
<td>7 ml</td>
<td>2 ml</td>
<td>3 ml</td>
<td>7 ml</td>
<td>2 ml</td>
<td>3 ml</td>
<td>7 ml</td>
</tr>
</tbody>
</table>
h. Repeat steps c through g for all agar dilution plates needed. Once the plates have solidified (5–10 min), they need to be dried. It is important to make sure that the plates are completely dry before inoculation. This one step will ensure that the inoculum is rapidly absorbed into the agar. This will also help to prevent running of the inoculum spots into one another, which makes the plates hard to read after the incubation. Drying the prepared agar plates can be done in several ways:

1) Let the plates sit overnight at room temperature. **Note:** This cannot be done with less stable antibiotics such as imipenem, meropenem, and any drug combination containing sulbactam or clavulanic acid.

2) Invert (agar side up) and leave the lids ajar in a 35°C incubator for no more than 45 min.

3) Invert and leave the lids ajar in a laminar flow hood or biological safety cabinet for 30–45 min.
   - Agar dilution runs can be planned so that the agar plates are prepared several days before they will be used for routine and research testing. This should be avoided if at all possible, especially with antibiotics that are known to be less stable (i.e., imipenem, meropenem, and any drug combination containing sulbactam or clavulanic acid).
   - If using agar dilution plates that have been stored at 2°C–4°C, allow them to warm to room temperature on the bench before inoculation. If the plates appear wet, repeat the drying process described in 1), 2), or 3).
   - It is not recommended that tubes that were used to make the agar dilution plates or antibiotic dilutions be reused. It is very labor intensive and difficult to clean the glass tubes to ensure that there is no carryover of antibiotics between runs. In addition, it has been shown that some antibiotics (i.e., quinolones) are very efficient at sticking to glass surfaces even after cleaning in an automated dishwasher (industrial strength).

   - Make a list of the isolates and quality control organisms to be tested.
   - Direct colony suspension.
     a. Inoculum is prepared from pure culture growth on brucella blood agar incubated for 24–72 h at 35°C.
        - Sufficient growth can be obtained at 24 h for rapid growers like *B. fragilis*.
        - Incubation can be 72 h for slow-growing species.
        - Whenever possible, 48-hour plates are preferred.
     b. Lightly touch several colonies of similar colony morphology using a loop or swab.
     c. Suspend the bacteria in brucella broth.
     d. Adjust the turbidity by adding more organism or more brucella broth until the suspension has a density equivalent to a 0.5 McFarland standard.
        - All work done with inoculum preparation can be done on the laboratory bench outside an anaerobic chamber.
        - Inoculum preparation should be done as quickly as possible, and bacterial oxygen exposure should be kept to a minimum. If possible, the preparation of all inocula for one run should be completed within 30 min.
        - If isolates do not grow on the growth control plate on an agar dilution run, those organisms should be repeated with the inoculum preparation done inside of an anaerobic chamber.
        - Not all anaerobic bacteria species grow well on standard agar media. If poor growth is obtained on the growth control plate, the MIC values recorded may not be accurate.
Isolates that are being tested that were previously frozen must be passed at least twice before susceptibility testing is performed to obtain accurate results.

Growth method.

a. Inoculum should come from brucella blood agar plates with 24–72 h growth.

b. Lightly touch several colonies of similar colony morphology using a loop or swab.

c. Suspend the bacteria in thioglycolate broth or supplemented brucella broth.
   - If using thioglycollate broth, boil for 5 min before use.
   - One liter of thioglycollate medium should be supplemented with 1 mL hemin stock solution, and 1 mL vitamin K₁ working solution.
   - One or two small marble chips can be added before sterilization. (Marble chips are available from Fisher and VWR.)

d. Incubate under anaerobic conditions for 6–24 h or until adequate turbidity is achieved.

e. Adjust the turbidity by adding more broth until the suspension has a density equivalent to a 0.5 McFarland standard.

7. Inoculation of plates.

a. The agar dilution plates should be organized before the inoculum has been prepared to reduce the amount of time the isolate will be out of an anaerobic environment.

b. The agar dilution plates should be stamped starting with the bacteriostatic antibiotics followed by the bactericidal antibiotics or starting with the least active agent followed by the most active agents (see Table 8.3).

c. Within the dilution range of one antibiotic, inoculate beginning with the lowest concentration to be tested and move to the highest concentration. Growth control plates should be included at the beginning and end of each antibiotic (see step f further on).

d. No more than four antimicrobial dilution sets of plates should be used with one replicator. Doing more than four sets increases the chance of antibiotic carryover and contamination of the seed tray of the replicator.

e. It is also important to note the order that the plates were stamped, for follow-up with contamination or no-growth problems. One option is to label the control plates as follows: end of antibiotic X and start of antibiotic Y.

f. Four types of control plates should be used with each run: positive growth control plates, negative growth (contamination) control plates, inoculum control plates, and Steers replicator sterility control plate. None of these control plates contains any antibiotics. They should be prepared just like the agar dilution plates but with the addition of 2 mL of water rather than antibiotic. It is not recommended that manufactured plates be used for this purpose.

1) Positive growth control plates should be placed at the start and end of each antibiotic being tested. These plates are incubated with the agar dilution plates in an anaerobic environment.
   - One plate can serve as the control at the end of one antibiotic and the beginning of a second antibiotic.
   - Using two plates can be more convenient to the workflow if more than one person is reading a set of plates (i.e., each antibiotic dilution can have its own before and after control plates).
   - Using two control plates can reduce the amount of antibiotic carryover within a run.

2) Negative growth (contamination) control plates should be plated at the start and end of each antibiotic being tested. These plates are incubated in an aerobic incubator.
   - One plate can serve as the control at the end of one antibiotic and the beginning of a second antibiotic.

3) Inoculum control plates should be done at least once for each replicator run. These plates are stored at 4°C during the incubation period. These plates are very helpful...
in determining the difference between wimpy growth of an organism and inoculum dried on the agar with no growth.

4) A Steers replicator sterility control plate is a plate that is stamped before the inoculator head is introduced into the seed tray with the bacterial suspension. This control plate is not described in the CLSI M11-A7 document [4].

g. Be sure to mark the plates with some indication as to the proper orientation of the plate with regard to the inoculation. This can be done by filling one well in the replicator with diluted India ink or by marking the side on the petri dish with a sharpie to indicate its orientation.

h. The order of inoculation of plates is as follows:
   1) Growth control plate
   2) Negative growth (contamination) control plate
   3) Inoculum control plate.
   4) Antibiotic agar dilution set #1
   5) Growth control plate(s)
   6) Negative growth (contamination) control plates.
   7) Antibiotic agar dilution set #2
   8) Growth control plate(s)
   9) Negative growth (contamination) control plate.
  10) Antibiotic agar dilution set #3
  11) Positive growth control plate
  12) Negative growth (contamination) control plate.

i. Carefully inoculate the agar plates (see sections 8.3.1.2.3 and 8.3.1.4.7 for the procedure).

j. Allow the inoculated plates to remain on the bench with the lids in place until the inoculum has been absorbed into the medium. This should take no longer than 5–10 min. Handle plates as little as possible to avoid running of the inoculum on the plate. Adequate drying of the plates for inoculation will allow absorption to occur. If the plates are wet, it will take significantly longer for the inoculum to be absorbed, which will result in no growth of some isolates or running of the inoculum, creating plates that are difficult to interpret.

k. The appropriate agar dilution plates should be placed as soon as possible into the anaerobic environment that is being used. All anaerobic incubated plates should be in an anaerobic environment within 1 h of inoculum suspension preparation completion. This time is more crucial if anaerobic jars are being used because of the additional time these systems take before anaerobiosis is achieved.

8. Incubation.
   a. Agar dilution plates and growth control plates are placed in the anaerobic environment for incubation. Incubation is for 46–48 h at 35°C–37°C.
   b. Negative (contamination) control plates are incubated in an aerobic incubator for 46–48 h at 35°C–37°C (a CO₂ incubator is not required).
   c. Inoculum control plates should be stored at 2°C–8°C until the agar dilution run is to be read.
   d. All plates should be incubated in the inverted position (agar side up).

9. Colony count.
   a. Transfer 0.1 mL from either the replicator block well or the inoculum suspension and add it to 9.9 mL of saline.
   b. Transfer 1 mL from the first tube to a second tube containing 9.9 mL of saline.
   c. Transfer 0.1 mL from the second tube to a third tube containing 9 mL of saline.
   d. Plate 0.1 mL from the third tube onto a brucella blood agar plate (or other medium that adequately supports the growth of the organism being used) and spread it with a
loop or bent glass tube. The plate count should be approximately $10^2$ organisms (approximately 100 colonies per plate).
e. Incubate these plates under anaerobic conditions at 35°C–37°C for 48 h.

8.3.1.5 Results

1. Reading.
   a. The agar dilution plates should be read in the order that they were stamped.
      • This is important if a contaminate is found during the run because one can determine the point at which the results are no longer valid.
   b. The agar dilution plates should be read against a dark nonreflecting background.
   c. Record the growth control plate results inoculated at the beginning and end of each set for an antibiotic.
   d. Record a (+) for growth and a (–) for no growth for each isolate on each plate.
   e. The endpoint, i.e., the first (–), is to be read at the point where a marked change in growth appears as compared with the growth on the control plate.
   f. Marked change in growth can be a change to a haze, lighter growth, multiple tiny colonies, or a few small colonies. Figure 2 in the M11-A7 document is a good reference for learning how to determine appropriate end points [4]. Determination of end points for anaerobic agar dilution is more difficult than with aerobic bacteria. If performing this procedure for the first time, one should perform the procedure with someone experienced in reading anaerobic agar dilution.
   g. If no growth is seen on the positive growth control plate, no MIC value can be determined for that organism for that antimicrobial agent, even if growth is observed on the antimicrobial agent containing plates.

2. Interpreting.
   • Consult the latest edition of the M11 CLSI document for the appropriate break points for the antimicrobial agents being tested for interpretation of MIC values (Table 8.3).
   • Consult the colored Figure 2 in M11-A7 [4] for assistance in the interpretation of broth microdilution for anaerobes.

3. Reporting.
   MIC values should be reported with their appropriate categorical interpretation: (R) resistant, (I) intermediate, or (S) susceptible. All quality control values for a given antimicrobial agent must be within range to report susceptibility values for that antimicrobial agent.

8.3.1.6 Work Flow

8.3.1.6.1 Before the Run

1. Read the procedure.
2. Check the supplies and order items needed.
3. Prepare the agar deeps.
4. Label the dilution tubes.
5. Add the appropriate amount of water or appropriate diluent to dilution tubes.
6. Label the petri dishes with the appropriate information.
7. Prepare the antibiotic stocks.
8. Autoclave the Steers replicator.
9. Lake the whole sheep blood.
10. Thaw the blood (24 h before the run).
11. Subculture the isolates (48 h before the run).
12. Check the water bath (set to 48°C ± 2°C).
8.3.1.6.2 Day 1
1. Remove antibiotic stocks from the freezer.
2. Warm the blood to room temperature.
3. Prepare antibiotic dilutions.
4. Melt agar deeps.
5. Disinfect the work area.
6. Mix agar deeps + blood + antibiotic, and pour the plates.
7. Dry the plates.
8. Prepare the inoculum.
9. Stamp the plates, and allow the inoculum to be absorbed.
10. Incubate the plates.
11. Disinfect the Steers replicator.

8.3.1.6.3 Day 3
1. Check the growth control plate.
2. Check the negative growth (contamination) control plate.
3. Read the plates, and record the results.

8.3.1.7 Advantages
1. Agar dilution has the ability to test multiple isolates at once.

8.3.1.8 Limitations
1. The agar dilution procedure is not ideal for testing small numbers of isolates.
2. The agar dilution procedure is a very labor intensive procedure and difficult to interpret, even for the experienced microbiologist.
3. The agar dilution procedure requires specialized equipment (Steers replicator).
4. The inoculum is based on the turbidity equivalent 0.5 McFarland standard; however, anaerobic bacteria vary greatly in size and shape, resulting in colony counts that differ from the established $1\times 10^8$ with E. coli ATCC 25922 or B. fragilis ATCC 25285 [4,13].

8.3.1.9 Conclusions
This is definitely a procedure that should not be attempted without guidance from someone with experience. It is very labor intensive, and the interpretation of the results is difficult for the untrained eye. However, this is the CLSI reference method that all new testing procedures must be compared with.

8.3.2 Microbroth Dilution
8.3.2.1 Introduction
Of the procedures described by the CLSI, this is the most practical one for the clinical microbiology laboratory to use when testing a small number of isolates against a given set of antibiotics. The current CLSI M11-A7 document restricts testing with this method to B. fragilis group isolates only [4]. This procedure involves a microtiter tray containing standard twofold dilutions of several antibiotics to be inoculated with one standardized bacterial suspension. These microtiter trays can be prepared fresh, in advance, and frozen or purchased from a manufacturer in either a frozen or lyophilized format (Remel [IDS], PML, TREK [Sensititer — Investigational Use Only]). If using a commercial product, the manufacturer’s instructions should be followed.

The procedure described in this chapter is for preparing panels in-house.
8.3.2.2 Materials

1. Media
   a. Antibiotic sources
      1) United States Pharmacopeia (USP)
      2) Sigma Chemical Company
      3) The antimicrobial agent manufacturer
         • Many antimicrobial agents can be obtained in small amounts from the drug
           manufacturer at no charge by request through the drug company’s customer
           service department or equivalent department. (Consult the PDR or your local
           pharmaceutical representatives for telephone numbers.)
   b. Agar medium
      1) Brucella blood agar plates supplemented with 5% sheep blood, vitamin K₁, and
         hemin.
      2) These plates are used to grow isolates for inoculum suspension if using the direct
         growth method and for purity plates.
   c. Broth medium
      1) Supplemented brucella broth is the only basal medium recommended in the CLSI
         M11-A7 [4] document. The media should be made following the manufacturer’s
         instructions (Becton Dickinson, Remel, Accmedia).
      2) The media should be supplemented with 1 mL/L of hemin stock solution (5 mg/mL)
         and 1 mL/L of working vitamin K₁ stock solution (1 mg/mL) before autoclaving
         or dispensing of the media.
      3) Broth medium should be dispensed in 7.5 mL volumes into screw cap tubes.
      4) As soon as the tubes have been dispensed, they should be autoclaved at 121°C for
         15 min with a slow exhaust.
      5) The tubes can be stored at 4°C for 1 month or at room temperature for 1 week.
   d. Sterile distilled water
   e. Laked horse blood
      1) Sterile whole defibrinated horse blood can be purchased from several manufacturers
         (Remel, PML, Hemostat, Hardy Diagnostics).
         • Sterile laked blood can be purchased from Hardy Diagnostics, PML, and
           Hemostat.
      2) To prepare laked blood in the laboratory from whole blood, completely freeze the
         whole horse blood at 20°C overnight or longer. When the blood is thawed, the red
         blood cells will lyse, and the blood is “laked.” With horse blood, the freeze–thaw
         cycle needs to be repeated five to seven times. The blood then needs to be clarified
         by centrifuging at 12,000 g for 20 min. Carefully decant the supernatant. The superna-
         tant may need to be centrifuged a second time if it is not free of flocculent material.
      3) Laked blood can be stored in the freezer at 20°C for up to 1 year.
         • Once thawed, laked blood has a 2½-week shelf life at 2°C–8°C and should be
           labeled with a new expiration date when thawed.
   f. Hemin stock solution (5 mg/mL)
      1) Weigh out 0.5 g of hemin (Sigma # H2250).
      2) Dissolve the hemin powder in 10 mL of 1 mol/L NaOH (ACS certified).
      3) Make sure that the hemin powder is completely dissolved.
         • If the water is added to the solution before the hemin is completely dissolved,
           the hemin will remain unsuspended.
         • If the hemin will not go into solution in the NaOH, the solution can be heated to
           help dissolve the hemin. This does not work if it is done after the addition of water.
      4) Bring the volume of the solution to 100 mL with distilled water.
5) Make sure that the solution is in a brown bottle or wrapped in foil to protect it from light. Sterilize the hemin solution at 121°C for 15 min.
6) Cool to room temperature and store at 4°C–8°C.
7) This solution has a 1-month self-life and should be labeled with an expiration date when made.
8) Add 1 mL of the hemin stock solution (5 mg/mL) to 1 L of media.
g. Vitamin K₁ solution (10 mg/mL)
  1) Stock solution (10 mg/mL)
     a) Add 0.2 mL of vitamin K₁ solution (3-phytylmenadione, Sigma # V3501) to 20 mL of 95% ethanol.
     b) Mix the ethanol and the vitamin K₁ solution.
     c) Place in a sterile dark bottle and store at 4°C–8°C.
     d) The solution has a 2-year shelf life and should be labeled with an expiration date when made.
        • This solution has not been sterilized; therefore, sterile technique should be used when making this solution.
        • Always inspect the solution for possible contamination before use.
        • Vitamin K₁ can be autoclaved; however, other forms of vitamin K₁ cannot.
  2) Working solution (1 mg/mL)
     a) Add 1 mL of stock vitamin K₁ solution (10 mg/mL) to 9 mL of sterile distilled water.
     b) Place in a sterile dark bottle and store at 4°C–8°C.
     c) This solution has a 1-month shelf life and should be labeled with an expiration date when made.
     d) Add 1 mL of the working vitamin K₁ stock solution (1 mg/mL) to 1 L of media.
        • This solution is not sterilized after making it, and therefore sterile technique should be used.
        • Always inspect the solution for possible contamination before use.
        • Medium can be autoclaved after the addition of the vitamin K₁.
h. Sterile buffers at various pHs (Table 8.1).
3. Supplies
   a. Test tubes
      • Any standard laboratory sterile screw cap test tube that holds at least 10 mL can be used for making antibiotic dilutions.
      • It is not recommended that the tubes that were used to make antibiotic dilutions be reused. It is very labor intensive and difficult to clean the glass tubes to ensure that there is no carryover of antibiotics between runs. In addition, it has been shown that some antibiotics (i.e., quinolones) are very efficient at sticking to glass surfaces even after proper cleaning in an automated dishwasher (industrial strength).
   b. Microtiter trays
      • Ninety-six-well microtiter trays are available from the following manufacturers: Evergreen Scientific and Nalgene Nunc International.
   c. Pipettes (1-, 5-, 10-, and 25-mL sterile serological pipettes)
   d. Pipetting aid for serological pipettes
   e. 0.5 McFarland standard (see Section 8.3.1.2 for more details)
   f. Sterile swabs
   g. Sterile cover trays
   h. Inoculum reservoirs
4. Equipment
   a. Autoclave
   b. Water bath 48°C–50°C
c. Replicating device
   1) Pipetter (single or multichannel)
   2) Disposable multipoint inoculators, available from Dynex Technologies

d. Incubator
   • The incubator should be set at 35°C–37°C.
   • Either the incubator is in an anaerobic chamber or is a non-CO₂ incubator for use with anaerobic jars.

e. Anaerobic chamber or anaerobic jars
   • The following is a list of manufacturers of anaerobic systems: Coy, EM, Forma, Microbiology International, Mitsubishi, Oxoid, Plas-Labs, Sheldon Manufacturing, Toucan Technologies.

f. Freezer (−70°C)

g. Reading device
   • Light box
   • Mirror reader

8.3.2.3 Quality Control

1. Quality control strains:
   • Bacteroides fragilis ATCC 25285
   • Bacteroides thetaiotaomicron ATCC 29741
   • Eubacterium lentum ATCC 43055

2. Frequency of quality control testing
   • At least one of the above isolates should be tested with every microbroth dilution run. If testing more than 10 isolates, it is recommended to test all three quality control strains.
   • Quality control isolates should be tested following the same procedure used for the clinical isolates.
   • All quality control strains should be maintained following procedures described by the CLSI [12].
   • All three quality control isolates should be tested with each new lot of panels before testing clinical specimens.

3. Evaluating results
   • The MIC values for a quality control organism tested in one run must be within acceptable ranges for the antimicrobial agent being tested with the broth microdilution method. These ranges are listed in the current CLSI M11 document and in Table 8.4.
   • It is important to keep in mind the function of these quality control organisms, which is to check that the antibiotic concentrations were made properly, meaning that the amount of antibiotic in the broth microdilution tray really matches what you think is there. These quality control organisms do not test the quality of the media or the anaerobic environment.

8.3.2.4 Procedure

1. Antibiotic stock preparation (see Section 8.3.1.3 for more details)

2. Preparation of trays
   a. Commercially available systems (Dynex Technologies, Inc.) are available to make broth microdilution trays in the laboratory.
   b. Label tubes containing 7.5 mL of the appropriate supplemented broth with the appropriate antibiotic name and antibiotic concentration.
   c. Place the tubes in an organized fashion going from highest to lowest concentration.
   d. Add 0.5 mL laked blood to each broth tube.
e. Add 1 mL of the antibiotic dilution to the broth tube.
f. Mix by gently inverting the test tube three times (turning the tube upside down and back to the upright position counts as one inversion).
   • Try to avoid making any bubbles during this process.
   • Do not shake the tubes violently.
g. Using a dispensing device, transfer 0.1 mL from each of the broth–antibiotic tubes to the appropriate well in each microtiter tray.
   • It is not recommended that volumes smaller than 0.1 mL be used in the microtiter trays because of evaporation.
h. Two wells in the microtiter tray should contain only 0.1 mL of the supplemented broth. These two wells will serve as the positive and negative control wells for the microtiter tray.
i. The prepared trays should be sealed in a plastic bag and placed in a -70°C freezer until needed. Prepared trays should not be stored in a self-defrosting freezer as this type of freezer will shorten the shelf life of the antimicrobial agents in the tray. Prepared trays should not be refrozen after being thawed.
j. The shelf life of the panel is the shortest expiration date on the antibiotics being used or 4–6 months.
k. Appropriate quality control should be done to monitor the performance of the panel throughout the shelf life.
l. Quality control should be performed on each new lot of broth microtiter trays using all three quality control organisms before being used with clinical specimens.

### TABLE 8.4
Quality Control Ranges (μg/mL) (broth microdilution)

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th><em>Bacteroides fragilis</em> ATCC 25285</th>
<th><em>Bacteroides thetaiaotaomicron</em> ATCC 29741</th>
<th><em>Eubacterium lentum</em> ATCC 43055</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>0.25–1</td>
<td>0.25–1</td>
<td>—</td>
</tr>
<tr>
<td>Ampicillin/sulbactam (2:1)</td>
<td>0.5/0.25–2/1</td>
<td>0.5/0.25–2/1</td>
<td>0.5/0.25–2/1</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>1–8</td>
<td>16–128</td>
<td>16–64</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>2–8</td>
<td>8–64</td>
<td>2–16</td>
</tr>
<tr>
<td>Ceftriaxime</td>
<td>—</td>
<td>—</td>
<td>8–32</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>4–16</td>
<td>8.32</td>
<td>4–16</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.5–2</td>
<td>2–8</td>
<td>0.06–0.25</td>
</tr>
<tr>
<td>Doripenem</td>
<td>0.12–0.5</td>
<td>0.12–1</td>
<td>—</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>—</td>
<td>2–8</td>
<td>2–16</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>0.06–0.5</td>
<td>0.5–2</td>
<td>0.5–4</td>
</tr>
<tr>
<td>Faropenem</td>
<td>0.015–0.06</td>
<td>0.12–1</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Garenoxacin</td>
<td>0.06–0.25</td>
<td>0.25–2</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.03–0.25</td>
<td>0.25–1</td>
<td>0.25–2</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2–8</td>
<td>2–8</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.03–0.25</td>
<td>0.06–0.5</td>
<td>0.125–1</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.25–2</td>
<td>0.5–4</td>
<td>0.125–0.5</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.125–0.5</td>
<td>1–8</td>
<td>0.125–0.5</td>
</tr>
<tr>
<td>Penicillin</td>
<td>8–32</td>
<td>8–32</td>
<td>—</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>4–16</td>
<td>8–64</td>
<td>8–32</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>0.03/4–0.25/4</td>
<td>2/4–16/4</td>
<td>8/4–32/4</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>0.06–0.5</td>
<td>0.5/2–2/2</td>
<td>8/2–32/2</td>
</tr>
<tr>
<td>Trovafoxacin</td>
<td>0.125–0.5</td>
<td>0.5–2</td>
<td>0.25–2</td>
</tr>
</tbody>
</table>

Values were taken from the CLSI M11-A7, 2007 document.
3. Inoculum preparation (see Section 8.3.1.4 for more details)

4. Inoculation of frozen microtiter trays
   a. Remove the number of microtiter test panels to be used from the freezer. Allow the panels to thaw at room temperature.
   • If thawed in a hood unstacked, it will take at least 45 min to thaw. If thawed stacked 4 high with a clean cover tray (cover trays do not need to be sterile), it will take close to 2 h to thaw.
   • Remove the sealing tape as soon as the panels are removed from the freezer, and put a clean cover tray on the top of the panels.
   • Thawed trays should be inoculated and placed in the chamber within 1 h of thawing.
   b. Transfer 3 mL of the adjusted inoculum suspension (equal to a 0.5 McFarland standard) into 27 mL of sterile saline.
   c. Carefully invert the tube several times or vortex to thoroughly mix the suspension (introduce as few bubbles as possible).
   d. Transfer 10 μL of the inoculum to each well of the microtiter tray using a single or multichannel pipette. A multipin inoculator can also be used for this purpose. If using one of these devices, follow the manufacturer’s instructions as to what inoculum concentration should be added to the inoculum reservoir.
   e. Inoculated panels should be covered with a tray lid or empty tray.
   f. The microtiter trays should be inoculated and placed into the anaerobic chamber as soon as possible with a maximum total time of 30 min. Panels should not be stacked more than four high. (Stacks higher than four can lead to uneven heat distribution between the panels.)
   g. A purity plate and a negative (contamination) control plate should be prepared for every isolate being tested. If colony counts are being done with an isolate, these plates can serve as the purity plate. Transfer one 10 μL loopful of inoculum from the positive control well and streak for isolated colonies on a brucella agar plate (purity plate) and a tryptic soy agar (TSA) blood agar plate (negative [contamination] control plate).

5. Incubation
   a. Microtiter trays and purity plates should be incubated in an anaerobic environment stacked no more than four high. Incubation is for 46–48 h at 35°C–37°C.
   b. Negative (contamination) control plates should be incubated in an aerobic incubator for 46–48 h at 35°C–37°C. (A CO2 incubator is not required.)

6. Colony count
   a. Colony counts should be performed periodically to verify the inoculum concentration.
   b. Transfer 10 μL from the positive control well and add it to 10 mL of saline (1:1,000 dilution).
   c. Plate 0.1 mL of the dilution onto a brucella blood agar plate (or other medium that adequately supports the growth of the organism being tested) and spread it with a loop or bent glass tube. The plate count should be approximately 10² organisms (approximately 100 colonies per plate).
   d. Incubate these plates under anaerobic conditions at 35°C–37°C for 48 h.

8.3.2.5 Results

1. Reading
   a. Remove the trays and quality control plates from the incubator.
   b. Check the purity plate and evaluate for contamination. If contamination is observed, repeat the test. If no contamination is apparent, continue with reading the panel.
   c. Wipe the bottom of the tray with a damp lint-free tissue to remove any condensation.
   d. Place the tray on a reading device (light box or mirror reader).
e. The positive control growth well must have good growth.
   • If adequate growth in not achieved in the positive control growth well, the test
     must be repeated.
   • “Adequate growth” will depend on the species being tested. Isolates that produce
     small colonies on blood agar plates will usually produce small buttons with the
     microbroth procedure.
   • If adequate growth is not achieved, consider adding supplements to the medium
     or performing the entire procedure under anaerobic conditions.

f. Record a (+) for growth and a (–) for no growth for each well on the microtiter tray.
g. The end point, that is, the first (–), is to be read at the point where marked change in
   growth appears as compared with the growth in the positive control well.
h. If no growth is seen in the positive control growth well, MIC values cannot be
   determined for that isolate for any of the antibiotics tested.

2. Interpreting
   • Consult the latest edition of the M11 CLSI document for the appropriate break points
     for antimicrobial agents being tested for interpretation of the MIC values (Table 8.3).
   • Consult the colored Figure 3 in M11-A7 [4] for assistance in the interpretation of
     broth microdilution for anaerobes.

3. Reporting
   • MIC values should be reported with their appropriate categorical interpretation: (R) resis-
     tant, (I) intermediate, or (S) susceptible. All quality control values for a given antimicrobial
     agent have to be within range to report susceptibility values for that antimicrobial agent.

8.3.2.6 Work Flow

8.3.2.6.1 Before the Run
   1. Read the protocol carefully.
   2. Make sure all the items needed are in stock.
   3. Make or purchase microtiter plates.
   4. Subculture the isolates 48 h before the run.
   5. Check the purity of the isolates.

8.3.2.6.2 Day 1
   1. Disinfect a flat, level area where the work is going to be done.
   2. Remove microtiter trays from the freezer, and allow them to thaw at room temperature
      (30–45 min if they are not stacked, and 2 h if they are in stacks of four or five).
   3. Prepare the inoculum.
   4. Inoculate the trays.
   5. Inoculate the purity plates (anaerobic and aerobic).
   6. Incubate the trays and plates.

8.3.2.6.3 Day 3
   1. Read the trays, and record the results.
   2. Check the purity plates.
   3. Check the quality control results.

8.3.2.7 Advantage
   1. The broth microdilution procedure has the flexibility to test any number of isolates desired
      at one time.
   2. This procedure is easy to perform. If the laboratory prepares the panels in-house, the
      procedure then becomes a little more complicated and time consuming.
8.3.2.8 Limitations

1. The CLSI M11-A7 document only recommends this method with *B. fragilis* group isolates [4]. The procedure has not been validated with other species by the CLSI. Many anaerobic bacterial species do not grow well in broth, even after supplementation.
2. This procedure requires specialized equipment (dispensing equipment) if the panels are going to be prepared by the laboratory.

8.3.2.9 Conclusions

This procedure is easy to perform with minimal labor, especially if manufactured panels are used. Interpretation of the results can be challenging to the untrained eye. This procedure is described and endorsed by the CLSI for *B. fragilis* group isolates only.

8.3.3 E TEST

8.3.3.1 Introduction

Etest (AB Biodisk, Solna, Sweden) is made of a nonporous plastic strip immobilized with a predefined gradient of a given antibiotic on one surface and printed with an MIC scale on the other side. This concept of susceptibility testing was designed by Bolmstrom et al. [14] and was first presented at the ICAAC meeting in 1988.

When the Etest strip is placed on an inoculated agar plate, a continuous, stable, and exponential antibiotic gradient is established along the side of the strip. After incubation, the MIC value (μg/mL) can be read directly from the MIC scale printed on the Etest strip. The resulting MIC is equivalent to the CLSI agar dilution reference values; thus, the MIC interpretive criteria for anaerobic bacteria established by the CLSI in M11-A7 is directly applicable for interpretation of Etest results [4]. This technology is similar to a disk diffusion assay but with a longer stability time for the antibiotic gradient.

8.3.3.2 Materials

1. Etest strips.
   Etest strips can be purchased directly from AB Biodisk or through Remel. Each strip, on average, costs $2.15 and they are sold in packages of 100 strips per antibiotic. The strips are to be stored at −20°C or −70°C. Once a set of strips is removed from the manufacturer’s packaging, the unused strips should be stored in an airtight container with a desiccant and returned to −20°C. Etest strips have a shelf life of 2 to 5 years depending on the antibiotic. Etest strips should not be used after the expiration date indicated by the manufacturer. The antibiotics that have obtained FDA clearance for *in vitro* diagnostic use in the United States for anaerobes are listed in Table 8.5. Depending on the antibiotic, the MIC ranges on the Etest strips are either from 0.016–256 μg/mL or 0.002–32 μg/mL, which represents 15 twofold dilutions.
2. A brucella blood agar plate, made in-house or obtained commercially, that meets the following criteria can be used: 4 mm ± 0.5 mm brucella agar with 5% lysed sheep blood supplemented with vitamin K₁ and hemin (either a 90 mm or 150 mm diameter petri dish can be used). These plates should be stored at 2°C–8°C, unless otherwise stated by the manufacturer.
3. Brucella broth is used for making bacterial suspensions. The broth should be in a usable volume, such as 1–5 mL. This broth should be stored at 2°C–8°C.
4. Swabs (sterile, nontoxic).
5. Test tubes (for brucella broth).
6. Pipettes.
TABLE 8.5
Etest Strips with FDA Clearance for Anaerobic Bacteria

<table>
<thead>
<tr>
<th>Antibiotic</th>
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<tr>
<td>Amoxicillin/clavulanic acid (2/1)</td>
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<td>Ampicillin/sulbactam (2/1)</td>
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</tr>
<tr>
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<td>PG</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CT</td>
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<td>Clindamycin</td>
<td>CM</td>
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<td>ETP</td>
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<td>Imipenem</td>
<td>IM</td>
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<td>Meropenem</td>
<td>MP</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>MZ</td>
</tr>
<tr>
<td>Piperacillin/tazobactam (4 μg/mL)</td>
<td>PTc</td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid</td>
<td>TLc</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>TGC</td>
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</tbody>
</table>

7. Scissors.
8. Forceps or Etest applicator kit.
9. 1 McFarland standard, which can be made following CLSI instructions or purchased from microbiology product manufacturers such as PML, Hardy, or Remel. (See Section 8.3.1.2 for more details.)
10. Air tight storage containers with desiccant (silica gel) for extra Etest strips once the manufacturer’s packaging has been opened.
11. Anaerobic system (anaerobic chamber, anaerobic gas generator, or anaerobic bag).
   • The new gas generators made by Oxoid, EM Science, and Mitsubishi can be used for the anaerobic system; however, bicarbonate systems are not recommended for use with Etest because of the time required to generate adequate anaerobiosis.
12. Freezer (–20°C or –70°C) for storage.
13. Vortex.
14. 35°C ambient air or CO₂ incubator (if using anaerobic jars).
15. Etest technical information.
   • Etest package insert.
   • Etest technical guide no. 1.
   • Etest application sheet no. 7.
   • Customer information sheet no. 3.
   • Etest technical manual.

8.3.3.3 Quality Control

1. Quality control strains:
   • Bacteroides fragilis ATCC 25285.
   • Bacteroides thetaiotaomicron ATCC 29741.
   • Eubacterium lentum ATCC 43055.
   • All quality control strains should be maintained following the procedures described by the CLSI [12].
2. Frequency of quality control testing.
   • Quality control should be run with every new lot of Etest strips.
   • Each antibiotic should be tested within the last 7 days of use if proficiency has been
documented following the CLSI procedure of testing for 30 consecutive days. Otherwise, quality control should be done with each occasion of testing.

3. Evaluating the results.
   • AB Biodisk publishes an anaerobe application sheet that lists the quality control ranges
for appropriate antimicrobial agents. The values are the same as agar dilution quality
control ranges in the current CLSI M11 document (also see Table 8.2).

8.3.3.4 Procedure

1. Obtain a pure culture of the isolate to be tested on a nonselective medium such as brucella
   blood agar supplemented with vitamin K₁ and hemin.
   • The inoculum should be prepared from plates incubated for 24–72 h, depending on
the species being tested.
   • Frozen or lyophilized isolates should be subcultured at least twice prior to suscepti-
bility testing.

2. Remove the strips from the freezer (30 min for −20°C and 60 min for −70°C) before use
to allow them to equilibrate to room temperature prior to removing them from the original
package or storage container.

3. Using a sterile swab, emulsify enough isolated colonies in sterile brucella broth.
   • If log phase inoculum is desired, add less organism to the broth and allow the
suspension to grow until the desired turbidity is acquired (4–6 h).

4. Mix gently to introduce as little oxygen as possible.

5. Adjust the suspension turbidity to be equivalent to a 1 McFarland standard. Add more
   colonies to increase the turbidity or more broth to decrease the turbidity (photometric
devices can also be used to measure the turbidity).

6. The inoculum suspension should be inoculated onto the agar plate within 15 min after
   preparation. Dip a cotton swab into the bacterial suspension, remove excess liquid by
pressing against the test tube wall, and swab the entire surface of a dry agar plate. To
cover the entire plate, rotate the plate 120°, swab the entire surface a second time, rotate
the plate another 120°, and swab the entire surface for a third time.
   • Make sure that coverage of the inoculum is spread evenly over the entire surface of
the plate.
   • A correctly inoculated plate will result in an even lawn of confluent growth.

7. Allow the inoculum to be absorbed completely into the agar (at least 15–20 min). The
   plate must be completely dry before application of the Etest strip. If moisture is present
on the agar surface when the Etest strip is applied, it will affect the performance of the
antibiotic gradient. For fastidious anaerobic species, this entire process should be done
in an anaerobic chamber.

8. The strip is designed to have the MIC scale facing away from the agar surface and the
antibiotic gradient touching the agar surface (you want to be able to see the writing on
the strip after applying it to the agar plate). Apply the “E” (highest concentration of
antibiotic) at the edge of the petri dish to the agar surface and gently lay down the strip
with the lowest concentration end of the strip placed toward the center of the plate.
   *Once the strip has touched the agar surface, do not move the strip.* If any large bubbles
appear under the strip, gently remove the bubbles by pressing on the Etest strip with a
forceps or a swab. Small bubbles under the strip will not affect the performance of the
Etest strip.
• One to two strips can be placed on a 90-mm plate with opposite orientation to each other (you do not want the “E” on the same side of the plate for two strips since the gradients will overlap).
• Up to six strips can be placed on a 150-mm agar plate in a spoke wheel pattern (Figure 8.3). (Templates are available from AB Biodisk to optimize the positioning of the strips.)
• When using the Etest applicator, refer to the instructions in the product insert.
• Do not remove the Etest strip during any part of the process.

9. Incubate the plate in the inverted position (agar side up) at 35°C in an anaerobic environment (80%–85% N₂, 5%–10% H₂, 5%–10% CO₂) as soon as possible after the placement of Etest strips. (Try to minimize the exposure time of anaerobic bacteria to oxygen as much as possible throughout the working procedure.)
• Use an anaerobic indicator to verify that anaerobiosis has been achieved (i.e., 1–2 h). Resazurin has a lower redox potential (Eh 450 mV) than methylene blue, making it a more sensitive indicator to the presence of oxygen in an environment.

10. Incubate the plate for 24 h for the Bacteroides fragilis group and Clostridium spp., with the exception of clindamycin, which requires 48 h. Other fastidious anaerobic species should be incubated for 48–72 h until clear visible growth is seen.

8.3.3.5 Results

1. Reading.
   a. Do not read the results if the incubation exceeds 72 h.
   b. Evaluate the growth of the isolate before reading it. If the lawn of growth appears as no more than a thin haze or if the density of colonies is sparse, do not attempt to read the MIC; reincubate and/or repeat.
   c. Read the MIC where the edge of the inhibition ellipse intersects the MIC scale on the Etest strip (Figure 8.4).
   • Bacteriostatic antimicrobial agents tend to form diffuse edges. These should be read at the so-called 80% inhibition region, that is, the first point of significant inhibition as judged by the naked eye, ignoring the haze and microcolonies.
Bactericidal antimicrobial agents tend to form sharp edges. With these agents, hazes and isolated colonies are indicative of growth, and the MIC should be read where these are completely inhibited.

For more detailed descriptions, see the AB Biodisk product insert. AB Biodisk has very useful colored handouts available that show guidelines for interpreting the results.

If a thin line of growth occurs along the side of the Etest strip within the ellipse, it should not be used to interpret the results; the MIC value is located where the ellipse of growth intersects with the Etest strip.

If a “lightbulb” shape of inhibition ellipse occurs, read the ellipse at the top of the dip. This is often observed with clindamycin.

Growth of large colonies within the zone of inhibition is an indication of a mixed culture or two resistant variants, and the test should be repeated with a pure culture.

d. Do not use hemolysis of the blood in the agar medium as an indication of the MIC; bacterial growth is the only indicator used for interpretation of the MIC value.

e. If there is complete growth and no zone appears on the plate around a strip, the MIC value is recorded as greater than the highest antibiotic concentration of the strip.

f. If the inhibition ellipse falls below the lowest value on the strip, the MIC value is recorded as less than the lowest value on the strip.

2. Interpreting.

a. The MIC results from an Etest strip are based on a continuous gradient; therefore, MIC values between the standard twofold dilution can be obtained. These values can be reported as such, if desired, or rounded up to the next appropriate twofold dilution value, i.e., 0.19 μg/mL becomes 0.25 μg/mL.

b. To interpret Etest MIC results as susceptible (S), intermediate (I), or resistant (R) categories, always round up the in-between values to the next higher twofold dilution.

• For example, if CLSI break points are S ≤0.064 μg/mL and R ≥0.25 μg/mL, an Etest value of 0.19 μg/mL is rounded up to 0.25 μg/mL and the category is reported as resistant.

• Etest MIC values are directly proportional to the CLSI reference agar dilution values. Therefore, CLSI breakpoints are directly applicable to the Etest MIC values. Consult
the latest edition of the M11 document for the appropriate agar dilution break points for antimicrobial agents (Table 8.3).

3. Additional considerations.
   a. Clindamycin should not be read before 48 h with any anaerobic bacteria because of delayed expression of inducible resistance genes.
   b. Achievement of an anaerobic environment (Eh 450 mV) early in the incubation time (within the first few hours) is needed when testing metronidazole. MIC values may be falsely elevated if anaerobiosis is incomplete because metronidazole is not sufficiently reduced to its active metabolite. Therefore, the use of bicarbonate anaerobic generators may not be appropriate.

8.3.3.6 Advantages

1. Etest is easy to perform and requires minimal training.
2. Etest can be used for many species, including the more fastidious anaerobic species.
3. Many species of anaerobic bacteria grow better on agar media than broth. Furthermore, contamination can be easily recognized on agar.
4. Etest can be easily set up for a small number of clinical isolates.
5. There is minimal labor involved with Etest as compared with agar dilution if testing a small number of isolates with a small number of antimicrobial agents.
6. Interpretation of the Etest is fairly easy.

8.3.3.7 Limitations

Cost is the most significant limitation; however, if careful consideration is given to which antibiotics to test, for fewer than five strips per isolate, the Etest methodology can be cost effective.

8.3.3.8 Conclusions

The Etest is relatively easy to set up and perform and lends itself well to laboratories that do not test large volumes of clinically significant anaerobes.

8.4 GENERAL CONCLUSIONS

Currently, the gold standard for susceptibility testing for anaerobic bacteria approved by the CLSI is the agar dilution method. This procedure is used typically by research laboratories or large clinical laboratories that can “batch” isolates for large scale research or surveillance studies. This method is very labor intensive and does not lend itself to testing small numbers of clinical isolates. If a manufactured product is proven to be equivalent to the agar dilution procedure, it can be used in the clinical laboratory even though CLSI has not described the use of the product in a document (the CLSI does not endorse products). Of the procedures approved by the CLSI, microbroth dilution is best suited for routine testing in the routine clinical laboratory. It can be used with any number of isolates and is less labor intensive than agar dilution. Microbroth dilution panels can be prepared in bulk and frozen in the laboratory, or they can be purchased from a manufacturer. However, some anaerobic bacterial species do not grow well in broth. If either of the CLSI methods is being used, it is strongly recommended that the most recent CLSI M11 document be viewed. The anaerobe working group has added color figures to help aid in the interpretation of these methods with anaerobes. The other practical method for testing anaerobic bacteria is the Etest. It has similar advantages to the microbroth dilution procedure but is more expensive per antibiotic tested. As antibiotic resistance continues to increase, the need for the clinical laboratory to be able to routinely
perform anaerobic susceptibility testing will become more important.

**APPENDIX — MANUFACTURERS**

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<th>Telephone</th>
<th>Fax</th>
<th>Product inquiries and orders</th>
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<tr>
<td>AB Biodisk North America Inc.</td>
<td>200 Centennial Avenue, Piscataway, NJ 08854-3910</td>
<td>732-457-0408</td>
<td>732-457-8980</td>
<td>800-874-8814</td>
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<tr>
<td>Dalvagen 10</td>
<td>S-169 56 Solna, Sweden</td>
<td>+ 46-8-730 07 60</td>
<td>+ 46-8-83 81 58</td>
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<tr>
<td>AB Biodisk</td>
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<tr>
<td>BD Biosciences</td>
<td>P.O. Box 243, Cockeysville, MD 21030</td>
<td>410-771-0100</td>
<td>800-638-8663</td>
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<tr>
<td>CMI-Promex (Steers Replicator)</td>
<td>7 Benjamin Green Road, P.O. Box 418, Pedricktown, NJ 08067</td>
<td>609-351-1000</td>
<td></td>
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<tr>
<td>Coy Laboratory Products, Inc.</td>
<td>14500 Coy Drive, Grass Lake, MI 49137</td>
<td>313-475-2200</td>
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<tr>
<td>Dynex Technologies, Inc.</td>
<td>14340 Sullyfield Circle, Chantilly, VA 20151</td>
<td>800-336-4543</td>
<td>703-631-7800</td>
<td>703-631-7816</td>
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<tr>
<td>EM Science</td>
<td>480 S. Democrat Road, Gibbstown, NJ 08027-1297</td>
<td>800-222-0342</td>
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<tr>
<td>Evergreen Scientific</td>
<td>2300 E. 49th St., Los Angeles, CA 90058</td>
<td>213-583-1331</td>
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<td>Fisher Scientific Co.</td>
<td>711 Forbes Ave., Pittsburgh, PA 15219</td>
<td>412-490-8300</td>
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<td>Forma Scientific, Inc.</td>
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<td>614-374-1851</td>
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<td>Gelman Science</td>
<td>600 South Wanger Rd., Ann Arbor, MI 48103</td>
<td>313-665-0651</td>
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<td>805-346-2766</td>
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<td>Hemostat Laboratories</td>
<td>P.O. Box 790, 515 Industrial Way, Dixon, CA 95620</td>
<td>707-678-9594</td>
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REFERENCES

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*Ana Espinel-Ingroff and Emilia Cantón*

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9.1 INTRODUCTION

Since 1956 and until the introduction of fluconazole in 1990, amphotericin B was the only available antifungal agent for the treatment of severe fungal infections; consequently, there was little need for routine susceptibility testing. Furthermore, the emergence of the AIDS pandemic, modern patient management technologies and therapies such as bone marrow and solid-organ transplants, and the more aggressive use of chemotherapy have resulted in a rapidly expanding number of patients highly susceptible to mycotic infections. With the increased incidence of fungal infections and the parallel development of resistance to established agents, the laboratory’s role in the selection of antifungal therapy has gained greater attention. The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) has approved standard broth dilution methodologies for susceptibility testing of Candida spp. and Cryptococcus neoformans to amphotericin B, fluconazole, ketoconazole, fluconazole, itraconazole, voriconazole, and posaconazole [1]. The CLSI antifungal susceptibility testing assays (broth dilution and disk diffusion) and two commercial methods for yeasts are described in this chapter. The endpoints determined by these methods are the minimum inhibitory concentration or the MIC and the inhibition zone diameter (disk testing).
The European Subcommittee on Antifungal Susceptibility Testing (EUCAST) has recently published a standard method for determination of MICs of fermentative yeasts (EUCAST 7.1 document). Although this broth microdilution assay is similar to the CLSI method, medium composition (standard RPMI-1640 supplemented with 2% dextrose), inoculum size (approximately 10^5 CFU/mL), and incubation time (24 h) are quite different; CLSI breakpoints should not be used to interpret EUCAST MICs. Description of this method is not included in this chapter but can be found elsewhere [2].

9.2 RELEVANCE

According to the CLSI recommendations, yeast susceptibility testing should be performed to determine patterns of susceptibility of Candida spp. and C. neoformans to available antifungal agents by conducting periodic testing in individual medical centers. For a yeast recovered from a clinical sample antifungal susceptibility testing should be performed when a pathogen is recovered from an invasive infection, when therapeutic failure has occurred, in instances where appropriate therapy is critical to the clinical outcome for a patient with a severe life-threatening infection, when long-term therapy is necessary, and when enough information is not available to make an empirical judgment about a therapy [1]. The CLSI M27-A2 document describes broth macro- and microdilution tests. For the development of the M27-A2 document, the Subcommittee on Antifungal Susceptibility Tests conducted several multicenter studies [3–6] to evaluate the best medium, inoculum size, temperature, duration of incubation, and endpoint definition. The test medium recommended is RPMI-1640 broth (RPMI) with [3-(N-morpholino)-propanesulfonic acid (MOPS) buffer. However, this broth does not appear to discriminate between resistant and susceptible strains to amphotericin B, and growth of C. neoformans is poor in this medium. The CLSI subcommittee has established breakpoints for fluconazole, itraconazole, voriconazole, and 5-FC (fluocytosine), as well as MIC ranges for two American Type Culture Collection (ATCC) quality control strains [1,6–10]. To appreciate the clinical value of the in vitro antifungal testing result, one must understand that the predictor value of in vitro susceptibility in bacterial infections has been accurately summarized as the “90–60 rule.” Infections due to susceptible isolates respond to therapy approximately 90% of the time, whereas infections due to resistant isolates respond approximately 60% of the time. There is now a considerable body of data indicating that standardized antifungal susceptibility testing for Candida spp. and some triazoles provides results that have a predictive utility consistent with this rule. However, host factors, rather thanazole resistance, can be responsible for clinical failure. For voriconazole, pharmacokinetics and pharmacodynamic parameters (PK/PD) indicate that a 24-h free-drug AUC/MIC (where AUC is the area under the curve) ratio of 20 is predictive of efficacy and recommended doses would produce free-drug AUCs of ~20 mg [11]. Therefore, it can be predicted that current voriconazole recommended dosing regimens could be used successfully to treat patients infected with isolates for which voriconazole MICs are ≤1 mg/mL as determined by the reference method. The PD parameter that predicts efficacy for fluconazole is approximately 25 (AUC/MIC). For itraconazole, an MIC within the susceptible-dose dependent (S-DD) range indicates the need for plasma concentrations >0.5 mg/mL for an optimal response. Actual breakpoints are listed in Table 9.1.

The CLSI group has made no recommendations for other yeasts or yeastlike organisms besides Candida spp. and C. neoformans. Since the publication of the M27-A document, several additional methods have been developed (CLSI disk test for yeasts and commercial Etest and colorimetric YeastOne methods, among others); however, these methods are not included in the CLSI M27-A2 document.

In Sections 9.3 and 9.4, the design of the CLSI broth macro- and microdilution methods for susceptibility testing of yeasts, respectively, are described. This is followed by descriptions of Etest, the colorimetric YeastOne method, and the disk diffusion method.
9.3 BROTH MACRODILUTION METHOD

9.3.1 INTRODUCTION

The design of the CLSI broth macro- and microdilution methods for susceptibility testing of yeasts is similar to that for antibacterial agents. These procedures involve preparing a series of drug dilutions, each containing a specific concentration of an antifungal agent. If a more practical method should yield results similar to those obtained by using the CLSI methods, it is considered an acceptable method for use in the clinical laboratory. Validation of an alternative method is the responsibility of the laboratory.

9.3.2 MATERIALS

1. Antimicrobial sources:
   b. Sigma Chemical Company.
   c. The antifungal agent manufacturer.
      • Some antifungal agents can be obtained from the drug manufacturer at no charge through the pharmaceutical company’s customer service department, or equivalent department, or your local pharmaceutical representatives.

2. Agar media:
   a. Sabouraud dextrose agar (SAB) plates.
   b. CHROMagar plates.
      • SAB plates may be used to grow isolates for inoculum preparation and to validate the density of the inoculum (colony counts). CHROMagar plates can be used to verify the purity of each isolate.

3. Test medium.
   a. RPMI (with glutamine, without bicarbonate, and with a pH indicator) is the broth medium recommended for the susceptibility testing of yeasts. The medium should be buffered with MOPS (0.164 M/L) mol/L. For the composition of the medium, see Table 9.2.
   b. Procedure for making the medium:
      • 10.4 g powdered medium (Sigma catalog # R 6504).
      • 34.53 g MOPS buffer (Sigma catalog # M 3183 or other manufacturer).

### TABLE 9.1
(CLSI) MIC Interpretive Criteria (breakpoints [µg/mL])

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Susceptible</th>
<th>S-DD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤8</td>
<td>16–32</td>
<td></td>
<td>≥64</td>
</tr>
<tr>
<td>Itraconazole&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.12</td>
<td>0.25–0.5</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5-FC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>≤4</td>
<td>8–16</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Voriconazole&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1</td>
<td>2</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> S-DD, susceptible-dose dependent on achieving maximum dosages or blood level to obtain optimal response, e.g., ≥400 mg/day of fluconazole or ≥0.5 µg/mL of itraconazole blood levels measured by high-pressure liquid chromatography (HPLC).

<sup>b</sup> Based on mucosal infections and limited data from invasive infections [1]. C. krusei are intrinsically resistant to fluconazole.

<sup>c</sup> Based on mucosal infections. Breakpoints for invasive infections are not available [1].

<sup>d</sup> Based on historical data and drug pharmacokinetics.

<sup>e</sup> Based on mucosal and invasive infections (Phase III clinical trials) and listed in M27-S2 [7].
TABLE 9.2
Composition of RPMI-1640 Medium

<table>
<thead>
<tr>
<th>Constituent</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Arginine (free base)</td>
<td>200</td>
</tr>
<tr>
<td>l-Aspartine</td>
<td>50</td>
</tr>
<tr>
<td>l-Aspartic acid</td>
<td>20</td>
</tr>
<tr>
<td>l-Cystine. 2HCl</td>
<td>65.2</td>
</tr>
<tr>
<td>l-Glutamic acid</td>
<td>300</td>
</tr>
<tr>
<td>Glycine</td>
<td>10</td>
</tr>
<tr>
<td>l-Histidine (free base)</td>
<td>15</td>
</tr>
<tr>
<td>l-Hydroxypropoline</td>
<td>20</td>
</tr>
<tr>
<td>l-Isoleucine</td>
<td>50</td>
</tr>
<tr>
<td>l-Leucine</td>
<td>50</td>
</tr>
<tr>
<td>l-Lysine. HCl</td>
<td>40</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>15</td>
</tr>
<tr>
<td>l-Phenylalanine</td>
<td>15</td>
</tr>
<tr>
<td>l-Proline</td>
<td>20</td>
</tr>
<tr>
<td>l-Serine</td>
<td>30</td>
</tr>
<tr>
<td>l-Threonine</td>
<td>20</td>
</tr>
<tr>
<td>l-Tryptophan</td>
<td>5</td>
</tr>
<tr>
<td>l-Tyrosine. 2Na</td>
<td>28.83</td>
</tr>
<tr>
<td>l-Valine</td>
<td>20</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2</td>
</tr>
<tr>
<td>n-Pantothenic acid</td>
<td>0.25</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>3</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1</td>
</tr>
<tr>
<td>Myoinositol</td>
<td>35</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>1</td>
</tr>
<tr>
<td>Para amino benzoic acid</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>1</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.2</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.005</td>
</tr>
<tr>
<td>Calcium nitrate H₂O</td>
<td>100</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>400</td>
</tr>
<tr>
<td>Magnesium sulfate (anhydrous)</td>
<td>48.84</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6,000</td>
</tr>
<tr>
<td>Sodium phosphate, dibasic (anhydrous)</td>
<td>800</td>
</tr>
<tr>
<td>n-Glucose</td>
<td>2,000</td>
</tr>
<tr>
<td>Glutathione, reduced</td>
<td>1</td>
</tr>
<tr>
<td>Phenol red, Na</td>
<td>5.3</td>
</tr>
</tbody>
</table>

1) Dissolve the powdered medium in 900 mL distilled water.
2) Add MOPS and stir until dissolved.
3) Adjust the pH to 7.0 at 25°C using 10 mol/L NaOH.
4) Add additional water to bring the medium to a final volume of 1 L.
5) Sterilize by filtration using a 0.22-μm filter, and dispense 100 or 500 mL volumes (depending on the volume needed per run) into sterile glass screw top bottles.
6) Store at 4°C (this solution has a 3-month shelf life, and bottles should be labeled with the preparation and expiration dates).
7) Check the sterility of the medium by incubating five tubes, each containing 2 mL of the medium per liter, at 35°C. In addition, visually inspect the solution for obvious contamination before using it
• The medium can be purchased as a powder (Sigma) or ready to use (Bio-whittaker catalog # 04-525F)

4. Sterile saline (8.5 g/L NaCl; 0.85% saline)

5. Supplies:
   a. Test tubes
   • Standard laboratory sterile screw or snap cap test tubes (e.g., 12 × 75 mm plastic) that hold at least 5 mL can be used for making drug dilutions (13 tubes per antifungal agent and strain) and 16 × 150 mm tubes for other purposes.

b. Petri dishes
   • Standard 100 × 15 mm round petri dishes (two dishes per strain, one with SAB for preparing the inoculum and another to count the number of CFU/mL)

c. Pipettes (0.1-, 0.25-, 0.5-, 1-, and 5-mL sterile serological pipettes)

d. Pipetting aid for serological pipettes

e. Swabs and loops

f. A 0.5 McFarland standard

1) Procedure for making McFarland standards [1].
   a) Combine 0.5 mL of 0.048 mol/L BaCl₂ (1.175% w/v BaCl₂ 2H₂O) and 99.5 mL of 0.18 mol/L H₂SO₄ (1%v/v)
   b) Mix the solution thoroughly
   c) Using the same size screw-cap tubes that are to be used for the preparation of yeast inoculum suspensions, aliquot 4–6 mL into each tube
   d) Seal the caps with tape or Parafilm®
   e) Store the tubes in the dark at room temperature
   f) Mix the standard solution well prior to each use
       • Commercially available McFarland standards can be obtained from a number of vendors, including PML, Hardy Diagnostics, and Remel

2) Validation of the McFarland standard turbidity
   a) Absorbance
       • Read the optical density (OD) of the standard on a spectrophotometer with a 1-cm light path using a matched set of cuvettes
       • The absorbance should be read at 530 nm
   b) Colony count
       • The density of the McFarland standard is validated by performing a colony count of a suspension of either Candida parapsilosis ATCC 22019, C. krusei ATCC 6258, or C. albicans ATCC 90028 that is equivalent to the turbidity of the standard
       • The density of the McFarland standard should be verified monthly by the absorbance or colony count method
       • A 0.5 McFarland standard is equivalent to 1–5 × 10⁶ yeast/mL (the density recommended by the CLSI for the stock inoculum)

3) Other turbidity standards
   a) Manufactured latex standards
       • Latex standards have a two year shelf life and are not light sensitive
       • Latex standards are available from several manufacturers, including Remel (Lenexa, KS; catalog product # 20-410). The density of latex standards should be verified monthly as described for the McFarland standard
   b) Turbidity meters
       • Available from Vitek and MicroScan Systems
6. Equipment:
   a. Autoclave
   b. Water bath (48°C–50°C)
   c. Vortex mixer
   d. Incubator set at 35°C ± 1°C
   e. Spectrophotometer

9.3.3 Quality Control

Quality control organisms are used to verify that the antifungal concentrations were prepared properly, but their use does not test the quality of the medium.

1. Quality control strains:
   • C. parapsilosis ATCC 22019
   • C. krusei ATCC 6258

2. Frequency of quality control testing
   • At least one of the above isolates should be tested with every run and should be performed using the same procedure used for the clinical isolates
   • Quality control strains should be preserved following the procedures described by the CLSI [1]

3. Evaluating quality control MIC results
   • The MIC values for quality control organisms must be within the acceptable ranges for the antifungal agent being tested for the results to be considered valid. These ranges are listed in the current CLSI M27 document and in Table 9.3 and Table 9.4.

9.3.4 Procedure

The purity and viability of the organism should be validated before testing is performed. Isolates that are being tested must be streaked at least twice onto a SAB plate before susceptibility testing is performed to ensure the purity and viability of the test organism.

1. Technical notes.
   • It is important to completely plan out the entire procedure because it involves many steps over several days. It is also important to understand all the steps of the procedure before beginning. The technologist is advised to read and understand the CLSI M27 document before proceeding.

<p>| TABLE 9.3 |
| Quality Control MIC Ranges of Antifungal Agents for Broth Macrodilution (48 h) |</p>
<table>
<thead>
<tr>
<th>MIC Ranges (µg/mL)</th>
<th>C. parapsilosis ATCC 22019</th>
<th>C. krusei ATCC 6258</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.25–1</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>2–8</td>
<td>16–64</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.06–0.25</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.06–0.25</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td>5-FC</td>
<td>0.12–0.5</td>
<td>4–16</td>
</tr>
</tbody>
</table>

Based on the M27-A method described in [1].

* 5-FC = 5-flucytosine.
Determine the number of isolates to be tested, the number of antifungal agents, and the range of concentrations for each antifungal.

The antifungal range should include drug concentrations that are at least two dilutions below and two dilutions above the breakpoint values for that antifungal. The breakpoints and the concentration ranges are listed in the CLSI M27-A2, CLSI M27-S2 (voriconazole by microdilution method) documents [1,7] and in Table 9.1.

2. Tubes
   a. Based on the number of strains and the antifungal agents to be tested, calculate the number of tubes needed (# strains × number of antifungal dilutions + controls = # of tubes/antifungal).
   b. Calculate the total amount of RPMI needed (# of strains × 12 mL = total volume/strain + 12 mL to prepare antifungal dilutions).

3. Antifungal stock solution preparation
   a. Antifungal powders should be stored according to the manufacturer’s instructions. Powders are assayed by the manufacturer to determine the potency of the antifungal agent. This information must be used when making stock solutions as follows:

   \[
   \text{Weight (mg)} = \frac{\text{volume (mL)} \times \text{desired concentration (mg/mL)}}{\text{antifungal potency (mg/mg)}}
   \]

   b. When possible, at least 10 mg of the antifungal powder should be weighed out at one time.
   c. All stock solutions should be made at the same concentration. Stock solutions of 1,280 μg/mL work well for most standard susceptibility testing ranges for water-soluble agents and 1,600 μg/mL for water-insoluble ones.
   d. The antifungal powder should be dissolved in water or other appropriate solvent as shown in Table 9.5.
   e. Stock solutions should be stored carefully sealed in either glass, polypropylene, or polyethylene sterile tubes at −70°C (or lower) in small aliquot volumes (approximately 1.5 mL). Stock solutions should not be stored in frost-free freezers.
TABLE 9.5
Solvents and Diluents for Preparation of Dilutions of Antifungal Agents

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>DMSO or PEG200</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Ravuconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Micafungin</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>5-FC</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
</tbody>
</table>

a 5-FC = 5-flucytosine.
b DMSO = dimethyl sulfoxide; PEG200 = polyethylene glycol, mol. wt. of 200.

f. Stock solutions should be accurately labeled with the antifungal’s name and concentration, the amount in the tube, and the preparation and expiration dates (either six months after the stock solution was made or the manufacturer’s expiration date for the powder).
g. Stock solutions should be discarded at the end of each day and never refrozen.
h. Because of the high concentration of antifungal in the stock solution, sterilizing the solution is not necessary.
i. If there is a need to sterilize the antifungal stock solution, it should be done using a membrane such as a 0.22-µm membrane.
   • Paper, asbestos, or sintered glass filters should not be used because they may bind to the agent.
   • Filter sterilization is not recommended because the antifungal agent may be retained in the filter.

a. Preparation of water-soluble antifungal agents (fluconazole, 5-FC, caspofungin, micafungin). (Figure 9.1) [12].
   1) Label 10 (16 × 150 mm) tubes 2–11 with the antifungal name and the appropriate concentration.
   2) Add appropriate amounts of sterile RPMI to each tube (see Figure 9.1 and M27-A2) as follows:
      • Add 1 mL of RPMI to tubes 2 and 11.
      • Add 0.5 mL of RPMI to tubes 3, 5, and 8.
      • Add 0.75 mL of RPMI to tubes 6 and 9.
      • Add 1.5 mL of RPMI to tube 4.
      • Add 1.75 mL of RPMI to tubes 7 and 10.
   3) Add 1 mL of the stock solution (1,280 µg/mL) to the first antifungal dilution tube (tube 2).
   4) Transfer from tube 2, 0.5 mL to tubes 3 and 4.
   5) Transfer from tube 4, 0.5 mL to tube 5 and 0.25 mL to tubes 6 and 7.
   6) Transfer from tube 7, 0.5 mL to tube 8 and 0.25 mL to tubes 9 and 10.
   7) Transfer from tube 10, 1 mL to tube 11.
8) Finally, discard 1 mL from tube 11.
   • Vortex each tube before the dilution step.
   • Notice that when dilutions have been prepared, each tube contains 1 mL.
   • Nine sets of MIC tubes may be prepared with these volumes.

b. Preparation of MIC tubes for water-soluble agents (Figure 9.2) [12].
   1) Place the tubes containing the drug dilutions (1 mL each) in an organized fashion,
      going from the highest to the lowest drug concentration.
   2) Label rows of 10 tubes (12 × 75 mm) with the final drug concentrations (64–0.12 µg/mL).
   3) Dispense 0.1 mL from each drug dilution tube into the bottom of each corresponding
      MIC tube using a repetitive or reservoir pipette with a syringe of 1 mL.
      • Beginning with the lowest concentration, the same tip can be used.
   4) Close the tubes with screw caps (plastic or metal caps), and store them at –70°C for
      up 6 months.
   5) On the day of the test, allow the tubes to thaw at room temperature before inoculation
      (about 1 h). Each tube is inoculated with 0.9 mL of the corresponding diluted
      inoculum.

c. Preparation of antifungal dilutions for water-insoluble agents (Figure 9.3) [12].
   • The dilutions of water-insoluble antifungal agents (amphotericin B, itraconazole,
      ketoconazole, posaconazole, ravuconazole, voriconazole, and anidulafungin) must
      be prepared by using a solvent such as dimethyl sulfoxide (DMSO) (see Table 9.5).
   • It is important to prepare the stock solution and drug dilutions in the solvent (follow
      the scheme of the dilution and instructions below) to avoid dilution artifacts that
      can result from the precipitation of a compound of low solubility (e.g., itraconazole)
      in aqueous media, thereby producing erroneous drug dilutions.

   1) All the test tubes should be labeled with the antifungal’s name and the appropriate
      concentration as described here for the broth macrodilution method.
2) Add the appropriate amounts of DMSO to each labeled tube 3–11 (see Figure 9.3 and M27-A2 [12]) as follows:
   - Add 0.5 mL of DMSO to tubes 3, 6, and 9.
   - Add 0.75 mL of DMSO to tubes 4, 7, and 10.
   - Add 1.75 mL of DMSO to tubes 5, 8, and 11.

3) Label the stock solution tube (1,600 µg/mL) as tube 2.
4) Transfer from tube 2, 0.5 mL to tube 3 and 0.25 mL to tubes 4 and 5.
5) Transfer from tube 5, 0.5 mL to tube 6 and 0.25 mL to tubes 7 and 8.
6) Transfer from tube 8, 0.5 mL to tube 9 and 0.25 mL to tubes 10 and 11.
7) Finally, discard 1 mL from tube 11.
   • Vortex each tube before the dilution step.
   • Notice that when dilutions have been prepared, all tubes contain 1 mL.

d. Preparation of MIC tubes for water-insoluble antifungal agents (Figure 9.4) [12].
1) Place the tubes containing the drug dilutions (1 mL) in an organized fashion, from
   the highest to the lowest drug concentration and label rows of 10 tubes (12 × 75
   mm) with the final drug concentrations (16–0.03 µg/mL).
2) Prepare a 1:10 dilution by mixing 0.9 mL of RPMI with 0.1 mL from each drug
   concentration, and mix well with a vortex mixer.
3) Nine sets of MIC tubes may be prepared with these volumes.
4) Dispense 0.1 mL from each concentration into the bottom of each sterile plastic
   test tube (12 × 75 mm), using a repetitive or reservoir pipette with a syringe of 1 mL.
   • Beginning with the lowest concentration, the same tip can be used for all the
     test tubes.
5) Close the tubes with plastic or metal screw caps, and store them at −70°C for up
   to 6 months.
6) On the day of the test, allow the tubes to thaw at room temperature before inocu-
   lation (about 1 h). Each tube is inoculated with 0.9 mL of the corresponding
   inoculum. When inoculated, the final concentration of DMSO is 1%.

5. Inoculum preparation.
   • Direct colony suspension (Figure 9.5) [12].
   • The inoculum is prepared from pure cultures grown on SAB at 35°C for 24 h (Candida
     spp.) and 72 h (C. neoformans).
Antifungal Susceptibility Testing of Yeasts

1. Lightly touch five colonies (at least 1 mm-diameter) of similar morphology using a loop or swab.
2. Suspend the yeast cells in 5 mL of sterile 0.85% NaCl, and mix the cell suspension using a vortex mixer for 15–20 s.
3. Adjust the turbidity of the inoculum to the density of a 0.5 McFarland standard at 530 nm wavelength. This procedure yields a yeast stock suspension of 1–5 × 10^6 cells/mL.
4. Prepare the working suspension by making a 1:100 dilution of the stock suspension followed by a 1:20 dilution with RPMI, which results in 5 × 10^2 to 2.5 × 10^3 CFU/mL.
   - Vortex each tube before the dilution step.
   - If possible, the preparation of inocula for one run should be completed within 30 minutes, otherwise, the suspensions may be held at 2°C–8°C for up to 2 h.
5. Inoculation of the tubes.
   - The drug concentration tubes should be organized before the preparation of the inocula in ascending order.
   - Include two tubes, for positive (growth control) and negative (sterility) controls.
   - Calculate the volume of the standardized inoculum–broth suspension needed for each isolate, including the growth control tubes (for example, for one antifungal agent and

---

**FIGURE 9.5** Inoculum preparation for micro- and macrodilution methods.

a. Lightly touch five colonies (at least 1 mm-diameter) of similar morphology using a loop or swab.
b. Suspend the yeast cells in 5 mL of sterile 0.85% NaCl, and mix the cell suspension using a vortex mixer for 15–20 s.
c. Adjust the turbidity of the inoculum to the density of a 0.5 McFarland standard at 530 nm wavelength. This procedure yields a yeast stock suspension of 1–5 × 10^6 cells/mL.
d. Prepare the working suspension by making a 1:100 dilution of the stock suspension followed by a 1:20 dilution with RPMI, which results in 5 × 10^2 to 2.5 × 10^3 CFU/mL.
   - Vortex each tube before the dilution step.
   - If possible, the preparation of inocula for one run should be completed within 30 minutes, otherwise, the suspensions may be held at 2°C–8°C for up to 2 h.
6. Inoculation of the tubes.
   a. The drug concentration tubes should be organized before the preparation of the inocula in ascending order.
   b. Include two tubes, for positive (growth control) and negative (sterility) controls.
   c. Calculate the volume of the standardized inoculum–broth suspension needed for each isolate, including the growth control tubes (for example, for one antifungal agent and
yeast, 10 mL of inoculated RPMI are needed), at the desired final concentration (5 × 10^2 to 2.5 × 10^3 CFU/mL).

- Begin inoculating the lowest drug concentration to be tested and move to the highest concentration.

d. The two control tubes contain 0.1 mL of RPMI (or the solvent) rather than the antifungal and either 0.9 mL of the diluted inoculum (growth control) or 0.9 mL of RPMI (sterility control).

- The growth control tube ensures that the inoculum is viable and actively growing, and indicates the 100% growth of the isolate.
- The negative control tube provides evidence of medium sterility.
- The inoculum control plate confirms that the inoculum is properly standardized.

e. Inoculum control plates should be prepared for each strain on either SAB or CHROMagar medium. The latter medium can determine both the purity and the density of the inoculum.

1) Remove 10 µL from the growth control tube to a CHROMagar plate or SAB before the incubation step.
2) Incubate for 24–48 h at 35°C, and count the CFUs (5–25 CFUs).

7. Incubation.

- MIC tubes and growth control plates and tubes are incubated in an aerobic incubator without CO₂ at 35°C for 48 h (Candida spp.) to 72 h (C. neoformans). The 24-h MIC results may also be obtained, and the tubes are then immediately reincubated [13,14].

9.3.5 MACRODILUTION MIC DEFINITIONS

9.3.5.1 MIC Definition for Azoles, echinocandins, and 5-FC

The MIC is the lowest concentration of the antifungal agent that produces an 80% (see Section 9.3.4) or more growth inhibition (light turbidity or optically clear) as visually compared with the growth control after 48 h (or 24 h for echinocandins) of incubation. For the azoles (fluconazole, itraconazole, ketoconazole, posaconazole, ravuconazole, and voriconazole) and echinocandins, there is usually a slight turbidity at concentrations above the MIC (mostly with C. albicans and C. tropicalis). This is known as the trailing effect.

9.3.5.2 MIC Definition for Amphotericin B

The MIC is the lowest drug concentration that prevents any discernible growth (optically clear) after 48 h of incubation.

9.3.6 BROTH MACRODILUTION RESULTS

9.3.6.1 Reading

The tubes should be shaken manually before reading to homogenize the turbidity.

Compare the amount of growth in the growth-control tubes with the amount of growth in each MIC tube. The 80% turbidity can be estimated by diluting 1:5 the contents of the growth control tube (0.2 mL plus 0.8 mL of medium). For this 1:5 dilution, use the negative control tube.

9.3.6.2 Interpretation

For the interpretation of MIC values, consult the latest edition of the CLSI M27 and M27-S2 (voriconazole by the microdilution method) documents [1,7] for the appropriate breakpoints for the antifungal agent being tested (also see Table 9.1).
9.3.6.3 Reporting

Quality control values for a given antifungal agent must be within range to report susceptibility values for that agent. MIC values should be reported with their appropriate categorical interpretation: resistant (R), intermediate (I), susceptible dose dependent (S-DD), or susceptible (S).

9.3.7 Advantages

1. Since susceptibility testing is not an assay that must be performed for all yeasts, the broth macrodilution has the advantage of testing only a single isolate and agent.
2. This procedure does not require specialized equipment.
3. The macrodilution procedure may give a less subjective measurement of the growth inhibition than does the microdilution method described in Section 9.4.

9.3.8 Limitations

1. The macrodilution procedure cannot be used for Malassezia spp. isolates because some of these species require oil overlay for growth.
2. The macrodilution procedure is not an ideal method for routine testing or for testing multiple isolates because it is labor-intensive and technically demanding.
3. Even for an experienced microbiologist, it is difficult to interpret MICs by this procedure because of the trailing growth of azoles.
4. C. neoformans isolates do not grow well in RPMI and must be read after 72 h of incubation.
5. Amphotericin B resistance is not well detected by this method.
6. Breakpoints are only available for fluconazole, itraconazole, 5-FC, and voriconazole against Candida spp.

9.3.9 Conclusions

This procedure should be attempted only with the guidance of an experienced individual; if an alternative method is chosen, its results should match those of the CLSI standard.

9.4 Broth Microdilution Method

9.4.1 Introduction

Of the two procedures described by the CLSI, the broth microdilution method is the most practical one for use in the clinical laboratory or for use when testing a large number of isolates against a given set of antifungal agents. This procedure involves a microtiter tray containing standard twofold dilutions of antifungal agents to be inoculated with standardized yeast suspensions. Each microtiter tray can be prepared with several antifungal agents or with only a single agent. The latter is the procedure described in this chapter.

9.4.2 Materials

1. Antimicrobial sources (refer to Section 9.3.2)
2. Agar media (refer to Section 9.3.2)
3. Test medium, RPMI-1640 and sterile saline (refer to Section 9.3.2).
4. Supplies
   The same supplies listed under the broth macrodilution method are needed (refer to Section 9.3.2), plus the following items.
a. Microtiter trays
   • Sterile 96 U-shaped-well microtiter trays (M27-A2 document [1]) are available from a number of manufacturers, including Dynatech and Nalgene Nunc International.

b. Micropipettes (single and multichannel) and sterile tips for 100-µL volumes

5. Equipment.
a. Reading devices
   • Mirror reader or plate reader

9.4.3 Quality Control

1. Quality control strains:
   • *C. parapsilosis* ATCC 22019
   • *C. krusei* ATCC 6258

2. Frequency of quality control testing
   • See instructions under the broth macrodilution method, and, in addition, test one of these isolates with each new lot of microdilution trays prior to testing clinical specimens.

3. Evaluating quality control MIC results
   • As for the broth macrodilution method, the MIC values for a quality control organism tested must be within acceptable ranges for the antifungal agent being tested. These ranges are listed in the current CLSI M27 and M27-S documents and in Table 9.4.

9.4.4 Procedure

1. Antifungal stock preparation (see the broth macrodilution method in Section 9.3.4).
2. Preparation of water-soluble antifungal agents (Figure 9.1) [12].
a. Water-soluble antifungal agents
   1) Label 10 (16 × 150 mm) tubes 2–11
   2) Add the appropriate amounts of sterile RPMI to each tube as follows:
      • Add 1 mL of RPMI to tubes 2 and 11.
      • Add 0.5 mL of RPMI to tubes 3, 5, and 8.
      • Add 0.75 mL of RPMI to tubes 6 and 9.
      • Add 1.5 mL of RPMI to tube 4.
      • Add 1.75 mL of RPMI to tubes 7 and 10.
   3) Add 1 mL of the stock solution (1,280 µg/mL) to the first antifungal dilution tube (tube 2).
   4) Transfer from tube 2, 0.5 mL to tubes 3 and 4.
   5) Transfer from tube 4, 0.5 mL to tube 5 and 0.25 mL to tubes 6 and 7.
   6) Transfer from tube 7, 0.5 mL to tube 8 and 0.25 mL to tubes 9 and 10.
   7) Transfer from tube 10, 1 mL to tube 11.
   8) Finally, discard 1 mL from tube 11.
      • Vortex each tube before the dilution step.
      • Notice that when dilutions have been prepared, each tube contains 1 mL.

b. Preparation of microdilution trays for water-soluble antifungal agents (Figure 9.6) [12].
   1) Place tubes containing the drug dilutions (1 mL each) in an organized fashion, going from the highest to the lowest drug concentration.
   2) Prepare a 1:5 dilution by adding 4 mL of RPMI to the drug dilution tubes, and mix each tube well with a vortex mixer.
3) Using a dispensing device, transfer 0.1 mL from each of the drug dilution tubes to the appropriate wells of each microtiter tray (Figure 9.6) [12].
   • The concentration of the antifungal agent in each well is 2 times the concentration needed after this step.
   • It is not recommended to use volumes smaller than 0.1 mL because the medium can evaporate during the incubation step.
4) Add 0.2 mL of RPMI to the wells in column 1 and 0.1 mL to the wells in column 12. These wells will serve as the negative (sterility) and positive (growth control) control wells, respectively.
5) The prepared tray should be sealed in a plastic bag and placed at –70°C until needed. Prepared trays should not be stored in a self-defrosting freezer as this type of freezer will shorten the shelf life of the antifungal agent in the tray. Prepared trays should not be refrozen after being thawed.
6) The shelf life of the tray is the expiration date of the antifungal being used or 6 months, whichever one comes first.

**FIGURE 9.6** Preparation of microdilution trays for water-soluble antifungal agents.

3) Using a dispensing device, transfer 0.1 mL from each of the drug dilution tubes to the appropriate wells of each microtiter tray (Figure 9.6) [12].

4) Add 0.2 mL of RPMI to the wells in column 1 and 0.1 mL to the wells in column 12. These wells will serve as the negative (sterility) and positive (growth control) control wells, respectively.

5) The prepared tray should be sealed in a plastic bag and placed at –70°C until needed. Prepared trays should not be stored in a self-defrosting freezer as this type of freezer will shorten the shelf life of the antifungal agent in the tray. Prepared trays should not be refrozen after being thawed.

6) The shelf life of the tray is the expiration date of the antifungal being used or 6 months, whichever one comes first.

c. Preparation of water-insoluble antifungal agents (Figure 9.3) [12].

- The dilutions of water-insoluble antifungal agents (amphotericin B, itraconazole, ketoconazole, posaconazole, ravuconazole, voriconazole, and anidulafungin) must be prepared by using a solvent such as DMSO or another appropriate solvent (Table 9.5).
- It is important to prepare the stock solution and drug dilutions in the solvent (follow the scheme of dilution and instructions below) to avoid dilution artifacts that can
result from the precipitation of a compound of low solubility (e.g., itraconazole) in aqueous media, thereby producing erroneous drug dilutions.

1) Label 9 (12 × 75 mm) tubes 3–11.
2) Add the appropriate amounts of DMSO to each tube as follows:
   - Add 0.5 mL of DMSO to tubes 3, 6, and 9.
   - Add 0.75 mL of DMSO to tubes 4, 7, and 10.
   - Add 1.75 mL of DMSO to tubes 5, 8, and 11.
3) Label the stock solution tube (1,600 µg/mL) as tube 2.
4) Transfer from tube 2, 0.5 mL to tube 3 and 0.25 mL to tubes 4 and 5.
5) Transfer from tube 5, 0.5 mL to tube 6 and 0.25 mL to tubes 7 and 8.
6) Transfer from tube 8, 0.5 mL to tube 9 and 0.25 mL to tubes 10 and 11.
7) Finally, discard 1 mL from tube 11.
   - Vortex each tube before the dilution step.
   - Notice that when dilutions have been prepared, all tubes contain 1 mL.

d. Preparation of microdilution trays for water-insoluble antifungal agents (Figure 9.7) [12].
1) Place the tubes containing the drug dilutions (1 mL) in an organized fashion, from the highest to the lowest drug concentration. Label a row of ten tubes with the final drug concentrations.
2) Prepare a 1:50 dilution by mixing 4.9 mL of RPMI with 0.1 mL from each drug dilution tube, and mix well with a vortex mixer.

FIGURE 9.7 Preparation of microdilution trays for water-insoluble antifungal agents.
3) Using a dispensing device, transfer 0.1 mL from each of the final drug dilution tubes to the appropriate well in each microtiter tray.

4) Add 0.2 mL of RPMI plus 2% DMSO (or the solvent used) to column 1 of the microtiter tray and 0.1 mL of RPMI plus 2% DMSO (or the solvent that was used) to column 12. These two wells will serve as the negative (sterility control) and positive (growth control) control wells, respectively.
   • The concentration of the antifungal agents in the wells is now 2 times the concentration needed.
   • The concentration of DMSO in the wells is now 2%
   • It is not recommended to use volumes smaller than 0.1 mL because the medium can evaporate during the incubation step
   • The prepared trays should be sealed and stored in a plastic bag at −70°C as described above for water-soluble agents.

3. Inoculum preparation
   a. Prepare the stock inoculum suspension as described above under the broth macrodilution method (see Section 9.3.4 and Figure 9.5).
   b. Prepare the working suspension by making a 1:50 dilution, and further diluting 1:20 with the medium to obtain two times the test inoculum concentration (1–5 × 10³ CFU/mL). Mix with a vortex mixer before the dilution step.

4. Inoculation of the microtiter trays
   a. Allow the microdilution trays to thaw at room temperature under a hood. It will take approximately 2 h to thaw if they are stacked four trays high or 45 min if unstacked.
   b. Remove the sealing tape as soon as the panels are taken from the freezer and cover them with a clean lid to avoid contamination.
   c. Thawed trays should be inoculated within 1 h of thawing.
   d. Fill each row of the microtiter tray from wells 2–12 with 0.1 mL of the working inoculum suspension (well mixed with a vortex mixer) using a single or a multichannel pipette. If using another device, follow the manufacturer’s instructions as to what inoculum concentration should be added to the inoculum reservoir.
   • Eight clinical isolates or seven isolates and one of the quality control isolates can be tested in one tray.
   e. A purity control agar plate should be prepared for every isolate being tested. Colony count plates can also serve as the purity control. For colony counts, transfer 10 µL of inoculum to an agar plate (CHROMagar or SAB) from the positive control well before the incubation step and streak for isolated colonies. The CFU per plate must range from 5–30.

5. Incubation
   Microtiter trays and purity agar plates should be incubated in an aerobic environment stacked no more than four high at 35°C for 46–48 h for Candida spp. and 72 h for C. neoformans.

9.4.5 Microdilution MIC Definitions

9.4.5.1 Microdilution MICs of Azoles and 5-FC

The MIC is the lowest concentration of the antifungal agent that substantially inhibits growth (about half, or 50%, or less growth than in the growth control) of the organism as detected visually after 24 h (caspofungin and other echinocandins) or 48 h of incubation.

For azoles, echinocandins, and 5-FC, there is slight turbidity at concentrations above the MIC (trailing effect), mainly for C. albicans and C. tropicalis.

9.4.5.2 Microdilution MICs of Amphotericin B

The MIC is the lowest drug concentration that prevents any discernible growth (optically clear) after 48 h of incubation.
9.4.6 **BROTH MICRODILUTION RESULTS**

9.4.6.1 **Reading**

It is important to disperse (or homogenize) the growth in each well by pipetting, vortexing, or stirring before reading. This step is helpful for strains that have abundant trailing growth at 48 h.

1. **Visual reading**
   a. Remove the trays and colony count and/or purity plates from the incubator.
   b. Check the purity plate. If contamination is observed, purify the isolate and repeat the MIC procedure. If no contamination is apparent, read the MIC trays.
   c. Place the tray on a reading device (light box, concave mirror reader, or plate reader).
   d. The growth control well must have sufficient growth.
      • If adequate growth is not achieved, the test must be repeated. Adequate growth will depend on the species being tested. Isolates that produce small colonies on agar plates will usually produce small buttons in the microdilution trays.
   e. Determine the MIC endpoint as described in Sections 9.4.5.1 and 9.4.5.2 (Table 9.6).

2. **Spectrophotometric reading**
   • This procedure is not included in the CLSI document, but sometimes it is helpful; it has been demonstrated that it provides results that are similar to those obtained by visual reading [13].
     a. Set the spectrophotometer at 405 nm and determine the optical density (OD) of each well.
        • Similar results are obtained using 450-, 492-, 550-, or 600-nm filters.
     b. Before reading, agitate the trays to ensure a uniform turbidity. The agitation can be made by either pipetting or with a microtiter shaker.
     c. Subtract the OD of the blank (uninoculated wells, column 1 wells) from the OD of the inoculated wells.
     d. The MIC is the lowest drug concentration that produces a $\geq 50\%$ growth reduction for azoles and 5-FC and $\geq 95\%$ growth reduction for amphotericin B.
        • Caution: With some brands of microtiter plates, bubbles may appear after incubation that will preclude an accurate MIC determination by this method. Burst the bubbles with a heated insuline needle to prevent these erroneous results. Alternatively, the Greiner-Bio-One microtiter plates (reference # 650180) will also prevent this problem.

9.4.6.2 **Interpretation**

See the description for the broth macrodilution method (Table 9.1).

9.4.6.3 **Reporting**

See the description for the broth macrodilution method.

Although the CLSI recommends reading after 48 h incubation, some species have sufficient growth at 24 h. Because it has been reported that reading at 24 h correlates with *in vivo* results in animal models [14,15], it is recommended to read MICs at both 24 and 48 h to establish the incubation time that better correlates with *in vivo* results. It has been demonstrated that the 24-h results correlate well with the 48-h endpoints [14,15].

9.4.7 **ADVANTAGES**

1. The broth microdilution procedure can test a number of isolates at one time.
2. This procedure is easy to perform and requires less space in the laboratory freezer and incubator than does the broth macrodilution procedure.
3. It does not require specialized equipment.
9.4.8 LIMITATIONS

1. The CLSI M27 document recommends this method only for *Candida* spp. and *C. neoformans* [1]; it has not been validated for other yeast species.
2. *C. neoformans* isolates do not grow well in RPMI and must be read after 72 h of incubation.
3. Amphotericin B resistance is not well detected by this method.
4. Azole MICs are difficult to interpret due to trailing growth.
5. Breakpoints are only available for fluconazole, itraconazole, 5-FC, and voriconazole against *Candida* spp.

9.4.9 CONCLUSIONS

The CLSI microdilution procedure is easy to perform with minimal labor. The interpretation of MIC results can be challenging to the untrained eye. This procedure is described by the CLSI for *Candida* spp. and *C. neoformans* isolates only.

9.5 ETEST

9.5.1 INTRODUCTION

Etest (AB Biodisk, Solna, Sweden) is a nonporous plastic strip immobilized with a predefined gradient of a given antimicrobial agent on one side and printed with an MIC scale on the other
When the Etest strip is placed on an inoculated agar plate, a continuous, stable, and exponential antimicrobial gradient is established along the side of the strip. After incubation, the MIC value (µg/mL) can be read directly from the MIC scale printed on the Etest strip. If the resulting MIC is equivalent to the CLSI reference values, the CLSI criteria for yeasts are directly applicable for interpretation of Etest results [1]. This technology is similar to a disk diffusion assay but with a longer stability time for the antimicrobial gradient.

### 9.5.2 MATERIALS

1. Etest strips can be purchased directly from AB Biodisk or through Remel. Strips (approximate cost ≥$2 per strip) are sold in packages of 100 strips per antifungal agent and are stored at −20°C or −70°C (see Table 9.7 for Etest drug dilution ranges). Once a set of strips is removed from the manufacturer’s package, the unused strips should be stored in an airtight container with a desiccant and returned to −20°C. Etest strips should not be used after the expiration date indicated by the manufacturer.

2. Plates consisting of RPMI-1640 supplemented with 2% glucose and 1.5% agar, prepared in-house or obtained commercially (Remel or others), that have an agar depth of 4 mm ± 0.5 mm can be used. Plates should be stored at 2°C–8°C. Modified casitone agar or antibiotic medium 3 (AM3) agar can also be used [16] as recommended by the manufacturer in the Etest technical guide No. 4.

3. Saline solution for making yeast suspensions

4. Swabs (sterile, nontoxic)

5. Test tubes (for inoculum preparation)

6. Pipettes

7. Scissors

8. Forceps or Etest applicator kit

9. A 0.5 McFarland standard (this can be made following CLSI instructions or purchased from microbiology product manufacturers such as PML, Hardy, or Remel).

10. Airtight storage containers with desiccant (silica gel) for extra Etest strips once the manufacturer’s packaging has been opened. These storage containers are available from AB Biodisk and Remel.

11. Freezer (−20°C or −70°C) for storage.

12. Vortex mixer

13. 35°C ambient air incubator

14. Etest technical information
   - E-test package insert
   - E-test technical guide No. 4

### TABLE 9.7

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Code</th>
<th>Concentrations (µg/mL)</th>
</tr>
</thead>
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<td>Amphotericin B</td>
<td>AP</td>
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</tr>
<tr>
<td>Fluconazole</td>
<td>FL</td>
<td>0.016–256</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>IT</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>FC</td>
<td>0.002–32</td>
</tr>
<tr>
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<td>KE</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Voriconazole</td>
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</tr>
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<td>Caspofungin</td>
<td>CAS</td>
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</tr>
<tr>
<td>Posaconazole</td>
<td>POS</td>
<td>0.002–32</td>
</tr>
</tbody>
</table>
9.5.3 Quality Control

1. Quality control strains:
   - *C. parapsilosis* ATCC 22019.
   - *C. krusei* ATCC 6258.
   - All quality control strains should be maintained following procedures described by CLSI [1].

2. Frequency of quality control testing.
   - A quality control organism should be run for every new lot of Etest strips prior to use.
   - Each antifungal agent should have quality control testing within the last 7 days of use, following the CLSI procedure for testing during 30 consecutive days [1]. Otherwise, quality control should be conducted on each occasion that testing is performed.

3. Evaluating results.
   - The E-test antifungal application sheet lists the quality control ranges for antifungal agents. These values are not included in the CLSI M27-A2 document.

9.5.4 Procedure

1. Obtain a pure culture of the isolate to be tested on a nonselective medium as described for the CLSI methods.
   - The inoculum should be prepared from 24 h (*Candida* spp.) and 48–72 h (*C. neoformans*) cultures as described for the CLSI methods.
   - Frozen or lyophilized isolates should be subcultured at least twice prior to susceptibility testing.

2. Remove the strips from the original package or storage container from the freezer 30 (–20°C) or 60 min (–70°C) before use to allow them to equilibrate at room temperature.

3. Emulsify five or more isolated colonies in saline by using a sterile swab.

4. Vortex for 15 s

5. Adjust the suspension turbidity to a 0.5 McFarland standard for *Candida* spp. and to a 1 McFarland standard for *C. neoformans*. Add more colonies to increase the turbidity or more saline to decrease the turbidity (photometric devices can also be used to measure the turbidity).

6. The inoculum suspension should be inoculated onto the agar plate within 15 min after preparation. Dispense 150 µL to the center of a 90-mm plate or 400 µL to a 150-mm plate. Swab carefully to obtain an even growth over the entire surface of the agar in three different directions.
   - Make sure that the inoculum is spread evenly over the entire surface of the plate. A correctly inoculated plate will have an even lawn of confluent growth.
   - Plates can be inoculated automatically with the AB-Biodisk spiral autoinoculator CR10.

7. Allow the inoculum to be absorbed completely into the agar (at least 15–20 min). The plate must be completely dry before the application of the Etest strip. If moisture is present on the agar surface when the Etest strip is applied, it will affect the performance of the antifungal gradient.

8. The strip is designed to have the MIC scale facing away from the agar surface and the antifungal gradient touching the agar surface (you should be able to see the writing on the strip after applying it to the agar plate). Apply the “E” labeled area of the strip (highest concentration of antifungal) at the edge of the Petri dish to the agar surface and gently place the end of the strip with the lowest toward the center of the plate. Once the strip
has touched the agar surface do not move the strip. If any large bubbles appear under the strip, gently remove the bubbles by pressing on the Etest strip with a forceps or a swab. Small bubbles under the strip will not affect the performance of the Etest strip.

- One to two strips can be placed on a 90-mm plate with opposite orientation to each other (you do not want the “E” end of the strip on the same side of the plate for two strips, i.e., the gradients will overlap).
- Up to six strips can be placed on a 150-mm agar plate in a spoke wheel pattern. (Templates are available from AB Biodisk to optimize the positioning of the strips.)
- When using the Etest applicator, refer to the instructions in the product insert.
- Do not remove the Etest strip during any part of the process.

9. Incubate the plate in the inverted position (agar side up) at 35°C in an aerobic environment as soon as possible after the placement of Etest strips.

10. Incubate the plate until growth is clearly seen (24–48 h for the Candida spp. and 48–72 h for C. neoformans) with the exception of amphotericin B, which requires at least 48 h for Candida spp. and 48 to 72 h for C. neoformans.

### 9.5.5 ETEST RESULTS

#### 9.5.5.1 Reading

1. Evaluate the growth of the isolate before reading. If the lawn of growth appears as a thin haze or if the density of colonies is sparse, do not attempt to read the MIC, reincubate the plate and/or repeat the test validating the inoculum size.

2. Read the MIC where the edge of the inhibition ellipse intersects the MIC scale on the Etest strip (use of the technical guide No. 4 for endpoint selection is highly recommended).
   - Fungistatic antifungal agents, such as azoles, tend to form diffuse edges. These should be read at the so-called 80% inhibition region, for example, the first point of significant inhibition as judged by the naked eye, ignoring the haze and microcolonies. However, if large colonies are detected inside the ellipse, the MIC should be read where these large colonies are inhibited.
   - Fungicidal agents such as amphotericin B tend to form sharp edges. With these agents, hazes and any isolated colonies inside the ellipse are indicative of growth, and the MIC should be read where these are completely inhibited.
   - If a thin line of growth occurs along the side of the Etest strip within the ellipse, it should not be used for interpretation because the MIC is where the ellipse of growth intersects with the Etest strip.
   - If there is complete growth and no zone appears on the plate around a strip, the MIC value is recorded as greater than the highest antifungal concentration of the strip.
   - If the inhibition ellipse does not fall above the lowest value on the strip, the MIC value is recorded as less than the lowest value on the strip.

#### 9.5.5.2 Interpreting

1. Etest MICs are based on a continuous gradient; therefore, MIC values between the standard two-fold dilution schema can be obtained. These values can be reported as such, if desired, or rounded up to the next appropriate twofold dilution value. For example, 0.19 µg/mL becomes 0.25 µg/mL.

2. To interpret Etest MIC results as susceptible, intermediate, susceptible-dose dependent, or resistant categories, always round up the in-between values to the next higher twofold dilution.
   - For example, the CLSI fluconazole breakpoint for resistant is ≥64 µg/mL. An Etest value of 48 µg/mL is rounded up to 64 µg/mL, and the category is reported as resistant.
3. Etest MIC values are directly proportional to the CLSI reference dilution values. Therefore, CLSI breakpoints are directly applicable to the Etest MIC values. Consult the most current M27 and M27-S documents for the appropriate breakpoints for antifungal agents (or see Table 9.1).

9.5.5.3 Additional Considerations

Amphotericin B should not be read before 48 h.

9.5.6 ADVANTAGES

1. Etest is easy to perform and requires minimal training for test performance, however, end points can be difficult to determine for the azoles against Candida spp.
2. Contamination can be easily recognized.
3. Etest can be easily set up for a small number of clinical isolates.
4. There is minimum labor involved with Etest as compared with broth dilution methods.
5. It is an adequate method to detect potentially resistant strains to amphotericin B [17].

9.5.7 LIMITATIONS

1. Cost is the most significant limitation. Careful consideration should be given to which antifungal agents to test because the Etest methodology can be cost effective for fewer than five strips per isolate.
2. Agreement between Etest and M27 results is species and antifungal agent dependent. The lowest correlation is for the combinations: C. glabrata and fluconazole; C. tropicalis, fluconazole, and itraconazole; and C. neoformans and amphotericin B [18].
3. Etest is not suitable for C. neoformans.

9.6 SENSITITRE YEASTONE COLORIMETRIC METHOD

9.6.1 INTRODUCTION

The Sensititre YeastOne colorimetric antifungal panel (TREK International, Westlake, OH) consists of a disposable tray that contains individual wells dried in serial twofold dilutions of up to six established antifungal agents in RPMI-1640 medium supplemented with 1.5% dextrose. The wells also contain the color indicator alamar blue. In the United States, antifungal agents included in the FDA-approved panel are fluconazole, itraconazole, and 5-FC. Panels for other antifungal agents are also available (amphotericin B, ketoconazole, voriconazole, and caspofungin). In Europe, antifungal agents included are amphotericin B, fluconazole, itraconazole, 5-FC, voriconazole, and caspofungin. A high degree of intra- and interlaboratory reproducibility of MIC endpoints has been demonstrated with this method [19]. Reading of the azoles and 5-FC is performed at 24 h and for amphotericin B at 48 h. The agreement with the CLSI M27 method for azoles and 5-FC ranged between 92–100% and for yeast species other than C. albicans from 87–90%. For amphotericin B, the agreement ranged from 92–99%. The exception is C. neoformans with a 76% agreement [19].

9.6.2 MATERIALS

1. Yeast susceptibility test panels.
2. Sensititre yeast-susceptibility inoculum broth.
3. Autoclaved demineralized water (5 mL volumes).
4. Panel layout template.
6. Plate seals.
7. Plate view-box light or mirror type.
8. A 0.5 McFarland turbidity standard.
9. SAB plates.
10. Sterile wooden applicator sticks or a loop.
11. Non-CO₂ incubator set at 35°C.
12. Vortex mixer.
13. 20-µL pipette.
14. Multichannel pipette for 100-µL volumes.
15. Disposable pipette tips.

9.6.3 Quality Control

1. Quality control strains:
   - C. parapsilosis ATCC 22019.
   - C. krusei ATCC 6258.
   - Quality control strains should be preserved following procedures described for the CLSI methods [1].
2. Frequency of quality control testing.
   - Quality control should be run in parallel with every run. The ranges of the quality control MIC results are the same as those listed in the M27-S2 [7] (or see Table 9.4). The Sensititre YeastOne package insert also provides the acceptable ranges for the antifungal agent being tested.

9.6.4 Procedure

1. Remove the panels to be used from storage. Panels should not be used if the desiccant is not present or if the integrity of the packing is compromised.
2. The inoculum should be prepared from 24 h (Candida spp.) or 48–72 h (C. neoformans) cultures as described for the CLSI method. Prepare a working suspension (1.1–8 × 10³ cells/mL) by adding 20 µL of the stock yeast suspension to 11 mL of YeastOne inoculum broth.
3. Panels can be inoculated manually using a pipetting device or automatically with the Sensititre autoinoculator. Fill each well of the Sensititre panel with 100 µL of the working suspension. The excess of the working suspension can be used to inoculate SAB or CHROMagar plates to check purity and ensure inoculum density.
4. Cover the inoculated plates with the plate seals.
5. Place the plates in a stack of no more than three panels high.
6. Incubate panels for 24–48 h at 35°C in a non-CO₂ incubator and read either after 24 h (Candida spp. andazole agents) or 48 h (Candida spp. and amphotericin B). C. neoformans requires 48–72 h of incubation.

9.6.5 Sensititre YeastOne MIC Results

1. The MIC of azoles and 5-FC is the lowest concentration of antifungal solution changing from red to purple (prominent growth inhibition) or blue (no growth) after 24–72 h of incubation.
2. The MIC of amphotericin B is the lowest concentration of antifungal solution changing from red (growth) to blue (no growth) after 48 h of incubation.
3. Reading
   - Panels may be read visually by using a view box and in normal laboratory lighting. If the growth control well is red, the endpoints for antifungal agents can be interpreted. If after incubation the well is only weakly purple, reincubate for several additional hours until it turns red. Do not read the turbidity in Sensititre YeastOne panels.
4. Interpreting and reporting Sensititre YeastOne MIC results
   As the agreement with the M27-A2 broth microdilution method is >90% [19], CLSI MIC interpretations can be applied (Table 9.1).

9.6.6 ADVANTAGES
   1. Sensititre YeastOne is easy to perform and requires minimal training for test performance.
   2. Sensititre YeastOne can be easily set up for a small number of clinical isolates.
   3. Interpretation of the MIC is more objective.
   4. Sensititre YeastOne panels are stored at room temperature.

9.6.7 LIMITATIONS
   1. This method is suitable for *Candida* spp. and other rapid growing yeast species; it is not suitable for *C. neoformans* [19].
   2. It does not discriminate well between amphotericin B-resistant and -susceptible strains.
   3. Breakpoints are only available for fluconazole, itraconazole, 5-FC, and voriconazole.

9.6.8 CONCLUSIONS
   The Sensititre YeastOne procedure is easy to perform with minimal labor. The interpretation of MIC results is more objective.

9.7 DISK DIFFUSION METHOD (CLSI M44-A DOCUMENT)

9.7.1 INTRODUCTION
   To make antifungal susceptibility testing more readily available to clinical microbiology laboratories, the CLSI has proposed a standardized disk diffusion method for susceptibility testing of *Candida* spp. to the triazoles, fluconazole, voriconazole, and posaconazole [20]. The subcommittee has only established zone interpretative criteria (break points) for fluconazole and voriconazole (Table 9.8) and quality control parameters for fluconazole, voriconazole, and posaconazole (Table 9.9) [20,21, CLSI annual meeting minutes, 2006]. Therefore, the clinical relevance for other than fluconazole and voriconazole drug–organism combinations is uncertain. The design of the disk diffusion method for yeasts is similar to that for bacteria using the same medium (Mueller–Hinton agar) supplemented with glucose and methylene blue dye (Table 9.8, Table 9.9, and Table 9.10).

9.7.2 MATERIALS
   1. Autoclave
   2. Water bath (48–50°C)
   3. Refrigerator
   4. Non–frost-free freezer (−14°C or below)
   5. Incubator set at 35°C ± 1°C with ambient air
   6. Metric vernier calliper
   7. Vortex mixer
   8. Swabs (sterile, nontoxic)
   9. Test tubes (for inoculum preparation)
   10. Plastic or glass petri dishes (150-mm or 90–100-mm diameter)
   11. Saline solution
   12. Pipettes
   13. Forceps or disk-dispensing apparatus
   14. A McFarland 0.5 turbidity standard. (Can be made following CLSI recommendations or purchased from a product manufacturer such as, PML, Hardy, or Remel.)
TABLE 9.8
Inhibition Zone Diameter Interpretative Standards and Equivalent MIC Break Points for Candida spp.

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Disk content</th>
<th>Zone Diameter (Nearest Whole mm)</th>
<th>Equivalent MIC Break Points (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R¹</td>
<td>S-DD</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>25 µg</td>
<td>≤14</td>
<td>15–18</td>
</tr>
<tr>
<td>Voriconazoleb</td>
<td>1 µg</td>
<td>≤13</td>
<td>14–16</td>
</tr>
</tbody>
</table>

¹ S = susceptible; S-DD = susceptible-dose dependent; R = resistant.
² Listed in the CLSI M27-S2 and M44-51 documents [7,21].

TABLE 9.9
Recommended Quality Control Inhibition Zone Diameter (mm) Ranges

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Disk Content</th>
<th>C. krusei ATCC 6258</th>
<th>C. parapsilosis ATCC 22019</th>
<th>C. albicans ATCC 90028</th>
<th>C. tropicalis ATCC 750</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>25 µg</td>
<td>—</td>
<td>22–33</td>
<td>28–39</td>
<td>26–37</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>1 µg</td>
<td>16–25</td>
<td>28–37</td>
<td>31–42</td>
<td>—</td>
</tr>
<tr>
<td>Posaconazoleb</td>
<td>5 µg</td>
<td>23–31</td>
<td>25–36</td>
<td>24–34</td>
<td>23–33</td>
</tr>
</tbody>
</table>

¹ Quality control ranges have not been established for these strain and antifungal combinations because of their extensive interlaboratory variability during initial quality control studies.
² CLSI annual meeting minutes, January 2006.

TABLE 9.10
Summary of M44-4 Document

| Test Medium | Mueller–Hinton agar + 2% glucose +0.5 µg/mL methylene blue |
| pH          | 7.2–7.4                                                   |
| Inoculum size | 0.5 McFarland standard and swab plates                  |
| Duration and temperature | 35°C for 20 to 24 h                                       |
| of incubation | Some strains of C. glabrata and C. krusei often require 48 h. |
| Disk content | Fluconazole 25 µg                                       |
|               | Voriconazole 1 µg                                        |
|               | Posaconazole 5 µg                                        |
| Measurement of zone inhibition | Measure to the nearest whole millimeter at which there is prominent reduction in growth |
| Breakpoints | Fluconazole: R ≤ 14 mm; S-DD 15–18 mm; S ≥ 19 mm |
|               | Voriconazole: R ≤ 13 mm; S-DD 14–16 mm; S ≥ 17 mm |
| Quality control strains (zone inhibition) | C. parapsilosis ATCC 22019 (fluconazole: 22–33 mm; voriconazole: 28–37 mm; posaconazole: 25–36 mm) |
|               | C. krusei ATCC 6258 (voriconazole: 16–25 mm; posaconazole: 23–31 mm) |
|               | C. albicans ATCC 90028 (fluconazole: 28–39 mm; voriconazole: 31–42 mm; posaconazole: 24–34 mm) |
|               | C. tropicalis ATCC 750 (fluconazole: 26–37 mm; posaconazole: 23–33 mm) |

CLSI annual meeting minutes, 2006 and references 20, 21.
9.7.3 **Antifungal Disks**

1. **Source and disk content**
   - Voriconazole 1 µg (Pfizer Inc.).
   - Posaconazole 5 µg (Schering-Plough Corporation, Becton Dickinson, BBL).

2. **Storage of antifungal disks**
   Cartridges containing commercially prepared paper disks for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks should be stored as follows:
   a. Refrigerate the containers at 8°C or below, or freeze at –14°C or below in a non–frost-free freezer until needed. The disk may retain greater stability if stored frozen until the day of use.
   b. Remove the unopened disk containers from the refrigerator or freezer 1–2 h before use to equilibrate them to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts the cold disk.
   c. Once a cartridge of disks has been removed from its sealed packing, it should be placed in a tightly sealed, desiccated container.
   d. Disks should be discarded on the expiration date, or if the diameter range of the quality control strains is not within the established range, or if the problem cannot be attributed to another faulty methodology.

9.7.4 **Test Medium**

1. Mueller-Hinton agar plus 2% glucose and 0.5 µg/mL methylene blue dye (pH 7.2–7.4). The addition of glucose provides suitable growth for most commonly encountered yeast pathogens. The addition of methylene blue dye enhances the zone edge definition. This medium is considered the best medium for routine susceptibility testing of yeasts for the following reasons:
   - There is not much variation.
   - It shows acceptable batch-to-batch reproducibility.
   - It is readily available.
   - The base medium can easily be supplemented either pre- or postproduction to contain the final concentration of 2% glucose and 0.5 µg/mL methylene blue dye.
   Check the suitability of any new batch of Mueller-Hinton medium by following CLSI document M6 recommendations or by the expected inhibition zones for quality control isolates because some batches will not support adequate growth of some organisms. Therefore, zones obtained by a disk diffusion test will be usually larger than expected and may exceed the acceptable quality control limits.

2. **Procedure to prepare the medium.**
   The medium can be prepared and poured as the complete medium with supplements (glucose and methylene blue) or the supplements can be added to commercially prepared Mueller-Hinton agar plates. The latter technique enables the use of routine Mueller-Hinton agar plates from the bacteriology laboratory.
   a. Glucose stock solution (0.4 g/mL):
      - 40 g glucose and 100 mL distilled water.
      - Heat gently and mix to dissolve.
   b. Methylene blue dye (Merck) solution (5 µg/mL):
      - 0.1 g methylene blue and 20 mL distilled water.
      - Warm gently until the dye is dissolved, but do not overheat.
c. Glucose-methylene blue (GMB) stock solution:
   • Add 200 µL of methylene blue dye solution to 100 mL of the glucose stock solution.
   • Dispense GMB stock solution into bottles or individual-use small vials containing 3.5 mL aliquots for 150-mm plates or 1.5 mL aliquots for 90–100-mm plates.
   • Autoclave for 25 min at 121°C followed by slow exhaust.
   • Store at room temperature (maximum a year) and handle aseptically.

d. Preparation of supplemented Mueller-Hinton agar:
   Prepare Mueller-Hinton agar from a commercially available dehydrated Mueller-Hinton agar base following the manufacturer’s instructions.
   1) Add 100 µL of the methylene blue dye per liter of Mueller-Hinton agar.
   2) Add 20 g of glucose per liter of Mueller-Hinton agar.
   3) Autoclave as directed by the manufacturer’s instructions.
   4) After autoclaving, allow it to cool in a 45–50°C water bath.
   5) Pour the medium into glass or plastic petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm; the corresponding volumes are 67–70 mL of medium for 150-mm diameter plates and 28–30 mL for 100-mm diameter plates.
   6) Allow the medium to cool at room temperature, and store it at refrigerator temperature (2–8°C). Although the plates can be used the same day of preparation, it is better to use them the following day to obtain sharper edges of growth.
   7) A representative sample of each batch of plates (5%) should be examined for sterility by incubating at 30–35°C for 24 h.
   8) Plates should be used within seven days after preparation, unless adequate precautions have been taken to minimize drying of the agar.

e. Supplementation of commercially prepared Mueller-Hinton agar with glucose and methylene blue:
   Commercially prepared Mueller-Hinton agar is identical to that utilized by the bacteriology laboratory for performing Kirby-Bauer disk diffusion tests for bacteria. These plates can be obtained from a variety of manufacturers.
   a) Pour 3.5 mL of GMB supplement onto the surface of a 150-mm plate with a 70-mL fill or 1.5 mL onto a 90–100-mm plate with a 30-mL fill.
   b) Spread the supplement evenly on the agar with a sterile spreader or sterile bent glass rod.
   c) Allow the GMB solution to absorb completely at room temperature or in the refrigerator before inoculating the plate. The time required for the agar to absorb the solution depends on the humidity of the plate (16–18 h). It is advisable to prepare supplemented plates the day before performing the test to allow absorption of the solution overnight in the refrigerator.

9.7.5 Quality Control

A quality control culture can be used to monitor the precision (reproducibility) and accuracy of the disk diffusion test. An unexplained result suggests a change in the organism’s inherent susceptibility, and a fresh culture of the control strain should be obtained.

1. Quality control strains:
   • *C. parapsilosis* ATCC 22019
   • *C. krusei* ATCC 6258
   • *C. albicans* ATCC 90028
   • *C. tropicalis* ATCC 750
   • All quality control strains should be maintained following procedures described by CLSI [1,20].
2. Frequency of quality control testing:
   • A quality control organism should be run for each new lot of medium and prior to using each lot of antifungal disks.
   • At least one of the above isolates should be tested with every run, and testing should be performed using the same procedure used for the clinical isolates. If satisfactory performance is documented, quality control testing may be conducted weekly.

3. Evaluating quality control disk results:
   The inhibition zone values for the quality control organisms must be within the acceptable ranges for the antifungal agent being tested for the results to be considered valid. These ranges are listed in the current CLSI M44-A [20,21, CLSI annual meeting minutes, 2006] document and in Table 9.9.

9.7.6 Procedure

1. Obtain a pure culture of the isolate to be tested on a nonselective medium as described for CLSI microdilution methods.
2. Remove the disks from the original package or storage container from the refrigerator or the freezer (–14°C or below) 1–2 h before use and allow them to equilibrate at room temperature.
3. If a disk-dispensing apparatus is used, the apparatus should be warmed at room temperature before opening. When not in use, the dispensing apparatus (containing the disks) should always be refrigerated, fitted with a tight cover, and stored with an adequate desiccant.
4. Remove the supplemented Mueller-Hinton agar plates from the refrigerator and equilibrate them at room temperature. If excess surface moisture is present, place the plates in an incubator (35°C) or a laminar flow hood at room temperature (with the lids ajar) until the excess surface moisture has evaporated (10–30 min). The agar surface should be moist, but the petri dish covers and agar surface should be free of droplets of moisture when the plates are inoculated.
5. Inoculum preparation — direct colony suspension method.
   a. Lightly touch five colonies (at least 1-mm diameter) using a loop or swab.
   b. Suspend the yeast cells in 5 mL of sterile 0.85% NaCl, and mix the cell suspension using a vortex mixer for 15 s.
   c. Adjust the turbidity of the inoculum to the density of a 0.5 of McFarland standard with a spectrophotometer at 530 nm wavelength. This procedure yields a yeast stock suspension of 1–5 \times 10^6 cells per mL and should produce semiconfluent growth with most Candida spp. isolates.
      • The preparation of the inocula for one run should be completed within 15 min.
      • Never use undiluted overnight broth culture or other unstandardized inocula.
6. Inoculation and incubation of test plates
   a. Dip a sterile cotton swab into the well mixed with a vortex mixer-adjusted suspension, and rotate several times and press firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
   b. Streak the swab carefully over the entire sterile agar surface to obtain an even growth in three different directions. Finally, swab the rim of the agar.
      • A correctly inoculated plate will have an even growth.
      • Plates can also be inoculated with a rotary plate inoculator.
   c. Leave the lids ajar for 3–5 min, but no more than 15 min, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.
7. Application of disks to inoculated agar plates and incubation.
   a. Dispense the predetermined battery of disks onto the surface of the inoculated agar plate, and press down each disk to ensure its complete contact with the agar surface.
   b. Disks must be distributed no closer than 24 mm from center to center.
      • No more than 5 disks should be placed on a 100-mm plate.
      • Up to 12 disks can be placed on a 150-mm plate.
   c. Once the disk has touched the agar surface, do not move the disk.
   d. Place the plates in the inverted position in an incubator set at 35°C in an aerobic environment within 15 min of the placement of the disks.
   e. Incubate the plates for 18–24 h.
8. Reading and interpretation of results
   a. Examine the plates after 20–24 h of incubation. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition around the disk will be uniformly circular, and there will be a semiconfluent lawn of growth.
   b. Hold the plate a few inches above a black, nonreflecting background that is illuminated with reflecting light.
   c. Measure the zone diameter to the nearest whole millimeter at the point at which there is a prominent reduction in growth.
      • Reading is subjective, and experience results in greater accuracy.
      • The presence of pinpoint microcolonies at the zone edge or large colonies within an inhibition zone should be ignored.
   d. If insufficient growth occurs after 24-h incubation, reincubate the plates and read them at 48 h.

9.7.7 DISK DIFFUSION RESULTS

Disk diffusion inhibition zone diameters correlate inversely with MIC results by standard dilution tests. Table 9.8 lists the MIC breakpoint correlates that correspond to the inhibition zone diameter interpretative criteria. These MIC correlates were based on inhibition zones diameters versus MIC comparisons and are generally identical to MIC interpretative criteria defined in the CLSI documents M27 and M27-S [1,7].

9.7.8 ADVANTAGES

1. The disk diffusion test is easy to perform and requires minimal training for test performance; however, the endpoints can be difficult to determine.
2. Contamination can be easily recognized.
3. The disk diffusion test can be easily set up for a small number of clinical isolates.
4. There is minimum labor involved as compared with the standard broth dilution methods.
5. It is cost-effective for routine testing.
6. The disk diffusion test usually provides qualitative results 24 h sooner than the standard broth dilution methods.
7. It is a good method to use as a routine screening procedure to test fluconazole against Candida spp.

9.7.9 LIMITATIONS

1. The method has been standardized for Candida species and two antifungal agents only.
2. The inhibition zone measurement is subjective.
3. Zone interpretative criteria (breakpoints) are available only for fluconazole and voriconazole.
4. Quality control parameters are available only for fluconazole, voriconazole, and posaconazole.
5. *C. krusei* and *C. glabrata* and sometimes *C. parapsilosis* strains may require 48 h of incubation [22].

6. Comparisons of this method with the approved M27 methods are scarce for agents other than fluconazole and voriconazole [22].

### 9.7.10 CONCLUSIONS

The disk diffusion method is an easy method for routine testing of fluconazole, voriconazole, and posaconazole in microbiology laboratories because it requires the same technology and medium used for bacteria testing. The medium only requires a supplementation with glucose and methylene blue dye; both reagents are easily available. Reports correlating disk diffusion results with M27-A methods have concluded that the disk diffusion methodology does not adequately separate fluconazole-resistant from -susceptible dose-dependent isolates [22–27]. Therefore, MIC determination may be needed for those strains that have intermediate or resistant zones by the diffusion method.

### 9.8 GENERAL CONCLUSIONS

Currently, the gold standard for susceptibility testing of yeasts to antifungal agents is the CLSI broth microdilution methodology [1], but the simpler CLSI disk methodology is also available for three triazoles, fluconazole, posaconazole, and voriconazole. Research or large clinical laboratories that can “batch” isolates for large-scale research or surveillance studies use CLSI procedures (disk and broth dilution) [27]. The CLSI broth dilution methodology is labor intensive and does not lend itself to testing small numbers of clinical isolates. However, of the two procedures approved by the CLSI, the broth microbroth dilution is better suited and less labor intensive than the broth macrodilution method for testing in the routine clinical laboratory. Broth microdilution trays can be prepared in bulk and stored frozen. If a commercial method is proven to be equivalent to the CLSI broth dilution procedure, that method can be used in the clinical laboratory. The colorimetric method has similar advantages to the broth microdilution procedure. The most suitable method for the discrimination between susceptible and resistant strains to amphotericin B is the Etest [17]. However, endpoints are difficult to determine. As antifungal resistance continues to increase, the need for the clinical laboratory to routinely perform yeast susceptibility testing is becoming more important. Other methods have also been evaluated [28]. For a summary of CLSI broth dilution methods, see Tables 9.9 and 9.10 for disk diffusion method (CLSI M44-A).

### REFERENCES


**APPENDIX — MANUFACTURERS**

**AB Biodisk North America Inc.**
200 Centennial Avenue
Piscataway, NJ 08854-3910
www.abbiodisk.com
Telephone: 732-457-0408
Fax: 732-457-8980
Product inquires and orders: 800-874-8814

**Dynex Technologies, Inc.**
14340 Sullyfield Circle
Chantilly, VA 20151
www.dynextechnologies.com
Telephone: 703-631-7800
800-336-4543
Fax: 703-631-7816

**EM Science**
480 S. Democrat Road
Gibbstown, NJ 08027-1297
www.emscience.com
Telephone: 800-222-0342

**Evergreen Scientific**
2300 E. 49th St.
Los Angeles, CA 90058
Telephone: 213-583-1331

**Angus Biochemicals Inc.**
2236 Liberty Dr.
Niagara Falls, NY 14304-3796
Telephone: 716-283-1434
Fax: 716-283-1770

**Fisher Scientific Co.**
711 Forbes Ave.
Pittsburgh, PA 15219
www.fishersci.com
Telephone: 412-490-8300

**BD Biosciences**
P.O. Box 243
Cockeysville, MD 21030
www.bd.com
Telephone: 410-771-0100
800-638-8663

**Forma Scientific, Inc.**
P.O. Box 649
Marietta, OH 45750
www.forma.com
Telephone: 614-374-1851

**CMI-Promex (Steers Replicator)**
7 Benjamin Green Road
P.O. Box 418
Pedricktown, NJ 08067
Telephone: 609-351-1000

**Gelman Science**
600 South Wanger Rd.
Ann Arbor, MI 48103
Telephone: 313-665-0651

**Coy Laboratory Products, Inc.**
14500 Coy Drive
Grass Lake, MI 49137
www.coylab.com
Telephone: 313-475-2200

**Hardy Diagnostics, Inc.**
1430 West McCoy Lane
Santa Maria, CA 93455
www.hardydiagnostics.com
Telephone: 805-346-2766
800-266-2222
Fax: 805-346-2760
# Antifungal Susceptibility Testing of Filamentous Fungi

**Ana Espinel-Ingroff and Emilia Cantón**

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10.1 CLSI PROCEDURES FOR FILAMENTOUS FUNGI

10.1.1 INTRODUCTION

The laboratory’s role in guiding the selection of antifungal therapy has gained greater attention because of the increased incidence of fungal infections, the development of new antifungal agents, and the description of resistance to itraconazole and amphotericin B among Aspergillus spp. and other important emerging molds. The Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antifungal Susceptibility Tests has developed a reproducible reference testing methodology (M38-A document) for antifungal susceptibility testing of filamentous fungi (molds) [1]. CLSI procedures for molds and comparable commercial methods are described in this chapter; the end point is the minimum inhibitory concentration or the MIC.

10.2 RELEVANCE

Mold susceptibility testing should be performed to determine the susceptibility of common molds that cause invasive infections (Aspergillus spp., Fusarium spp., Rhizopus arrhizus, and other zygomycetes, Pseudallescheria boydii [Scedosporium apiospermum], and the mycelial form of Sporothrix schenckii) and other opportunistic pathogenic molds to itraconazole, voriconazole, posaconazole, ravuconazole, and amphotericin B as well as the new echinocandins, caspofungin, micafungin, and anidulafungin. Determination of the antifungal susceptibility endpoints for the echinocandins is not described in the CLSI document, but it is described later in this chapter. CLSI methodology has not been evaluated for the yeast forms of dimorphic fungi, such as Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum variety capsulatum, Penicillium marneffei, or Sporothrix schenckii. Susceptibility testing could also be performed to determine patterns of susceptibility by conducting periodic batch surveys to establish antibiograms within an institution when the pathogen is recovered from an invasive infection due to a mold, when the utility of the antifungal agent is uncertain, when monitoring resistance development during therapy, and when testing new pathogens and new drugs. Because interpretative breakpoints are not available for any species of filamentous fungi versus any antifungal agent, the clinical relevance of susceptibility testing for molds remains uncertain. For the development of the M38-A document, the Subcommittee on Antifungal Susceptibility Tests conducted multicenter studies [2–5] to evaluate the best medium, inoculum size, temperature, duration of incubation, and end-point definition; quality control MIC limits for a mold isolate have also been established. The test medium recommended is RPMI-1640 broth (RPMI) with [3-(N-morpholino)]-propanesulfonic acid (MOPS) buffer. Since the publication of the proposed CLSI document (M38-A) for molds, alternative methods have been developed (Etest, colorimetric, etc.); however, these methods are not described in the CLSI M38-A document. The CLSI group is currently evaluating a disk diffusion assay for mold testing; reference testing conditions also have been identified for testing dermatophytes (briefly described in Section 10.7).
The CLSI broth macro- and microdilution methods for susceptibility testing of molds involve preparing a series of drug dilutions, each containing a specific concentration of an antifungal agent. If a more practical method should yield results similar to those obtained using CLSI methods, it is considered an acceptable method for use in the clinical laboratory. Validation of an alternative method is the responsibility of the laboratory.

- Caution: Because the risk of producing aerosols is present, all steps in testing molds must be performed in a class IIA or IIB biological safety cabinet.

10.3 BROTH MICRODILUTION METHOD

10.3.1 INTRODUCTION

The design of the CLSI broth microdilution method for susceptibility testing of molds is similar to that for yeasts. It is an easier and more economical method than the broth macrodilution method. The purity and viability of the organism should be validated before any testing is performed.

1. Technical notes.
   - It is important to completely plan out the entire procedure since it involves many steps over several days. It is also important to understand all the steps of the procedure before beginning. The technologist is advised to read and understand the CLSI M38-A document before proceeding.
   - Determine the number of isolates to be tested, the number of antifungal agents, and the range of concentrations for each antifungal (Table 10.5).

10.3.2 MATERIALS

1. Antimicrobial sources:
   a. United States Pharmacopeia (USP)
   b. Sigma Chemical Company
   c. The antifungal agent manufacturer
      - Some antifungal agents can be obtained from the drug manufacturer at no charge through the pharmaceutical company’s customer service department or equivalent department or from your local pharmaceutical representatives.

2. Agar media:
   a. Sabouraud dextrose agar (SAB) plates
   b. Potato dextrose agar slant tubes
      - SAB plates should be used to determine the viable number of CFU per milliliter (inoculum density validation) and potato dextrose agar plates should be used to induce conidium and sporangiospore formation.

3. Test medium
   a. RPMI-1640 (with glutamine, without bicarbonate, and with a pH indicator) is the medium recommended for the susceptibility testing of filamentous fungi. The medium should be buffered with MOPS (0.164 mol/L). For the composition of the medium, see Table 10.1.
   b. Procedure for making RPMI-1640 medium:
      - 10.4 g powdered RPMI-1640 medium (Sigma catalog # R 6504).
      - 34.53 g MOPS buffer (Sigma catalog # M 3183 or other manufacturer).
      1) Dissolve powdered medium in 900 mL of distilled water.
      2) Add MOPS, and stir until dissolved.
      3) Adjust the pH to 7.0 at 25°C using 10 or 1 mol/L sodium hydroxide while stirring.
      4) Add additional water to bring the medium to a final volume of 1 L.
5) Sterilize by filtration using a 0.22-μm filter, and dispense into 100 or 500 mL volumes (depending on the volume needed per run) into sterile glass screw-top bottles.

6) Store at 4°C (this solution has a three-month shelf life and bottles should be labeled with the preparation and expiration dates).

7) Check the sterility of the medium by incubating five tubes, each containing 2 mL of the medium per liter, at 35°C. In addition, visually inspect the solution for obvious contamination before using it.

- The medium can be purchased as a powder (Sigma) or ready to use (Biowhit-taker catalog # 04525).

---

**TABLE 10.1**

**Composition of RPMI 1640 Medium**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine (free base)</td>
<td>200</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>50</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>20</td>
</tr>
<tr>
<td>L-Cystine, 2HCl</td>
<td>65.2</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>300</td>
</tr>
<tr>
<td>Glycine</td>
<td>10</td>
</tr>
<tr>
<td>L-Histidine (free base)</td>
<td>15</td>
</tr>
<tr>
<td>L-Hydroxyproline</td>
<td>20</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>50</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>50</td>
</tr>
<tr>
<td>L-Lysine, HCl</td>
<td>40</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>15</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>15</td>
</tr>
<tr>
<td>L-Proline</td>
<td>20</td>
</tr>
<tr>
<td>L-Serine</td>
<td>30</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>20</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>5</td>
</tr>
<tr>
<td>L-Tyrosine, 2Na</td>
<td>28.83</td>
</tr>
<tr>
<td>L-Valine</td>
<td>20</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2</td>
</tr>
<tr>
<td>D-Pantothenic</td>
<td>0.25</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>3</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>35</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>1</td>
</tr>
<tr>
<td>PABA*</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>1</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.2</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.005</td>
</tr>
<tr>
<td>Calcium nitrate × H2O</td>
<td>100</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>400</td>
</tr>
<tr>
<td>Magnesium sulfate (anhydrous)</td>
<td>48.84</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6,000</td>
</tr>
<tr>
<td>Sodium phosphate, dibasic (anhydrous)</td>
<td>800</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>2,000</td>
</tr>
<tr>
<td>Glutathione, reduced</td>
<td>1</td>
</tr>
<tr>
<td>Phenol red Na</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* PABA (Para amino benzoic acid)
4. Sterile saline (8.5 g/L NaCl; 0.85% saline).
5. Tween 20.
6. Supplies
   a. Test tubes
      • Standard laboratory sterile screw- or snap-cap test tubes (e.g., 12 × 75 mm and 16 × 150 mm plastic) that hold at least 5 mL
   b. Petri dishes
      • Standard 100 × 15 mm round petri dishes (one dish per strain)
   c. Pipettes (0.1-, 0.25-, 0.5-, 1-, and 5-mL sterile serological pipettes)
   d. Pipetting aids for serological pipettes
   e. Swabs and loops
   f. Microtiter trays
      • Sterile 96 U-shaped-well microtiter trays (M38-A document) are available from a number of manufacturers, including Dynatech and Nalgene Nunc International.
   g. Micropipettes (single and multichannel) and sterile tips for 100-μL volumes
   h. A 0.5 McFarland standard
      1) Procedure for making McFarland standards [1].
         a) Combine 0.5 mL of 0.048 mol/L BaCl$_2$ (1.175% w/v BaCl$_2$ 2 H$_2$O) and 99.5 mL of 0.18 mol/L (0.36 N) H$_2$SO$_4$ (1%/v/v).
         b) Mix the solution thoroughly
         c) Using the same size screw-cap tubes that are to be used for the preparation of conidial inoculum suspensions, aliquot 4–6 mL into each tube.
         d) Seal the caps with tape or Parafilm®. Store the tubes in the dark at room temperature
         e) Vigorously agitate the standard solution on a vortex mixer prior to each use
         f) Replace standards or validate densities three months after their preparation
            • Commercially available McFarland standards can be obtained from a number of vendors, including PML, Hardy Diagnostics, and Remel.
      2) Validation of the McFarland standard turbidity
         a) Absorbance
            i) Read the optical density (OD) of the standard on a spectrophotometer with a 1-cm light path using a matched set of cuvettes
            ii) The absorbance should be read at 625 nm (OD should be 0.08 to 0.10)
         b) Colony count
            • The density of the McFarland standard is validated by performing a colony count of a conidial suspension of either reference isolate Aspergillus flavus American Type Culture Collection (ATCC) 204304 or quality control isolate Paecilomyces variotii ATCC MYA-3630 (2) that is equivalent to the turbidity of the standard
            • The density of the McFarland standard should be verified monthly by the absorbance or colony count method.
            • A 0.5 McFarland standard is equivalent to 0.4–5 × 10$^6$ conidia or sporangiogiospores per mL (density recommended by the CLSI for the stock inoculum).
      3) Other turbidity standards
         a) Manufactured latex standards
            • Latex standards have a two-year shelf life and are not light sensitive
            • Latex standards are available from Remel (Lenexa, KS; catalog # 20-410) as well as other manufacturers. The density of latex standards should be verified monthly as described for the McFarland standard.
         b) Turbidity meters
            • Available from Vitek and MicroScan Systems
            • Turbidity meters should be calibrated using turbidity standards.
            • The frequency of calibration depends on the manufacturer.
7. Equipment
   a. Biological safety cabinet (class IIA or IIB)
   b. Autoclave
   c. Water bath (48°C–50°C)
   d. Vortex mixer
   e. Incubator set at 35°C ± 1°C
   f. Reading devices
      • A concave mirror reader or plate reader

10.3.3 Quality Control

Quality control organisms are used to verify that the antifungal concentrations were made properly, but their use does not test the quality of the media. Recently, one mold isolate (Paecilomyces variotii ATCC MYA-3630) has been selected for quality control purposes, and several other molds have been selected as reference strains [2]. The yeast quality control strains [6] and the reference molds listed here and in Table 10.2 can also be used.

1. Quality control strains
   • Paecilomyces variotii ATCC MYA-3630
   • Candida parapsilosis ATCC 22019
   • C. krusei ATCC 6258

2. Reference strains
   • Aspergillus flavus ATCC 204304
   • Aspergillus fumigatus ATCC MYA-3631
   • Aspergillus fumigatus ATCC MYA-3626
   • Aspergillus fumigatus ATCC MYA-3627
   • Aspergillus terreus ATCC MYA-3633
   • Fusarium moniliforme ATCC MYA-3629
   • Scedosporium apiospermum ATCC MYA-3635

3. Frequency of quality control or reference testing
   • At least one of the above isolates should be included each time testing is performed using the same procedure as for the clinical isolates.
   • Quality control and reference strains should be preserved following the procedures described by the CLSI [1].

4. Evaluating quality control MIC results
   • MIC values for quality control organisms must be within the acceptable ranges for the antifungal agent being tested for the results to be considered valid. These ranges are listed in the current CLSI M38-A document and in Table 10.2.

10.3.4 Procedure

1. Drug dilutions
   a. Antifungal stock solution preparation
      1) Antifungal powders should be stored according to the manufacturer’s instructions. Powders are assayed by the manufacturer to determine the potency of the antifungal agent. This information must be taken into account when making stock solutions as follows:

      \[
      \text{Weight (mg)} = \frac{\text{volume (mL) \times \text{desired concentration (mg/mL)}}}{\text{antifungal potency (mg/mg)}}
      \]
<table>
<thead>
<tr>
<th>QC Isolates</th>
<th>Antifungal Agent</th>
<th>MIC Range (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>48 h, Macro dilution</td>
</tr>
<tr>
<td>P. variotii ATCC MYA-3630</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td>Amphotericin B</td>
<td>0.25–1.0</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>2.0–8.0</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>0.06–0.25</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.06–0.25</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Ravuconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Flucytosine</td>
<td>0.12–0.50</td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Anidulafungin</td>
<td>NA</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>Amphotericin B</td>
<td>0.25–2.0</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>16–64</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Ravuconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Flucytosine</td>
<td>4.0–16</td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Anidulafungin</td>
<td>NA</td>
</tr>
</tbody>
</table>

(continued)
### TABLE 10.2
Quality Control (QC) and Reference MIC Ranges of Antifungal Agents for M38-A Microdilution and Macrodilution Methods (continued)

<table>
<thead>
<tr>
<th>QC or Reference Isolates</th>
<th>Antifungal Agent</th>
<th>MIC Range (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>48 h, Macrodilution</td>
</tr>
<tr>
<td><em>A. flavus</em> ATCC 204304</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Ravuconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td><em>A. flavus</em> ATCC MYA-3631</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td><em>A. fumigatus</em> ATCC MYA-3626</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td><em>A. fumigatus</em> ATCC MYA-3627</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td><em>A. terreus</em> ATCC MYA-3633</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td><em>F. moniliforme</em> ATCC MYA-3629</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. apiospermum</em> ATCC MYA-3635</td>
<td>Amphotericin B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* Caspofungin and anidulafungin are not described in the M38-A document; however, they will be in the M38-A2 document.

*b* Most MIC ranges have been obtained by the microdilution method [2,6]; incubation times for molds are 24 h (*Aspergillus* spp. and *Fusarium* spp.) and 48 h for *S. apiospermum*.
2) When possible, at least 10 mg of the antifungal powder should be weighed out at one time.

3) All stock solutions should be made at the same concentration. Stock solutions of 1,280 μg/mL work well for most standard susceptibility testing ranges of water-soluble agents and 1,600 μg/mL for water-insoluble ones.

4) The antifungal powder should be dissolved in water or other appropriate diluent as shown in Table 10.3.

5) Stock solutions should be stored carefully sealed in either glass, polypropylene, or polyethylene sterile tubes at –70°C (or lower) in small aliquot volumes (approximately 1.5 mL). Stock solutions should not be stored in frost-free freezers.

6) Stock solutions should be accurately labeled with the antifungal agent’s name, concentration, amount in tube, and the preparation and expiration dates (either six months after the stock solution was made or the manufacturer’s expiration date for the powder).

7) Stock solutions should be discarded at the end of each day and are never refrozen.

8) Because of the high concentration of antifungal in the stock solution, sterilizing the solution is not necessary.

9) If there is a need to sterilize the antifungal stock solution, it should be done using a membrane such as 0.22-μm membrane.

10) Paper, asbestos, or sintered glass filters should not be used because they may bind to the agent.

   • Filter sterilization is not recommended because the antifungal agent may be retained in the filter.

b. Drug dilution preparation

1) Preparation of water-soluble antifungal agents (Figure 10.1) [7]

   a) Label 10 (16 × 150 mm) tubes 2–11.

   b) Add the appropriate amounts of sterile RPMI to each tube as follows:

   • Add 1 mL of RPMI to tubes 2 and 11.
   • Add 0.5 mL of RPMI to tubes 3, 5, and 8.
   • Add 0.75 mL of RPMI to tubes 6 and 9.
   • Add 1.5 mL of RPMI to tube 4.
   • Add 1.75 mL of RPMI to tubes 7 and 10.

---

**TABLE 10.3**

Solvents and Diluents for Preparation of Dilutions of Antifungal Agents

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>DMSO or PEG200</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Ravuconazole</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Micafungin</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>DMSO</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>5-FC</td>
<td>Water</td>
<td>RPMI-1640</td>
</tr>
</tbody>
</table>

a 5-FC = 5-flucytosine.
b DMSO = dimethyl sulfoxide; PEG200 = polyethylene glycol, mol. wt. of 200.
c) Add 1 mL of the stock solution (1,280 μg/mL) to the first antifungal dilution tube (tube 2)

  d) Transfer from tube 2, 0.5 mL to tubes 3 and 4

c) Transfer from tube 4, 0.5 mL to tube 5 and 0.25 mL to tubes 6 and 7

  e) Transfer from tube 7, 0.5 mL to tube 8 and 0.25 mL to tubes 9 and 10

  f) Transfer from tube 10, 1 mL to tube 11

  g) Finally, discard 1 mL from tube 11

• Notice that when the dilutions have been prepared, each tube contains 1 mL.
  • Mix well with a vortex mixer before each dilution step.

2) Preparation of microdilution trays for water-soluble antifungal agents (Figure 10.2) [7].

  a) Place the tubes containing the drug dilutions (1 mL each) in an organized fashion, going from the highest to the lowest drug concentration.

  b) Prepare a 1:5 dilution by adding 4 mL of RPMI to each drug dilution tube and mix well with a vortex mixer

  c) Using a dispensing device, transfer 0.1 mL from each of the drug dilution tubes to the appropriate wells of each microtiter tray; mix well before performing the dispensing step

    • The concentration of the antifungal agent in each well is 2 times the concentration needed after this step.

    • It is not recommended to use volumes smaller than 0.1 mL because the medium can evaporate during the incubation step.

  d) Add 0.2 mL of RPMI to the wells in column 1 and 0.1 mL to wells in column 12. These wells will serve as the negative (sterility) and positive (growth) control wells, respectively.

  e) The prepared tray should be sealed in a plastic bag and placed at –70°C until needed. Prepared trays should not be stored in a self-defrosting freezer as this type of freezer will shorten the shelf life of the antifungal agent in the tray. Prepared trays should not be refrozen after being thawed

  f) The shelf life of the tray is the expiration date of the antifungal being used or 6 months, whichever comes first

3) Preparation of water-insoluble antifungal agents (Figure 10.3) [7].
The dilutions of water-insoluble antifungal agents (amphotericin B, itraconazole, ketoconazole, posaconazole, ravuconazole, voriconazole, and anidulafungin)
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must be prepared by using dimethyl sulfoxide (DMSO) or some other appropriate solvent (Table 10.3).

- It is important to prepare the stock solution and drug dilutions in the solvent (follow the scheme of dilution and instructions outlined here) to avoid dilution artifacts that can result from the precipitation of a compound of low solubility (e.g., itraconazole) in aqueous media, thereby producing erroneous drug dilutions.

a) Label 9 (12 × 75 mm) tubes 3–11.
b) Add appropriate amounts of DMSO to each tube as follows:
   - Add 0.5 mL of DMSO to tubes 3, 6, and 9
   - Add 0.75 mL of DMSO to tubes 4, 7, and 10
   - Add 1.75 mL of DMSO to tubes 5, 8, and 11
c) Label the stock solution tube (1,600 μg/mL) as tube 2
d) Transfer from tube 2, 0.5 mL to tube 3 and 0.25 mL to tubes 4 and 5
e) Transfer from tube 5, 0.5 mL to tube 6 and 0.25 mL to tubes 7 and 8
f) Transfer from tube 8, 0.5 mL to tube 9 and 0.25 mL to tubes 10 and 11
g) Finally, discard 1 mL from tube 11
   - Notice that when dilutions have been prepared, all the tubes contain 1 mL
   - Mix well with a vortex mixer before the transfer step

4) Preparation of microdilution trays for water-insoluble antifungal agents (Figure 10.4) [7].
a) Place the tubes containing the drug dilutions (1 mL) in an organized fashion, from the highest to the lowest drug concentration and label a row of 10 tubes with the appropriate drug concentration.

FIGURE 10.4 Preparation of microdilution trays for water-insoluble antifungal agents.

SC: sterility control (Tube 1)
GC: growth control (Tube 12)
Trays are now ready for the inoculation step.
b) Prepare a 1:50 dilution by mixing 4.9 mL of RPMI with 0.1 mL from each drug dilution tube; mix well with a vortex mixer.

c) Using a dispensing device, transfer 0.1 mL from each of the final drug dilution tubes to the appropriate well in each microtiter tray.

d) Add 0.2 mL of RPMI plus 2% DMSO (or the solvent that was used) to column 1 of the microtiter tray and 0.1 mL of RPMI plus 2% DMSO (or the solvent that was used) to column 12. These two wells will serve as the negative (sterility) and positive (growth control) control wells, respectively, for the microtiter tray.

e) The concentration of the antifungal agents in the wells is now 2 times the concentration needed.

f) The concentration of DMSO in the wells is now 2%.

g) Follow steps e) and f) (preparation of microdilution trays for water-soluble agents) for storage of prepared microdilution trays.

2. Inoculum preparation (Figure 10.5) [7]
a. For Aspergillus spp., R. arrhizus, S. apiospermum, and S. schenckii, inoculum must be prepared from a conidium or sporangiospore suspension obtained from 7-day cultures grown on potato dextrose agar at 35°C. Fusarium spp. should be grown on potato dextrose agar for 48–72 h at 35°C and then at 25°C–28°C until day 7. Some isolates need longer incubation (more than 7 days) to produce conidia.

b. Recover conidia by wetting a loop with Tween 20 and transfer the loopful of conidia into 3 mL of sterile saline.

FIGURE 10.5 Inoculum preparation of molds for micro- and macrodilution methods.
c. Vortex the conidia suspension vigorously for 15–20 s to prevent clumping of the spores. (Caution: Remove the cap carefully as liquid adhering to the cap may produce aerosols upon opening).

d. Allow the heavy particles to settle for 3–5 min and then transfer the upper suspension to a sterile tube and adjust using a spectrophotometer (530 nm) to the optical density (OD) that yields a stock suspension of $0.4–5 \times 10^6$ viable conidia or sporangiospores per milliliter. The OD at which the inoculum must be adjusted will depend on the size of conidia. Table 10.4 shows the OD for some species and the corresponding CFU/mL [1, 8].

e. Prepare a working suspension by diluting 1:50 of the conidia stock suspension in the standard medium; mix well with a vortex mixer. Inoculum suspensions of *S. apiospermum* may require a lower (50%) dilution factor. The 1:50 inoculum dilutions will correspond to double the density needed (approximately $0.4–5 \times 10^4$ CFU/mL).

f. Check or verify the final inoculum size by plating 10 μL of a 1:20 dilution of working suspension onto SAB plates (approximately 4–50 colonies per plate).

g. Incubate plates at 35°C (or 28°C–30°C) and observe daily for the presence of fungal colonies.

- Colonies should be counted as soon as possible after growth becomes visible (24 h or less for *R. arrhizus* to 5 days for *S. apiospermum*).

3. Inoculation of microtiter trays.

a. Allow the microtiter trays to thaw at room temperature; it will take approximately 2 h to thaw if they are stacked four trays high or 45 min if unstacked under a hood.

b. Remove the sealing tape as soon as the trays are taken from the freezer and cover them with a clean lid.

### TABLE 10.4
Optical Density (OD) Range and Inoculum Sizes for Common and Uncommon Molds

<table>
<thead>
<tr>
<th>Species</th>
<th>OD Range (%T)</th>
<th>$10^6$ CFU/mL Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. nidulans</em></td>
<td>0.09–0.11 (80–82)</td>
<td>1.1–2</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>0.09–0.11 (80–82)</td>
<td>0.4–4</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>0.09–0.11 (80–82)</td>
<td>0.6–5</td>
</tr>
<tr>
<td><em>A. terreus</em></td>
<td>0.09–0.11 (80–82)</td>
<td>0.9–5</td>
</tr>
<tr>
<td><em>Bipolaris hawaiensis</em></td>
<td>0.2–0.3</td>
<td>0.07–0.4</td>
</tr>
<tr>
<td><em>B. spicifera</em></td>
<td>0.2–0.3</td>
<td>0.3–3</td>
</tr>
<tr>
<td><em>Cladophialophora bantiana</em></td>
<td>0.15–0.17 (68–70)</td>
<td>0.4–3.1</td>
</tr>
<tr>
<td><em>Dactilaria constricta</em></td>
<td>0.15–0.17 (68–70)</td>
<td>0.4–1</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>0.15–0.17 (68–70)</td>
<td>0.8–5</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>0.15–0.17 (68–70)</td>
<td>0.5–5.9</td>
</tr>
<tr>
<td><em>Paecilomyces lilacinus</em></td>
<td>0.09–0.13</td>
<td>0.8–2.3</td>
</tr>
<tr>
<td><em>P. variotii</em></td>
<td>0.09–0.11 (80–82)</td>
<td>ND</td>
</tr>
<tr>
<td><em>Scedosporium apiospermum</em></td>
<td>0.15–0.17</td>
<td>0.4–3.2</td>
</tr>
<tr>
<td><em>R. arrhizus</em></td>
<td>0.15–0.17</td>
<td>0.4–2.6</td>
</tr>
<tr>
<td><em>S. prolificans</em></td>
<td>0.15–0.17</td>
<td>0.6–1.7</td>
</tr>
<tr>
<td><em>S. schenckii</em></td>
<td>0.09–0.11</td>
<td>ND</td>
</tr>
<tr>
<td><em>Trichoderma longibrachiatum</em></td>
<td>0.09–0.11</td>
<td>0.7–2.3</td>
</tr>
<tr>
<td><em>Wangiella dermatitidis</em></td>
<td>0.15–0.17</td>
<td>1.2–3.7</td>
</tr>
</tbody>
</table>

* %T = percent transmission.

Based on Refs. 4 and 8.
c. Thawed trays should be inoculated within 1 h of thawing
d. Fill each row of the microtiter tray from wells 2–12 with 0.1 mL of the working inoc-
ulm suspension using a single or a multichannel pipette; mix well with a vortex mixer
before dispensing. If using another device, follow the manufacturer’s instructions as to
what inoculum concentration should be added to the inoculum reservoir
  • Eight clinical isolates or seven isolates and one of the quality control or reference
isolates can be tested in one tray. The GC ensures that the isolate is viable and
indicates the 100% growth of the isolate.

4. Incubation
MIC trays are incubated without agitation in an aerobic incubator without CO₂ at 35°C for
46–50 h for most of the opportunistic molds (Aspergillus spp., Fusarium spp., and S.
schenkii). Rhizopus spp. and other zygomycetes will require 21–26 h of incubation, and
S. apiospermum and S. prolificans 70–74 h. Stack trays no more than three high.

5. Microdilution MIC definitions
  a. MIC definition for itraconazole, the new triazoles (voriconazole, ravuconazole and
posaconazole), and amphotericin B (MICs of 5-FC [5-flucytosine] and ketoconazole
can also be determined by this methodology) is as follows:
    • The MIC is the lowest drug concentration that prevents any discernible growth
(optically clear) as detected visually.

6. Reading
  a. Visual reading
    1) Remove the trays from the incubator.
    2) Place the tray on a reading device (light box, concave mirror reader, or plate reader).
    3) The growth control well must have sufficient growth.
    4) If adequate growth is not present, the trays must be reincubated. Adequate growth
will depend on the species being tested.
    5) Determine the MIC endpoint, using the definition described above.
    6) MICs for quality control isolates should be within the established limits (Table
10.3) [2,6]
  b. Spectrophotometric reading
    • This procedure is not included in the CLSI documents, but sometimes it is helpful.
    1) Set the spectrophotometer at 405 nm, and determine the OD of each well.
    2) The use of 450-, 492-, 550-, or 600-nm filters gives similar results.
    3) Do not agitate the trays before reading them.
    4) Subtract the OD of the blank (uninoculated column 1 wells) from the OD of the
inoculated wells.
    5) The MIC is the lowest drug concentration that produces ≥95% growth reduction
both for azoles and amphotericin B.
    • Caution: With some brands of the microtiter plates, bubbles may appear after
incubation that will preclude an accurate MIC determination or OD reading.
Use of the Greiner-Bio-One microtiter plates (reference # 650180) will avoid
this problem. Burst the bubbles with a heated needle.

10.3.5 INTERPRETATION OF THE RESULTS
Interpretative breakpoints have not been established for molds. The clinical relevance of testing
this group of fungal pathogens remains uncertain. Nevertheless, preliminary data indicate that high
itraconazole MICs (>8 μg/mL) are associated with clinical resistance to this agent [9,10] when the
MICs are determined by the M-38-A microdilution method [1]. For the new triazole antifungal
agents, data indicating a correlation between MICs and clinical outcome of treatment are not yet
available. The same applies to amphotericin B. However, correlation between clinical outcome and
amphotericin B MIC results suggests that MICs >2 μg/mL are associated with treatment failure and MICs below 2 μg/mL with clinical response [11].

Trailing endpoints with amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole against Aspergillus spp. and most other opportunistic pathogenic molds are not usually encountered. Such a pattern may reflect clinically relevant drug resistance [9,10].

10.3.6 Advantages
1. The broth microdilution procedure can test several isolates in the same microtiter tray.
2. Microtiter plates can be prepared in advance and stored at –70°C for 6 months.
3. This procedure is easy to perform and requires less space in the laboratory freezer and incubator than does the macrodilution procedure.
4. It does not require specialized equipment.
5. It is the optimal standard procedure to detect azole resistance in Aspergillus spp. [5].

10.3.7 Limitations
1. The method has only been standardized for conidium-forming molds with azoles and amphotericin B. It has not been evaluated for the yeast forms of dimorphic fungi, such as B. dermatitidis, C. inmitis, H. capsulatum variety capsulatum, P. marneffei, or S. schenckii.
2. Breakpoints are not yet available.
3. The OD for preparation of inocula varies with the species to be tested.
4. There is a risk that drug dilutions in the microdilution trays could dry out when testing slow-growing molds.

10.3.8 Conclusions
This procedure requires the utilization of a biological safety cabinet and should be attempted only with the guidance of an experienced individual; if an easier method is chosen, its results should match those of the CLSI standard. The medium, inoculum size, time and temperature of incubation can affect the MICs for filamentous fungi. The RPMI medium yields reproducible results and facilitates the detection of itraconazole and amphotericin B resistant strains. The minimum effective concentration (MEC) is a more reliable susceptibility end point to evaluate the echinocandins (see Section 10.3.10); although testing conditions for this class of antifungal agents are not described in the CLSI M38-A document.

10.3.9 Minimum Fungicidal (or Lethal) Activity
The M38-A document does not describe testing conditions for the determination of minimum fungicidal (or lethal) concentrations (MFCs). It has been demonstrated that the MFC may be a better predictor of therapeutic failure than the MIC in disseminated trichosporosis [12,13]. A CLSI collaborative study has identified certain testing conditions for determining the MFCs of azoles (itraconazole, posaconazole, voriconazole and ravuconazole) and amphotericin B [14]. These testing conditions are:

1. MFC determination.
   a. Subculture 20 μL from each MIC well that showed complete inhibition (100% or an optically clear well) after 72 h of incubation onto SAB plates.
      • Do not agitate the well prior to removal of the specified volumes.
   b. Subculture 20 μL from the growth control well (drug-free medium) onto SAB plates.
      • Do not agitate the well prior to removal of the specified volumes.
   c. Incubate the plates at 35°C until growth is seen in the growth control subculture (usually before 48 h).
2. MFC definition.
   The MFC is the lowest drug concentration that shows either no growth or fewer than three colonies to obtain an approximately 99–99.5% killing activity.

3. Additional considerations
   a. The clinical relevance of the MFC needs to be established.
   b. Preliminary data suggest that the poor in vitro fungicidal activity of amphotericin B appears to correspond to the refractory nature of *A. terreus* infections to amphotericin B therapy [15].
   c. The method has good reproducibility for azoles and amphotericin B (91%–98% to 96%–100%, respectively) [8].

10.3.10 Broth Microdilution Alternative Approach for Molds versus the Echinocandins: Evaluation of Morphologic Changes

Trailing growth is not a problem when testing azoles against most molds. However, when testing the echinocandins, most *Aspergillus* isolates show trailing growth and conventional MIC determination could categorize these trailing isolates as resistant. A more careful examination of microdilution wells reveals the presence of compact, round microcolonies. Under microscopic examination, these microcolonies correspond to significant morphologic alterations. The hyphae grow abnormally as short, highly branched filaments with swollen germ tubes. The concentration of drug producing these morphologic changes is defined as the minimum effective concentration (MEC) to distinguish it from conventional MICs. A multicenter study has demonstrated that caspofungin MECs were reliable endpoints in 14 of 17 laboratories [16]; similar results were obtained in another study in the 8 laboratories that participated in the study when evaluating anidulafungin against a variety of mold species.1

MICs and MECs of the echinocandins are usually <1.0 μg/mL for *Aspergillus* spp. but higher for other molds (MECs and MICs >8 μg/mL). However, MICs <1.0 μg/mL have been reported for some isolates of the dimorphic fungi, *Phialophora* spp. and *S. apiospermum*. MEC methodology will be introduced in the revised M38-A2 CLSI document.

10.4. Broth Macrodilution Procedure

10.4.1 Introduction

The design of the CLSI broth macrodilution method is similar to that for yeasts with some variation. This method has been assayed by a number of laboratories and has been found to correlate well with the microdilution method [3,4]. It is a practical method for use in the clinical laboratory when testing slow-growing fungi that require a long incubation time (more than 72 h).

1. Technical notes.
   • It is important to completely plan out the entire procedure since it involves many steps over several days. It is also important to understand all the steps of the procedure before beginning. The technologist is advised to read and understand the CLSI M38-A document before proceeding.
   • Determine the number of isolates to be tested, the number of antifungal agents, and the range of concentrations for each antifungal.
   • The concentration ranges for drugs are the same as for yeast susceptibility testing (see Table 10.5).

---

TABLE 10.5
Summary of M38-A Method

| Test medium | RPMI 1640 with glutamine, without bicarbonate and with a pH indicator |
| pH          | 6.9–7.1 |
| Buffer      | [3-(N-morpholino)]-propanesulfonic acid (MOPS) at a concentration of 0.164 mol/L (34.53 g/L) |
| Inoculum size | 0.4–5 × 10^6 CFU/mL |
| Duration and temperature of incubation | Rhizopus spp.: 21–26 h at 35°C, Aspergillus spp., Fusarium spp., S. schenckii: 46–50 h at 35°C, S. apiospernum: 70–74 h at 35°C |
| Concentrations to be assayed | Amphotericin B, itraconazole, voriconazole, posaconazole, ravuconazole: 16–0.03 μg/mL |
| MIC definition | Amphotericin B, itraconazole, voriconazole, posaconazole, ravuconazole: the lowest drug concentration that prevents any discernible growth (clear tubes) after 48 to 72 h of incubation; echinocandins, MEC determination at 24 to 48 h |
| Breakpoints | Not established for molds |
| Quality control strains | P. variotii ATCC MYA-3630, Candida krusei ATCC6258 and C. parapsilosis ATCC22019 |
| Reference strains | Aspergillus flavus ATCC 204304 and others listed in Table 10.2 |

10.4.2 MATERIALS

1. Antimicrobial source (refer to Section 10.3.2)
2. Agar media (refer to Section 10.3.2)
3. Test medium is RPMI 1640 medium (refer to Section 10.3.2 for medium preparation)
4. Sterile saline (8.5 g/L NaCl; 0.85% saline)
5. Tween 20
6. Supplies
   - The same supplies listed in Section 10.3.2 are needed with the exception of microtiter trays and multichannel micropipettes.
7. Equipment
   - The same equipment listed in Section 10.3.2 is needed with the exception of the microdilution plate reader.

10.4.3 QUALITY CONTROL

Quality control organisms verify that the antifungal concentrations were made properly, but they do not test the quality of the media. Paecilomyces variotii ATCC MYA-3630 has been selected for quality control purposes, and several other molds have been selected as reference strains [2]. The yeast quality control strains [6] described in Chapter 9 or the reference molds listed here and in Table 10.2 can also be utilized; however, most quality control data have been obtained by the microdilution method.

1. Quality control strains
   - Paecilomyces variotii ATCC MYA-3630.
   - Candida parapsilosis ATCC 22019.
   - C. krusei ATCC 6258.
2. Reference strains
   - Aspergillus flavus ATCC 204304.
   - Aspergillus fumigatus ATCC MYA-3631.
   - Aspergillus fumigatus ATCC MYA-3626.
   - Aspergillus fumigatus ATCC MYA-3627.
• *Aspergillus terreus* ATCC MYA-3633.
• *Fusarium moniliforme* ATCC MYA-3629.
• *Scedosporium apiospermum* ATCC MYA-3635.

3. Frequency of quality control or reference testing.
   - At least one of the above isolates should be included each time testing is performed using the same procedure used for the clinical isolates.
   - Quality control and reference strains should be preserved following procedures described by the CLSI [1].
   - See instructions given in Section 10.10.4 under “Inoculation of MIC tubes” for a description of how to perform the inoculations. In addition, test one of these isolates with each new lot of macrodilution tubes before testing clinical specimens.

4. Evaluating quality control and reference strain MIC results.
   - The MIC values for a quality control or reference organism tested must be within acceptable ranges for the antifungal agent being tested. These ranges are listed in Table 10.2 [2,6].

### 10.4.4 Procedure

1. Tubes
   - Based on the number of strains and the antifungal agents to be tested, calculate the number of tubes needed (# strains × number of antifungal dilutions + controls = # of tubes per antifungal).
   - Calculate the total amount of RPMI needed (# of strains × 12 mL = total volume per strain plus 12 mL to prepare antifungal agent dilutions).

2. Drug dilutions
   - Antifungal stock solution preparation (see Section 10.3.4).
   - Drug dilution preparation
     1) Preparation of the water-soluble antifungal agents (Figure 10.6) [7].
        a) Label 10 (16 × 150 mm) tubes 2–11.

![Diagram of dilutions of water-soluble antifungal agents](image)

*Volume of the RPMI before the drug dilution step.

**FIGURE 10.6** Scheme to perform the dilutions of water-soluble antifungal agents.
b) Add the appropriate amounts of sterile RPMI to each tube as follows:
   - Add 1 mL of RPMI to tubes 2 and 11.
   - Add 0.5 mL of RPMI to tubes 3, 5, and 8.
   - Add 0.75 mL of RPMI to tubes 6 and 9.
   - Add 1.5 mL of RPMI to tube 4.
   - Add 1.75 mL of RPMI to tubes 7 and 10.

c) Add 1 mL of the stock solution (1,280 µg/mL) to the first antifungal dilution tube (tube 2)

d) Transfer from tube 2, 0.5 mL to tubes 3 and 4

e) Transfer from tube 4, 0.5 mL to tube 5 and 0.25 mL to tubes 6 and 7

f) Transfer from tube 7, 0.5 mL to tube 8 and 0.25 mL to tubes 9 and 10.

g) Transfer from tube 10, 1 mL to tube 11

h) Finally, discard 1 mL from tube 11

   - Notice that when dilutions have been prepared, each tube contains 1 mL.
   - Mix well with a vortex mixer before each transfer or dilution step.

1) Preparation of MIC tubes for water-soluble agents (Figure 10.7) [7].

   a) Place the tubes containing the drug dilutions (1 mL each) in an organized fashion, going from the highest to the lowest drug concentration

   - Nine sets of MIC tubes may be prepared with these volumes.

   b) Label the rows of 10 tubes (12 × 75 mm) with the final drug concentrations (64 to 0.12 µg/mL)

   c) Dispense 0.1 mL from each drug dilution tube into the bottom of each corresponding MIC tube using a repetitive or reservoir pipette with a syringe of 1 mL; mix well before dispensing

   - Beginning with the lowest concentration, the same tip can be used throughout.

   - Mix well with a vortex mixer before each dispensing step.

   d) Close the tubes with screw caps (plastic or metal caps), and store them at –70°C for up 6 months

   e) On the day of the test, allow the tubes to thaw at room temperature before inoculation (about 1 h). Each tube is inoculated with 0.9 mL of the corresponding diluted inoculum

   ![Illustration of MIC tube preparation](image-url)

   **Final concentration after inoculation:**
   64–0.12 µg/mL

   **SC:** sterility control (Tube 1)
   **GC:** growth control (Tube 12)

**FIGURE 10.7** Preparation of MIC tubes for water-soluble antifungal agents.
2) Preparation of the water-insoluble antifungal agents (Figure 10.8) [7].
   • The dilutions of water-insoluble antifungal agents (amphotericin B, itraconazole,
     anidulafungin, ketoconazole, posaconazole, ravuconazole, and voriconazole) must
     be prepared by using a solvent such as DMSO or other appropriate solvent
     (Table 10.3).
   • It is important to prepare the stock solution and drug dilution in the solvent
     (follow the scheme of dilutions and instructions here) to avoid dilution artifacts
     that can result from the precipitation of a compound of low solubility (e.g.,
     itraconazole) in aqueous media, thereby producing erroneous drug dilutions.

    a) Label 10 (12 × 75 mm) tubes 3–11.
    b) Add appropriate amounts of DMSO to each tube as follows:
       • Add 0.5 mL of DMSO to tubes 3, 6, and 9.
       • Add 0.75 mL of DMSO to tubes 4, 7, and 10.
       • Add 1.75 mL of DMSO to tubes 5, 8, and 11.
    c) Label the stock solution tube (1,600 µg/mL) as tube 2.
    d) Transfer from tube 2, 0.5 mL to tube 3 and 0.25 mL to tubes 4 and 5.
    e) Transfer from tube 5, 0.5 mL to tube 6 and 0.25 mL to tubes 7 and 8.
    f) Transfer from tube 8, 0.5 mL to tube 9 and 0.25 mL to tubes 10 and 11.
    g) Finally, discard 1 mL from tube 11.
     • Notice that when dilutions have been prepared, all the tubes contain 1 mL.
     • Mix well with a vortex mixer before each transfer step.

3) Preparation of MIC tubes for water-insoluble antifungal agents (Figure 10.9) [7].
   a) Place tubes containing the drug dilutions (1mL) in an organized fashion, from the
      highest to the lowest drug concentration and label rows of 10 tubes (12 × 75 mm)
      with the final drug concentrations (16 to 0.03 µg/mL).
   b) Prepare a 1:1 dilution by mixing 0.9 mL of RPMI with 0.1 mL from each drug
      concentration, and mix well with a vortex mixer.
   c) Nine sets of MIC tubes may be prepared with these volumes.
   d) Dispense 0.1 mL from each concentration into the bottom of each sterile 12 × 75 mm
      plastic test tube, using a repetitive or reservoir pipette with a syringe of 1 mL.
   e) Beginning with the lowest concentration, the same tip can be used throughout.
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f) Mix well with a vortex mixer before each dispensing step.
g) Close the tubes with screw caps (plastic or metal caps), and store them at –70°C for up to 6 months.
h) On the day of the test, allow the tubes to thaw at room temperature before the inoculation (about 1 h). Each tube is inoculated with 0.9 mL of the corresponding inoculum, and the final concentration of DMSO is 1%.

3. Inoculum preparation (Figure 10.10) [7]
a. For *Aspergillus* spp., *R. arrhizus*, *S. apiospermum*, and *S. schenckii*, inoculum must be prepared from a conidium or sporangiospore suspension obtained from 7-day cultures grown on potato dextrose agar at 35°C. *Fusarium* spp. should be grown on potato dextrose agar for 48–72 h at 35°C and then at 25°C–28°C until day 7. Some isolates need longer incubation (more than 7 days) to produce conidia.
b. Recover conidia by wetting a loop with Tween 20 and transfer the loopful of conidia into 3 mL of sterile saline.
c. Vortex the conidia suspension vigorously for 15–20 s to prevent clumping of the spores. (Caution: Remove the cap carefully as liquid adhering to the cap may produce aerosols upon opening).
d. Allow the heavy particles to settle for 3–5 min, and then transfer the upper suspension to a sterile tube and adjust using a spectrophotometer (530 nm) to the OD that yields a stock suspension of 0.4–5 × 10^6 viable conidia or sporangiospores per milliliter. The OD at which the inoculum must be adjusted will depend on the size of conidia. Table 10.4 shows the OD for some species and the corresponding CFU per milliliter [1,8].
e. Prepare a working suspension by diluting 1:100 the conidia stock suspension in the test medium. Inoculum suspensions of *S. apiospermum* may require a lower (50%)
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dilution factor. The 1:100 inoculum dilutions will correspond to the density needed (approximately \(0.4–5 \times 10^4\) CFU/mL).

f. Check or verify the final inoculum size by plating 10 \(\mu\)L of a 1:20 dilution of working suspension onto SAB plates (approximately 4–50 colonies per plate).

g. Incubate SAB plates at 35°C (or 28°C–30°C) and observe daily for the presence of fungal colonies.
   • Colonies should be counted as soon as possible after growth becomes visible (24 h or less for \(R.\) arrhizus to 5 days for \(S.\) apiospermum).

4. Inoculation of MIC tubes
   a. Allow the MIC tubes to thaw at room temperature; it will take approximately 1 h to thaw.
   b. Thawed MIC tubes should be inoculated within 1 h of thawing.
   c. The drug concentration tubes should be organized before the preparation of the inocula in ascending order.
   d. Include two tubes, for positive (growth control) and negative (sterility) controls per isolate.
   e. Calculate the volume of standardized inoculum-broth suspension needed for each isolate, including the growth control tubes (for example, for one antifungal agent and mold, 10 mL of inoculated RPMI are needed), at the desired final concentration (0.4–5 \(\times 10^4\) CFU/mL).

FIGURE 10.10 Inoculum preparation for micro- and macrodilution methods.

SS: saline solution
• Begin inoculating the lowest drug concentration to be tested and move to the highest concentration.

f. The two control tubes contain 0.1 mL of RPMI (or solvent) rather than the antifungal and either 0.9 mL of the diluted inoculum (growth control) or 0.9 mL of RPMI (sterility control).

• The growth control tube ensures that the inoculum is viable and actively growing and indicates 100% growth of the isolate.

• The negative control tube provides evidence of medium sterility.

g. Add 0.9 mL of the working suspension to the MIC tubes with the antifungal agent; mix this suspension well with a vortex mixer before the inoculation step.

5. Incubation.

• MIC tubes are incubated at 35°C (without agitation) in an aerobic incubator and observed for the presence or absence of visible growth. The incubation time depends on the species. Most opportunistic filamentous fungi (Aspergillus spp., Fusarium spp., and S. schenkii) require 46–50 h of incubation, while Rhizopus spp. require 21–26 h and S. apiospermum, 70–74 h.

6. Reading

a. Visual reading

1) Remove MIC tubes from the incubator.

2) The growth control tube must have sufficient growth.

3) If adequate growth is not present, the tubes must be reincubated. Adequate growth will depend on the species being tested.

4) Determine the MIC end point as described here.

5) MICs for quality control isolates should be within established ranges (Table 10.3) [2,6].

7. Macrodilution MIC definition and interpretation

The MIC definition for itraconazole, the new triazoles (voriconazole, ravuconazole and posaconazole), and amphotericin B (MICs of 5-FC and ketoconazole can also be determined by this methodology) is as follows:

• The MIC is the lowest drug concentration that prevents any discernible growth (optically clear) as detected visually.

• Interpretative breakpoints have not been established for molds. The clinical relevance of testing this group of fungal pathogens remains uncertain.

10.4.5 Advantages

1. Since susceptibility testing is not an assay that must be performed for all filamentous fungi, the broth macrodilution has the advantage of testing only a single isolate and agent.

2. It does not require specialized equipment.

3. It is the best method for slower growing organisms or for testing those fungi that require more than 72 h of incubation to produce enough growth for MIC determination. As with the microdilution method, there is a risk of evaporation and thus may yield erroneous MIC results.

10.4.6 Limitations

1. The method has only been standardized for conidium-forming molds. It has not been used in the yeast forms of dimorphic fungi such as B. dermatitidis, C. immitis, H. capsulatum variety capsulatum, P. marneffei, or S. schenckii.

2. Broth macrodilution is not an ideal method for routine testing or for testing multiple isolates because it is labor-intensive and technically demanding.
3. The incubation period is variable, depending on the type of fungal species involved. Nevertheless, most pathogenic filamentous fungi show enough growth after 48–72 h of incubation.
4. Breakpoints are not yet available. There is a need to document *in vitro* versus *in vivo* correlations.
5. The OD for inoculum densities varies with the species to be tested.

### 10.4.7 Conclusions

This procedure should be attempted only with the guidance of an experienced individual; if an easier method is chosen, its results should match those of the CLSI standard. MICs for filamentous fungi can be affected by varying the conditions outlined here. Careful attention must be given to the OD required for each mold species and each spectrophotometer. The RPMI medium provides good reproducibility and allows the detection of itraconazole resistant strains. For a summary of CLSI methods, see Table 10.5.

### 10.5 Etest Procedure

#### 10.5.1 Introduction

Etest (AB Biodisk, Solna, Sweden) is a nonporous plastic strip immobilized with a predefined gradient of a given antimicrobial on one side and printed with an MIC scale on the other side. When the Etest strip is placed on an inoculated agar plate, a continuous, stable, and exponential antimicrobial gradient is established along the side of the strip. After incubation, the MIC value (µg/mL) can be read directly from the MIC scale printed on the Etest strip. This technology is similar to a disk diffusion assay but with a longer stability time for the antimicrobial gradient. The equivalency of the resulting MIC with the CLSI reference values has not been well established. Preliminary results show that the correlation improves if the Etest MICs are read after 24 h of incubation rather than after 48 h or when sufficient growth is visible [17].

#### 10.5.2 Materials

1. Etest strips can be purchased directly from AB Biodisk or through Remel (Table 10.6). Strips (approximate cost ≥$2/strip) are sold in packages of 100 strips per antifungal agent and are stored at −20°C or −70°C. Once a set of strips is removed from the manufacturer’s package, the unused strips should be stored in an airtight container with a desiccant and returned to −20°C. Etest strips should not be used after the expiration date indicated by the manufacturer.

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Code</th>
<th>Concentrations (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>AP</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>FL</td>
<td>0.016–256</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>IT</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>FC</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>KE</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>VO</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>PO</td>
<td>0.002–32</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>CS</td>
<td>0.002–32</td>
</tr>
</tbody>
</table>
2. Plates consisting of RPMI-1640 supplemented with 2% glucose and 1.5% agar, prepared in-house or obtained commercially (Remel or others), that have a depth of 4 mm ± 0.5 mm can be used. Plates should be stored at 2°C–8°C. Modified casitone agar or antibiotic medium 3 (AM3) agar can also be used [17] as recommended by the manufacturer.

3. Saline solution for making conidium suspensions
4. Swabs (sterile, nontoxic)
5. Test tubes (for inoculum preparation)
6. Pipettes
7. Scissors
8. Forceps or Etest applicator kit
9. A 0.5 McFarland standard (this can be made following CLSI instructions or purchased from microbiology product manufacturers such as PML, Hardy, or Remel).
10. Airtight storage containers with desiccant (silica gel) for extra Etest strips once the manufacturer’s packaging has been opened. Storage containers may be purchased from AB Biodisk or Remel.
11. Freezer (–20°C or –70°C) for storage
12. Vortex mixer
13. 35°C ambient air incubator
14. Etest technical information
   • Etest package insert
   • Etest technical guide No. 10
   • Etest application sheet EAS 012
   • Customer information sheet (CIS) No. 5 for preparation of the media

10.5.3 Quality Control

1. Quality control strains:
   • C. parapsilosis ATCC 22019
   • C. krusei ATCC 6258
   • P. variotii ATCC 22319
   • All quality control strains should be maintained following the procedures described by CLSI [1].

2. Frequency of quality control testing
   • A quality control organism should be run with every new lot of Etest strips prior to their use.
   • Each antifungal agent should have quality control testing for 30 consecutive days [1]. Otherwise, quality control should be conducted on each occasion that testing is performed.

3. Evaluating the results
   • The Etest antifungal application sheet EAS 012 lists the quality control ranges for appropriate antifungal agents (also see Table 10.7); P. variotii ATCC MYA-3630 isolate has not been evaluated by the Etest.
   • These values are not included in the CLSI M38-A document.

10.5.4 Procedure

• Caution: All steps in the testing of molds must be performed in a class IIA or IIB biological safety cabinet.

1. Remove the strips from the freezer 30 min (–20°C) or 60 min (–70°C) before use from the original package or storage container to allow them to equilibrate at room temperature.
2. Use mature growth from SAB or potato dextrose agar slants, 5–7 days, depending on the genus.

3. Carefully pour 1 mL of sterile saline over the growth, and homogenize the conidia and hyphal particles into suspension using a Pasteur pipette.

4. Pipette out the suspension, and transfer to a sterile screw cap tube (13 × 100 mm).

5. Vortex gently for 15 s, and allow the particles to settle for 15 minutes.

6. Adjust the turbidity of the supernatant to a 0.5 McFarland standard or correct the percent transmission at 530 nm to obtain colony counts of approximately 10^6 CFU/mL (see Table 10.4). Add more conidia to increase the turbidity or more saline to decrease the turbidity (photometric devices can also be used to measure the turbidity).

7. Dip a swab into the inoculum suspension and streak the whole surface of the plate carefully in three different directions to obtain an even growth; mix well before the inoculation step.
   • Make sure that the inoculum is spread evenly over the entire surface of the plate. A correctly inoculated plate will have an even lawn of confluent growth.
   • Plates can be inoculated automatically with the AB–Biodisk spiral autoinoculator CR10.

8. Allow the inoculum to be absorbed completely into the agar (at least 15–20 min). The plate must be completely dry before the application of the Etest strip. If moisture is present on the agar surface when the Etest strip is applied, it will affect the performance of the antifungal gradient.

9. The strip is designed to have the MIC scale facing away from the agar surface and the antifungal gradient touching the agar surface (you should be able to see the writing on the strip after applying it to the agar plate). Apply the “E” labeled area of the strip (highest concentration of antifungal) at the edge of the petri dish to the agar surface and gently lay down the strip with the lowest concentration end of the strip placed toward the center of the plate. Once the strip has touched the agar surface do not move the strip. If any large bubbles appear under the strip, gently remove the bubbles by pressing on the Etest strip with a forceps or a swab. Small bubbles under the strip will not affect the performance of the Etest strip.
   • One to two strips can be placed on a 90-mm plate with opposite orientation to each other (you do not want the “E” end of the strip on the same side of the plate for two strips, that is, the gradients will overlap).
   • Up to six strips can be placed on a 150-mm agar plate in a spoke wheel pattern. (Templates are available from AB Biodisk to optimize the positioning of the strips.)
   • When using the Etest applicator, refer to the instructions in the product insert.
   • Do not remove the Etest strip during any part of the process.

10. Incubate the plate, in plastic bags, in the inverted position (agar side up) at 35°C in an aerobic, non-CO2 environment.

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Candida parapsilosis ATCC 22019</th>
<th>Paecilomyces variotii ATCC 22319</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.25–2</td>
<td>0.24–1</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.064–0.25</td>
<td>0.032–1</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.032–0.125</td>
<td>0.064–0.5</td>
</tr>
</tbody>
</table>

Based on Etest application sheet EAS 012.
11. Incubate the plate until growth is clearly seen (24–72 h for most Zygomycetes) [17]. Incubation conditions are genus-dependent, for example, *Fusarium* spp. are incubated at 35°C for 24–48 h followed by room temperature (approximately 25°C) for 24–48 h. For slow-growing clinical isolates, extend the incubation time as needed and inspect the plates daily.

10.5.5 Etest Results

1. Reading
   a. Evaluate the growth of the isolate before reading, and read as soon as possible. Reading times are species-dependent, for example, *Rhizopus* spp. for 16–24 h and *Aspergillus* spp. for 24–48 h. A longer incubation gives overgrowth.
   b. Make the first reading at 16–24 h and the second at 48 h. Slow-growing isolates may require up to 72 h or longer before sufficient growth is obtained.
   c. Read the MIC where the edge of the inhibition ellipse intersects the MIC scale on the Etest strip (use the technical guide No. 10 for endpoint selection assistance).
   d. Ignore filaments bending over into the ellipse; they are usually caused by overgrowth when incubation is prolonged.
   e. If there is complete growth and no zone appears on the plate around a strip, the MIC value is recorded as greater than the highest antifungal concentration of the strip.
   f. If the inhibition ellipse does not fall below the lowest value on the strip, the MIC value is recorded as less than the lowest value on the strip.

2. Interpreting
   a. Etest MICs are based on a continuous gradient; therefore, MIC values between the standard twofold dilutions can be obtained. These values can be reported as such, if desired, or rounded up to the next appropriate twofold dilution value, for example, 0.19 μg/mL becomes 0.25 μg/mL.
   b. Because CLSI interpretative criteria are not available, only report the MIC values.

10.5.6 Advantages

1. Etest is easy to perform and requires minimal training for test performance.
2. Contamination can be easily recognized.
3. Etest can be easily set up for a small number of clinical isolates.
4. There is minimum labor involved with Etest as compared with broth dilution methods.
5. The Etest methodology is cost-effective for two antifungal agents.
6. Etest is a flexible methodology because the antifungal agent, medium, incubation time, and inoculum size can be adapted to each organism.
7. Itraconazole *in vitro* resistant strains may be distinguished by the Etest method [17].

10.5.7 Limitations

1. The cost is the most significant limitation. Careful consideration should be given to which antifungal agents are tested because for fewer than two strips per isolate, the Etest methodology can be cost-effective.
2. A low level of reproducibility has been reported: 67% for amphotericin B and 87% for itraconazole [18].
3. End points can be difficult to determine.
4. The agreement between Etest and M38-A results is dependent on the species, antifungal agent, and incubation time; results are usually comparable (for example, using itraconazole, from 83.3% for *Aspergillus nidulans* to >90% for other species). For amphotericin B,
the lowest agreement is for *F. solani* (70%) and *A. flavus* (75%) and the highest is for *A. niger* (100%) [19].

5. Amphotericin B MIC values can substantially change from 24 to 48 h of incubation for some strains.

6. Etest strips are not U.S. FDA approved for mold testing at this time.

### 10.5.8 Additional Considerations

1. The inhibition ellipses of amphotericin B can be narrower than those of voriconazole and itraconazole.

2. The MIC values obtained by the Etest method are generally higher than those obtained by the M38-A method.

   For amphotericin B, itraconazole, and posaconazole, the agreement improves if Etest MICs are determined at 24 h [18,19] or as soon as sufficient growth allows it (before 48 h) [17]. Major discrepancies between amphotericin B MICs determined by the Etest at 48 h and M38-A have been reported for *Aspergillus* spp. and *F. solani* [17].

3. For voriconazole, the agreement is less dependent on the incubation time (24 or 48 h) [19].

### 10.5.9 Conclusions

The Etest is easy to perform. However, the changes in MIC values from 24 to 48 h warrant further evaluation to identify optimal conditions for its use and to establish which results (24 vs. 48 h) correlate better with *in vivo* results. Reproducibility studies are also needed.

### 10.6 Sensititre YeastOne Colorimetric Method

#### 10.6.1 Introduction

The Sensititre YeastOne colorimetric antifungal plate (TREK International, Westlake, OH) consists of a disposable tray that contains dried serial twofold dilutions of seven (in Europe) or three (in the United States) established antifungal agents in RPMI-1640 medium supplemented with 1.5% dextrose in individual wells. The wells also contain the color indicator Almar blue (TREK). YeastOne panels have been evaluated with filamentous fungi [18,20–24]. Good intra- and interlaboratory reproducibility of MIC endpoints has been demonstrated with this method [18,19], for example, using *Aspergillus* spp., 83–93% for itraconazole and >93% for amphotericin B after 24 and 48 h of incubation [18]. Agreement (within ±2 log₂ dilutions) with the CLSI M38-A method for itraconazole is poor (84.6%); however, it is better with amphotericin B (99.6%) [18]. In the United States, the FDA has approved this panel only for yeast testing for three antifungal agents. In Europe, antifungal agents included in the panel are amphotericin B, fluconazole, itraconazole, ketoconazole, 5-FC, caspofungin, and voriconazole.

#### 10.6.2 Materials

1. YeastOne susceptibility test panels
2. YeastOne susceptibility inoculum broth
3. Autoclaved demineralized water (5-mL volumes)
4. Panel layout template
5. Manual worksheet
6. Plate seals
7. Plate viewbox light or mirror type
8. A 0.5 McFarland turbidity standard
9. SAB plates
10. Sterile wooden applicator sticks or a loop
11. Non-CO₂ incubator set at 35°C
12. Vortex mixer
13. 20-μL pipette
14. Multichannel pipette for 100-μL volumes
15. Disposable pipette tips

10.6.3 Quality Control

1. Quality control strains:
   • *C. parapsilosis* ATCC 22019
   • *C. krusei* ATCC 6258
   • *P. variotii* ATCC MYA-3630
   • Quality control strains should be preserved following the procedures described by CLSI [1].

2. Frequency of quality control testing
   • Quality control should be run in parallel with every run. The MIC ranges are the same as those in the M38-A document (see Table 10.2). Quality control strains confirm the potency of the antifungal agent dilutions. YeastOne provides the acceptable ranges for the antifungal being tested [1], but the *P. variotii* ATCC MYA-3630 isolate has not been evaluated by the YeastOne method.

10.6.4 Procedure

1. Remove the panels to be used from storage. Panels should not be used if the desiccant is not present or if the integrity of the packing is compromised.
2. The inoculum should be prepared as described for the microdilution method (see Section 10.3.4) in TREK demineralized water.
3. Prepare a working suspension by adding 20 μL of the stock suspension to 11 mL of YeastOne inoculum broth.
4. Panels can be inoculated manually using a pipetting device or automatically with the Sensititre autoinoculator. Fill each well of the Sensititre panel with 100 μL of the working suspension. The excess of the working suspension should be used to inoculate SAB plates to check the inoculum density.
5. Cover the inoculated panels with the panel seals.
6. Stack the panels no more than three high.
7. Incubate the panels for 24–72 h at 35°C in a non-CO₂ incubator, and read when the control well (A1) is red.

10.6.5 YeastOne MIC Results for Molds

1. The MIC of azoles, 5-FC, and amphotericin B is the lowest concentration of antifungal solution changing from red (growth) to blue (no growth) after 48 h of incubation.
2. Reading.
   Panels may be read visually by using a view box and in normal laboratory lighting.
   If the growth control well is red, the end points for antifungal agents can be interpreted. If after incubation the well is only weakly purple, re-incubate for several additional hours until it turns red. Do not read the turbidity in Sensititre YeastOne panels.
3. Interpreting and reporting YeastOne MIC results.
   As no CLSI interpretative criteria are available (see the microdilution method), only report MIC values.
10.6.6 ADVANTAGES

1. YeastOne is easy to perform and requires minimal training for test performance.
2. YeastOne can be easily set up for a small number of clinical isolates.
3. This method discriminated well two itraconazole-resistant strains [23].
4. Panels are stored at room temperature.

10.6.7 LIMITATIONS

1. Low reproducibility MIC values and low agreement with the M38-A method for itraconazole have been reported [18].
2. Breakpoints are not available.
3. There is only limited experience with this method for mold testing.

10.6.8 CONCLUSIONS

The YeastOne procedure is easy to perform with minimal labor and compares well with M38-A for amphotericin B, voriconazole, and posaconazole [24]. Other uses and the clinical relevance of results by the panel are yet to be determined.

10.7 BROTH MICRODILUTION METHOD FOR DERMATOPHYTES

The M38-A broth microdilution method has also been successfully adapted, with minor modifications, to the testing of dermatophytes [25]. These modifications include the use of oatmeal agar for inoculum preparation when testing *Trichophyton rubrum* to induce conidium formation and incubation at 35°C for 4–5 days for MIC determination (80% growth inhibition end points). Two isolates of *Trichophyton* spp. have been selected as reference strains by the subcommittee for testing this group of fungi.

10.8 CONCLUSIONS

Currently, the gold standard for susceptibility testing for molds is the CLSI M38-A broth dilution methodology [1]. This methodology is used typically by research laboratories or large clinical laboratories that can “batch” isolates for large-scale research or surveillance studies. The CLSI methods are labor intensive and do not lend themselves well to testing small numbers of clinical isolates. If a commercial product is proven to be equivalent to the broth dilution procedure, it can be used in the clinical laboratory even though CLSI does not describe alternative methods. Of the two procedures approved by the CLSI, the microdilution method is best suited for routine testing in the clinical laboratory. It can be used with any number of isolates and is less labor intensive than is the broth macrodilution method. Broth microdilution panels can be prepared in bulk and frozen. For molds that require more than 72 h of incubation to achieve visual growth, the macrodilution method is a more suitable procedure. Other alternative methods for testing molds are the colorimetric (YeastOne and others) and Etest procedures; reproducibility and agreement with the M38-A are controversial for these tests. At the present time, the M38-A is a reproducible method that discriminates azole-resistant strains, but the overall relevance of *in vitro* results with clinical outcomes (breakpoint development) is not yet established [26]. As antifungal resistance continues to increase, the need for the clinical laboratory to routinely perform mold susceptibility testing has become more important. For a summary of CLSI methods, see Table 10.5.
REFERENCES


11 Susceptibility Testing of Mycobacteria

Barbara A. Brown-Elliott, Samuel Cohen, and Richard J. Wallace, Jr.

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11.1 INTRODUCTION

This chapter reviews the principles and procedures of mycobacterial susceptibility testing for the nontuberculous mycobacteria (NTM), including *Mycobacterium avium* complex (MAC) and the
rapidly growing mycobacteria (RGM). A review of the advances in susceptibility testing of the M. tuberculosis complex (M. tuberculosis, M. bovis, M. africanum, M. bovis BCG, and M. canetti) is also included. Conventional methodology using critical concentrations in agar proportion method is discussed along with newer methods, including broth-based systems such as BACTEC 460 TB and MGIT 960, (Becton Dickinson Biosciences, Sparks, MD), ESPII system (Trek Diagnostic Systems, Westlake, OH), and the BacT/ALERT 3D System (bioMérieux, Durham, NC).

11.2 PRINCIPLES OF MYCOBACTERIUM TUBERCULOSIS COMPLEX SUSCEPTIBILITY TESTING

Mycobacteriology susceptibility testing should be performed by experienced technologists who process enough specimens and see enough positive cultures to handle them promptly and correctly. The American Thoracic Society (ATS) has recommended that laboratories culture at least 20 specimens per week to maintain proficiency as well as continuing and frequent performance of test procedures [1]. Problems such as contamination, mixed cultures, slow growth, or improbable results should be recognized by the technologist. Some of the media is expensive and has a relatively short shelf life, a consideration for laboratories that do minimal mycobacterial susceptibility testing. The laboratory must have quality control strains to cover all species tested, and the strains must be stored and subcultured appropriately [2]. The laboratory director or supervisor must be willing and able to provide consultation and feedback to physicians and public health officials, and technologists must troubleshoot problems in a timely manner. In this era of overnight air courier service and electronic communication, patients may be better served when small, localized laboratories send their clinical specimens to a reference laboratory that is specialized in the identification and susceptibility testing of Mycobacterium species.

When is susceptibility testing warranted? As recently as the mid-1980s, susceptibility testing of M. tuberculosis complex initial isolates in the United States was not recommended unless a “high probability of drug resistance is expected” [3]. Issues such as HIV, homelessness, prison conditions, and immigration changed the official position on susceptibility testing of initial M. tuberculosis complex isolates, making it mandatory in the United States [4]. The recent Clinical Laboratory and Standards Institute (CLSI, formerly The National Committee for Clinical Laboratory Standards, NCCLS) M24-A document [2] states that susceptibility testing should be performed on the first isolate of M. tuberculosis complex cultured from a patient sample. Testing should be repeated if the patients fail to convert to negative after 3 months of therapy or there is evidence of failure to respond to therapy.

Until the development of streptomycin and p-amino salicylic acid (PAS), there were no drugs to treat tuberculosis. Other agents such as isoniazid (INH) were subsequently introduced. Subsequently, drug resistance began to develop and the need to detect drug resistance became evident. Eventually, international committees (i.e., International Working Groups for Mycobacteria Taxonomy, IWGMT) met to set standards for susceptibility testing and to define drug-susceptible and drug-resistant M. tuberculosis complex so that the results of testing could be interpreted consistently.

“Susceptible” strains are defined as “those that have never been exposed to the main antituberculosis drugs ("wild" strains) and respond to these drugs, generally in a uniform manner” [5]. The definition was based on in vitro studies showing that growth of such strains was inhibited by a narrow range of concentrations of drugs such as INH, PAS, and streptomycin. The goal of susceptibility testing was to determine whether a patient’s isolate conformed to the pattern of wild strains and if so treat with a standard regimen that generally provided a cure. The critical concentration was close to the lowest concentration that inhibited the growth of wild strains in culture medium. When an isolate did not conform to the pattern of wild strains it was considered resistant. According to IWGMT, “resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain is different from a sample of wild strains of human type that have never come
Susceptibility Testing of Mycobacteria

into contact with the drug” [6]. This historic definition of susceptibility and resistance applies only
to isolates of the *M. tuberculosis* complex. Testing of the nontuberculous mycobacteria utilizes
definitions more in-line with those used by the CLSI for bacterial species. An unanswered question
with this definition is whether mutations that confer minor changes in the minimum inhibitory
concentrations (MICs) that are still within achievable serum levels (e.g., isolates resistant to 0.1
µg/mL but susceptible to 1.0 µg/mL of INH) will still respond to therapy with that drug.

We now know that most resistance to the antituberculous drugs relates to mutations in the genes
that encode for one or more enzymes that are inhibited by the drug. These mutations generally
interfere with the binding of the drug such that it no longer inhibits the enzymes. Specific enzyme
systems inhibited include catalase-peroxidase (KatG gene for INH), DNA-dependent RNA poly-
merase (rpoB gene for rifampin), arabinose transferases (embCAB operon for ethambutol), and
pyrazinamidase (pncA gene for pyrazinamide). Other resistance relates to mutations in the ribosomal
RNA genes that encode protein synthesis (e.g., streptomycin, amikacin).

Today, the goal of susceptibility testing of *M. tuberculosis* complex isolates remains to differ-
entiate resistant from wild strains so that the physician is able to make informed choices about
chemotherapy. Because of the continued significant incidence of resistant strains in the United
States, including multiple drug resistant (MDR-TB) strains, defined as strains resistant to two or
more of the primary antituberculosis agents (isoniazid and rifampin), the laboratory is under
increased pressure to provide rapid susceptibility results so that patients infected with these strains
can be placed on individualized treatment regimens designed to exclude the drugs to which the
isolate is resistant. This was emphasized in the early 1990s outbreak of MDR-TB in New York
City in which delays in obtaining susceptibility results almost certainly contributed to poor patient
outcomes, especially among HIV-infected patients [7,8].

Current therapy for suspected or proven disease due to *M. tuberculosis* complex generally
involves administering four drugs until drug susceptibility reports are available. In the United
States, these drugs are isoniazid, rifampin, pyrazinamide, and ethambutol. In countries outside the United
States, streptomycin is sometimes used in place of ethambutol, primarily for cost reasons. If the
isolate is found to be susceptible, the ethambutol (or streptomycin) is discontinued. Pyrazinamide
is discontinued after 2 months, and therapy is continued with isoniazid and rifampin for the
remaining 4 months.

This 6-month regimen is currently the approved regimen (by the ATS and CDC) for treating
tuberculosis disease in the United States [9] and it is referred to as the standard short course therapy.
Patients whose isolates are resistant to one or more of these primary drugs may then be treated
with one or more secondary drugs that include amikacin, streptomycin, ethionamide, cycloserine,
and the fluorinated quinolones such as ofloxacin or levofloxacin. Recent studies suggest that some
of the newer fluorquinolones, such as moxifloxacin, may have better activity against *M. tubercu-
losis* complex as well as other *Mycobacterium* species [10].

There are two types of drug resistance as detected in clinical isolates; primary or initial drug
resistance occurs when the patient has been infected with a strain that is already resistant to one
or more drugs; acquired drug resistance stems from the proliferation of naturally occurring resistant
mutants when they have been exposed to a drug during suboptimal or inadequate chemotherapy.
Patient’s noncompliance with the treatment regimen also leads to acquired resistance. Clinical trials
with some of the first antituberculosis drugs, especially streptomycin, showed the inability of
monotherapy to prevent the emergence of resistance to the administered drug. It then became clear
that multidrug regimens were necessary to obviate acquired (mutational) drug resistance and to
successfully treat tuberculosis. Therefore, by using multiple agents with different targets of activity,
one can minimize the emergence of resistance. Consequently, it is standard practice to test at least
the four primary drugs, also called first-line drugs: isoniazid, rifampin, ethambutol, and pyrazina-
mide (PZA). Streptomycin, once considered a first-line drug, is rarely used in the United States
except when treating patients with drug resistant isolates.
For susceptibility testing of *M. tuberculosis* complex, qualitative assays are standard. These may be performed in a broth-based system (e.g., BACTEC MGIT 960 system [11–13]) or by an agar proportion method using Middlebrook 7H10 or 7H11 agar plates (Table 11.1) [12,14–16]. If testing is performed by the agar proportion method, one may choose to perform susceptibility testing to both the primary and the secondary agents (e.g., aminoglycosides, capreomycin, cycloserine) at the same time. Performing both simultaneously may be more cost effective and improve turnaround time for results. The agar proportion method may be accomplished easily using commercially available plates (REML, Lenexa, KS; Hardy Diagnostics, Santa Maria, CA), or preparing them in-house.

The Centers for Disease Control and Prevention (CDC) recommends that laboratories work toward the goal of reporting first-line susceptibility results within 15–30 days from receipt of the initial clinical specimen [4]. Ideally, susceptibility results should be available within 7–14 days following the isolation and identification of *M. tuberculosis* complex from a clinical specimen. This is generally possible only if primary isolation and susceptibility testing is performed using a broth culture system and if the initial specimen is acid-fast bacillus (AFB) smear positive. Timely laboratory results allow changing the short-course standard regimen if indicated and preclude the physician’s prescribing drugs to which the isolate is resistant. A short description of the various methods for performing susceptibility testing for *M. tuberculosis* complex is listed here.

### TABLE 11.1
Comparison of Mycobacterial Growth Indicator Tube, BACTEC 460 TB, and Conventional Method of Proportion Antimycobacterial Susceptibility Testing

<table>
<thead>
<tr>
<th>MGIT</th>
<th>BACTEC 460 TB</th>
<th>LJMOP</th>
<th>7H10MOP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Time to Reporting for AST (Days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>Not given</td>
<td>21</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>5.38/7.9</td>
<td>7.33</td>
<td></td>
<td>24, 25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.2–6.9(^a)</td>
<td>13.7–21(^b)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>5.58/5.47(^a)</td>
<td>7.4</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4–8</td>
<td>21</td>
<td>31, 32</td>
<td></td>
</tr>
<tr>
<td>Mean Detection Time for Mycobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td></td>
<td>24, 25</td>
<td></td>
</tr>
<tr>
<td>8.3</td>
<td>19.4</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>5.2 (NTM)</td>
<td>17.8 (NTM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>19.5</td>
<td></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Radiometric Instrumentation Required</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

*Note: Unless specified, data refers to testing of *M. tuberculosis*.  
\(^a\) MGIT = mycobacterial growth indicator tube; MOP = method of proportion; AST = antimycobacterial susceptibility testing;  
\(^b\) Includes *M. tuberculosis* or nontuberculous mycobacteria.  
\(^c\) Range given; no mean turnaround time is available.  
\(^d\) Testing of ethambutol and streptomycin, respectively.
11.2.1 Broth-Based Systems [17]

1. BACTEC 460TB (Becton Dickinson Biosciences [BD], Sparks, MD). The radiometric qualitative susceptibility test in the BACTEC 460 TB system is a broth-based, FDA-approved method [12,15,16,18]. This system is a rapid and highly reliable method for susceptibility testing of \textit{M. tuberculosis} complex. With this system, growth in the presence of the drug is compared with that in a drug-free culture representing 1% of the bacterial population. The BACTEC procedure for drug susceptibility testing of mycobacteria is based on the same principle employed in the conventional agar method. The main difference is that a liquid medium is used and instead of counting colonies, the growth is monitored radiometrically. The critical proportion for resistance is read as 1% for all antituberculous agents. This means that if $\geq 1\%$ of the test mycobacterial population is resistant, the isolate is considered resistant. Results are based on the radiometric growth index (GI) as a measure of radiolabeled CO$_2$ generated by the multiplying organisms. Currently, five drugs—streptomycin, isoniazid, rifampin, ethambutol (SIRE) and PZA—are recommended for this method. This method has been used to test other agents (e.g., levofloxacin and others) in laboratories that have validated its reliability. The shortcomings of the method are: There is limited experience with testing of second-line drugs, it utilizes radiolabeled substrate and the disposal of the vials may be problematic, and finally, the instrument used to monitor the vial is “old”—parts are not readily available and the manufacturer has been phasing out its use. Therefore, over the past 5–10 years, BD has developed a nonradiolabeled broth system that will address these shortcomings. Susceptibility testing including PZA with BACTEC is rapid and reliable [19,20].

2. BACTEC MGIT 960 system (Becton Dickinson [BD] Biosciences, Sparks, MD). This nonradiometric system for susceptibility testing of \textit{M. tuberculosis} complex is FDA approved for testing SIRE and PZA [16,18,21–25]. The tubes contain enriched 7H9 broth with an O$_2$-sensitive fluorescence sensor indicating microbial growth. Positivity is indicated by a bright orange fluorescence on the bottom of the tube and an orange reflection at the meniscus; negative tubes show little or no fluorescence [13]. A fully automated MGIT 960 instrument allows for the continuous monitoring of positive tubes [21]. Macondo and colleagues [22] reported that the MGIT 960 could be used as an alternative to the BACTEC 460TB system for rifampin and isoniazid; however, they, like previous investigators [23], reported discrepancies with streptomycin and ethambutol, which gave impetus to additional studies [21,22]. Since then, multiple studies have demonstrated that the BACTEC 460TB and the MGIT 960 have equivalent sensitivity and reliability when performing susceptibility testing for the primary agents and PZA [18,23–26]. Recently, a multicenter validation study of the MGIT 960 technique for susceptibility testing of the second-line drugs and newer agents has been published [27].

3. ESPII system (Trek Diagnostics Inc., Westlake, OH). The ESPII (an FDA approved method) combines a liquid culture media, a growth supplement, and specific antibiotics with a detection system that automatically incubates and continuously monitors culture bottles. Sponges in the bottles provide growth support and increase the surface area exposed to headspace oxygen. The technology for the ESPII is based upon detection of headspace pressure changes within a sealed bottle. These pressure changes are the result
of gas consumption or gas production due to mycobacterial growth. Susceptibility testing of *M. tuberculosis* complex isolates is performed by inoculating a suspension of organisms into five ESP Mycoculture bottles supplemented with ESP Myco GS and one of the four primary drugs. One control bottle without drug is also inoculated. An ESP connector is attached to each bottle to establish a closed system enabling the bottles to be monitored on the ESP instrument. A hydrophobic membrane in the connector prevents aerosols released into the laboratory. Bergmann and Woods reported the ESP II system as a rapid and reliable method for susceptibility testing of *M. tuberculosis* complex [28]. A similar study by LaBombardi and Lukowski concluded the ESP was in agreement with the agar reference method [29]. However, both authors indicated that susceptibility results for ethambutol and streptomycin may be discrepant and need further study [29]. Ruiz, Zerolo, and Casal reported good agreement with the BACTEC 460TB for testing rifampin, isoniazid, streptomycin, and ethambutol with the lowest agreement between the two methods (88.6%) for isoniazid [30]. LaBombardi also showed that the PZA results were reliable [20].

4. The BacT/ALERT system (bioMérieux Inc., Durham, NC). The system is a rapid, sensitive, fully automated method continuously monitored for growth and detection of mycobacteria. Recently, investigators have studied the potential of the system for antimycobacterial susceptibility testing of *M. tuberculosis* complex. They found correlation of sensitivity with the BACTEC 460 TB system for isoniazid, ethambutol, streptomycin, and rifampin of 96%, 100%, 78%, and 92%, respectively [31–33]. Brunello and Fontana reported the median turn around time was 8.5 days (range of 5–11 days) for the MB/BacT versus 6 days (range of 4–8 days) for the BACTEC 460 TB system [32] while other investigators reported a range of 2.5–10.7 days with a 7 day average for the MB/BacT compared to 21 days by agar proportion [31]. The authors stated, however, that further development was necessary to improve the sensitivity of drug resistance. Thus, the BacT/ALERT 3D system for susceptibility testing of mycobacteria is not available currently.

### 11.2.2 Agar-Based Systems

Agar-based tests are carried out in quadrant Petri dishes in which one quadrant contains drug-free medium while the other quadrants contain antituberculosis drugs incorporated at the critical concentrations (Table 11.2) [2]. The CLSI M24-A previously recommended 7H10 agar or 7H11 (which is 7H10 supplemented with casein hydrolysate to promote better growth of drug-resistant strains) [2]. The critical concentration of some of the drugs is different for 7H10 and 7H11 media (see Table 11.2) [2]. With these media, “susceptible” is defined as less than 1% growth in the presence of the drug compared to the number of colonies on the drug-free control [3,34]. Two types of susceptibility testing are possible on agar plates: the “direct” test in which an AFB smear-positive specimen concentrate is inoculated directly onto the plates and the “indirect” test in which a previously grown culture is inoculated onto the media. Plates for this assay can be made in-house or purchased from a commercial source. When preparing plates, this can be accomplished using specially designated elution disks or by adding diluted antibiotic solutions that are made from purchased powder (Table 11.4). Results of direct and indirect tests are reported as the percent of bacteria resistant to each drug. When there is no growth in the presence of the drug, the result “zero percent resistant” indicates the strain is fully susceptible [34].
### 11.3 SUSCEPTIBILITY TESTING FOR MYCOBACTERIUM TUBERCULOSIS COMPLEX

#### 11.3.1 PROCEDURE 1: QUALITATIVE RADIOMETRIC DRUG SUSCEPTIBILITY TESTING FOR *M. TUBERCULOSIS* [15,35]

1. **Principle.**

   Susceptibility testing for the four first-line drugs—isoniazid, streptomycin, rifampin, and ethambutol—can be performed as a qualitative test in the BACTEC 460 TB system. This broth-based susceptibility method is based on the proportion method, testing a “critical concentration” of the drug, just as in the direct and indirect agar plate proportion methods. In this test, the radiometric growth index in the presence of the critical concentration is compared with that representing 1% of the bacterial population in the 1:100 control. At the end of the test, if the change in GI of a drug containing vial is greater than that of the 1:100 control, the isolate is considered to be resistant to that drug. If the change in GI of a drug-containing vial is less than that of the 1:100 control, the isolate is susceptible.

<table>
<thead>
<tr>
<th>Primary Drugs</th>
<th>7H10 Agar</th>
<th>7H11 Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethambutol (EMB)</td>
<td>5.0</td>
<td>7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isoniazid (INH)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Rifampin&lt;sup&gt;b&lt;/sup&gt; (RMP)</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Pyrazinamide (PZA)</td>
<td>NR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Drugs&lt;sup&gt;d&lt;/sup&gt;</th>
<th>7H10 Agar</th>
<th>7H11 Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capreomycin</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Ethambutol&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Kanamycin&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Ofloxacin&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>p-Aminosalicylic acid</td>
<td>2.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Rifabutin&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>


<sup>a</sup> Data supporting equivalency with 7H10 (5.0 µg/mL) are limited.

<sup>b</sup> Rifampin is the class agent for rifapentine.

<sup>c</sup> NR = not recommended.

<sup>d</sup> All secondary drugs should be tested on isolates of *M. tuberculosis* complex that are resistant to rifampin or resistant to any two primary drugs. Testing cycloserine is not recommended due to technical problems.

<sup>e</sup> Kanamycin is the class agent for amikacin.

<sup>f</sup> Ofloxacin is the class agent for the older fluoroquinolones (ciprofloxacin and ofloxacin) but not moxifloxacin.

<sup>g</sup> Some investigators have included a higher concentration, usually 1.0–2.0 µg/mL.
2. **Specimen.**
   A 7H12 broth culture ("seed culture") grown to GI of 500 or greater, or a smooth suspension of a *M. tuberculosis* complex culture is adjusted to the density of a 1 McFarland standard.

3. **Reagents, media, and supplies.**
   See the manufacturer’s suggestions.

4. **Procedure.**
   See the manufacturer’s technical procedure manual [15,35].

5. **Reporting and calculations.**
   See the manufacturer’s technical procedure manual [15].

6. **Quality control** [15,35].

7. **Limitations.**
   This test is limited to qualitative results. Percent resistant cannot be determined from GI values.

8. **Method validation** [3,15,35].

### 11.3.2 Procedure 2: Pyrazinamide Susceptibility

1. **Principle.**
   Pyrazinamide, one of the first-line drugs, is more active at an acidic rather than at neutral pH. It is necessary, therefore, to test this drug at low pH (5.9–6.0) to obtain accurate results. Agar-based methods such as the agar proportion method have not proved satisfactory because of the failure of many isolates to grow at an acidic pH. Susceptibility testing of PZA in the BACTEC 460 TB system has proven to be satisfactory and as of 2003 was considered the reference method [2]. The procedures in the manufacturer’s technical procedure manual should be followed carefully [36,37]. More recently, a study by Piersimoni et al. evaluated current nonradiometric detection systems as an option for replacing the BACTEC 460 TB radiometric method. The authors concluded that although all of the currently available systems were acceptable, the BACTEC MGIT 960 appeared to be the most reliable option [17].

2. **Specimen.**
   A 7H12 broth culture grown to GI of 500 or greater ("seed culture") or a smooth suspension of a *M. tuberculosis* complex culture adjusted to the density of a 1 McFarland standard.

3. **Reagents, media, and supplies.**
   See the manufacturer’s technical procedure manual [36].

4. **Procedures.**
   See the manufacturer’s technical procedure manual [36].

5. **Reporting and calculations.**
   The categories for reporting PZA resistance include “susceptible,” “resistant,” or “borderline” [2].

6. **Quality control.**
   See the manufacturer’s technical procedure manual [36].

7. **Expected values.**
   See the manufacturer’s technical procedure manual [36].

8. **Limitations.**
   See the manufacturer’s technical procedure manual [36].

9. **Method validation.**
   See the manufacturer’s technical procedure manual [36].

Isolates that yield uninterpretable results in the BACTEC PZA test system should be retested with and without polyoxyethylene stearate (POES), which has been reported to cause inhibition of growth of some isolates [38].
11.3.3 Procedure 3: Direct Drug Susceptibility Test in 7H10 or 7H11 Agar Plates

1. Principle.
This test determines the percentage of drug resistant organisms in the patient’s specimen by inoculating a concentrated specimen directly onto 7H11 agar plates containing various antituberculous drugs (see Table 11.3). It is preferentially performed on specimens from known or suspected TB patients.

2. Specimen.
Digested, decontaminated specimen concentrate
If the concentrated specimen is AFB negative by smear examination (200–400 × magnification), it should be inoculated undiluted. The following dilutions are necessary if the specimen is smear-positive:
- Up to 25 AFB per field—dilutions 1 and 0.1.
- From 50 to 250 AFB per field—dilutions 1 and 100 (1:100).
- Greater than 250 per field—dilutions 100 and 1000 (1:100 and 1:1000).

Note: If <5 AFB seen per field, increase the inoculum to 0.2mL.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Solvent</th>
<th>Working Concentration (µg/mL) for 7H10 Agar</th>
<th>Volume to Add to 200 mL of 7H10 Agar</th>
<th>Final Concentration of Drug in 7H10 Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-Line Agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>SDW a</td>
<td>1,000</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>SDW</td>
<td>200</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Rifampin</td>
<td>DMSO</td>
<td>1,000</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Second-Line Agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capreomycin</td>
<td>SDW</td>
<td>1,000</td>
<td>2.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>DMSO</td>
<td>1,000</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>SDW</td>
<td>1,000</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>SDW</td>
<td>200</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>Methanol</td>
<td>100</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>SDW</td>
<td>1,000</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>p-Aminosalicylic acid</td>
<td>SDW</td>
<td>1,000</td>
<td>0.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>


a SDW = sterile distilled water; DMSO = dimethyl sulfoxide (should not be filter sterilized using synthetic filters; solutions made in DMSO should be left at room temperature in the dark for 30 minutes for self-sterilization; personal communication, author, SC).

Calculate the weight based on potency if less than 100%.
Sterilize stock solutions by filtration through 0.22-µm pore membrane, and dispense into sterile vial before freezing. Thaw the vials to room temperature, and use immediately. The excess should be discarded and never refrozen!
3. Procedure.
   a. At the same time the media for primary isolation is being inoculated, inoculate 0.1 mL of the specimen (diluted as above if necessary) on each quadrant of a set of labeled drug susceptibility plates. Tilt plates gently to spread the inoculum. Allow plates to stand for 1 hour under the hood for absorption of the inoculum. If there are only a few AFB on the smear, the quadrants can be inoculated with 0.2 mL each and left under the hood to dry.
   b. Place the plates top-side up individually into CO₂-permeable polyethylene bags and seal the bags. Incubate in the CO₂ incubator at 37°C. Check the growth appearance, at 1, 2, 3, and 6 weeks of incubation. Read at 3 weeks if the growth is adequate.
   c. Remove the plates from the incubator and leave upside down (agar-side up) at room temperature until the condensate disappears from the cover. Examine the plates macroscopically and with a dissecting microscope. Record the number of colonies on each quadrant onto a laboratory worksheet.

4. Reporting and calculations.
   Calculate the percentages of bacteria resistant to the various drug concentrations based on the number of colonies in the drug-free control and the number in the drug-containing quadrants.

\[
\text{Percent resistant} = \frac{\text{number of colonies in drug-containing quadrant}}{\text{number of colonies in the drug-free control quadrant}} \times 100
\]

Most laboratory computer systems can be programmed to perform the calculations and print a report showing the percent resistant for each drug.

5. Quality control.
   Each day of testing, inoculate a set of plates with the CLSI (NCCLS) quality control \textit{M. tuberculosis} strain American Type Culture Collection (ATCC) 27294 (H37Rv) [2].

6. Expected values.
   Colony counts should be within the expected range for the test to be valid. Thus, precise quantitation may be difficult when direct testing is performed.

7. Limitations.
   The test is valid only when there is no contaminant overgrowth and there are >50 colonies on the drug-free controls. A comment should be noted in the laboratory report about the limited validity of the results if:
   a. Sufficient growth in the drug-free controls did not appear within 3 weeks of incubation.
   b. There are fewer than 50 colonies per drug-free quadrant.
   State that the drug susceptibility test should be repeated as an indirect test. In case of heavy contamination, request a repeat specimen. If there is no growth on the control plates, perform an indirect susceptibility test from the primary isolate if positive.

8. Method validation [3,14].
   Validate by performing the indirect susceptibility test, colony count of the control quadrant, and by use of ATCC reference quality control strains.

11.3.4 Procedure 4: Indirect Drug Susceptibility Test in 7H10 or 7H11 Agar Plates

1. Principle.
   This test shows the percentage of drug resistant organisms for \textit{M. tuberculosis} complex isolates. The indirect test may be performed if the results of the direct test are not valid due to contamination, insufficient numbers of colonies in the drug-free quadrants, or insufficient growth after 3 weeks of incubation. The preparation of this inoculum is critical because variations in the number of AFB in the suspension can alter the test interpretation [34].
2. Specimen.
   The following cultures can be used:
   a. A suspension made from surface of the medium (L-J, 7H10, 7H11, etc.). Primary cultures, rather than subcultures, should be used when available.
   b. A BACTEC 12B vial at GI of 500 or greater [15,35].
   c. Isolates from other continuous monitoring systems (see the manufacturers’ guidelines) [15].
   d. 7H9 broth at the optical density equal to a 1 McFarland standard.
3. Procedure.
   a. If the only culture available contains only a few colonies on solid media, use these colonies to inoculate 7H12 (BACTEC 12B) or 7H9 broth. Incubate the 12B vial until the GI is 500 or greater, or the growth in the 7H9 broth is equal to a McFarland 1 standard.
   b. If there is sufficient growth on solid media, prepare a 7H9 suspension made directly from the culture. Lightly inoculate a sterile tube containing four to five small glass beads and 5 mL of 7H9 broth. Incubate the tube at 37°C for a total of 48–72 hours or until the turbidity in the tube matches that of a 1 McFarland standard. Inside a biological safety cabinet (BSC), vortex the tube vigorously, twice daily (in the AM and PM) to facilitate smooth bacterial suspension.
   c. Dilute the bacterial suspension to $10^2$ (1:100) and $10^4$ (1:10,000) in serial 1:10 dilutions made in the 4.5 mL blanks. The plates must be inoculated the same day the dilutions are made.
   d. BACTEC 12B vials can be used directly, undiluted, if the GI is 500 or $10^2$ and $10^4$ if GI is 999. Store the vials at room temperature (22°C–24°C) if the test cannot be done the same day the desired GI is reached. Do not refrigerate.
   e. Inoculate each quadrant of a set of labeled plates with 0.1 mL of the bacterial suspension. Tilt the plates for even distribution of the inoculum, and allow them to stand under the hood for 1 hour to allow the inoculum to be absorbed.
   f. Place the plates, agar side up, individually into CO$_2$-permeable polyethylene bags and seal the bags. Incubate in the CO$_2$ incubator at 37°C. Check the growth appearance, at 1, 2, and 3 weeks of incubation. Discard the plates that do not grow adequately within 3 weeks of incubation. (Hold plates for up to three additional weeks if the organism growth is suspected to be dysgonic as in some multiple drug-resistant strains. If there is still inadequate growth, repeat the test.)
   g. Read at 3 weeks if the growth is adequate. Count the number of colonies in each quadrant and record the results on a laboratory worksheet as above for the direct test.
4. Reporting and calculations.
   As for the direct test, calculate the report percent resistant for each drug.
5. Quality control.
   Each day of testing, inoculate a set of plates with the CLSI recommended $M. tuberculosis$ ATCC 27294 [2].
6. Expected values.
   N/A.
7. Limitations.
   Results of the direct and indirect test for the same drug-resistant isolate may not agree. Selection at the time of subculture may change the proportion of the drug-resistant subpopulations within the culture as a whole. Sometimes a direct susceptibility from a relapsed patient may show greater susceptibility because the raw specimen originated in a recently broken down cavity that has been protected from drugs and therefore does not contain the acquired resistant populations in areas of the lung that have been previously accessible.
8. Method validations [3,34].

The indirect test is currently the “gold standard” for tuberculosis susceptibility testing. However, the current trend is to perform broth susceptibility testing for first-line agents.

11.4 PRINCIPLES OF ANTIMYCOBACTERIAL SUSCEPTIBILITY TESTING OF NONTUBERCULOUS MYCOBACTERIA

Just as mycobacterial susceptibility testing for the *M. tuberculosis* complex should be performed by experienced technologists, so also should susceptibility testing of the nontuberculous mycobacteria be performed by qualified laboratories. Many of the recommendations that are made for laboratories that test *M. tuberculosis* complex should be followed by laboratories that test isolates of NTM. Problems such as contamination, mixed cultures, unusual growth rates, or aberrant results should also be recognized.

To answer the question of when it is necessary to perform susceptibility testing of NTM, the laboratory must consider the type of specimen, number of positive cultures, and the clinical setting. The ATS and the CLSI have published diagnostic recommendations that should be followed in determining whether or not an isolate is clinically significant and thus requires susceptibility testing [1,2,9].

For instance, the CLSI recommendation is not to perform susceptibility testing on respiratory isolates of *M. gordonae* unless recovered from multiple samples [2]. Most experts agree that *M. gordonae* is rarely, if ever, a respiratory pathogen [2,9]. For some species of the rapidly growing mycobacteria in respiratory samples (e.g., *M. fortuitum* group, *M. mucogenicum* and pigmented rapid growers), this advisement is also true. Because many of the species are common environmental organisms, the physician must evaluate the situation carefully. However, susceptibility testing is indicated for any NTM considered clinically significant from blood, tissue, and skin and soft tissue sources. The likelihood that the NTM is a pathogen is increased when the isolate is recovered from multiple specimens in large numbers or in a specimen that is smear positive for AFB. Clinical failure to eradicate the NTM from almost any site (except respiratory) after 6 months of appropriate antimicrobial therapy emphasizes the need to confirm species identification and perform repeat susceptibility testing [1,2,9]. For respiratory specimens, the ATS (2006) recommends reliance on specific criteria for determining clinical significance of the NTM [9]. At least two positive sputum cultures or one culture positive bronchial wash or lavage usually confirm clinical significance of the isolate. Single positive sputum cultures (e.g., one of three or four) that are AFB smear negative and grown only in the broth medium and not solid agar are likely to be clinically insignificant [9].

The MIC value obtained when performing susceptibility testing with NTM indicates the concentration of agent necessary to inhibit the growth of the NTM. The MIC, however, does not represent an “absolute value.” The “true” MIC is a specific value somewhere between the lowest test concentration that inhibits the organism’s growth (that is, the MIC reading) and the next lower test concentration [2].

MICs are generally derived from serial two-fold dilutions indexed to base 1 (e.g., 1, 2, 4, 8, 16, etc.) although other dilution schemes may provide MICs between these values. When there is inhibition of growth at the lowest concentration, the true MIC can not be determined and must be reported as “less than or equal to the lowest MIC.”

MIC results are typically reported as “susceptible,” “intermediate,” or “resistant” [2]. The “susceptible” category implies that an infection can be effectively treated with the dosage of the antimicrobial agent recommended for that type of infection and species of NTM unless otherwise contraindicated. The “intermediate” category includes isolates with MICs that approach attainable blood and tissue levels and for which response rate may be slower than for susceptible isolates. This category also allows for small, uncontrolled technical factors that might cause large discrepancies in interpretations, especially with narrow pharmacotoxicity margins. Finally, the “resistant”
category contains those isolates that are not inhibited by the usual achievable systemic concentrations of the agent [2]. Note that these definitions are in agreement with other CLSI definitions for susceptibility of bacterial species, and quite different from the definition of resistance used for MTB.

Preparation of microtiter panels may be time consuming, may require considerable experience, and may be an expensive undertaking for the average laboratory, especially if the number of NTM isolates seen is low. Thus, many laboratories choose to use a reference laboratory for testing isolates of NTM. Only recently have the methods for testing NTM been approved by the CLSI [2]. Laboratory quality control should include the CLSI recommended isolates to be tested, and the strains must be stored and subcultured appropriately [2]. Panels are now available commercially; however, these should be used by technologists experienced in working with nontuberculous mycobacteria.

11.4.1 Rapidly Growing Mycobacteria (RGM)

1. Introduction.
   Four different methods have been used for susceptibility testing of the RGM. These methods include agar disk diffusion, broth microdilution, E-test, and agar disk elution. Each method has advantages and disadvantages, but until recently, none has been compared in multicenter trials or been standardized by the CLSI. Recently, following publication of a multicenter trial [39], members of the Antimycobacterial Susceptibility Subcommittee of the CLSI recommended the determination of MICs by the broth microdilution as the “gold standard” for susceptibility testing of the RGM [2].

   Details of the broth microdilution MIC follow this brief review of the following nonstandardized methods (2a–c).

   a. Agar disk diffusion. This method applies the Bauer-Kirby technique of bacterial susceptibility testing to the susceptibility testing of RGM. This method has not been recommended for several years due to major technical problems such as interpretation of “partial zones” and lack of validation of the newer drugs (e.g., fluoroquinolones, clarithromycin, imipenem and linezolid) against an MIC method. Although its major advantage is the simplicity of the procedure, the problems outweigh the advantages, and this method can only be recommended as a “screening tool” prior to the performance of broth MICs, as a taxonomic aid, or as a supplement to broth microdilution. As an adjuvant to broth microdilution, it allows for rapid recognition of bacterial contamination, for recognition of mixed susceptible/resistant organism populations (presence of double zones), and for crude assessment of the validity of results obtained by broth microdilution. Disk diffusion should not be used as the sole testing method for NTM in the laboratory.

   b. Agar disk elution. This method is used most often by laboratories who test limited numbers of isolates infrequently. This method uses commercial susceptibility disks from which the drug is eluted into the Middlebrook enrichment into the OADC (oleic acid-albumin-dextrose-catalase) and mixed with molten agar to produce specific final drug concentrations. Interpretation of the results is similar to the proportion method. This method is not practical and is not recommended for the following reasons: (1) tedious preparation of the plates, (2) difficulty in the adjustment of inoculum to avoid too heavy suspensions resulting in “overinoculation”, (3) lack of validation studies with MICs for the newer antimicrobials such as the fluoroquinolones, imipenem, clarithromycin and linezolid, (4) some desired concentrations are not practically attainable due to the amount of drug in the commercial disk, and (5) “trailing endpoints” (seen by the appearance of “microcolonies”) especially with the macrolides [40]. Moreover, this method is also considered a nonstandardized method for testing of the RGM.
c. **E-test.** The E-test (AB Biodisk) or gradient diffusion combines the simplicity of the agar diffusion method with an exponential gradient of antimicrobials to produce MIC results. However, a recent interlaboratory study by members of the Subcommittee on Antimycobacterial Susceptibility Testing of the CLSI showed that interpretation and reproducibility of the E-test MICs with the RGM were difficult. Trailing endpoints and diffuse elliptical edges made determination of precise MICs difficult with several drugs, including ciprofloxacin, clarithromycin, imipenem, and cefoxitin. No break points utilizing this method were established and no control strains could be suggested since no standard RGM strain exhibited reproducible MICs for all of the drugs tested. Thus, the CLSI determined that further studies were needed before a recommendation for adopting its use for susceptibility testing of the RGM could be made [41]. In addition, E-test is not a cost effective method when one considers testing multiple agents for a given isolate. However, the E-test is an appealing alternative when the laboratory is asked to perform susceptibility testing for an agent not found on a standard panel (e.g., tigecycline) or when new and/or investigational agents become available.

d. **Broth microdilution MIC.** This method is currently the CLSI recommended susceptibility procedure for testing the RGM and is described below [2,42].

The procedure described in this section is intended for the susceptibility testing of RGM based on studies that have included *Mycobacterium fortuitum* group (*M. fortuitum, M. peregrinum*, and the former *M. fortuitum* third biovariant complex that includes species such as *M. porcinum, M. houstonense*, and *M. senegalense*), *M. chelonae* complex (defined as strains including formerly *M. chelonae* subsp. *chelonae* and other novel species that have similar 16S gene sequences but are currently uncertain if they can be identified by conventional methods), and *M. abscessus* [39,43–54]. They should also apply to other species such as *M. mucogenicum, M. smegmatis* group (*M. smegmatis, M. wolinskyi*, and *M. goodii*) and clinically significant pigmented RGM.

Most RGM species will grow well in unsupplemented, cation-adjusted Muller-Hinton broth (CAMHB). However, on rare occasion, supplementation with OADC may be necessary to support the growth of some isolates. When supplementation is added, it should be disclosed by stating that “susceptibility testing was performed by non-CLSI standardized methodology.”

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**TABLE 11.4**

**Drugs and Drug Concentrations for Agar Disk Elution Susceptibility Testing of *Mycobacterium tuberculosis* Complex**

<table>
<thead>
<tr>
<th>Antimycobacterial Agent</th>
<th>Disk Content (µg)</th>
<th>Final Drug Concentration in Well (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>25</td>
<td>5.0</td>
</tr>
</tbody>
</table>


The use of a six-well tissue culture plates (round instead of square wells) is recommended for this method; 5 mL of Middlebrook 7H10 agar is added to each well. The control well contains no disks.
1) Specimen. Suspensions are prepared from viable RGM colonies on solid media that has been incubated at 28°C–30°C in ambient air. An alternative option is to subculture isolates in 7H9 or CAMHB, incubate the tube at 28°C–30°C for 24–48 hours, and use this suspension to inoculate MIC panels.

2) Procedure.
   a) Sweep the confluent portion of growth with a sterile loop or swab, and transfer the colonies to 4.5 mL of sterile water containing glass beads until the turbidity matches the density of a 0.5 McFarland standard. (If large clumps remain, allow them to settle and use the supernatant for the inoculum suspension.)
   b) Vortex suspensions vigorously for 15–30 seconds or invert 8–10 times to mix.
      i. Freeze-dried panels [2].
         (a) Prepare the standardized inoculum to match the 0.5 McFarland standard as described previously.
         (b) Transfer 50 µL of the standardized suspension into a 10 mL tube of CAMHB and mix well by vortexing and/or inverting the tube several times.
         (c) Pour the broth suspension into a sterile trough.
         (d) Transfer 100 µL of broth suspension into each well of the 96 well plate and inoculate the plate using a multichannel pipettor with sterile pipette tips.
         (e) A periodic check of the colony count should be performed by sampling the positive control well. Isolates should have an inoculum of $5 \times 10^5$ CFU/mL (range $1 \times 10^4$ to $1 \times 10^6$).
         (f) Cover wells with plastic lid or adhesive seal. If using an adhesive seal, press all wells firmly to assure adequate sealing and avoid creasing of the seal.
         (g) Incubate aerobically at 30°C in a non-CO₂ incubator for 72 hours. Check for growth at 72 hours and if inadequate, reincubate up to 5 days.
   b) Vortex suspensions vigorously for 15–30 seconds or invert 8–10 times to mix.
      i. Freeze-dried panels [2].
         (a) Prepare the standardized inoculum to match the 0.5 McFarland standard as described previously.
         (b) Transfer 50 µL of the standardized suspension into a 10 mL tube of CAMHB and mix well by vortexing and/or inverting the tube several times.
         (c) Pour the broth suspension into a sterile trough.
         (d) Transfer 100 µL of broth suspension into each well of the 96 well plate and inoculate the plate using a multichannel pipettor with sterile pipette tips.
         (e) A periodic check of the colony count should be performed by sampling the positive control well. Isolates should have an inoculum of $5 \times 10^5$ CFU/mL (range $1 \times 10^4$ to $1 \times 10^6$).
         (f) Cover wells with plastic lid or adhesive seal. If using an adhesive seal, press all wells firmly to assure adequate sealing and avoid creasing of the seal.
         (g) Incubate aerobically at 30°C in a non-CO₂ incubator for 72 hours. Check for growth at 72 hours and if inadequate, reincubate up to 5 days.
   b) Vortex suspensions vigorously for 15–30 seconds or invert 8–10 times to mix.
      i. Freeze-dried panels [2].
         (a) Prepare the standardized inoculum to match the 0.5 McFarland standard as described previously.
         (b) Transfer 50 µL of the standardized suspension into a 10 mL tube of CAMHB and mix well by vortexing and/or inverting the tube several times.
         (c) Pour the broth suspension into a sterile trough.
         (d) Transfer 100 µL of broth suspension into each well of the 96 well plate and inoculate the plate using a multichannel pipettor with sterile pipette tips.
         (e) A periodic check of the colony count should be performed by sampling the positive control well. Isolates should have an inoculum of $5 \times 10^5$ CFU/mL (range $1 \times 10^4$ to $1 \times 10^6$).
         (f) Cover wells with plastic lid or adhesive seal. If using an adhesive seal, press all wells firmly to assure adequate sealing and avoid creasing of the seal.
         (g) Incubate aerobically at 30°C in a non-CO₂ incubator for 72 hours. Check for growth at 72 hours and if inadequate, reincubate up to 5 days.
   b) Vortex suspensions vigorously for 15–30 seconds or invert 8–10 times to mix.
      i. Freeze-dried panels [2].
         (a) Prepare the standardized inoculum to match the 0.5 McFarland standard as described previously.
         (b) Transfer 50 µL of the standardized suspension into a 10 mL tube of CAMHB and mix well by vortexing and/or inverting the tube several times.
         (c) Pour the broth suspension into a sterile trough.
         (d) Transfer 100 µL of broth suspension into each well of the 96 well plate and inoculate the plate using a multichannel pipettor with sterile pipette tips.
         (e) A periodic check of the colony count should be performed by sampling the positive control well. Isolates should have an inoculum of $5 \times 10^5$ CFU/mL (range $1 \times 10^4$ to $1 \times 10^6$).
         (f) Cover wells with plastic lid or adhesive seal. If using an adhesive seal, press all wells firmly to assure adequate sealing and avoid creasing of the seal.
         (g) Incubate aerobically at 30°C in a non-CO₂ incubator for 72 hours. Check for growth at 72 hours and if inadequate, reincubate up to 5 days.
      ii. RGM plates containing antimicrobials in broth.
         (a) Alternatively, if inoculating prepared RGM plates containing antimicrobials in 100 µL of broth, the volume of standardized suspension to be added to 36 mL of water diluent to obtain a final organism concentration of $1–5 \times 10^5$ CFU/mL ($1–5 \times 10^4$ CFU per well in 0.1 mL volume) is calculated. The volume depends on the delivery system that is being used. For example, when using a disposable plastic multi-pronged inoculating device that delivers 0.01 mL (10 µL) per well, the inoculum is prepared as follows [2,42]:
            (i) Prepare 0.5 McFarland suspension (approximately $1–5 \times 10^4$ CFU/mL) and invert or vortex to mix.
            (ii) Add 0.5 mL of the 0.5 McFarland standardized suspension to 4.5 mL of water diluent (1:10 dilution) = $1–5 \times 10^7$ CFU/mL, and vortex.
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(iii) Add 4 mL from previous step to 36 mL of water diluent (1:10 dilution) = 1–5 × 10⁶ CFU/mL.

(iv) Prongs deliver 0.01 mL into 0.1 mL in well (1:10 dilution) = 1–5 × 10⁵ CFU/mL or 1.5 × 10⁴ per well.

3. Reagents, media, and supplies.
   a. Mueller–Hinton broth aliquots, stored at ambient temperature.
   b. Microtiter panels.
   c. Sterile pipette troughs.
   d. Multi (8 or 12) channel pipettor.
   e. Sterile pipette tips.
   f. Sterile water blanks, 4.5 mL tubes.
   g. 0.5 McFarland (BaSO₄) turbidity standard.

4. Reading, reporting, and interpretation of MIC values for RGM.
   At 72 hours, trays are examined for growth. If the growth is sufficient (i.e., at least 2+ based on the following criteria: ± growth = few flecks in bottom of the well; 1+ = light growth in the well; 2+ = moderate growth for the particular species in the well; 3+ to 4+ = readily visible button in the bottom of the well, maximum growth seen for the RGM), MICs are recorded (see Figure 11.1). Otherwise, trays are reincubated and read

FIGURE 11.1 Legend:
1E Control growth (4+)
3A ± Few countable colonies; no turbidity (also wells 5B and 2D are ±)
5C ± Few countable colonies, hazy turbidity (also well 3E is ±)
5D 1+ Definite haze or turbidity
5E 2+ Definite turbidity and “clumpy” growth
1B 2+ Moderate countable colonies; slight turbidity
1D 3+ Heavy colonial growth
4E 3+ Heavy growth, turbidity
1C 4+ Heavy confluent growth comparable to control (also wells 2A and 2E are 4+)
Wells 4A, 5A, 2B, 4B, 4C, 3D, and 4D are all negative.
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daily thereafter (for up to 5 days) until growth is sufficient. For all antibiotics recommended by the CLSI for susceptibility testing except sulfonamides, the MIC is recorded as the lowest concentration of drug that inhibits macroscopic growth [2]. Sulfonamide MICs are read at 80% inhibition of growth compared to the growth control well (Table 11.5) [2].

5. Quality control and proficiency testing.

*M. peregrinum* ATCC 700686 (preferred) or *Staphylococcus aureus* ATCC 29213 and/or *Enterococcus faecalis* ATCC 29212 [2,55].

If plates are prepared in-house, susceptibility testing should be validated by performing the MIC on a series of isolates with known MICs. For laboratories that encounter RGM only rarely, referral to an established reference laboratory is recommended. Currently, no proficiency testing program from the College of American Pathologists (CAP) regularly includes these organisms in their program, although the CDC performance evaluation does include at least one NTM in each shipment. Therefore, the best alternative is comparison of the test results with those from an established reference laboratory on initial validation and at regular intervals thereafter to evaluate performance proficiency.

6. Expected values. See Table 11.6 for acceptable MIC ranges.

7. Notes.

a. When testing isolates of *M. chelonae* complex and *M. abscessus*, incubation for 4–5 days is sometimes necessary to obtain sufficient growth.

b. Given the known temporal instability of imipenem, the frequent need for prolonged incubation, and the modal MICs (i.e., 8–16 µg/mL) being at the resistance break point, imipenem MICs tend to result in major category changes of susceptibility for

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**TABLE 11.5**

Antimicrobial Agents and MIC Break Points Using Broth Microdilution for Rapidly Growing Mycobacteria

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>≤16</td>
<td>32</td>
<td>≥64</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>≤16</td>
<td>32–64</td>
<td>≥128</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤1</td>
<td>2</td>
<td>≥4</td>
</tr>
<tr>
<td>Clarithromycin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Doxycycline/Minocycline&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤1</td>
<td>2–8</td>
<td>≥16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤4</td>
<td>8</td>
<td>≥16</td>
</tr>
<tr>
<td>Linezolid</td>
<td>≤8</td>
<td>16</td>
<td>≥32</td>
</tr>
<tr>
<td>Sulfamethoxazole&lt;sup&gt;c&lt;/sup&gt;</td>
<td>≤32</td>
<td></td>
<td>≥64</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>≤4</td>
<td>8</td>
<td>≥16</td>
</tr>
</tbody>
</table>

Trimethoprim/Sulfamethoxazole<sup>b,c</sup>


<sup>a</sup> Isolates of *M. fortuitum* group with a trailing endpoint should be considered resistant. Recent studies suggest this trailing reflects the presence of an inducible erythromycin (macrolide) methylase (erm) gene that confers macrolide resistance [Nash, K.A., et al. *J. Antimicrob. Chemother.*, 55, 170, 2005]. Results for *M. chelonae* and *M. abscessus* should be read at 3 days. Clarithromycin is the class agent for the newer macrolides.

<sup>b</sup> No CLSI standardized break points have been proposed yet. These break points are based on the authors’ (BBE, SC, RJW) personal experience with RGM. Break points given for TMP/SMX are the closest value to the sulfamethoxazole value.

<sup>c</sup> MIC is 80% inhibition of growth.
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M. chelonae/M. abscessus group. Imipenem categorization (susceptible, intermediate, or resistant) is not reproducible for the M. abscessus/chelonae groups because the modal MIC is at the intermediate break point, and hence CLSI recommends not reporting MICs currently for these species [2]. The drug may be clinically useful, however, and the other useful beta lactam (cefoxitin) is often not available. Hence, imipenem may be reported with a disclaimer in situations such as these. All other groups of the RGM, including isolates of the M. fortuitum group, M. mucogenicum, and the M. smegmatis group are intermediate or susceptible in vitro to imipenem and have not shown problems with reproducibility; therefore, imipenem MICs should be reported for these species.

c. Because tobramycin is the aminoglycoside of choice only for isolates of M. chelonae complex, tobramycin MICs are reported for this species but not for other RGM [2].

d. Testing of antimicrobials other than those recommended by the CLSI may be desired, especially when encountering multidrug resistant isolates. (For example, the new 8 methoxy-fluoroquinolones, such as moxifloxacin and the new glycolcycline, tigecycline, may be useful to test since they may be used as part of a treatment regimen, and susceptibility to these agents is not be predicted by ciprofloxacin, levofloxacin, or the tetracyclines (including doxycycline or minocycline) [56,57]. For laboratory

### TABLE 11.6
Recommended Quality Control Strains and Their Ranges of MICs (µg/mL) for Testing Rapidly Growing Mycobacteria

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Mycobacterium peregrinum ATCC 700686 (Modal Value)</th>
<th>Staphylococcus aureus ATCC 29213</th>
<th>Enterococcus faecalis ATCC 29212</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>≤1–4 (1)</td>
<td>1–4</td>
<td>64–256</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>16–32 (16)</td>
<td>1–4</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤0.12–0.5 (1.125)</td>
<td>0.12–0.5</td>
<td>0.25–2</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>≤0.06–0.5 (0.25)</td>
<td>0.12–0.5</td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.12–0.5 (0.25)</td>
<td>0.12–0.5</td>
<td>2–8</td>
</tr>
<tr>
<td>Minocycline</td>
<td>≤0.5</td>
<td>0.15–0.5</td>
<td>1–4</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2–16 (8)</td>
<td>0.015–0.06</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Linezolid</td>
<td>≤2–4 (1)</td>
<td>1–4</td>
<td>1–4</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>≤1–4 (1)</td>
<td>32–128</td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>4–8 (4)</td>
<td>0.12–1</td>
<td>8–32</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>≤0.5/9.5</td>
<td>≤0.5/9.5</td>
<td>≤0.5/9.5</td>
</tr>
</tbody>
</table>


Revision of break points proposed for M. peregrinum ATCC 700686: 4–32 µg/mL for cefoxitin and 1–8 µg/mL for linezolid [Holliday, N., et al. 105th ASM General Meeting, Atlanta, GA, June 5–9, 2005, C-020].

No CLSI standardized break point has been established for M. peregrinum ATCC 700686. Personal communication from authors (BBE, SC, RJW).

No CLSI standardized break point has not been established for M. peregrinum ATCC 700686. Personal communication from authors (BBE, RJW).
testing of these agents, currently the recommendation is to use the CLSI bacterial break points provided in the CLSI documents [55], Supplemental Tables, until further studies have been done with the RGM [2,55]. The laboratory should note that these drugs have not yet been validated or approved by the CLSI for use with mycobacteria.

e. For linezolid, the proposed CLSI break points for the RGM are ≤8 µg/mL, susceptible; 16 µg/mL, intermediate; and ≥32 µg/mL, resistant (Table 11.5).

8. Limitations.

a. The control well must show sufficient growth in order to interpret reading of the drug wells. This is especially important when reading the MICs of sulfonamides. Because sulfonamide susceptibility is read as 80% inhibition, insufficient growth in the wells can be misinterpreted and the MIC may be read as lower than the expected value. This may also be true for some newer antimicrobials such as linezolid and tigecycline for which data is sparse. The positive control well may also be used to perform colony counts to ensure the inoculum is appropriate.

b. The purity or negative control well, if present, should show no growth.

c. Skipped wells occur occasionally that may indicate a pipetting or some other technical or procedural error. The first well in which there is no growth after the last well with growth should be regarded as the MIC. If skipped wells occur more than once in the plate or at the break point concentration, the MIC determination should be repeated.

d. If bacterial contamination occurs in the wells, the MIC should be repeated. On a rare occasion, when one or two wells are contaminated with bacteria but the purity check plate is pure and the contaminated wells do not interfere with reading the MICs, the contaminated wells may be ignored.

e. Initial transfers of isolates into broth and preparation of dilutions into panels should be performed within 30 minutes.

f. Plates containing high concentrations of doxycycline or minocycline (≥32 µg/mL) may show a precipitate after incubation. To avoid this problem, these wells (≥32 µg/mL) should be reconstituted with 5 µL of sterile distilled water before broth is added.


A multicenter CLSI study of broth microdilution MICs with RGM was conducted to assess the interlaboratory reproducibility of the method against eight antimicrobials including amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, imipenem, sulfamethoxazole, and tobramycin. The recent interpretative guidelines were based on the data obtained from this study [2,39].

11.4.2 SLOWLY GROWING NTM: MYCOBACTERIUM AVIUM COMPLEX

1. Principle.

The only antimicrobials for which a correlation between in vitro susceptibility tests for MAC and clinical response has been demonstrated in controlled clinical trials are the macrolides (clarithromycin and azithromycin) [58–61]. Therefore, routine susceptibility testing should be performed for these drugs only. Furthermore, since clarithromycin and azithromycin are cross-susceptible and cross-resistant, clarithromycin may serve as the “class agent” for both drugs so that it should be necessary to test clarithromycin only [2].

Recently, the CLSI recommended susceptibility testing of macrolides (clarithromycin or azithromycin) with MAC using a broth-based method, either macrodilution or microdilution (Table 11.7). However, since solubility in microvolumes may be a problem with azithromycin, macrodilution is the preferred method when testing this agent. As for the RGM, the broth microdilution MIC panels may be purchased commercially as freeze-dried or frozen or they may be prepared in-house.
2. Specimen.
Inoculum is prepared as for the RGM broth microdilution. Care should be taken to pick transparent colony-types when present as they tend to be more drug resistant than the pigmented colonies [61].

3. Reagents, media, and supplies.
Broth microdilution.
- Sterile water, 4.5 mL tube.
- Mueller-Hinton broth (CAMHB, 10 mL) with 5% OADC.
- Sterile pipette trough.
- Microtiter panel with drug.
- Sterile pipette tips.

Broth macrodilution (see the manufacturer’s procedure manual).
- BACTEC 12B medium.
- BACTEC diluting fluid.
- Sterile pipette tips.

4. Drug concentrations.

   a. Broth microdilution. For clarithromycin, the minimum range of concentrations (doubling dilutions) is 1–64 µg/mL; the optimum range is 0.25–256 µg/mL [2].

   b. BACTEC 460 TB macrodilution. For clarithromycin, concentrations of 2, 4, 8, 16, 32, and 64 µg/mL are proposed. Alternately, testing only 4, 16, and 64 µg/mL in BACTEC 12B medium at pH 6.8 or 4, 8, and 32 µg/mL in 12B medium at pH 7.3–7.4 may provide adequate data for patient management and be more cost effective. For azithromycin, the optimal concentrations for testing patient isolates by the radiometric macrodilution method are 16, 32, 64, 128, 256, and 512 µg/mL. However, testing only 32, 128, and 512 µg/mL may provide adequate data for patient management and be more cost effective [2].

When using the radiometric macrodilution method, the concentrations of clarithromycin and azithromycin used for quality control differ from those used to test patient isolates. Clarithromycin quality control concentrations are 1, 2, and 4 µg/mL at pH 6.8 and 0.5, 1, and 2 µg/mL at pH 7.3–7.4 when using 12B medium. For azithromycin, they are 8, 16, and 32 µg/mL in 12B medium (pH 6.8) [2,61].

### TABLE 11.7
Susceptibility and Resistance Break Points for Macrolide Testing in Broth against *Mycobacterium avium* Complex

<table>
<thead>
<tr>
<th>Drug</th>
<th>Method</th>
<th>pH</th>
<th>Broth*</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>BACTEC 460 TB or 12B or CAMHB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8</td>
<td>≤16</td>
<td>≥32</td>
<td>≥64</td>
<td></td>
</tr>
<tr>
<td>BACTEC 460 TB</td>
<td></td>
<td>7.3–7.4</td>
<td>12 B</td>
<td>≤8</td>
<td>16–32</td>
<td>≥32</td>
</tr>
<tr>
<td>Broth microdilution</td>
<td>CAMHB</td>
<td>7.3–7.4</td>
<td>8–16</td>
<td>≥32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azithromycin</td>
<td>BACTEC 460 TB</td>
<td>6.8</td>
<td>≤128</td>
<td>256</td>
<td>≥512</td>
<td></td>
</tr>
</tbody>
</table>


5. Procedure.
   Broth microdilution.
   a. Inoculate a tube of sterile demineralized water with several colonies from the primary agar plate. Adjust the organism suspension to a 0.5 McFarland standard. Vortex for 15–30 seconds.
   b. Transfer 50 µl of the organism suspension to 10 mL of Mueller Hinton broth (CAMHB with 5% OADC). Vortex. Once the final inoculum has been prepared, the plate must be inoculated within 30 minutes to prevent organism overgrowth (it is recommended to streak an agar plate from the final inoculum to act as a purity check).
   c. Transfer 100 µl of inoculated broth to each well of the plate. A final organism density of approximately $1 \times 10^5$ CFU/mL is recommended for testing.
   d. Place the plastic lid on the plate or if using plates with adhesive seals, apply the adhesive seal. After applying the seal, press firmly and carefully to ensure adequate sealing and prevent creasing of the seal.
   e. Incubate aerobically at 35–37°C for 7 days. If growth in the control well is 1+ or less, reincubate and read again at 14 days.

6. Reading and reporting of results.
   Test results are best read using a viewer that displays the underside of the wells.
   a. It is not necessary to remove the adhesive seal or lid for reading when the plate is read from the bottom.
   b. Read the growth control well first. If the control well does not exhibit satisfactory growth (2+ or greater), the plates should be reincubated until satisfactory growth appears (up to 2 weeks for most slowly growing NTM). However, some fastidious species may require longer incubation times, and note should be made if this occurs as some results may be invalidated by lengthy incubation.
   c. Growth appears as turbidity or as a button of cells at the bottom of a well. The growth control well should always be used as the reference for interpreting growth patterns in the plate.
   d. The MIC is recorded as the lowest concentration of antimicrobial that inhibits macroscopic growth.
   e. MIC values and an interpretation should be reported as “susceptible,” “intermediate,” or “resistant.” Break points are provided in Table 11.7.
   f. For an isolate with an “intermediate” level of susceptibility to one of the macrolides, a comment should be added to the report of the susceptibility result, indicating that the isolate should be monitored for possible emerging macrolide resistance. Additional testing of other isolates may also be warranted.
   g. Trailing endpoints with macrolides and isolates of MAC should be disregarded.

7. Quality control.
   M. avium ATCC 700898 is recommended for quality control of broth dilution testing of MAC [2].

8. Expected value.
   See Table 11.8.

   a. Procedures for alkalization of 12B broth can be found in the CLSI, M24-A, 2003, Appendix E, p. 5 [2].
   b. Working quality control cultures of MAC should be subcultured weekly or each time testing is performed (if done less often than weekly).
   c. For storage, stock cultures should be maintained at less than 20°C in an appropriate culture medium although the working culture may be stored at ambient temperatures for up to 1 month.
d. The interpretive criteria for MAC are based in part on a monotherapy clarithromycin trial of disseminated disease in humans [58]. Untreated MAC isolates are uniformly susceptible to macrolides, but macrolide resistance develops within a few months with monotherapy and may also occasionally occur in combination therapy. This resistance correlates with clinical relapse and the emergence of a base pair mutation at the adenine at position 2058 or 2059 in the 23S ribosomal RNA macrolide binding site. Untreated strains of MAC that always have adenine at these two positions (“wild type”) should always be macrolide susceptible [62].

e. While testing first-line antituberculous agents with MAC is not recommended, testing of newer antimicrobials such as moxifloxacin and/or linezolid may be reasonable especially for isolates that are macrolide resistant or are from patients who failed therapy with standard drugs since it is not yet known if these predict clinical outcome (results of testing the antituberculous drugs rifampin and ethambutol do not and hence are not tested). These drugs should be tested using a broth microdilution method and established bacterial break points as described in the CLSI Supplemental Tables [55] until further studies with MAC are available. For linezolid, the break points as proposed in the CLSI document for the RGM may be used [2].

f. The status of testing aminoglycosides such as amikacin and streptomycin against isolates of MAC has not been addressed by the CLSI. If MICs are requested for either of these agents, each laboratory must validate their method and results and appropriate quality control must be performed.

### 11.4.3 BACTEC 460 Drug Susceptibility for Nontuberculous Mycobacteria

1. Principle.

The BACTEC 460 radiometric susceptibility test is based on the modified proportion method in that when >1% of a test population is resistant to the drug, the culture is considered resistant. The inoculum in the control is adjusted 100-fold less as compared to the inoculum in the drug vial to achieve 1% proportion.

Studies have shown that if the appropriate inoculum is used, susceptibility testing of NTM including MAC, *M. kansasii* (except streptomycin), and *M. marinum* correlates well with conventional drug susceptibility testing.

Generally, in 12B medium, the NTM show greater susceptibility to streptomycin, rifampin, and ethambutol than when tested by the proportion method on 7H10 agar and are resistant to the concentrations of INH used for testing of *M. tuberculosis*. Susceptibility with NTM has been reported by multiple investigators with antituberculosis drugs as well as newer agents [35,60,63,64,66–69].

---

**TABLE 11.8**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Media pH</th>
<th>Acceptable MIC Range (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>6.8</td>
<td>0.5–2</td>
</tr>
<tr>
<td></td>
<td>7.3–7.4</td>
<td>1–4</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>6.8</td>
<td>8–32</td>
</tr>
</tbody>
</table>

Susceptibility Testing of Mycobacteria

2. Specimen.
   Due to the more rapid growth of the NTM, the drug susceptibility testing in the BACTEC
   460 uses a more dilute inoculum so that the GI in the control vial reaches about 30 in
   4–8 days [15,35].

3. Reagents, media, and supplies.
   a. BACTEC 460 12B medium.
   b. BACTEC diluting fluid.
   c. McFarland standards.

4. Procedure.
   a. Set up the test with an actively growing culture in 12B medium. The original culture
      may be growing on solid or liquid medium. In 12B medium, the GI should be >500.
      If it is in another broth, the turbidity should approximate a 1 McFarland standard. If
      from solid medium, make the suspension in BACTEC diluting fluid with the turbidity
      approximately equal to a 1 McFarland standard.
   b. Subculture 0.1 mL into a 12B medium vial. Incubate and test daily on the BACTEC
      460 instrument. Once the GI reaches 999 (usually within 1–3 days), use as the
      inoculum for testing as follows:
      1) M. kansasii. Prepare 1:20 dilution by mixing 0.5 mL culture with 9.5 mL BACTEC
         diluting fluid.
      2) M. avium complex. Prepare a 1:100 dilution by mixing 0.1 mL into 9.9 mL
         BACTEC diluting fluid.
      3) RGM. Prepare a 1:1000 dilution in BACTEC diluting fluid.
   c. Inoculate 0.1 mL of the diluted culture into the drug containing 12B vial. For the
      control, dilute again 1:100 and inoculate 0.1 mL into the 12B vial without antimicro-
      bial. Test the vial daily until the GI of the control vial >30 (4–10 days). If GI 30 is
      achieved in <3 days, do not interpret the results.

5. Reading and reporting results.
   Interpretations are made as for M. tuberculosis susceptibility tests. The radiometric method
   using BACTEC 460 is accurate and reliable [15,35] for susceptibility testing of MAC. See
   previous sections for information regarding other commercial systems (i.e., MGIT, ESP, etc.)

6. Quality control [2,15,17,35].
   Quality control must be validated for each species of NTM as susceptibility with NTM
   other than MAC is currently nonstandardized [2].

7. Expected results.
   a. Compared to testing on solid media, drug susceptibility tests performed by the
      BACTEC method usually yields more susceptible results to streptomycin, rifampin,
      and ethambutol.
   b. Variation in drug susceptibility levels with the same species of NTM, especially MAC,
      is not uncommon.

8. Limitations.
   See the manufacturer’s technical procedure manual [15,35].
   The clinical efficacy (i.e., reproducibility, correlation with the agar method of propor-
   tion, and relationship to clinical outcome) of the antituberculosis drugs with NTM in
   BACTEC 460 susceptibility testing has not been fully determined [2,15,35].

11.4.4 SLOWLY GROWING NTM: MYCOBACTERIUM KANSASII

1. Modified proportion method in Middlebrook 7H10 or 7H11 agar [2].
   a. Drug to be tested routinely:
      1) Rifampin only.
b. Additional agents to test against RMP resistant *M. kansasii*:
   1) Rifabutin.
   2) Ethambutol.
   3) Isoniazid.
   4) Streptomycin.
   5) Clarithromycin.
   6) Amikacin.
   7) Ciprofloxacin.
   8) Trimethoprim-sulfamethoxazole or sulfamethoxazole.
   9) Linerolod

   1) 7H10 or 7H11 agar prepared from dehydrated base as recommended by manufacturer.
   2) After the agar is autoclaved and cooled to 50°C–60°C in a water bath, add OAD or OADC supplement (warmed to room temperature) and the appropriate antitubercular agent.
   3) The antituberculous agent is then added to plastic quadrant petri plates, so that three quadrants contain different concentrations of one or more of the agents to be tested and one quadrant in each plate is filled with 7H10 agar medium without drug (growth control quadrant).
   4) For the agar dilution method, thaw a tube of frozen stock of drug and dilute with sterile distilled water to yield a working concentration (usually 200–10,000 µg/mL). To achieve the final concentration (see Table 11.3), add the appropriate volume of working solution to sterile 7H10 agar (cooled to 50°C–60°C) to reach a volume of 180 mL.
   5) Mix the agar thoroughly with OAD or OADC (20 mL for 200 mL total volume) and the antituberculous drug solution.
   6) Dispense 5 mL amounts into labeled quadrants of a series of sterile plastic petri plates, reserving one quadrant for 7H10 medium without any added drug.
   7) Dry the plates thoroughly (preferably under a laminar flow hood for several hours or overnight) with the lids partially removed.
   8) After drying, store in sealed plastic bags in the refrigerator (protected from light) for no more than 28 days.
   9) To test several samples of each batch of plates for sterility by incubating at 35°C for 48 hours; discard these test samples.

d. Preparation of inoculum for the agar proportion method.
   1) Scrape colonies (not more than 4–5 weeks old) from surface of medium, taking care not to remove medium.
   2) Transfer the mycobacterial mass to a sterile 16 × 125 mm screw-cap tube containing 3-mm glass beads and Middlebrook 7H9 broth.
   3) Emulsify the growth and vortex for 1–2 minutes.
   4) Allow the tube to stand for >30 minutes (less than 4 hours) to allow large particles to settle.
   5) Withdraw the supernatant suspension and transfer into another sterile glass tube.
   6) Adjust the broth to a 1 McFarland standard density.

e. Inoculation and incubation of media.
   1) Prepare 10⁻² and 10⁻⁴ dilutions of the standardized suspensions in sterile water, sterile saline, or 7H9 broth.
   2) Inoculate 0.1 mL of the 10⁻² dilution onto the control quadrant and onto each of the drug containing quadrants.
   3) Inoculate 0.1 mL of the 10⁻⁴ dilution in the same fashion.
4) Allow the plates to remain at room temperature until the inoculum (three drops each quadrant) spots are absorbed and dry.
5) Seal the plates in CO₂ permeable polyethylene bags.
6) Incubate the plates medium-side down at 37°C ± 2°C in CO₂ or ambient air. (Incubation in CO₂ is not necessary for growth of M. kansasii. Do not incubate in CO₂ when testing macrolides.)
7) Examine plates carefully at the end of 7 days. If growth in the control well is 1+ or less, reincubate an additional 7 days.

f. Interpretation.
1) MICs to INH, RMP, EMB for untreated strains generally fall within a narrow range, and mutational resistance to INH and EMB are only seen in association with RMP resistance. Hence, routine susceptibility testing to agents other than RMP is not recommended by the CLSI [2,68,70–72].
2) The critical concentrations of RMP (1 µg/mL) and EMB (5 µg/mL) used to test M. tuberculosis also inhibit untreated (wild) strains of M. kansasii [2].
3) MICs for INH for wild strains of M. kansasii range from 0.5 to 5 µg/mL so that the standard MTB critical concentration of 0.2 µg/mL in 7H10 agar shows resistance and the 1.0 µg/mL concentration gives variable results. Thus susceptibility testing of INH at either of these concentrations is not recommended [2,69].
4) Isolates susceptible to rifampin are also susceptible to rifabutin.
5) For isolates resistant to RMP (1 µg/mL), secondary agents should be tested (see Table 11.9).

g. Quality control strains and expected ranges [2].
1) M. kansasii ATCC 12478 (RMP ≤1 µg/mL)
2) M. marinum ATCC 927 (RMP ≤0.25–1 µg/mL)
3) Enterococcus faecalis ATCC 29212 (RMP ≤0.5–4 µg/mL)
   Note: Either or all three of the above strains may be used until a standardized reference strain is CLSI approved and available for testing.
   The strain(s) should be tested weekly or each time the test is performed, if less often than weekly.

2. Agar disk elution method of preparation of drug medium (see agar proportion method for additional details) [2,40,73].
   a. Dispense the commercially prepared antimycobacterial agent disks aseptically into each well of a 6-round well tissue culture plate.
   b. Prepare the 7H10 agar with OAD or OADC supplement. Dispense 5 mL of this medium into each well overlaying the disk and keeping the disk centered. Allow to solidify at room temperature.
   c. Before use, dry plates as in step 7) for the agar proportion method and store as in step 5) to 6) for the agar proportion method.
   d. Examine sample plates as in step 7) of agar proportion method.

   a. Inoculate a tube of sterile demineralized water with three to five colonies from the primary agar plate. Adjust organism suspension to a 0.5 McFarland standard. Invert 8 to 10 times to mix or vortex 15 to 30 seconds.
   b. Transfer 50 µL of the organism suspension to 10 mL of CAMHB (with 5% OADC). Invert 8 to 10 times to mix. Once the final inoculum has been prepared, the plate must be inoculated within 30 minutes to prevent organism overgrowth. It is recommended to streak a noninhibitory agar plate (such as blood, tryptic soy agar, etc.) from the final inoculum to act as a purity check.
   c. Transfer 100 µL of inoculated broth to each well of the plate. A final organism density of approximately 5 × 105 CFU/mL is recommended for testing.
d. Place the lid on top of the plate, or if using adhesive seals, apply the seal. After applying the seal, press firmly and carefully to assure adequate sealing and prevent creasing of the seal.

e. Incubate aerobically at 35°C–37°C. Read and interpret results as described earlier for broth microdilution method for MAC. Read at 7 days; if not readable, reincubate and read at 14 days.

4. Reading and reporting of results. Test results are best read using a viewer that displays the underside of the wells, and does not require removal of the adhesive seal.

5. Quality control. See the discussion of quality control and expected ranges for the agar proportion method.)

6. Expected values. See Table 11.9.

<table>
<thead>
<tr>
<th>Antimycobacterial Drugs</th>
<th>Intermediate and Resistance Break Points (µg/mL)</th>
<th>7H10/7H11 Agar Proportion or Agar Disk Elution</th>
<th>BACTEC 460 TB</th>
<th>Broth Microdilution (MHB + 5% OADC or 7H9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Test Drug</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (+)</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary Test Drugs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>5 (+)</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>2–4</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Rifabutin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (+)</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 (+)</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4–8</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
</tbody>
</table>

**Antibacterial Drugs**

<table>
<thead>
<tr>
<th>Antimycobacterial Drugs</th>
<th>Intermediate and Resistance Break Points (µg/mL)</th>
<th>7H10/7H11 Agar Proportion or Agar Disk Elution</th>
<th>BACTEC 460 TB</th>
<th>Broth Microdilution (MHB + 5% OADC or 7H9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>32</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Clarithromycin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16 (+)</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td>16</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Moxifloxacin&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole&lt;sup&gt;f&lt;/sup&gt;</td>
<td>32</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>2/38</td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
</tbody>
</table>


<sup>a</sup> MHB + OADC = cation-adjusted Mueller-Hinton broth + 5% oleic acid-albumin-dextrose-catalase.

<sup>b</sup> No intermediate break point.

<sup>c</sup> Class agent for older fluoroquinolones (ciprofloxacin, ofloxac, levofloxacin). Ciprofloxacin is not as active in vitro as moxifloxacin; thus, it cannot be used to predict susceptibility to moxifloxacin.

<sup>d</sup> Class agent for macrolides (clarithromycin, azithromycin, roxithromycin) [Woods, G.L., et al. NCCLS, 2003].

<sup>e</sup> The CLSI has not yet addressed the susceptibility break points for slowly growing nontuberculous mycobacteria other than MAC to moxifloxacin. Therefore, break points used here are bacterial break points [CLSI. Sixteenth Informational Supplement, M100-S16, 2006].
TABLE 11.10
Proposed Antimycobacterial Agents, Resistance Break Points, and Methodology for Susceptibility Testing of *Mycobacterium marinum*

<table>
<thead>
<tr>
<th>Resistance Break Point (µg/mL)</th>
<th>7H10 Agar Proportion</th>
<th>Agar Disk Elution</th>
<th>Broth Microdilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimycobacterial Drugs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>5 (+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>4 (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampin</td>
<td>1 (+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
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<td><strong>Antibacterial Drugs</strong></td>
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<td>Amikacin</td>
<td>12 (+)</td>
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<td></td>
<td>32 (+)</td>
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<tr>
<td>Clarithromycin*</td>
<td>16 (+)</td>
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<td>Doxycycline/Minocycline</td>
<td>6 (+)</td>
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<td>4 (+)</td>
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<tr>
<td>Sulfamethoxazole</td>
<td>20 (+)</td>
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<td></td>
<td>32 (+)</td>
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<tr>
<td>Trimethoprim sulfamethoxazole</td>
<td>2/32 (+)</td>
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* Class agent for macrolides (clarithromycin, azithromycin, roxithromycin).

11.4.5 *SLOWLY GROWING NTM: M. MARINUM*

Generally, routine MICs are not required as there is little variability in susceptibility to antimicrobial agents and the number of organisms present at the infected site is low. Thus there is little risk for the development of mutational resistance. Unless a patient fails therapy, or is intolerant of antibiotics, the CLSI recommends not performing susceptibility testing on isolates of *M. marinum* [2].

1. Methodology. See *M. kansasii* except that incubation temperature requires 28°C–30°C and the incubation period is 7 days) [2,73].
2. Drugs to be tested in cases of therapy failure (Table 11.10):
   a. Rifampin.
   b. Ethambutol.
   c. Doxycycline/Minocycline.
   d. Clarithromycin.
   e. Amikacin.
   f. Trimethoprim sulfamethoxazole or sulfamethoxazole.

11.4.6 *SLOWLY GROWING NTM: MISCELLANEOUS (M. TERRAE COMPLEX, M. XENOPI, M. SIMIAE, M. MALMOENSE, ETC.)*

Follow guidelines for other primary and secondary drugs for *M. kansasii*. Too few isolates from each of these species have been tested to recommend a specific method for these species [2,66,74].
11.4.7 SUSCEPTIBILITY TESTING OF MYCOBACTERIUM HAEMOPHILUM

1. Currently there is no CLSI standardized method of susceptibility testing for \textit{M. haemophilum}.
2. Methods of susceptibility testing which have been previously reported include the broth microdilution MIC method, E-test, and agar disk elution [75–80]. For any of these methods, supplementation with ferric ammonium citrate or hemin is necessary to support growth. Incubation may require 2–3 weeks for adequate growth.
3. In-house validation of the method should be established as a quality control measure. Currently, no optimal reference strain is available for quality control of susceptibility testing of \textit{M. haemophilum}.

11.4.8 SUSCEPTIBILITY TESTING OF OTHER FASTIDIOUS SPECIES (\textit{M. genavense}, \textit{M. ulcerans})

Currently there is no CLSI standardized method of susceptibility testing for these fastidious NTM. Too little is known about these species to make recommendations for susceptibility testing.

1. \textit{M. genavense} requires more than 6 weeks using the radiometric system [81–83].
2. \textit{M. ulcerans} requires incubation for 4–6 weeks [2,65,84].
3. Currently no optimal reference strain is available for quality control of susceptibility testing of these species.

REFERENCES

29. LaBombardi, V.J. and Lukowski, C. Anti-tubercular susceptibility testing using the DIFCO ESP Myco System, presented at the 97th ASM General Meeting, Miami Beach, FL, May 4-8, 1997, U-75, p. 556.

Methods for Determining Bactericidal Activity and Antimicrobial Interactions: Synergy Testing, Time-Kill Curves, and Population Analysis

Punam Verma

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12.1 **CLINICAL RATIONALE**

It is common to use antibiotic combinations in the practice of today’s clinical medicine. Antimicrobial combinations are used for a number of reasons: expansion of antimicrobial spectrum, minimization of drug toxicity, minimization of antimicrobial resistance, and antimicrobial synergism [1–3]. Combination therapy has become the empirical choice to treat severe bacterial infections until the etiologic agent has been established. Antimicrobial combinations are also used to treat polymicrobial infections when treatment with a single agent may be inadequate. Increased failure rates have been reported with the use of a single agent to treat serious infections such as acute bacterial endocarditis, osteomyelitis, or septic shock. Reduction in antimicrobial dosage-related toxicity might also be theoretically achieved by antimicrobial combinations, permitting the use of drugs at lower doses than if the drugs were administered independently [1–3]. In practice, this is almost impossible to accomplish because drug levels attained in the blood cannot predict the achievable levels at the infected site. Combination therapy may also avert or delay the emergence
of antibiotic-resistant subpopulations. This tactic has been used in the treatment of tuberculosis because the use of monotherapy inevitably results in the development of resistance. With escalating numbers of immunocompromised patients in recent years and concurrent documented cases of endocarditis, meningitis, and osteomyelitis there may be a pressing need to achieve bacterial killing or cidal activity with an antimicrobial agent(s). Routine susceptibility tests used in a clinical microbiology laboratory usually measure inhibitory activity of a single antimicrobial agent. This information is sufficient in most clinical situations to curtail the spread of the etiologic agent and augments the activity of the host immune system to eradicate the agent, achieving clinical cure [1–4]. In cases of clinical failure, anticipation of clinical failure due to immunosuppression or other underlying conditions requiring multidrug prophylaxis, the clinician in concert with the infectious disease specialist might wish to revisit the complex role of polyantimicrobial therapy. Obtaining meaningful information about the interaction of antimicrobials in combination, singly or in synergy may require in vitro testing in the clinical laboratory. Determination of frank resistance, susceptibility, identification of resistant clones, antagonism, synergism, and resistance attributable to genotypic induction need to be assessed by sensitive laboratory methods. Assessment of drug interactions in the laboratory is, because of its very complexity and sensitivity, an expensive and time consuming approach to therapy. Approaching the issues involved necessitates the use of consulting specialists to provide advice on the proper assays to be performed, their timing, and ultimate usefulness [4]. It cannot be stressed enough that proper performance of many assays discussed in this chapter needs highly trained and certified laboratory personnel under the direction of a clinical microbiologist trained in the interpretation of these specialized testing results.

12.2 METHODS FOR ASSESSING DRUG INTERACTIONS

12.2.1 CHEckerBOARD Titration

12.2.1.1 Principle

Checkerboard titration is one of the most frequently used techniques to assess drug interactions. The term checkerboard arises from the pattern that is generated by the multiple dilutions of the two antimicrobial agents that are being tested [1]. The range of concentrations tested for each antimicrobial agent is four to five dilutions lower than the minimal inhibitory concentration (MIC) and at least two dilutions higher than the MIC (if antagonism is suspected). Twofold dilutions of each antimicrobial agent are used. Tubes are arranged in a column and row format. Each row has a constant amount of one drug and twofold doubling dilutions of the second drug. In other words, the first row has doubling dilutions of drug A and the columns have twofold doubling dilutions of the second drug. Each tube has a unique combination of the two antimicrobial agents being tested [1]. Historically, this test was performed in an array of tubes using broth macrodilution that was cumbersome, time consuming, and costly. Testing is now most often performed utilizing microtiter trays. If testing is desired using broth macrodilution, the assay is performed using a limited series of tubes. The results are calculated mathematically and expressed in terms of a fractional inhibitory concentration (FIC) index equal to the sum of the FICs for each drug. The FIC for a drug is defined as the MIC of the drug in combination divided by the MIC of the drug used alone. If the FIC index is ≤0.5, the antimicrobial combination is interpreted as being synergistic; between 1 and 4 as indifferent; and >4 as antagonistic. Isobolograms can also be used to represent these interactions [1,2,5].

12.2.1.2 Specimen

Test organisms must be in pure culture and may be removed from trypticase soy agar plates with or without 5–7% sheep blood. Test organisms should be 18–24-h old, and if taken from frozen stocks, must be consecutively subcultured twice before testing.
12.2.1.3 **Materials**

12.2.1.3.1 **Broth Microdilution Method**

12.2.1.3.1.1 **Media and Reagents**

1. Cation-supplemented Mueller-Hinton broth (CSMH)
2. Trypticase soy agar with or without 5% sheep blood
3. Sterile 0.85% sodium chloride
4. Sterile stock antibiotic solutions (frozen)

12.2.1.3.1.2 **Supplies**

1. Sterile 75 × 12 mm, and 17 × 100 mm glass or plastic tubes
2. Sterile microtiter plates, 96 wells (U bottom)
3. 0.5 McFarland turbidity standard
4. Sterile 1-, 5-, and 10-mL disposable pipettes
5. Pipette bulb or automatic pipette device
6. Sterile cotton-tipped swabs
7. Tray-sealing tape
8. Plastic storage bags

12.2.1.3.1.3 **Equipment**

1. Vortex mixer
2. Multichannel pipette, 50 µL capacity with sterile tips
3. 100-µL adjustable micropipette with sterile tips
4. 1-µL and 10-µL calibrated loops
5. Plate viewer
6. 35°C ± 2°C ambient-air incubator (or other growth conditions as required by some organisms)

12.2.1.3.2 **Broth Macrodilution Method**

12.2.1.3.2.1 **Media and Reagents**

1. Cation-supplemented Mueller-Hinton broth
2. Trypticase soy agar with 5% sheep blood (BAP)
3. Sterile 0.85% sodium chloride
4. Sterile stock antibiotic solutions (frozen)

12.2.1.3.2.2 **Supplies**

1. Sterile 75 × 12 mm, and 17 × 100 mm capped glass tubes
2. Sterile polypropylene 50-mL screw-capped tubes
3. 0.5 McFarland turbidity standard
4. Sterile 1-, 5-, and 10-mL disposable pipettes
5. Pipette bulb or automatic pipette device
6. Sterile cotton-tipped swabs

12.2.1.3.2.3 **Equipment**

1. Vortex mixer
2. Multichannel pipette 50 µL capacity with sterile tips
3. 100-µL adjustable micropipette with sterile tips
4. 1- and 10-µL calibrated loops
5. 35°C ± 2°C ambient-air incubator (or other growth conditions as required by some organisms)

12.2.1.4 Quality Control

1. Choose appropriate quality control strains depending on the antimicrobial agent being tested. The quality control strain should be susceptible to the antimicrobial agent being tested.
2. The following quality control strains are recommended:
   a. *Escherichia coli* ATCC 25922.
   b. *Pseudomonas aeruginosa* ATCC 27853.
   c. *Staphylococcus aureus* ATCC 29213.
   d. *Enterococcus faecalis* ATCC 29212.
3. Quality control strains must be tested in parallel with the clinical isolate following the same procedure.
4. The growth control should show 3+ to 4+ turbidity.
5. The sterility control should show no growth.
6. The inoculum control should show 75–150 colonies with no contaminating organisms.
7. Acceptable results:
   a. The growth medium supports the growth of control and test organisms.
   b. MIC values obtained for quality control strains should be within published limits for the individual antimicrobial agent.
   c. The growth, inoculum, and sterility controls should show appropriate results.
8. If unacceptable results are obtained, repeat the quality control testing. If unacceptable results are obtained when the test is repeated, inform a supervisor and determine the reason for the failure. Do not report any patient results if the quality control results are unacceptable.

12.2.1.5 Procedure

12.2.1.5.1 Broth Microdilution Method

12.2.1.5.1.1 Preparation of Checkerboard Microdilution Panels

1. Determine the MIC of individual antibiotics for the test isolate.
2. Construct a panel configuration by completing the worksheet as shown in Appendix 12.1 in the following manner:
   a. Place the MIC value of antibiotic A in Cell A7 of the worksheet.
   b. Place the MIC value of antibiotic B in cell E1 of the worksheet.
   c. Complete the worksheet by following the example shown in Appendix 12.2.
3. Determine the number of combination panels to prepare: one for each isolate, two for the quality control isolates, and one for the dilution of the second antibiotic.
4. Prepare sufficient quantities of antimicrobial solutions. The initial concentration should be four times the desired concentration in the first well for each drug. For example, if the initial concentration for antibiotic A is 512 µg/mL and for antibiotic B is 64 µg/mL, then it is necessary to start with a concentration of 2,048 µg/mL for antibiotic A and 256 µg/mL for antibiotic B.
5. For each panel, the following quantities of antibiotic solutions will be required:
   a. Antibiotic A: 16 wells × 0.05 mL = 0.8 mL.
   b. Antibiotic B: 24 wells × 0.05 mL = 1.2 mL.
6. For one isolate:
   a. Prepare two panels; one is for the combination drug panel (panel 1) and the other
      (panel 2) is used for making antimicrobial dilutions for the second antibiotic (antibiotic B).
      1) For panel 1:
         a) Dispense 50 µL of sterile CSMHB into every well except those of column 1
            and column 12.
         b) Add 50 µL of sterile CSMHB to G12.
         c) Add 50 µL of antibiotic solution A to wells A12–G12 and wells A11–H11.
         d) Dilute serially from column 11 to column 2.
         e) Discard 50 µL from column 2.
         f) Take 50 µL from B1–B11 from panel 2 and dispense it in the corresponding
            row of panel 1.
         g) Repeat step f for the next higher concentration.
      2) For panel 2:
         a) Dispense 100 µL of CSMHB into every well except those of row A and row H.
         b) Add 100 µL of antibiotic solution B to H1–H11 and G1–G12.
         c) Dilute serially from row G to row B.
         d) Discard 100 µL from each well in row B (the final volume in each well is 100
            µL [0.1 mL]).

7. Prepared panels can be used immediately or can be stored at −70°C for up to 6 months.
   If the panels are to be used later, the trays should be covered with lids or sealed with
   tape and placed in sealed plastic bags.

12.2.1.5.1.2 Preparation of Inoculum

1. Select 3 to 5 colonies of the same morphologic type from a fresh (18–24 h) agar plate culture.
2. Touch the top of the colonies using a sterile loop or swab and transfer the test inoculum
   to 5 mL of sterile CSMHB.
3. Incubate at 35°C until the turbidity matches that of a 0.5 McFarland standard (1.5 × 10^8
   CFU/mL).
4. A total of at least 4.8 mL of diluted inoculum is required for one panel (50 µL× 96 wells
   = 4.8 mL). Include sufficient excess volume for the reservoir.
5. Add 0.1 mL of the adjusted inoculum suspension from step 3 to 9.9 mL of sterile CSMHB
   (1:100 dilution) to achieve approximately 10^6 CFU/mL.

12.2.1.5.1.3 Inoculation and Incubation of Checkerboard Panel

1. Using a multichannel pipette, add 50 µL of diluted inoculum to every well except H12.
2. Add 50 µL of diluted inoculum to growth control well A1.
3. Using a 0.001-mL (1-µL) calibrated loop, inoculate a purity plate by subculturing 0.001
   mL of inoculum from the reservoir onto a blood agar plate.
4. Prepare an inoculum count verification plate as follows:
   a. Add 50 µL of diluted inoculum to 0.45 mL of sterile 0.85% saline (dilution10^1).
   b. Transfer 0.1 mL from dilution 10^1 to 0.9 mL of sterile 0.85% saline and vortex well
      (dilution 10^2).
   c. Transfer 0.1 mL from dilution 10^2 to 0.9 mL of sterile 0.85% saline and vortex well
      (dilution 10^3).
   d. Plate 0.1 mL of dilution 10^3 onto a blood agar plate and spread for quantitation by
      streaking in several directions with a sterile loop or by using a sterile bent glass rod
      to spread the inoculum evenly.
5. Place the trays in plastic bags. Incubate at 35°C ± 2°C for 16–20 h.
6. Incubate purity and inoculum verification plates at 35°C ± 2°C for 16–20 h.
12.2.1.5.1.4 Reading MIC Panels

1. Examine the growth control well for organism viability. The growth control well should show heavy turbidity.
2. Examine the purity plate, and verify that the culture is not mixed. If it is mixed, repeat the test.
3. Count the number of colonies on the inoculum verification plates. The number of colonies should be between 75 and 150.
4. Record growth or no growth with the aid of a viewing device for all the wells on the worksheet.
5. Record the MIC results for antibiotic A from wells A2–A12.
6. Record the MIC results for antibiotic B from wells B–H in column 1.

12.2.1.5.2 Broth Macrodilution Method

12.2.1.5.2.1 Preparing Broth Macrodilution Limited Checkerboard

1. Determine the MIC of individual antibiotics for the test isolate by using the broth macrodilution methodology.
2. Use the template as shown in Appendix 12.3 to determine the concentrations and number of tubes needed for synergy testing.
3. Prepare sufficient quantities of antimicrobial solutions. The initial concentrations are four times the desired concentrations as shown in Appendix 12.3.
4. Calculate the volumes of individual concentrations of antibiotics needed for each organism (0.5 mL of each antibiotic concentration is required per tube).
5. Aliquot the antibiotic solutions into the desired tubes.
   a. Dispense 0.5 mL of antibiotic A to the appropriate tubes.
   b. Dispense 0.5 mL of antibiotic B to the appropriate tubes.
   c. The tubes now contain twice the desired concentration of the antibiotic solution.
   d. Dispense 1 mL of CSMHB to the growth control tube and the sterility control tube.
6. The prepared antibiotic concentration, growth control, and sterility control tubes can be capped and stored at –70°C for up to 6 months.

12.2.1.5.2.2 Preparation of Inoculum

1. Using a sterile loop or swab, transfer 3–5 colonies of the bacterial isolate to 5 mL of sterile CSMHB.
2. Incubate at 35°C until turbidity equivalent to a 0.5 McFarland standard is achieved (1.5 × 10^8 CFU/mL).
3. Make a 1:100 dilution of the standardized suspension. (Add 0.25 mL of standard suspension to 25 mL of CSMHB to achieve 1.5 × 10^6 CFU/mL.)
4. Mix by inverting five or six times, or gently vortex the suspension.
5. Using a sterile serologic pipette, add 1 mL of the organism suspension to each tube (7.5 × 10^5 CFU/mL; antibiotic concentration is diluted 1:2). Make sure that organisms are inoculated below the fluid meniscus without touching the tube sides with the pipette.
6. Prepare an inoculum count verification plate as follows:
   a. Add 0.1 mL from the growth control tube to 0.9 mL of sterile 0.85% saline (dilution 10^-1).
   b. Transfer 0.1 mL from dilution 10^-1 to 0.9 mL of sterile 0.85% saline and vortex well (dilution 10^-2).
   c. Transfer 0.1 mL from dilution 10^-2 to 0.9 mL of sterile 0.85% saline and vortex well (dilution 10^-3).
   d. Plate 0.1 mL of dilution 10^-3 onto a blood agar plate and spread for quantitation by streaking in several directions with a sterile loop or by using a sterile bent glass rod to spread the inoculum evenly.
7. Incubate the tubes at 35°C ± 2°C for 16–20 h in an ambient air incubator.
8. Incubate the purity and inoculum verification plates at 35°C ± 2°C for 16–20 h in an ambient air incubator.

12.2.1.5.2.3 Reading and Recording the Results
1. Examine the growth control tube for organism viability. The growth control tube should show heavy turbidity.
2. Examine the purity plate, and verify that the culture is not mixed. If it is mixed, repeat the test.
3. Count the number of colonies on the inoculum verification plates. The number of colonies should be between 75 and 150.
4. Record growth or no growth for all the tubes on the worksheet as shown in Appendix 12.3.
5. Record the MIC results for antibiotic A.
6. Record the MIC results for antibiotic B.

12.2.1.5.3 Calculations
1. Calculate the FIC for each antibiotic as follows:
   \[
   \text{FIC for antibiotic } A = \frac{\text{MIC of antibiotic A in combination}}{\text{MIC of antibiotic A alone}}
   \]
   \[
   \text{FIC for antibiotic } B = \frac{\text{MIC of antibiotic B in combination}}{\text{MIC of antibiotic B alone}}
   \]
2. Calculate the summation of FIC (ΣFIC) index for each combination as follows:
   \[
   \Sigma \text{FIC} = \text{FIC for antibiotic } A + \text{FIC for antibiotic } B
   \]

12.2.1.5.4 Interpretation
1. If acceptable quality control parameters are obtained, interpret and record each ΣFIC.
   Interpretation of the summation is as follows:
   - Synergism = ΣFIC is ≤0.5.
   - Antagonism = ΣFIC is >0.5 and ≤4.
   - Indifference = ΣFIC is >4.
2. Report results in terms of the organism tested, single antibiotic MICs, and interpretation of the summation of the FIC index, that is, synergism, antagonism, or indifference.

12.2.1.6 Limitations
This technique is usually used in a research setting and is not standardized completely [1,2,5]. There are significant flaws in the methodology. First, it does not examine bactericidal activity. Second, the FIC index calculation assumes a linear dose–response curve for all antimicrobial agents. Because this method only provides all-or-none responses (growth or no growth), it is incapable of measuring the graded responses necessary to define dose–response curves. Third, it provides a static view of the antimicrobial interaction since the results are examined only at one time interval. Results obtained by the checkerboard method may disagree with time-kill methods because each method measures different parameters [1,2,5–9].
12.2.2 SYNERGY TESTING BY DISK DIFFUSION TESTING

12.2.2.1 Principle

Qualitative measurement of drug interaction can be performed using the disk diffusion method. The advantages of this technique are ease of performance in a clinical laboratory and utilization of commercially available antimicrobial-impregnated disks and media. This technique utilizes the same inoculum and Mueller-Hinton agar as routine Bauer-Kirby susceptibility testing. Disks impregnated with individual antimicrobial agents are placed at a distance equal to the sum of the zone radii of inhibition of drugs when tested separately. After overnight incubation, the interface of zone of inhibition is examined. Synergism shows an enhancement or bridging at or near the junction of the two zones of inhibition, or inhibition of growth only due to the combined effects of both antimicrobial agents. Truncation of zones near the junction of the two zones represents antagonism. An indifferent antimicrobial combination shows no change. Refer to Figure 12.1 for examples. Antagonism is best studied by this methodology [1,2,5].

FIGURE 12.1 Assessing drug interactions using disk-diffusion. Shaded areas represent bacterial growth and clear areas represent zones of inhibition. (Adapted and reprinted with permission from Eliopoulos, G.M. and Moellering, R.C., Jr. Antibiotics in Laboratory Medicine, Lorain, V., Ed., Williams Wilkins, Baltimore, 1996, figure 9.10, p. 344.)
12.2.2.2 Specimen

Refer to section 12.2.1.2.

12.2.2.3 Materials

12.2.2.3.1 Media and Reagents

1. Mueller-Hinton agar medium
2. Sterile cation-supplemented Mueller-Hinton broth
3. Antimicrobial disks

12.2.2.3.2 Supplies

1. Sterile cotton-tipped swabs
2. Biohazard waste bag
3. 0.5 McFarland turbidity standard
4. 13 × 100 mm test tubes with snap caps
5. Test tube racks
6. Millimeter ruler
7. Sterile transfer pipette
8. Forceps
9. Inoculating loop or needle

12.2.2.3.3 Equipment

1. 35°C ± 2°C ambient-air incubator (or other growth conditions as required by some organisms)
2. Incandescent light
3. Vortex mixer

12.2.2.4 Quality Control

1. Choose the appropriate quality control strains depending on the antimicrobial being tested. The quality control strain should be susceptible to the antimicrobial agent being tested.
2. The following quality control strains are recommended:
   a. *E. coli* ATCC 25922.
   b. *P. aeruginosa* ATCC 27853.
   c. *S. aureus* ATCC 29213.
   d. *E. faecalis* ATCC 29212.
3. Quality control strains must be tested in parallel with the clinical isolate following the same procedure.
4. The zone measurements for a given antimicrobial for a specific quality control strain should be within limits published by Clinical and Laboratory Standards Institute (CLSI) (refer to the current M100 document).
5. Acceptable results:
   a. The growth medium supports the growth of control and test organisms.
   b. The zone measurements obtained for quality control strains should be within published limits for the individual antimicrobial agent.
   c. The culture is not mixed with contaminants. If it is mixed, repeat the test.
6. If unacceptable results are obtained, repeat the quality control testing. If unacceptable results are obtained when the test is repeated, inform a supervisor and determine the reason for failure. **Do not** report any patient results if the quality control results are unacceptable.
12.2.2.5 Procedure

1. Allow the agar and antimicrobial disks to warm to room temperature.
2. Inoculum preparation:
   a. Using a sterile loop or swab, transfer three to five colonies of the bacterial isolate to 5 mL of sterile CSMHB.
   b. Incubate at 35°C until a turbidity that is equivalent to a 0.5 McFarland standard is achieved (1.5 × 10^8 CFU/mL).
3. Inoculation of agar plate:
   a. Dip a sterile cotton-tipped swab into the inoculum within 15 min after adjusting the turbidity of the inoculum suspension, and rotate it several times.
   b. Remove excess inoculum by pressing the swab against the wall of the tube above the liquid.
   c. Streak the swab over the entire agar surface three times, rotating the plate approximately 60° each time to ensure even distribution of inoculum.
   d. Finally, swab the rim of the agar.
   e. Allow the inoculated plate to stand for 3–15 min before the application of the disks.
4. Application of the disks to inoculated agar plates:
   a. Using a sterile forceps, place disks separated by a distance equal to the sum of the zone of radii for each disk tested separately.
   b. Press down each disk to ensure complete contact with the agar surface.
   c. Do not move a disk once it has made contact with the agar surface.
5. Incubation:
   a. Incubate inoculated plates at 35°C ± 2°C for 16–18 h in an ambient air incubator.
   b. Invert plates (lids facing down), and do not stack more than five high.
6. Reading the plates:
   a. Examine plates for a confluent lawn of growth. Read the plates only if the growth is confluent or nearly confluent.
   b. Observe the interface of the zones of inhibition.

12.2.2.6 Interpretation

If the quality control is within limits, record the pattern of the zones of inhibition as follows (see Figure 12.1):

1. Indifference: Two independent circles
2. Synergism: Enhancement or bridging at or near the junction of the two zones of inhibition or inhibition of growth only due to the combined effects of both antibiotics A and B.
3. Antagonism: Truncation of zones is observed near the junction of the two zones.

12.2.2.7 Limitations

This methodology yields only qualitative information about the antimicrobial agent combination. Using this technique, it may be difficult to distinguish indifferent from synergistic interaction [1,2,5].

12.2.3 Time-Kill Curves

12.2.3.1 Background

The killing effect of an antimicrobial agent can be expressed as the rate of killing by a fixed concentration of drug under controlled conditions [1,4,10,11]. This rate is determined by measuring the number of viable bacteria at various time intervals. The resulting graphic depiction is known
as a time-kill curve. Historically, time-kill curves have been used in the evaluation of new drugs.
It is rarely used to guide chemotherapy in an individual patient. Both concentration-dependent and
time-dependent bactericidal activities of antimicrobial agents can be studied using time-kill curves.
Time-kill curves may be used to verify the presence of persisters, paradoxical effect, and tolerance
[4]. They are also used to determine synergy between two or more antimicrobial agents. Several
studies have shown that results obtained for synergy testing by the checkerboard method may differ
from the time-kill method [6,8,9,12]. The reason for this discrepancy is that in the checkerboard
methodology results are examined only at one time interval whereas killing curves measure changes
over time. Time-kill curve results correlate best with cure in animal models. A steep negative slope
of the curve indicates a faster rate of decline of survivors. The rate of decline of survivors at different
concentrations of antimicrobial agent provides more information than the concentration at which
99.9% killing of the final inoculum occurs.

Because of the selection of resistant mutants, antimicrobial degradation, or unaffected bacterial
cells, colony counts may increase after initial reduction in organism numbers [1,2,4,10]. The
important aspect of this unexplained rebound effect is to isolate the survivors, determine the MIC,
and make a determination whether a resistant clone has been selected as a result of selective pressure.
Determination of whether the antimicrobial agent has been inactivated versus selection of resistant
mutants may be determined by filter sterilization of the original antimicrobial containing broth and
performance of MIC determinations of a known ATCC strain with a defined MIC value. If antibiotic
degradation has occurred, the MIC value for the known ATCC strain will increase. If resistant
subpopulations have been selected, the MIC of the survivors will be several-fold higher than the
parent strain.

Various published protocols exist for this methodology including a CLSI protocol, but none
have been standardized [4,10]. The protocol described here is a recommendation only.

12.2.3.2 Principle

Time-kill curves are used to study the efficacy of an antimicrobial agent to a particular bacterial
isolate [1]. A standardized inoculum of the bacterial isolate is grown in the presence and absence
of the antimicrobial agent. Viable cells are counted by performing serial dilutions on an aliquot
removed at different time intervals. The results are plotted on semilog paper with the time intervals
on the abscissa (x axis) and the number of survivors on the ordinate (y axis). Bactericidal activity
can be determined from a time-kill curve if a greater than 3 log 10-fold decrease in the number of
survivors is noted. This is equivalent to 99.9% killing of the inoculum [1,10]. Time-kill curves
can also be used to study drug interactions. Synergy is defined as a ≥2 log 10(CFU/mL)-fold
decrease by the combination compared with the most active single agent. Antagonism is defined
as a ≥2 log 10(CFU/mL)-fold increase by the combination compared with the most active single
agent [1,10].

12.2.3.3 Specimen

Refer to section 12.2.1.2.

12.2.3.4 Materials

12.2.3.4.1 Media and Reagents

1. Cation-supplemented Mueller-Hinton broth
2. Trypticase soy agar with 5% sheep blood
3. Sterile 0.85% sodium chloride
4. Sterile stock antibiotic solutions (frozen)
12.2.3.4.2 Supplies

1. Sterile 75 × 12 mm and 17 × 100 mm capped tubes
2. Sterile polypropylene 50-mL screw-capped tubes
3. 0.5 McFarland turbidity standard
4. Sterile 1-, 5-, and 10-mL disposable pipettes
5. Pipette bulb or automatic pipette device
6. Sterile cotton-tipped swabs

12.2.3.4.3 Equipment

1. Vortex mixer
2. 100-µL adjustable micropipette with sterile tips
3. 1-µL and 10-µL calibrated loops
4. 35°C ± 2°C ambient-air incubator (or other growth conditions as required by some organisms)

12.2.3.5 Quality Control

1. Choose appropriate quality control strains depending on the antimicrobial being tested. The quality control strain should be susceptible to the antimicrobial agent being tested.
2. The following quality control strains are recommended:
   a. *E. coli* ATCC 25922
   b. *P. aeruginosa* ATCC 27853
   c. *S. aureus* ATCC 29213
   d. *E. faecalis* ATCC 29212
3. Quality control strains must be tested in parallel with the clinical isolate following the same procedure.
4. Growth control should show 3+ to 4+ turbidity.
5. Sterility control should show no growth.
6. Inoculum control should show 75–150 colonies with no contaminating organisms.
7. Acceptable results:
   a. The growth medium supports the growth of control and test organisms.
   b. MIC values obtained for quality control strains should be within published limits for the individual antimicrobial agent.
   c. Growth, inoculum, and sterility controls show appropriate results.
8. If unacceptable results are obtained, repeat the quality control testing. If unacceptable results are obtained when the test is repeated, inform a supervisor and determine the reason for failure. Do not report any patient results if the quality control results are unacceptable.

12.2.3.6 Procedure

1. Time-kill curve for determining bactericidal activity
   a. Preparation of antimicrobial agent concentrations:
      1) Determine the MIC of the antimicrobial agent for the test isolate.
      2) Test several concentrations of antimicrobial agent, usually multiples of the MIC, for example, 1×, 2×, and 4× the MIC.
      3) Determine the number and quantity of antimicrobial agents that will be needed.
      4) Calculate the volume of antimicrobial agent stock solution needed for the assay.
      5) Preparation of working antibiotic concentrations:
          a) Dispense 9.9 mL of CSMHB to sterile 50-mL tubes for each antimicrobial concentration to be tested.
b) Add 100 µL of 100-fold-concentrated antimicrobial agent to achieve the final working concentration.
c) Mix by using a vortex.
6) Dispense 10 mL of CSMHB to the growth and sterility control tubes.
7) Perform a limited broth macrodilution MIC for the quality control strain.
b. Preparation of inoculum:
1) Using a sterile loop or swab, transfer three to five colonies of the bacterial isolate to 5 mL of sterile CSMHB.
2) Incubate at 35°C until a turbidity that is equivalent to a 0.5 McFarland standard is achieved (1.5 × 10^8 CFU/mL).
3) Make a 1:100 dilution of a standardized suspension. (Add 0.1 mL of the standard suspension to 9.9 mL of CSMHB to achieve 1.5 × 10^6 CFU/mL.)
4) Mix by inverting five or six times or gently vortex the suspension.
c. Inoculation and incubation:
1) Using a sterile serologic pipette, add 1 mL of organism suspension to each tube (7.5 × 10^8 CFU/mL). Make sure that organisms are inoculated below the fluid meniscus without touching the tube sides with the pipette.
2) Mix gently by inverting several times.
3) Remove 10 µL of the above suspension using a calibrated loop, and streak for isolation on a blood agar plate.
4) Remove another 100-µL aliquot from each tube, and return the tubes to the 35°C ± 2°C incubator.
5) Perform a 10-fold serial dilution from 10^{-1}–10^{-7} on the aliquot removed as follows:
   a) Take 7 tubes and label them from 1–7.
   b) Add 100 µL of saline to each tube.
   c) Remove a 100-µL aliquot, and add it to the tube marked as 1.
   d) Mix using a pipettor, aspirate 100 µL, and add it to the tube marked as 2.
   e) Repeat steps c and d.
   f) Remove 100 µL from tube number 7.
   g) Spot 10 µL each of the 10^{-2} to 10^{-7} dilutions (in duplicate) as shown in Figure 12.2 onto a single blood agar plate.

FIGURE 12.2 Spot inoculation of serial dilutions on agar plate for colony enumeration. (Adapted and reprinted with permission from Eliopoulos, G.M. and Moellering, R.C., Jr. in Antibiotics in Laboratory Medicine, Lorain, V., Ed., Williams Wilkins, Baltimore, 1996, figure 9.5, p. 339.)
Methods for Determining Bactericidal Activity and Antimicrobial Interactions

6) Incubate the plates at 35°C for 48 h.
7) Repeat sampling (steps b–f) at 4, 6, 8, and 24 h.

Reading and recording the results:
1) Count viable colonies from those dilutions containing 5–50 CFU.
2) Multiply colony counts with the dilution factor to obtain CFU per milliliter.
3) Tabulate the results as CFU per milliliter (mean; y axis) versus time (x axis) on semilog paper (CFU per milliliter can be converted to log_{10} values, e.g., log_{10} 2.5 × 10^7 Å 7.4).
4) Determine the antimicrobial agent concentration that shows a 3 log_{10}(CFU/mL)-fold decrease when compared with the growth control.
5) Determine the time when the 3 log_{10}(CFU/mL)-fold decrease occurs when compared with the growth control. Refer to Appendix 12.5.

2. Time-kill curves for determining antimicrobial agent interactions.

a. Preparation of antimicrobial agent concentrations:
1) Determine the MIC of the antimicrobial agent for the test isolate.
2) Use the template as shown in Appendix 12.5 to determine the concentrations and number of tubes needed for this assay.
3) Calculate the volumes of individual antimicrobial agent stock solution needed for each organism tested.
4) Preparation of working antibiotic concentrations:
   a) Dispense 9.8 mL of CSMHB to sterile 50-mL tubes for each antimicrobial concentration to be tested.
   b) Add 100 µL of each 100-fold-concentrated antimicrobial agent to achieve the final working concentration.
   c) Mix by using a vortex.
5) Dispense 10 mL of CSMHB to the growth and sterility control tubes.
6) Perform a limited broth macrodilution MIC for the quality control strain.

b. Preparation of inoculum:
1) Using a sterile loop or swab, transfer three to five colonies of the bacterial isolate to 5 mL of sterile CSMHB.
2) Incubate at 35°C until turbidity equivalent to a 0.5 McFarland standard is achieved (1.5 × 10^8 CFU/mL).
3) Make a 1:100 dilution of standardized suspension. (Add 0.1 mL of standard suspension to 9.9 mL of CSMHB to achieve 1.5 × 10^6 CFU/mL.)
4) Mix by inverting five or six times, or gently vortex the suspension.

c. Inoculation and incubation.
1) Using a sterile serologic pipette, add 1 mL of the organism suspension to each tube (7.5 × 10^5 CFU/mL). Make sure that organisms are inoculated below the fluid meniscus without touching the tube sides with the pipette.
2) Mix gently by inverting several times.
3) Remove 10 µL of the above suspension using a calibrated loop and streak for isolation on a blood agar plate.
4) Remove another 100-µL aliquot from each tube and return the tubes to the 35°C ± 2°C incubator.
5) Perform 10-fold serial dilutions from 10^{-1} to 10^{-7} on the aliquot removed as follows:
   a) Label seven tubes as 1–7.
   b) Add 100 µL of saline to each tube.
   c) Remove a 100-µL aliquot, and add it to the tube marked as 1.
   d) Mix using a pipettor, aspirate 100 µL, and add it to the tube marked as 2.
   e) Repeat steps c and d.
   f) Remove 100 µL from tube number 7.
g) Spot 10 µL each of the $10^{-2}$ to $10^{-7}$ dilutions (in duplicate) as shown in Appendix 12.5 onto a single blood agar plate.

6) Incubate the plates at 35°C for 48 h.
7) Repeat sampling (steps b–f) at 4, 6, 8, and 24 h.

d. Reading and recording the results:
1) Count viable colonies from those dilutions containing 5–50 CFU.
2) Multiply the colony counts with the dilution factor to obtain CFU per milliliter.
3) Tabulate results as CFU per milliliter (mean; y axis) versus time (x axis) on semilog paper (CFU milliliter can be converted to $\log_{10}$ values, for example, $\log_{10} 2.5 \times 10^7$ Å 7.4).
4) Determine the antimicrobial agent combination that shows a 2 $\log_{10}$ (CFU/mL)-fold decrease (synergy) and increase (antagonism) when compared with the most active single agent.
5) Determine the time at which the 2 $\log_{10}$ (CFU/mL)-fold decrease (synergy) and increase (antagonism) occurs when compared with the most active single agent.

12.2.3.7 Interpretation

1. Time-kill curve for determining bactericidal activity in the presence of an antimicrobial agent.

   Bactericidal activity absent = <$3 \log_{10}$ (CFU/mL) decrease

2. Time-kill curve for determining antimicrobial interactions by a combination relative to the most active single antimicrobial agent.

   Antagonism = $\geq 2 \log_{10}$ (CFU/mL) increase

12.2.3.8 Limitations

This technique is cumbersome and laborious, and therefore only a few drug combination concentrations can be effectively tested. The concentrations of the antimicrobial agent combination tested should be carefully planned and should be those that are achievable at the infected site. Based on the time-kill curve, if one suspects inactivation of antimicrobial agent or selection of resistant mutants, then appropriate testing must be performed to confirm this. For antimicrobial agents that exhibit concentration-dependent killing, organisms may be killed faster at concentrations greater than the MIC, requiring sampling at shorter time intervals [1,2,4,10].

12.2.4 Population Analysis

12.2.4.1 Principle

Population analysis is employed to determine the mechanism of phenotypic resistance and bactericidal activity of a given bacterial isolate. A checkerboard is created with serial dilutions of inoculum on the ordinate (y axis) and serial dilutions of the antimicrobial agent on the abscissa (x axis). A pour-plate agar procedure is performed, and colonies are counted after incubation. Results are tabulated as CFU per milliliter versus concentration of antimicrobial agent. If the isolate expresses homogeneous resistance, then a steep decline in the number of survivors will be noted
and no growth will be seen at a concentration above the MIC. If the test isolate consists of a heterogeneous population, then a subpopulation of survivors will be seen at increasing concentrations of antimicrobial agent [13,14]. Similar to time-kill curves, an antimicrobial is said to have bactericidal activity if a $\geq 3 \log_{10} (\text{CFU/mL})$-fold decrease at a given antimicrobial concentration is obtained when compared with counts obtained on plates without antibiotic.

12.2.4.2 Specimen
Refer to section 12.2.1.2.

12.2.4.3 Materials

12.2.4.3.1 Media and Reagents
a. Cation-supplemented Mueller-Hinton broth
b. Cation-supplemented Mueller-Hinton agar
c. Brain–heart infusion (BHI) broth
d. Trypticase soy agar with 5% sheep blood
e. Sterile 0.85% sodium chloride
f. Sterile stock antibiotic solutions (frozen)

12.2.4.3.2 Supplies
a. Sterile 75 $\times$ 12 mm and 17 $\times$ 100 mm capped tubes
b. Sterile polypropylene 50-mL screw-capped tubes
c. Sterile Erlenmeyer flasks
d. Sterile 100-mm petri dishes
e. 0.5 McFarland turbidity standard
f. Sterile 1-, 5-, and 10-mL disposable pipettes
g. Pipette bulb or automatic pipette device
h. Sterile cotton-tipped swabs
i. Semilog paper

12.2.4.3.3 Equipment
a. Vortex mixer
b. Orbital shaker incubator, 35°C ± 2°C
c. Spectrophotometer with appropriate filters
d. 35°C ± 2°C ambient-air incubator (or other growth conditions as required by some organisms)

12.2.4.4 Quality Control
Refer to section 12.2.3.4.

12.2.4.5 Procedure

12.2.4.5.1 Preparation of Inoculum
1. Grow the isolates in Brain Heart Infusion (BHI) broth and incubate overnight in an orbital shaker at 35°C.
2. Adjust the turbidity with fresh BHI broth until it is equivalent to a 0.5 McFarland standard.
3. Return the flasks to the orbital shaker, and incubate further at 35°C until the culture is grown to an absorbance of 0.5–0.55 optical density (OD) units at 620 nm.
4. Prepare serial dilutions from $10^{-1}$ to $10^{-8}$ of the inoculum as follows:
   a. Assemble eight 50-mL polypropylene tubes.
   b. Label the tubes from 1–8.
   c. Using a 25-mL disposable pipette, dispense 18 mL of sterile saline in each tube.
   d. Add 2 mL of inoculum to tube 1.
   e. Vortex.
   f. Using a sterile disposable pipette, transfer 2 mL from tube 1 to tube 2.
   g. Vortex.
   h. Repeat steps f and g to dilute from tube 2 to tube 8.
   i. Discard 2 mL from tube 8 (final volume in each tube is 18 mL).

12.2.4.5.2 Preparation of Antimicrobial Dilutions in Melted Agar

1. Assemble 13 50-mL polypropylene tubes for each culture dilution to be tested.
2. Label the tubes with twofold increasing concentrations of the antibiotic.
3. Place the tubes in a test tube rack.
4. Place the rack in a water bath at 50°C.
5. Using a 25-mL sterile disposable pipette, dispense 14 mL of melted BHI agar cooled to 50°C into all the tubes except tube 2 (64 µg/mL).
6. Dispense 28 mL of melted BHI agar to tube 2 (64 µg/mL).
7. Add 1.9 mL of prepared antibiotic stock solution (1,028 µg/mL) to the tube marked 128 µg/mL.
8. Add 3.8 mL of prepared antibiotic stock solution to the tube marked 64 µg/mL.
10. Using a 25-mL sterile disposable pipette, transfer 14 mL of melted agar from tube 2 (64 µg/mL) into tube 3 (32 µg/mL).
11. Vortex.
12. Repeat steps 10–11 to dilute serially from tube 3 to tube 12.
13. Discard 14 mL of melted agar from tube 12.
14. The final concentrations of antimicrobial agent in this series will be from 128 µg/mL to 0.06 µg/mL after the addition of the inoculum.

12.2.4.5.3 Inoculation and Incubation

1. With the agar still melted, add 1 mL of the culture from each serial dilution to each tube in the twofold increasing antimicrobial concentration series.
2. Mix by either gently inverting the tube or by drawing solution back and forth into the pipette six to eight times without causing air bubbles or splashes.
3. Pour the mixture into 100-mm empty sterile petri dishes.
4. Allow the agar to solidify at room temperature.
5. Incubate plates at 35°C for 48 h.

12.2.4.5.4 Reading and Recording the Results

1. After incubation, count the number of colonies at each concentration.
2. Tabulate the results as CFU per milliliter versus concentration of antimicrobial agent on semilog graph paper.
3. Determine the antimicrobial agent concentration that shows a $[3 \log_{10} (\text{CFU/mL})$]-fold decrease when compared with the growth control.
4. Determine the slope of the curve if the mechanism of phenotypic resistance is being elucidated.
12.2.4.6 Interpretation

1. Bactericidal activity at a given antimicrobial concentration relative to the control.

   Bactericidal activity $\geq 3\log_{10}$CFU/mL decrease

   Bactericidal activity $< 3\log_{10}$CFU/mL decrease

2. Expression of phenotypic resistance.
   a. Homogeneous: A steep negative slope
   b. Heterogeneous: A gradual slope indicating the presence of a subpopulation at higher concentration of the antibiotic

12.2.4.7 Limitations

The limitations of this assay are similar to those noted for time-kill curves.
### APPENDIX 12.1 — WORKSHEET TEMPLATE FOR BROTH MICRODILUTION CHECKERBOARD PANEL

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**Sterility control**
APPENDIX 12.2 — EXAMPLE OF A BROTH MICRODILUTION CHECKERBOARD PANEL

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<td>H</td>
<td>+</td>
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<td>B64</td>
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<td>B64</td>
<td>B64</td>
<td>B64</td>
<td>B64</td>
</tr>
</tbody>
</table>

(Drugs A and B are tested at 1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC.

APPENDIX 12.3 — WORKSHEET TEMPLATE FOR BROTH MACRODILUTION LIMITED-SERIES CHECKERBOARD FORMAT

<table>
<thead>
<tr>
<th>DRUG A</th>
<th>1/8 MIC</th>
<th>¼ MIC</th>
<th>½ MIC</th>
<th>MIC</th>
<th>2 X MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DRUG A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/8 MIC</td>
<td>SYN</td>
<td></td>
<td></td>
<td>ANT</td>
<td>(2 MIC A+ 1/8 MIC B)</td>
</tr>
<tr>
<td></td>
<td>(1/8 MIC A+ 1/8 MIC B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¼ MIC</td>
<td>SYN</td>
<td></td>
<td></td>
<td>ANT</td>
<td>(2 MIC A+ 1/4 MIC B)</td>
</tr>
<tr>
<td></td>
<td>(1/4 MIC A+ 1/4 MIC B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>½ MIC</td>
<td>SYN</td>
<td></td>
<td></td>
<td>ANT</td>
<td>(2 MIC A+ 1/2 MIC B)</td>
</tr>
<tr>
<td></td>
<td>(1/2 MIC A+ 1/2 MIC B)</td>
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</tr>
<tr>
<td>MIC</td>
<td>ANT</td>
<td></td>
<td></td>
<td>ANT</td>
<td>(MIC A+ 2 MIC B)</td>
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<tr>
<td></td>
<td>(1/2 MIC A+ 2 MIC B)</td>
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<td></td>
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<tr>
<td>2 X MIC</td>
<td>ANT</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1/8 MIC A+ 2 MIC B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The concentrations in shaded boxes are tested. Concentrations designated as SYN and ANT are tested for synergism and antagonism, respectively. (Adapted and reprinted with permission from Moody, J.A. Synergism testing: Broth microdilution checkerboard and broth macrodilution methods, in *Clinical Procedures Handbook*, Isenberg, H.D., Ed., ASM Press, Washington, DC, 1992, Appendix 8, p. 5.18.19.)
APPENDIX 12.4 — EXAMPLE OF TIME-KILL CURVE SHOWING SYNERGISM AND ANTAGONISM

Symbols: ◇, growth control; ●, agent A; ○, agent B; ●, agent A+B. (Adapted and reprinted with permission from Eliopoulos, G.M. and Moellering, R.C., Jr. Antimicrobial combinations, in Antibiotics in Laboratory Medicine, Lorain, V., Ed., Williams Wilkins, Baltimore, 1996, figure 9.6, p. 340.)
APPENDIX 12.5 — RESULTS OF CHECKERBOARD REPRESENTED AS ISOBOLGRAMS

(Adapted and reprinted with permission from Eliopoulos, G.M. and Moellering, R.C., Jr. Antimicrobial combinations, in Antibiotics in Laboratory Medicine, Lorain, V., Ed., Williams Wilkins, Baltimore, 1996, figure 9.2, p. 335.)
REFERENCES

13 Serum Bactericidal Testing

Harriette Nadler and Michael Dowzicky

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13.1 INTRODUCTION

A procedure for determining serum inhibitory titers (SITs) was developed by Schlichter and MacLean to provide guidance for the treatment of certain serious bacterial infections, such as endocarditis [1]. As more became known about the pharmacodynamic requirements (effect of drug against infecting organism) for successful treatment of endocarditis (bactericidal rather than bacteriostatic activity), the test procedure was modified by Fisher to enable determination of bactericidal end points, hence, the serum bactericidal titer (SBT) test [2].

There are very few clinical situations warranting conducting a SBT. Routine susceptibility tests, such as the minimum inhibitory concentration (MIC) test, are most often adequate in guiding therapy since the patient’s success depends largely on the patient’s own defense mechanisms that ultimately kill and eradicate infecting pathogens reduced in number by antibiotic agents. However, in certain clinical circumstances where host defenses are compromised or sites are difficult to
penetrate and bactericidal activity is required, it may be necessary to make a quantitative assessment of this effect. Bactericidal antibiotics most often tested have included the β-lactam agents and aminoglycosides. Quinolones, glycopeptides, streptogramins, and glycyclines, may also be assayed for bactericidal activity. The antimicrobial–organism combination most frequently assayed has been staphylococci and β-lactam agents because of lack of reproducibility and hence often artificially diminished killing observed with such assays [3]. It is not advisable to conduct an SBT on fastidious microorganisms, that is, isolates that will not grow in Mueller-Hinton broth with 24-h incubation.

When precise assessments of bactericidal activity are needed, the individual pharmacokinetics of the patient must be addressed (the patient’s absorption and elimination of the antibiotic and the binding of the drug to serum proteins). The SBT test uniquely incorporates both pharmacodynamic (intrinsic drug activity) and pharmacokinetic therapeutic considerations. The SBT test measures the interaction of pathogen, drug(s), and patient. This is most important when assaying a highly serum protein–bound drug, for example, nafcillin or ceftriaxone. The SBT also determines the effect of interactions with other drugs administered simultaneously with the antibiotic or when the dosing regimen is altered.

The SBT is a variation of the broth dilution MIC test and is intended only for aerobic organisms that grow well after overnight incubation in cation-adjusted Mueller-Hinton broth (CAMHB). Rather than an antibiotic solution, samples of the patient’s serum taken at the beginning (trough) and at the end (peak) of the dosing interval are diluted serially in twofold dilutions. After inoculation of the patient’s organism and incubation at 35°C, tubes with no visible growth are subcultured for colony counts. The SIT is useful to guide selection of samples for subculture. The SBT may then be defined as the greatest dilution of the patient’s serum that kills ≥99.9%, that is, ≥3 log_{10} (CFU/mL), of the inoculum. For this reason, the interim inoculum size used to inoculate the serum dilutions may be estimated by comparison of actively growing cultures with the 0.5 McFarland turbidity standard. However, the final inoculum size must be confirmed by a colony count for interpretation of killing end points.

Bactericidal activity determined by SBTs correlates with estimates based on minimum bactericidal concentrations (MBCs) assessed in conjunction with drug assays in blood provided that the SBTs and MBCs were determined in a physiological diluent such as CAMHB [4]. The SBT is more convenient to conduct than the two other procedures and has the additional capability of measuring the impact of drug interactions.

Determination of bactericidal activity reflects the intrinsic activity of the specific antimicrobial and microorganism being studied, but also depends on the procedures and test conditions used. Both SBTs and MBCs are methodologically dependent. Results obtained may vary with inoculum size, growth phase of the inoculum, choice of medium, volume of broth subcultured for colony counts, and extent of physical contact between the organism and drug [5]. SBTs may also vary with the accuracy of patient sample collection.

The two test conditions with the most impact on end points and interpretations include extent of contact between organisms and drug within the test system as well as the inoculum size and proportion of rapidly growing cells within the inoculum. Splashing of organisms and adherence to the test tube surface above the meniscus may lead to bacterial escape from drug action and hence invalid test results. Adherence may be minimized by the use of acid-washed borosilicate test tubes, rather than polypropylene plastic tubes. Optimal bactericidal activity of most antibiotics can be achieved with mid-logarithmic phase (rapidly growing), rather than stationary phase (slowly growing), cultures (Figure 13.1).

Mid-logarithmic phase inocula are optimally prepared in a flask, beaker, or bottle, and incubated in a shaker-incubator for up to 6 h for staphylococci and ≤6 h for gram-negative rods. It is equally important that final inocula contain the correct inoculum size both in terms of the concentration of
organisms per milliliter of broth and the absolute number of organisms in the test system to permit expression of resistance mechanisms or selection of mutants.

Results of both SBTs and MBCs are also subject to the influence of biological phenomena characteristic of certain antimicrobial–organism combinations tested such as the laboratory-induced tolerance of organisms to bactericidal activity of β-lactam agents (reduction in rapidity and magnitude of bactericidal activity of penicillins or cephalosporins) or are dependent on test conditions used (laboratory-induced resistance development during conduct of the test). Variability in SBT results can be minimized if a standardized methodology [6,7] is consistently followed, and results may be more relevant to the situation of human infection if a physiologic diluent with at least 50% serum supplement is used.

The use of human serum may, however, be problematic because of the presence of complement and other antibiotic-neutralizing activity that may negatively affect organism survival. Pooled human serum may offer advantages over use of serum from a single donor. These challenges may be overcome by the use of an ultrafiltrate of the patient’s serum [7]. Ultrafiltration separates the free antimicrobial agent present and allows the use of CAMHB as the diluent.

Although the SBT procedure has been used for almost 50 years, standardization efforts have been made only in the last decade — 1992 and 1999 — by the Clinical and Laboratory Standards Institute (CLSI) formerly NCCLS [6,7]. Differences in test methodology from laboratory to laboratory have led to poor reproducibility and difficult result interpretation. Furthermore, a drug’s capability in eradicating bacteria, when considered alone, has not been adequately predictive of successful treatment of patients. Other drug attributes beyond intrinsic bactericidal activity such as the postantibiotic effect (growth inhibitory effects determined after drug removal) and growth inhibitory effects at sub-MIC concentrations may also influence bacteriologic response. Difficulties in SBT interpretation have been further compounded by the effect of serious underlying diseases, the degree of heart or major organ failure, and the intensity of medical management on the outcome of infection treatment. Hence the clinical value of the SBT has been controversial.

Major indications for performing the SBT include endocarditis, osteomyelitis, or infections in seriously immunocompromised patients [7–10]. The SBT allows an estimate of bactericidal activity at only two specified time points, the beginning and the end of the dosing interval. If additional information is desirable, especially for newly introduced antibiotics, research parameters, such as the area-under-the-bactericidal-titer-curve and serum bactericidal rate, may also be determined [6,7]. These parameters will further describe the rapidity, magnitude, and duration of bactericidal activity,

![Bacterial growth curve](image-url)
and should only be conducted by experienced laboratory personnel having special expertise in medical microbiology.

13.2 APPROPRIATE TIMING, COLLECTION, AND HANDLING OF SERUM SPECIMENS

It is of great value to have an initial consultation with the ordering physician at the time of the SBT order and determine its clinical value and potential limitations. Inherent in the design of the SBT is the use of the patient’s serum to represent the physiologic concentration of the antimicrobial agent being tested. The details on the collection and handling of serum specimens must be followed for the SBT to be of any clinical value.

13.2.1 TIMING OF SERUM SPECIMENS

Generally, peak (maximal theoretical drug concentration) and trough (lowest theoretical drug concentration) samples are obtained and assayed for the serum bactericidal test. The optimal timing of samples for obtaining a peak level varies by drug and route of administration; therefore, the product labeling should be consulted to select the optimal sampling times [11]. A general guideline for obtaining a peak serum sample is as follows:

- Sixty minutes after completion of a 30-min intravenous infusion.
- Sixty minutes after giving an intramuscular dose.
- Ninety minutes after an oral dose [6,7,12,13].
- If more than one antibiotic is being used, then 60 min after giving the second antibiotic [7].

This timing is used to ensure that peak levels are obtained approximately 30–60 min after the antimicrobial agent has had a chance to be absorbed and/or distributed. The trough level is obtained immediately before the next dose.

13.2.2 COLLECTION AND HANDLING OF SPECIMENS

To avoid possible contamination from the skin, collect the serum sample aseptically. Because of the short half-lives of some antimicrobial agents, the timing of the collection is crucial, and the time the antimicrobial agent was given to the patient should be confirmed and documented. The following steps to ensure this process are recommended:

- Record the time that the previous dose was given.
- Record the time that the current dose was given or will be given.
- Record the duration of the infusion when obtaining peak levels for IV administered agents.

To ensure proper collection, the above information must be recorded on the laboratory requisition slip or entered into the computerized order process system by the appropriate persons (medications nurse or phlebotomist) [6,7].

After the specimen has been collected the following guidelines are recommended:

- The serum must be separated rapidly from the red blood cells to avoid hemolysis and subsequent interference with the assay.
- If a delay of more than 2 h in performing the SBT is expected, the serum collected from the specimen must be frozen.
- When submitting the serum sample for SBT testing to a reference laboratory, it must be placed in a sterile plastic vial and shipped in a frozen state on dry ice.
Of special note is the need for strict adherence to the pharmaceutical manufacturer’s guidelines [11] for the maximum storage periods of the antibiotic in frozen human serum prior to testing to avoid degradation of the drug [5–7].

13.3 PROCEDURES

13.3.1 ISOLATE PREPARATION FOR STORAGE AND SUBSEQUENT RETRIEVAL PRIOR TO SBT TEST

- The patient’s isolate must be stored frozen prior to the performance of the SBT procedure. The isolate may be kept frozen at −70°C in trypticase soy broth or in a glycerol (5–10%) suspension of a subculture from a trypticase soy agar plate or slant.
- It is necessary to subculture the isolate three times before testing to ensure that the organism has an optimal growth and metabolic status before performing the drug exposure [7].

13.3.2 SERUM DILUTION PROCEDURE

The SBT method is a variation of the MIC broth dilution test, and many of the precautions used to control the variables in the MIC test are applicable to the serum dilution procedure (see Chapter 4, Macro- and Microdilution Methods of Antimicrobial Susceptibility Testing).

13.3.2.1 Broth Medium

13.3.2.1.1 Broth and Supplements

- Many different broths have been used as diluents in the SBT test, that is, trypticase soy or brain–heart infusion, but cation adjusted Mueller-Hinton broth (CAMHB) is the most commonly used for isolates that do not have special growth requirements [14]. One advantage of CAMHB is that the Ca²⁺ and Mg²⁺ will approximate that found in serum, thereby facilitating closer approximation of the pH and osmolality of the serum. In addition, CAMHB conforms to the CLSI-recommended MIC procedure, especially for the testing of aminoglycosides against Pseudomonas aeruginosa and of tetracyclines versus all bacteria [15]. The use of salt-containing diluents must be avoided when testing staphylococci and β-lactam agents.
- For highly protein-bound drugs, for example, ceftriaxone and nafcillin, it is advisable that CAMHB combined with pooled human serum (at least a 1:1 ratio) be used as the diluent in the SBT [6,7,16]. Some of the key advantages of using human serum as part of the diluent are that it mimics more closely the physiological environment in the bloodstream of humans and allows one to assess the role of serum protein binding in the final results. It should be noted that for antimicrobial agents that are known to have relatively little protein binding (<90%, e.g., gentamicin and vancomycin), CAMHB alone can be used as the diluent [6,7,17,18].
- An ultrafiltrate of the patient’s own serum (instead of pooled human serum) may also be used as a diluent but the process of ultrafiltration may not be practical in a small laboratory. Ultrafiltration separates the free antimicrobial agent present, avoids the interfering substances, that is, complement, that may be in pooled serum, and allows the use of CAMHB as the diluent.

13.3.2.1.2 Diluent Monitoring

The chemical and performance characteristics of both the CAMHB and human serum must be monitored:
• The pH of each batch of CAMHB should be checked when the medium is prepared; the pH should be between 7.2 and 7.4.
• MIC and MBC characteristics using the CAMHB alone and the combination of CAMHB and pooled human serum should be evaluated periodically (monthly) with a standard set of quality control microorganisms and with an antibiotic from each major class. Select an ATCC quality control organism that most closely resembles the species of the test organism [6,7,14].

Pooled human serum can be obtained from commercial sources or from volunteers (outdated blood bank blood). This pooled human serum must have its quality confirmed like any other reagent in the clinical microbiology laboratory. It is critical to determine the presence of β-lactamase activity in the pooled human serum because these enzymes will neutralize some antimicrobial agents, for example, β-lactam agents [6,7].

• The serum from volunteers needs to be screened for hepatitis B virus antigen and for antibodies to HIV-1.
• The pooled human serum from either source (commercial preparation or volunteers) may be heated to 56°C for 1 h upon receipt. This inactivates any HIV, hepatitis B antigen, and complement that may be present and also ensures the safety of the laboratory staff performing the SBT test.
• The serum should then be adjusted with 0.1 mol/L NaOH or 0.1 mol/L HCl to a pH of 7.2 to 7.4. After this pH adjustment, the serum should be run through a 0.80-µm filter and a 0.22-µm filter.

Before using it, the pooled human serum should be tested for the presence of β-lactamase activity by using a cefinase disk (Becton Dickinson, Cockeysville, MD); drop serum on a test strip or disk and wait for a color change by 30 min. In addition, the serum should be screened for antimicrobial activity by putting 20 µL of the pooled human serum on blank paper disks, placing the disks onto nutrient agar that has been seeded with Bacillus subtilis ATCC 6633, and looking for zones of inhibition after incubation at 35°C for 24 h.

13.3.2.2 Dilution Procedure

Serum bactericidal titers can be determined by either:

• Macrodilution method (1–2 mL in each test tube).
• Microdilution method (100 µL in each microliter well).

In this chapter as in Chapter 4, we focus on the broth macrodilution SBT procedure.

• The macrodilution method allows for a larger volume of inoculum to be delivered, i.e., 10-fold more (absolute numbers of) microorganisms in each tube as compared with each tray well (microdilution method), and greater surface area for growth. This provides optimal growth and testing conditions to measure killing activity of most antibiotics [19–21].
• The broth microdilution method has been found by some workers to be more reproducible than the broth macrodilution method for SBT testing, especially for staphylococci tested against β-lactam agents; however, it can be difficult to determine 99.9% killing of the final inoculum because of the low absolute number of microorganisms in each tray well. Close adherence to the details of the broth macrodilution method can increase the reproducibility of the test method [22,23].

13.3.2.2.1 Macrodilution Method

The macrodilution method should be performed in sterile 13 × 100 mm acid-treated borosilicate glass test tubes. Bacteria adhere to the sides of plastic tubes. The procedure is as follows (Figure 13.2):
• Add 1.0 mL of pooled human serum: CAMHB (1:1 ratio) to tubes 2 through 10.
• Add 1.0 mL of the patient’s serum to each of the first two tubes.
• Make serial twofold dilutions of the serum from tubes 2 through 9 with a calibrated semiautomatic pipette, leaving a volume of 1.0 mL in each tube.
• Add 1.0 mL of CAMHB to each tube to yield a final volume of 2.0 mL in each tube.
• Tube 10 is a growth control tube.

Mix each transfer by flushing the pipette two or three times without splashing the sides of the tubes or creating air bubbles, and discarding the excess 1.0 mL in the pipette from the tube 9 transfer. There is no need to change pipette tips during the serial transfer because the concentration is being decreased during serial dilution and the between-sample contamination should be very small.

FIGURE 13.2 General serum bactericidal activity setup (macrodilution method).
13.3.3 Preparation of Inoculum

The patient’s isolate is used to prepare the inoculum. It is critical that proper preparation of the standardized inoculum be achieved because variations in the density of the inoculum may affect the end points by several-fold. The procedure is as follows (see Figure 13.3):

**STEP 1: Inoculum Setup**

1. **Inoculum Tube**
   - 5 mL

2. **Touch lightly**
   - 20–25 colonies

3. **Incubate 3–5 hours at 35°C**

4. **Adjust to 0.5 McFarland**
   - adjusted culture = 0.5 McFarland (~1.5 x 10^8 CFU/mL)
   - 7.5 x 10^6 CFU/mL

**STEP 2: Inoculum Delivery**

1. **Inoculum tube**
   - 7.5 x 10^6 CFU/mL

2. **Add 0.1 mL to each tube**
   - (well below meniscus)
   - containing 2 mL (except tube #9)
   - Delivery of ~ 4 x 10^5 CFU/mL.
   - final inoculum in each tube is achieved.

**STEP 3: Verify Final Inoculum Size**

1. **0.1 mL**
   - too numerous to count
   - > 200 colonies
   - ~60 colonies

**Figure 13.3** Inoculum preparation delivery.
• Touch lightly five to ten colonies of one single morphological type from a subculture plate (blood agar media) that has been incubated between 16–24 h. This addresses the possibility of heterogeneously distributed resistance among colonies.

• Place into prewarmed (35°C) enrichment broth containing 5.0 mL of CAMHB or trypticase soy broth. Note: Avoid the use of cultures that have recently undergone a change in test condition, for example, temperature shift or a change from agar to broth medium.

• Incubate this bacterial suspension until visibly turbid (usually takes no longer than 6 h at 35°C); for staphylococci and gram-negative rods, it may require less time, but for methicillin-resistant *Staphylococcus aureus* (MRSA) (slow growing), it may require a little more time.

• Adjust the turbidity of the actively growing culture (log phase) to the density of a 0.5 MacFarland standard.

• Dilute the adjusted culture in broth so that each tube contains ~5 x 10^5 CFU/mL (final inoculum), that is, an interim inoculum size estimated to be within the appropriate size. Important exception: when testing enterococci, the traditional inoculum is ~5 x 10^6 CFU/mL [10].

The dilution procedure to follow to obtain this final inoculum depends on the final volume of culture medium in each tube. For example, if 2 mL is the final desired volume in each tube, and the inoculum used is 0.1 mL, the following procedure is followed:

• Dilute the adjusted culture (~1.5 x 10^8 CFU/mL) 1:20 (one part of organism suspension and nineteen parts of broth) to yield ~7.5 x 10^6 CFU/mL.

• Since 0.1 mL of this suspension is inoculated into 2 mL of broth, the final inoculum in each tube will be ~4 x 10^5 CFU/mL.

• To verify the final inoculum size, one must serially dilute at 10^2, 10^3, and 10^4 dilutions of the positive growth control tube (tube 10) and spread the withdrawn aliquot (10 µL) onto agar surfaces, e.g., Mueller-Hinton (using a sterile loop). Colonies present after overnight incubation are counted and recorded on the laboratory worksheet. If pinpoint colonies are observed at 18–20 h, reincubate until colonies are more visible, allowing adequate time for colonies to grow (Figure 13.3).

### 13.3.4 Inoculating Broth

#### 13.3.4.1 Macrodilution method

• The serum dilutions, as detailed in Section 13.3.2.2, should be done at the start of the preparation and incubation of the inoculum.

• Once this is completed, take 0.1 mL of the final inoculum and release beneath the meniscus of the serum dilution in tubes 1–8 and 10. Mix by flushing the pipette two to three times without splashing the sides of the tubes or creating air bubbles. *Do not vortex or agitate the tubes.*

• Once the inoculation is completed, the final range of dilutions will be from 1:2 to 1:256 in tubes 1–8, respectively; the negative control will be in tube 9, and the positive control will be in tube 10. Alternatively, in lieu of a negative control in tube 9, an additional dilution can be made, expanding the range to 1:512.

#### 13.3.5 Incubation

• For the macrodilution method, incubate the test tubes at 35°C in air (or CO₂ if required) to ensure adequate growth of the patient’s isolate.
• Macrodilution tubes should be incubated for 20 h, vortexed, reincubated and then vortexed (4 s) again before sampling at 24 h.

13.3.6 ENDPOINT DETERMINATION

• Antibiotic carryover can be determined by streaking the sample just subcultured across the surface of a prewarmed agar plate and then allowing the inoculated plates to sit 20 min at room temperature to ensure antimicrobial absorption into the agar.
• Cross-streak this inoculum over the entire surface, and after 24 h of incubation look for inhibition of colony growth at the site of the initial streak [24]. Antibiotic carryover is extremely important to determine when testing staphylococci and β-lactams.

13.3.6.1 Determination of Macrodilution Endpoints

• Using a 0.01-mL (10-µL) calibrated pipettor, subculture each clear test tube onto an agar plate surface.
• Streak the broth subcultures throughout the agar plate. Because of the inherent variability of performing colony counts, it is advisable to prepare duplicate subcultures onto solid media to determine the endpoints.
• The number of colonies grown after 48 h of incubation is used to determine the lethal endpoint (≥99.9% kill) (Figure 13.4). The SBT is considered the highest titer of serum that prevented growth and also reduced the inoculum by ≥99.9%. Survivors at lower titers (usually having higher concentrations of drug) are ignored as long as they number below 0.1% of the original inoculum count. These are accounted for by their rejection values (Table 13.1).

Rejection values are determined by considering the final inoculum size, single versus duplicate sampling, pipetting error, and the Poisson distribution of sample responses. For example, if the final inoculum is ~6 × 10^5 CFU/mL, the dilution having fewer than 29 colonies is the lethal endpoint. It is this dilution that demonstrates ≥99.9% killing of the final inoculum and is the serum bactericidal titer.

After 24 hour incubation of tubes:

![Diagram of antibiotic testing](image)

**FIGURE 13.4** Determination of serum inhibitory (SIL) and bactericidal (SBL) end points.
13.4 RESULT INTERPRETATION AND REPORTING

Every test for bactericidal activity should be interpreted by someone knowledgeable in infectious diseases and bactericidal testing.

As a general guideline, it has been suggested that peak serum SBTs of 16 to 32 were frequently associated with more favorable clinical outcomes in patients with endocarditis and neutropenia [7,22,23]. More specifically, a SBT of 16 indicates that there will be some bactericidal activity in the serum for at least four half-lives of the drug(s) being tested. Since the precise interpretation of the SBTs has been very controversial, the interpretation of the results must be customized for the individual patient and the patient’s treatment regimen by the supervisory microbiologist or chief laboratory technologist in concert with the attending physician. SBTs will not accurately and reproducibly predict either favorable or unfavorable patient outcome. However, they will document whether therapeutic concentrations are present and if bactericidal activity is achieved.

The assessment of antibiotic combinations becomes important in infected immunocompromised patients, for example, neutropenic patients and activity of the combination can be assessed by use of the SBT.

The SBT has been reported to be useful in certain cases for patients with skeletal infections, for example, osteomyelitis and suppurative arthritis, although, in this situation, some workers have indicated that trough, rather than peak, SBTs (2 or greater) are more predictive of patient outcome [25]. Some workers have used the SBT for patients with meningitis. However, there is little information to evaluate clinical utility in that situation.

---

**TABLE 13.1**

<table>
<thead>
<tr>
<th>Final Inoculum (CFU/mL)</th>
<th>Rejection</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^5$</td>
<td>4</td>
<td>77</td>
<td>97</td>
</tr>
<tr>
<td>$2 \times 10^5$</td>
<td>8</td>
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<tr>
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<td>$2 \times 10^6$</td>
<td>91</td>
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<td>$3 \times 10^6$</td>
<td>136</td>
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<td>99</td>
</tr>
<tr>
<td>$6 \times 10^6$</td>
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<td>$7 \times 10^6$</td>
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<tr>
<td>$9 \times 10^6$</td>
<td>409</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>455</td>
<td>99</td>
<td>99</td>
</tr>
</tbody>
</table>

Source: Clinical and Laboratory Standards Institute, M26-A, 1999, p. 4.

Note: The sum of colonies from duplicate samples was equal to or less than the rejection value; the antibiotic was declared lethal.

* Number of colonies.
The SBT cannot be used to define what dosage regimens are ineffective. If the patient response is favorable, the dosage regimen need not be altered. In addition, an arbitrary SBT should not be targeted because there is a likelihood of increased toxicity, and surgical intervention may be indicated despite the observation of high SBTs.

13.5 TROUBLESHOOTING AND INDICATIONS FOR RETESTING AND PHYSICIAN CONSULTATION

13.5.1 CONSIDERATIONS

It is suggested that the attending physician be consulted when retesting of the patient’s isolate becomes necessary. Consultation with the nursing staff may also be warranted to ensure proper serum sample collection in association with drug dosing. In addition, it is highly recommended that the supervisory laboratory scientist discuss the interpretation of the final SBT results with the physician.

13.5.2 LABORATORY OBSERVATIONS

1. **Overestimated** bactericidal activity, for example, no growth in low and nearly all high dilutions:
   - Question of inadequate inoculum size or excess drug potency; recheck methods used for preparation.
   - Question of neutralizing activity within pooled or donor serum used for diluent; recheck source and qualification testing of serum.

2. **Underestimated** bactericidal activity, for example, growth in low and high dilution(s) or initial decrease followed by increase:
   - Question of drug inactivation during storage or handling of serum; recheck serum collection, serum sample labels (peak versus trough), and serum dilution procedures. If loss of drug potency is strongly suspected, especially for 24-h determinations, it is advisable to contact the manufacturer. The telephone numbers may be obtained from the Physicians’ Desk Reference [11]. It is important to be aware of drugs that are more easily degraded during laboratory storage and testing, for example, carbapenems and streptogramins. The reader may refer to Chapter 2, Antimicrobial Classifications: Drugs for Bugs, for a list of drugs by class.
   - Question of excess bacterial inoculum size or stationary, rather than logarithmic, growth; recheck method for preparation. Note: McFarland standards need periodic replacement and may become contaminated; they may thus become an invalid guide for inoculum adjustment.
   - Question of inadequate contact between organisms and drug and/or splashing of inoculum above the broth mixture; recheck methodology used to inoculate serum dilutions and determine if dilutions were vortexed at 20 h and again at 24 h.
   - Question of selection of resistant mutants from test population during procedures; check for increase in MIC. This is a common observation with some drug classes, for example, aminoglycosides.

3. **Skip** tubes (low dilutions with growth followed by interim dilution(s) without growth and then followed by the higher dilution(s) with recurrence of growth).
   - Question of error during addition of bacterial inoculum or dilution of serum; recheck procedures.

4. **Growth in highest**, but not lowest, dilutions beyond the SIT or growth inhibitory end point.
   - This is a common observation when testing cell wall-active agents, for example, penicillins, cephalosporins, and carbapenems, and can be ignored for staphylococci; however, it must be considered if it occurs with gram-negative rods, for example,
Serum Bactericidal Testing

Enterobacter spp. or P. aeruginosa. At the higher concentrations of cell wall active agents, protein synthesis may be substantially diminished so that the drug’s lethal effect cannot be expressed because it depends upon rapid growth and cell wall turnover.

5. **Discrepancies** between quality control strain and patient isolates when bactericidal activity is expected:
   - It is advisable to repeat the testing.
   - Notify laboratory supervisory personnel.

6. **Poor growth** in many dilutions:
   - It is advisable to optimize growth of the patient’s isolate in broth, with supplements as needed, before repeating the SBT. *Note*: Organisms from patients already undergoing antibacterial treatment may be damaged and require repeated subculture in nutritionally rich or hypertonic (sucrose-enriched) broth [6,7].

**13.5.3 Terms Associated with Bactericidal Testing**

See Table 13.2 [10].

<table>
<thead>
<tr>
<th>TABLE 13.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terms Associated with Bactericidal Testing</strong></td>
</tr>
<tr>
<td><strong>MIC</strong></td>
</tr>
<tr>
<td><strong>MBC (MLC)</strong></td>
</tr>
<tr>
<td><strong>SIT</strong></td>
</tr>
<tr>
<td><strong>SBT</strong></td>
</tr>
<tr>
<td><strong>Paradoxical effect (eagle phenomenon)</strong></td>
</tr>
<tr>
<td><strong>Skip tubes</strong></td>
</tr>
<tr>
<td><strong>Tolerance</strong></td>
</tr>
</tbody>
</table>

**ACKNOWLEDGEMENTS**

We acknowledge the technical assistance of Michael Pease.

**REFERENCES**

14 Bioassay Methods for Antimicrobial and Antifungal Agents

Donald H. Pitkin and Estrella Martin-Mazuelos

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14.1 INTRODUCTION

The concentrations of antimicrobial agents in human body fluids were originally determined to support toxicological, pharmacological, and pharmacokinetic studies related to regulatory requirements [1–4].

In the 1960s investigators began to determine the antimicrobial concentrations in body fluids from patients treated with drugs that have a narrow therapeutic range, such as aminoglycosides or vancomycin. The concentrations were monitored to minimize occurrence of possible concentration associated toxicity, including nephrotoxicity and ototoxicity [5–9].

Once the importance of antimicrobial agent concentrations in body fluids was established, it was demonstrated that both efficiency and toxicity of antimicrobial agents were related to drug concentration and its duration in body fluids. The importance of determining antibiotic concentrations in body fluids is now widely accepted [2,9–13]. Determinations of antimicrobial agent concentrations in patients receiving drugs with a narrow therapeutic range are made to avoid underdosing, with the likelihood of a poor therapeutic outcome, and to avoid overdosing, with the likelihood of concentration related toxicity [7,8]. Peak concentrations are monitored to ensure therapeutic efficacy and to avoid a large overdose [13,14]. Determinations of antimicrobial agent concentrations in serum are also used to evaluate the pharmacokinetic profile of a new antimicrobial agent.

Rapid, commercially available methods for the accurate quantification of antimicrobial agent concentrations in serum and other biological fluids were developed because of the need to correlate drug concentrations with therapeutic effectiveness and drug induced adverse events [1,2,15–17]. In the 1960s and 1970s, efforts to develop assays centered on microbiological assay systems for the major classes of antibiotics. Since then, substantial technological advances have provided a variety of alternative methods to replace the tedious and time-consuming bioassay, radioenzymatic assay, and radioimmunoassay methods [2,14–18]. However, bioassays remain an important component of modern therapy in many clinical settings.

14.2 CLINICAL SPECIMENS

14.2.1 OVERVIEW

Blood, serum, plasma, urine, and cerebrospinal fluid are commonly assayed for their concentration of antimicrobial agents. Serum is the most frequently assayed body fluid. The use of universal or standard precautions, to prevent exposure of the laboratory worker to infection, is advised when working with clinical specimens.

Proper specimen collection, accurate documentation of the time and route of drug administration, the dose administered, sampling times, and a list of other medications given to the patient are vital to the interpretation of assay results. Improper handling and storage of collected specimens may influence assay results. The effects of handling and storage conditions on the stability of the antimicrobial should be known. Antimicrobial agents must remain stable while specimens are processed and during storage prior to assay. If any procedure is performed incorrectly, the resulting data may be invalid and require additional patient specimens. Repeated testing consumes time and resources, and is unnecessarily discomforting and stressful to the patient.
When biological fluids are assayed, the determination of antimicrobial content may be influenced by factors such as serum protein binding, abnormal serum protein or serum biochemical profiles, specimen availability, and the diluent used to prepare the standard response line [11,19–21].

14.2.2 Specimen Collection Times

The appropriate postdose interval(s) to collect specimens of serum or other body fluids are dependent on the intent of the study, the route of drug administration, and the body fluid collected. A study to determine the pharmacokinetic properties of a new intravenously administered antimicrobial agent requires a completely different set of sampling intervals than does a study to routinely monitor peak and trough concentrations of the drug. Drugs administered intravenously, intramuscularly, and orally will each have unique sampling interval requirements [22–24]. Drugs administered intramuscularly or orally achieve their peak concentration later than an intravenously administered drug. The solubility of drug at the site of administration is a major variable in the time required to reach peak concentration. Poorly soluble drugs will take longer to reach their peak concentration, and the peak concentration achieved will be less than that of drugs with greater solubility [22,24].

Appropriate times for sample collections for routine monitoring of antimicrobial concentrations for toxicity and efficacy are when peak and trough concentrations of the antibiotic are present. Peak and trough concentrations are defined as the highest (peak) and the lowest (trough) concentration of drug achieved during the dosage interval. The timing of samples for trough concentrations is independent of the route of administration. Trough samples should be obtained immediately prior to administration of the next dose.

Determination of the peak concentration of an antimicrobial in serum may be important if efficacy is concentration dependent, for example, aminoglycosides or fluoroquinolones. It can also be important for drugs that have adverse reactions associated with high concentrations of drug in serum, such as, 5-flucytosine (5-FC) or chloramphenicol.

The steady state concentration of an antimicrobial may be measured 4–8 h after initiating a continuous intravenous infusion; for example, after administration of a β-lactam antibiotic that has a short half-life [25].

Urine specimens are usually obtained as pools containing all of the urine voided by the patient during a specified time interval. The total volume of urine in the pool must be documented and the volume made known to the laboratory to determine the amount of drug excreted. Thoroughly clean urine collection containers after use to eliminate specimen cross-contamination.

14.2.3 Specimen Processing and Storage

Specimens should be processed without delay to prevent possible loss of antimicrobial agent potency. Prior to processing, specimens can be stored at 2°C–8°C.

14.2.3.1. Plasma

Collect the patient’s blood in a tube containing a suitable anticoagulant. Plasma must be separated from the red blood cells. Centrifuge the blood specimen using conditions that permit separation of cells and plasma. Decant the plasma into a sterile container and label. If the expected concentration of antimicrobial in the plasma is greater than the highest concentration on the standard response line, dilute the specimen with plasma prior to assay. Do not dilute plasma specimens with serum; the diluted sample may clot.

14.2.3.2 Serum

Collect the patient’s blood in a tube without any anticoagulant. Serum is separated from blood cells after the blood has clotted and clot retraction has occurred. Permit clot retraction to occur in a
Antimicrobial Susceptibility Testing Protocols

14.2.3.3 Cerebral Spinal Fluid (CSF)

Specimens of CSF should be free from obvious contamination with blood. Contamination with blood is evidenced by the presence of a pink to red color. The assay of CSF samples contaminated with blood will provide unreliable data on the content of drug in the CSF. Assays of CSF specimens pose significant challenges to the analyst. The limited specimen volume available minimizes the number of replicate determinations that can be made and the volume available for each estimate. Assay formats that require small sample volumes are preferred. The difficulty in obtaining drug-free CSF affects the preparation of a standard response line. Preparation of the standard response line using serum diluted with an aqueous diluent to a protein content similar to that of the CSF specimen is a potential alternative. The serum protein content of the diluent is particularly important for drugs such as ceftriaxone that are highly bound to serum proteins.

14.2.3.4 Urine

Urine collection containers should be kept cold, for example, in an ice bath, during the collection interval to minimize loss of bioactivity. It is important to record the total volume of urine collected during each collection interval. Mix the total volume collected and submit a labeled aliquot to the laboratory for analysis. Processed specimens that are assayed the same day they are collected may be stored at 2°C–8°C until they are assayed. Store all other processed specimens at –70°C to –80°C. Some antimicrobials are stable at –20°C; however, others such as imipenem require colder storage temperatures to ensure multiple day stability. Urine specimens containing certain antimicrobials, including imipenem, should be diluted in the buffer suggested by their manufacturer to ensure stability in the frozen state. Frozen specimens must be completely thawed and mixed before they are assayed.

14.3 BIOASSAY METHODS

14.3.1 Overview

Most, if not all, of the bioassays run in a clinical laboratory to determine antimicrobial concentrations in body fluids are based on agar diffusion methods. The assays measure the total antimicrobial activity in the specimen to which the indicator organism responds. The presence of more than one antimicrobial in a specimen will invalidate the results unless the analyst takes special precautions to suppress the activity of the interfering antimicrobial agent(s) that are present.

The antimicrobial activity is measured by determining the diameter, in millimeters, of the zone of no indicator organism growth that surrounds the tested material. The clarity of the edge of the zone of inhibition is a critical variable in obtaining reproducible results. Subtle changes in the inoculum colony forming units per milliliter (CFU/mL), media composition, media pH, depth of agar in the petri dish, and duration of incubation can have pronounced effects on inhibition zone size and edge clarity [27–29].

Bioassays are quantified by comparing the response of the indicator organism to an unknown concentration of a known antimicrobial agent in the sample to the responses produced by known concentrations of the same antimicrobial agent in a standard response line.

In a clinical laboratory, any organism that is inhibited by the antimicrobial agent can be used as an indicator and any media that supports growth of the indicator can be used. The concentrations of antimicrobial used to prepare the standard response line will vary with the antimicrobial agent,
indicator organism, assay media, incubation conditions, and type of sample. The choice of indicator organism and growth media may, to a large extent, be varied to suit the required assay sensitivity and the needs of the laboratory.

The analyst must then determine the antimicrobial concentrations to use in preparing the standard response line. Determine the standard response line concentrations using data obtained in a preliminary assay. In preliminary assays, use widely separated concentrations of the antimicrobial, for example, 0.25, 1, 2.5, 10, 25, and 100 µg/mL. A plot of logarithm of the concentration against linear inhibition zone diameter should form a straight line without obvious deviations. Deviations in linearity of a response line are most likely to occur at concentration range extremes unless technical errors were made during preparation of the solutions, during application of the solutions to the assay plates, or measurement of zone diameters. The concentration range chosen must encompass the range of concentrations expected in the sample or dilution of the sample. When determining the drug concentrations for the standard response line, it is generally appropriate to avoid concentrations that produce small (<13 mm) or large (>27 mm) zones of inhibition. When calculating the drug concentration in a specimen, do not extrapolate the concentration using inhibition zones with a diameter that exceeds the boundaries of the standard response line. The response may not be linear.

The standard response line concentrations suggested in the U.S. Pharmacopeia and the Code of Federal Regulations Title 21 relate to bioassays of drug substances in aqueous solution and were intended for assays of dosage forms. The standard response line concentrations required for assays of clinical specimens, particularly those of blood, serum, or plasma, may not be the same as those for a drug substance in an aqueous environment. The concentrations required are often greater.

Development of inhibition zones with a clearly defined edge is a critical requirement for all bioassays. Poorly defined inhibition zone edges make it essentially impossible to run a reproducible bioassay. The analyst should run a preliminary trial of any new bioassay before using the assay to analyze clinical samples [20,21,30]. A trial run provides training for the analyst and permits subtle changes to be incorporated into the final procedure before clinical specimens are tested. The results of the trial assay will show the quality and sizes of the inhibition zone diameters and the assay sensitivity. Microbiological bioassays are labor intensive and slow, and have a low degree of specificity, but they require only a minimal amount of specialized equipment.

Aminoglycosides and glycopeptide antimicrobials, such as gentamicin and vancomycin, respectively, produce standard response lines with a steep slope, i.e., only a small difference in response is obtained for relatively large differences in concentration. Doubling the concentration of aminoglycoside or vancomycin may change the zone diameter by only 0.5–1.5 mm. Consistent and accurate measurement of zone diameters is mandatory. A change of only 0.2 mm in the average zone diameter may change an assayed concentration value by 15%. Replicate zones of inhibition should have zone diameters that are almost the same size. Large differences in replicate zone diameters, >1 or 1.5 mm for assays using a 6.3-mm disk, suggest technical errors may have occurred.

Bioassays should only be run by well trained technical staff. Strict control of analytical technique is necessary to minimize the likelihood of errors. Sequential carryover errors may occur when serial dilutions are used to produce standard response line concentrations. Standard response line preparation schema should minimize use of serial dilutions. Control samples of known antimicrobial content stored under the same conditions as the specimens should be tested with each batch of assays to evaluate assay quality and accuracy. Antibacterial bioassays typically have a sensitivity in the range of 0.1 to 5 µg/mL [3,30].

### 14.3.2 Assay Formats

Bioassays can be broadly classified into two formats: the self-contained and the internally normalized assay. All bioassays should be validated and written procedures strictly followed to ensure production of reproducible data.
14.3.2.1 Self-Contained Assay

In the self-contained assay, each assay dish includes a complete standard response line and sample(s). Assays can be run in a 90-mm petri dish with a two-point standard response line, two zones of each material on each plate. The statistical power of this assay format is low. Now that more powerful assay formats have been developed it is infrequently used and is not recommended.

Alternately, use a 150-mm petri dish with a five-point standard response line and a single sample, one zone of each material on each plate. Replicate estimates of each response are run on separate assay plates. Corrections for interplate variation in responses are not to be made using this assay format. Each of these assays can be run using a single layer of nutritive agar seeded with inoculum or on a plate containing two layers of nutritive agar, the unseeded bottom layer and an overlaid seeded top layer of nutritive agar. Preparation of uniform assay plates is mandatory and preparation of uniform two-layer plates requires extensive practice.

Assays run in the Nunc square dish, 295 mm [24,28,29], are a modification of the self-contained assay. In this modification, multiple replicates of each standard response line concentration and multiple samples are tested in a single plate. Tested materials may be applied using a matrix of eight materials in each of eight columns (8 × 8 matrix) or nine materials using a 9 × 9 application matrix. Each standard concentration and each sample is randomly assigned to a specified unique location in each column. Replication of each tested material is contained in a single dish; therefore, interplate variation in response is eliminated as a variable. Accurate placement of each solution in the correct location is necessary. Use of a template for placement of solutions and identification of zones is recommended.

14.3.2.2 Internally Normalized Assay

In the internally normalized bioassay using 90-mm petri dishes, each dish alternately contains three replicates of a single concentration of one standard or three replicates of one sample and three replicates of a solution common to all plates [31]. The solution common to all plates is frequently, but is not required to be, the midpoint of the standard response line. Additional replicate values for each standard and sample are on separate plates. Each standard response line concentration or sample is typically replicated on each of 3 or 4 plates to provide 9 to 12 estimates of the “true response.” Petri dishes of 150-mm diameter may also be used with an adjustment of agar volume based on the relative surface areas of the two plate sizes. The 150-mm petri dish format reduces the likelihood of zone overlap or interference when large, >27 mm, adjacent zones are produced.

The process of normalizing the responses of the common solution across all assay dishes and applying the correction to each set of plates containing a standard response line concentration or sample eliminates interplate variation in standard and sample responses.

14.3.3 Indicator Organism

The CFR Title 21 and current edition of the U.S. Pharmacopeia list suitable indicator organisms for assaying specimens containing most common single antimicrobial agents (Table 14.1). Spores of Bacillus subtilis ATCC 6633, or cells of Staphylococcus aureus 6538P (ATCC 29737), S. epidermidis ATCC 12228, Enterococcus faecalis ATCC 10541, or Micrococcus luteus ATCC 9341 are often used to assay antimicrobials active against gram-positive organisms. Escherichia coli ATCC 10536, Klebsiella pneumoniae ATCC 10031, and Pseudomonas aeruginosa ATCC 25619 are often used for antimicrobials active against gram-negative organisms.

The presence of multiple antimicrobial agents or combination drugs, such as a β-lactam antibiotic plus a β-lactamase inhibitor, may require selection of an alternate indicator organism. Assays of specimens containing multiple antimicrobial agents require the analyst to modify the media or sample by the addition of agents to selectively inactivate or remove the interfering
antimicrobial agents. A β-lactamase preparation such as the mixed β-lactamase from Bacillus cereus that contains both a serine active site β-lactamase and a zinc dependent β-lactamase can inactivate most available β-lactam antibiotics, with the possible exception of the monobactam aztreonam. This enzyme preparation is commercially available under the name of Genzyme®. Addition of 1% w/v cellulose phosphate or 3% to 5% w/v sodium polyanethol sulfonate (Sigma Chemical Co) to the media will suppress activities due to aminoglycosides. Adjustment of assay media pH will enhance or suppress activity of an antimicrobial whose activity is pH dependent. Differences in sensitivity to heat can be used to separate activities of some antimicrobial agents; β-lactam antibiotics are heat sensitive while aminoglycosides are considerably more heat resistant. The presence of urea and bilirubin in serum may interfere with bioassays, especially the determination of aminoglycoside concentrations [7]. Specimen dilution with normal human serum may resolve the interference if the concentration of antimicrobial in the specimen is sufficient to permit specimen dilution. Use of an indicator organism that does not respond to the interfering antimicrobial is another alternative. The chosen indicator organism must respond to concentrations of the antibiotic of interest that are expected to be present in the sample. Suitable control samples must be tested in parallel with the specimens to verify assay accuracy and selectivity when testing samples containing multiple antimicrobials.

TABLE 14.1
Microbiological Assay Methods: Indicator Organism Inocula

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Methoda/Methodb</th>
<th>Incubation (hr°C)</th>
<th>Suggested Dilution</th>
<th>Storage Periodc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Staphylococcus aureus ATCC 6538P</td>
<td>1/1</td>
<td>24/32–35</td>
<td>1:20</td>
<td>1 week</td>
</tr>
<tr>
<td>B-Micrococcus luteus ATCC 7468</td>
<td>1/1</td>
<td>24/32–35</td>
<td>1:30</td>
<td>2 weeks</td>
</tr>
<tr>
<td>C-Micrococcus luteus ATCC 9341</td>
<td>1/1</td>
<td>24/32–35</td>
<td>1:40</td>
<td>2 weeks</td>
</tr>
<tr>
<td>D-Staphylococcus epidermidis ATCC 12228</td>
<td>1/1</td>
<td>24/32–35</td>
<td>1:14</td>
<td>1 week</td>
</tr>
<tr>
<td>E-Saccharomyces cerevisiae ATCC 9763</td>
<td>6/19 or 7/19</td>
<td>48/29–31</td>
<td>1:30</td>
<td>4 weeks</td>
</tr>
<tr>
<td>F-Bordetella bronchiseptica ATCC 4617</td>
<td>1/1</td>
<td>24/32–35</td>
<td>1:20</td>
<td>2 weeks</td>
</tr>
<tr>
<td>H-Bacillus subtilis ATCC 6633</td>
<td>1/1</td>
<td>24/32–35</td>
<td>e</td>
<td>1 month</td>
</tr>
<tr>
<td>or 2/32</td>
<td>5–7 days/32–35</td>
<td>6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-Klebsiella pneumoniae ATCC 10031</td>
<td>1/1</td>
<td>24/36–37.5</td>
<td>1:25</td>
<td>1 week</td>
</tr>
<tr>
<td>J-Escherichia coli ATCC 10536</td>
<td>1/1</td>
<td>24/32–35</td>
<td>1:20</td>
<td>2 weeks</td>
</tr>
<tr>
<td>K-Enterococcus faecium ATCC 10541</td>
<td>5/...</td>
<td>24/36–37.5</td>
<td>/4/</td>
<td>1 week</td>
</tr>
<tr>
<td>T-Saccharomyces cerevisiae ATCC 2601 Cerevisiae4</td>
<td>7/3</td>
<td>48/29–31</td>
<td>1:30</td>
<td>4 weeks</td>
</tr>
<tr>
<td>W-Pseudomonas aeruginosa ATCC 25619</td>
<td>1/1</td>
<td>24/36–37.5</td>
<td>1:25</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Y-Pseudomonas aeruginosa ATCC 29336</td>
<td>9/36</td>
<td>24/36–37.5</td>
<td>1:50</td>
<td>1 week</td>
</tr>
<tr>
<td>Paecilomyces variotii ATCC 36257</td>
<td>10/3</td>
<td>24–48/29–31</td>
<td>1:1</td>
<td>1 day</td>
</tr>
<tr>
<td>Cryosporium pruinosum ATCC 36374</td>
<td>10/3</td>
<td>24–48/29–31</td>
<td>1:1</td>
<td>1 day</td>
</tr>
<tr>
<td>(5-flucytosine resistant) Saccharomyces cerevisiae ATCC 36375</td>
<td>6/19f</td>
<td>48/29–31</td>
<td>e</td>
<td>2 days</td>
</tr>
<tr>
<td>Candida tropicalis ATCC 13803</td>
<td>6/19f</td>
<td>24–48/29–31</td>
<td>e</td>
<td>2–5 days</td>
</tr>
</tbody>
</table>


... No media suggested

a Organisms are available from American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852.
b Methods are defined in Table 14.2, and media are defined in Table 14.3.
c Storage temperature 2°C–8°C.
d If the antibiotic to be tested is paromomycin, the dilution factor is 1:25.
e Determine dilution with test plates.
14.3.4 INOCULUM

The inocula for most one-layer bioassays will contain $2 \times 10^5$ to $10^6$ CFU/mL of nutritive agar. The inocula for two-layer bioassays generally contain a larger number of CFU per milliliter of nutritive agar than the inocula for a single-layer assay. It may contain as many as $1 \times 10^8$ CFU/mL of seeded agar (Table 14.2). Use inoculum cells in mid to late log phase of growth for the rapid determination of antibiotic concentrations. Spores of *B. subtilis* ATCC 6633 provide a readily available inoculum inhibited by many antibiotics, have a long shelf life, and are commercially available from media manufacturers. The spores may lack adequate sensitivity or produce unacceptable inhibition zone edges when used as an indicator for some assays.

14.3.5 CULTURE MEDIA

A universal culture media for bioassays does not exist. The CFR currently lists more than 17 media for bioassays (Table 14.3) [3]. The choice of media depends on the antibiotic assayed, the indicator microorganism, and pH requirements.

The volume of media to add to each petri dish is a variable [28,29] (Table 14.3 and Table 14.4). Small volumes enhance assay sensitivity while large volumes may clarify zone edge definition. Petri dishes of 90-mm diameter, for the single-layer assay format, may contain 7–25 mL of nutritive agar, depending on the required sensitivity and inhibition zone quality. Volumes of 10–15 mL of nutritive agar per plate frequently produce acceptable results. For two-layer assay plates, use 20 mL of sterile base agar and overlay with 3–5 mL of seeded nutritive agar. The relative temperatures of the solidified base layer and molten seeded agar overlay are important variables when preparing two-layer plates. A uniform thickness of seeded overlay cannot be obtained if the base layer is too cold. The seeded layer must be uniformly distributed on the surface of the base layer, and each plate must be overlaid with the same volume of seeded agar to provide interplate consistency.

Petri dishes of 150-mm diameter for the single-layer assay format may contain 15–50 mL of nutritive agar. Volumes of 25–30 mL of nutritive agar per dish frequently produce acceptable results. For two-layer assay plates, use 40 mL of sterile base agar and overlay with 7–8 mL of seeded agar following the same precautions as used for the smaller plate size.

The Nunc 295-mm square plates require 200–300 mL of seeded nutritive agar for one-layer plates. These plates are not recommended for two-layer assays because of the difficulty in evenly spreading the thin layer of seeded agar over the sterile base layer in a large plate.

14.3.6 ANTIBIOTIC STANDARDS

The antimicrobial agent used to prepare the standard response line must be prepared from a reference powder of known potency (purity). The use of a therapeutic preparation of an antimicrobial agent is not recommended because many therapeutic preparations contain antimicrobially inactive excipients. Each lot of antibiotic has its own potency [3]. Small amounts of reference grade antimicrobial can be obtained from the drug manufacturer, the U.S. Pharmacopia, or Sigma Chemical Company among other sources. Stock reference solutions of high concentration should be prepared using the reference powder. Equilibrate the temperature of a reference standard powder to room temperature before opening the container to minimize adsorption of moisture. Dissolve the powder in the recommended diluents, and store small volumes of the stock reference solution at the temperature recommended by the manufacturer, or as listed in the CFR (Table 14.5 and Table 14.6), or as suggested by the Clinical and Laboratory Standards Institute (CLSI) [31]. Stock reference solutions prepared from a reference powder can frequently be stored at $-70^\circ C$ for up to 3 months [3]. Do not refreeze a stock reference solution once it is thawed. During preparation of the stock reference solution, the analyst must take into consideration the potency of the antimicrobial. Some antimicrobials, especially some of the
TABLE 14.2
Microbiological Assay Methods: Preparation of Indicator Organism

Method 1
Maintain organisms on agar slants containing 10 mL of the appropriate medium. Incubate the slants at 32°C–35°C for 24 h. Using 3 mL of sterile U.S.P. saline, wash the growth from the agar slant onto a large agar surface, such as a Roux bottle, containing 250 mL of the appropriate medium. Spread the suspension of organisms over the entire surface of the Roux bottle with the aid of sterile glass beads. Incubate the Roux bottle at 32°C–35°C. Wash the resulting growth from the agar surface with 50 mL of sterile U.S.P. saline.

Standardization of suspension: Determine the dilution factor that will give 25% light transmission at a wavelength of 580 m\(\mu\) using a suitable photoelectric colorimeter and a 13-mm diameter test tube as an absorption cell. It may be necessary to adjust the suspension. Determine the amount of suspension to be added to each 100 mL of agar or nutrient broth by the use of test plates or test broth. Store the test organism suspension under refrigeration.

Method 2
Proceed as directed in method 1.

Standardization of the suspension: Centrifuge and decant the supernatant liquid, resuspend the sediment with 50–70 mL of sterile U.S.P. saline, and heat the suspension for 30 min at 70°C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 mL of agar. Maintain the spore suspension under refrigeration.

Method 3
Proceed as directed in method 1.

Standardization of the suspension: Heat the suspension for 30 min at 70°C, wash the spore suspension three times with 25–50 mL of sterile distilled water, resuspend the organisms in 50–70 mL of sterile distilled water and heat-shock again for 30 min at 70°C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 mL of agar. Maintain the spore suspension under refrigeration.

Method 4
Maintain the test organisms in 100-mL quantities of nutrient broth (Medium 3). For the test, prepare a fresh subculture by transferring a loopful of the stock culture to 100 mL of the same nutrient broth and incubate for 16–18 h at 37°C. Store this broth culture under refrigeration.

Method 5
Maintain the test organisms on agar slants containing 10 mL of the appropriate medium. Incubate the slants at 32°C–35°C for 24 h. Inoculate 100 mL of nutrient broth-Medium 13. Incubate for 16–18 hours at 37°C.

Standardization of the suspension: Follow the directions for method 1.

Method 6
Proceed as directed in method 1, except incubate the slants at 30°C for 24 h and incubate the Roux bottle at 30°C for 48 h.

Method 7
Maintain organisms on agar slants containing 10 mL of the appropriate medium and transfer to a fresh slant about once a week. Incubate the slants at 37°C for 48 h. Using 3 mL of sterile U.S.P. saline, wash the growth from the agar slant into a 500-mL Erlenmeyer flask containing 100 mL of medium 34 and 50 g of glass beads. Agitate the culture by rotation at a speed of 130 cpm and a radius of 3.5 cm at 27°C for 5 days. Determine the amount of suspension to be added to each 100 mL of agar by the use of test plates. Store the test organism suspension under refrigeration.

Method 8
Proceed as directed in method 1, except incubate the slant and Roux bottle at 37°C and wash the resulting growth from the agar surface with 50 mL of Medium 37.

Method 9
Proceed as directed in method 1, except standardize to 80%–90% light transmission at 360 m\(\mu\).
### TABLE 14.3
Microbiological Assay Methods: Culture Media

| Medium 1                      | Peptone: 6.0 g  
|                              | Pancreatic digest of casein: 4.0 g  
|                              | Yeast extract: 3.0 g  
|                              | Beef extract: 1.5 g  
|                              | Dextrose: 1.0 g  
|                              | Agar: 15.0 g  
|                              | Distilled water, q.s: 1,000 mL  
| pH 6.5–6.6 after sterilization |

| Medium 2                      | Peptone: 6.0 g  
|                              | Yeast extract: 3.0 g  
|                              | Beef extract: 1.5 g  
|                              | Agar: 15.0 g  
|                              | Distilled water, q.s: 1,000 mL  
| pH 6.5–6.6 after sterilization |

| Medium 3                      | Peptone: 5.0 g  
|                              | Yeast extract: 1.5 g  
|                              | Beef extract: 1.5 g  
|                              | Sodium chloride: 3.5 g  
|                              | Dextrose: 1.0 g  
|                              | Dipotassium phosphate: 3.68 g  
|                              | Potassium dihydrogen phosphate: 1.32 g  
|                              | Distilled water, q.s: 1,000 mL  
| pH 6.95–7.05 after sterilization |

| Medium 4                      | Peptone: 6.0 g  
|                              | Yeast extract: 3.0 g  
|                              | Beef extract: 1.5 g  
|                              | Dextrose: 1.0 g  
|                              | Agar: 15.0 g  
|                              | Distilled water, q.s: 1,000 mL  
| pH 6.5–6.6 after sterilization |

| Medium 5                      | Medium 5 is the same as medium 2, except adjust the final pH to 7.8–8.0 after sterilization. |

| Medium 8                      | Medium 8 is the same as medium 2, except adjust the final pH to 5.8–6.0 after sterilization. |

| Medium 9                      | Pancreatic digest of casein: 17.0 g  
|                              | Papaic digest of soybean: 3.0 g  
|                              | Sodium chloride: 5.0 g  
|                              | Dipotassium phosphate: 2.5 g  
|                              | Dextrose: 2.5 g  
|                              | Agar: 20.0 g  
|                              | Distilled water, q.s: 1,000 mL  
| pH 7.2–7.3 after sterilization |

(continued)
| Medium 11 | Medium 1 is the same as medium 1, except adjust the final pH to 7.8–8.0 after sterilization. |
| Medium 13 | Peptone: 10.0 g  
Dextrose: 20.0 g  
Distilled water, q.s: 1,000 mL  
P pH 5.6–5.7 after sterilization |
| Medium 19 | Peptone: 9.4 g  
Yeast extract: 4.7 g  
Beef extract: 2.4 g  
Sodium chloride: 10.0 g  
Dextrose: 10.0 g  
Agar: 23.5 g  
Distilled water, q.s: 1,000 mL  
P pH 6.0–6.2 after sterilization |
| Medium 32 | Prepare as medium 1, except add 300 mg of hydrated manganese sulfate to each liter of medium. |
| Medium 34 | Glycerol: 10.0 g  
Peptone: 10.0 g  
Beef extract: 10.0 g  
Sodium chloride: 3.0 g  
Distilled water, q.s: 1,000 mL  
P pH 7.0 after sterilization |
| Medium 35 | Same as medium 34, except add 17.0 g of agar to each liter of medium. |
| Medium 36 | Pancreatic digest of casein: 15.0 g  
Papaic digest of soybean: 5.0 g  
Sodium chloride: 5.0 g  
Agar: 15.0 g  
Distilled water, q.s: 1,000 mL  
P pH 7.3 after sterilization |
| Medium 37 | Pancreatic digest of casein: 17.0 g  
Soybean peptone: 3.0 g  
Dextrose: 2.5 g  
Sodium chloride: 5.0 g  
Dipotassium phosphate: 2.5 g  
Distilled water, q.s: 1,000 mL  
P pH 7.3 after sterilization |
| Medium 38 | Peptone: 15.0 g  
Papaic digest of soybean meal: 5.0 g |

(continued)
newer quinolone antibiotics, are light sensitive. These antimicrobials must be handled under conditions of low light intensity and stored in containers shielded from light.

The final dilutions of the stock reference solution that are used to prepare the standard response line concentrations are made in the same body fluid as the sample that will be assayed, when possible. For example, for the determination of antibiotic levels in blood, the final dilutions of each standard response line concentration should be made using blood as the diluent. Plasma samples are diluted in plasma, and serum samples are diluted in serum [24]. Urine samples may be diluted in drug-free urine if available [1] or 1% phosphate buffer pH 6–7. Standards used to assay CSF samples may require dilution in an aqueous dilution of serum to mimic the concentration of plasma proteins in the CSF because of difficulty in obtaining drug-free CSF and its scarcity. When the antibiotic is diluted in a liquid that is different than that of the test sample, large errors in the results may occur because of the pH or content of serum proteins [1].

Bioassays should include at least three concentrations to generate the standard response line and a minimum of five concentrations is recommended. Suggested standard response line concentrations for aqueous specimens may be found in the CFR or U.S. Pharmacopeia [3]. Each laboratory may prefer to develop its own standard response line concentrations based on available indicator organisms, antimicrobials of interest, and type of specimens tested. It is critical that the antibiotic standards be free of other antimicrobial substances.

14.3.7 APPLICATION OF TEST MATERIALS TO ASSAY PLATES

Solutions of antimicrobial agents can be applied to assay plates using filter paper disks (Schleicher & Schuell 740 E) of 6.25-mm or 12.5-mm diameter. The smaller disk readily adsorbs 20 µL of liquid. The larger disk readily adsorbs 80 µL of liquid. The liquid can be placed on disks using a micropipette, changing the pipette tip between solutions of higher concentration. Alternatively, disks can be carefully dipped into the liquid and the excess volume drained off (or place the disk on a screen to dry), before applying the disk to an assay plate. Application of the same absorbable volume of liquid to each disk, not the absolute volume applied, is critical.

Wells of consistent size, usually 6–9-mm in diameter, are cut into the agar surface of assay plates with a cutting tool such as a cork borer attached to a suction device to remove the agar plug. A well cutter with adjustable well spacing can be custom made to simplify and standardize the procedure. Cut the wells without causing fissures to develop around the well. A cutter sharpened (beveled) on the inner surface, rather than the outer surface, may be easier to use. The solution volume added to each well must be the same, but the absolute volume applied will vary with the well diameter and agar depth.

### TABLE 14.3
Microbiological Assay Methods: Culture Media (continued)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Sodium chloride</td>
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</tr>
<tr>
<td>Sodium sulfite</td>
<td>0.2 g</td>
</tr>
<tr>
<td>l-Cystine</td>
<td>0.7 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water, q.s.</td>
<td>1,000 mL</td>
</tr>
</tbody>
</table>

pH 7.0 after sterilization

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Media to Be Used Listed by Medium Number</th>
<th>mL of Media to Be Used in the Base and Seed Layers</th>
<th>Suggested Volume of Standardized Inoculum to Be Added to Each mL (mL)</th>
<th>Incubation Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base Layer</td>
<td>Seed Layer</td>
<td>Base Layer</td>
<td>Seed Layer</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>11</td>
<td>11</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Amphotericin B</td>
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<td>19</td>
<td>None</td>
<td>8</td>
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<tr>
<td>Ampicillin</td>
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<td>11</td>
<td>21</td>
<td>4</td>
</tr>
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<td>Bacitracin</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Bacitracin</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Carbenicillin</td>
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<td>10</td>
<td>21</td>
<td>4</td>
</tr>
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<td>Cefactor</td>
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<td>1</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>Cefadroxil</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Cefamandole</td>
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<td>1</td>
<td>21</td>
<td>5</td>
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<td>21</td>
<td>4</td>
</tr>
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<td>21</td>
<td>5</td>
</tr>
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<td>21</td>
<td>5</td>
</tr>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Cephaloglycin</td>
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<td>21</td>
<td>4</td>
</tr>
<tr>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
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<td>Cephamycin</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
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<td>Cephadine</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
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<td>Clindamycin</td>
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<td>11</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Cloxacillin</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Colistimethate, sodium</td>
<td>9</td>
<td>10</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Colistin</td>
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<td>10</td>
<td>21</td>
<td>4</td>
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<td>21</td>
<td>4</td>
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<td>Dactinomycin</td>
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<td>5</td>
<td>10</td>
<td>4</td>
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<td>Dicloxacillin</td>
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<td>1</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
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<tr>
<td>Erythromycin</td>
<td>11</td>
<td>11</td>
<td>21</td>
<td>4</td>
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(continued)
<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Media to Be Used Listed by Medium Number</th>
<th>mL of Media to Be Used in the Base and Seed Layers</th>
<th>Suggested Volume of Standardized Inoculum to Be Added to Each mL (mL)</th>
<th>Incubation Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>11 11</td>
<td>21 4</td>
<td>D</td>
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<td>Kanamycin B</td>
<td>5 5</td>
<td>21 4</td>
<td>H</td>
<td>b</td>
</tr>
<tr>
<td>Methicillin</td>
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<td>21 4</td>
<td>A</td>
<td>0.3</td>
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<td>Nafcillin</td>
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<td>21 4</td>
<td>A</td>
<td>0.3</td>
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<td>Natamycin</td>
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<td>None 8</td>
<td>E</td>
<td>0.8</td>
</tr>
<tr>
<td>Neomycin</td>
<td>11 11</td>
<td>21 4</td>
<td>A</td>
<td>0.4</td>
</tr>
<tr>
<td>Neomycin</td>
<td>11 11</td>
<td>21 4</td>
<td>D</td>
<td>1.0</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>11 11</td>
<td>20 5</td>
<td>D</td>
<td>0.25</td>
</tr>
<tr>
<td>Novobiocin</td>
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<td>21 4</td>
<td>D</td>
<td>4.0</td>
</tr>
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<td>Nystatin</td>
<td>None 19</td>
<td>None 8</td>
<td>T</td>
<td>1.0</td>
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<td>Oleandomycin</td>
<td>11 11</td>
<td>21 4</td>
<td>D</td>
<td>1.0</td>
</tr>
<tr>
<td>Oxacillin</td>
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<td>21 4</td>
<td>A</td>
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<td>Paromomycin</td>
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<td>21 4</td>
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<td>21 4</td>
<td>A</td>
<td>1.0</td>
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<td>Penicillin V</td>
<td>2 2</td>
<td>21 4</td>
<td>A</td>
<td>1.0</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>9 10</td>
<td>21 4</td>
<td>F</td>
<td>0.1</td>
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<td>Rifampin</td>
<td>2 2</td>
<td>21 4</td>
<td>H</td>
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<td>Sisomicin</td>
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<td>D</td>
<td>0.03</td>
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<tr>
<td>Streptomycin</td>
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<td>21 4</td>
<td>H</td>
<td>b</td>
</tr>
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<td>Ticarcillin</td>
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<td>Vancomycin</td>
<td>8 8</td>
<td>10 4</td>
<td>H</td>
<td>b</td>
</tr>
</tbody>
</table>

a Use dilution of the suspension that gives 25% light transmission in lieu of the stock suspension.

b Determine the amount of the inoculum by the use of test plates.
Stainless steel or glazed ceramic penicylinders can be placed on the inoculated agar surface of assay plates. Fill the cylinders with individual solutions. Each cylinder will hold at least 100 µL. Penicylinders must seat firmly on the agar surface, and all movement of the cylinders must be prevented after they are filled to prevent leakage of the solutions. Cylinders that leak produce
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Initial Solvent (Number)</th>
<th>Subsequent Diluent (µg/mL)</th>
<th>Stock Solution Concentration</th>
<th>Standard Response Line (µg/mL)</th>
<th>Storage Time at 2–8°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>3</td>
<td>DW</td>
<td>1.000</td>
<td>0.064, 0.08, 0.1, 0.125, 0.156</td>
<td>1 week</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>DMSO</td>
<td>DMSO</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>Use immediately</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>DW</td>
<td>DW</td>
<td>1.000</td>
<td>0.064, 0.8, 0.1, 0.125, 0.156</td>
<td>1 week</td>
</tr>
<tr>
<td>Bacitracin zinc</td>
<td>0.01 N HCl</td>
<td>100 units</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>Use immediately</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>12.8, 16, 20, 25, 31.2</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>3.2, 4, 5, 6.25, 7.81</td>
<td>1 day</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>12.8, 16, 20, 25, 31.2</td>
<td>Use same day</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>3</td>
<td>1</td>
<td>1.000</td>
<td>1.28, 1.6, 2, 2.5, 3.12</td>
<td>1 day</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>5 days</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>6.4, 8, 10, 12.5, 15.6</td>
<td>Use same day</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>12.8, 16, 2, 25, 31.2</td>
<td>Use same day</td>
</tr>
<tr>
<td>Cephalixin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>12.8, 16, 20, 25, 31.2</td>
<td>1 week</td>
</tr>
<tr>
<td>Cephaloglycin</td>
<td>DW</td>
<td>DW</td>
<td>100</td>
<td>6.4, 8, 10, 12.5, 15.6</td>
<td>1 week</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>5 days</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>5 days</td>
</tr>
<tr>
<td>Cephalxin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>5 days</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>6.4, 8, 10, 12.5, 15.6</td>
<td>5 days</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>EtOH</td>
<td>EtOH</td>
<td>1.000</td>
<td>4, 6, 8, 12, 16</td>
<td>1 week</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>DW</td>
<td>DW</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>1 month</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>3.2, 4, 5, 6.25, 7.81</td>
<td>1 week</td>
</tr>
<tr>
<td>Colistimethate</td>
<td>6</td>
<td>6</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>Use same day</td>
</tr>
<tr>
<td>Colistin</td>
<td>6</td>
<td>6</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Cyclacillin</td>
<td>DW</td>
<td>DW</td>
<td>1.000</td>
<td>0.64, 0.8, 1, 1.25, 1.56</td>
<td>1 day</td>
</tr>
<tr>
<td>Drug</td>
<td>Derivative</td>
<td>Concentration (mg/mL)</td>
<td>Dilution</td>
<td>Storage</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------</td>
<td>-----------------------</td>
<td>----------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>Dactinomycin</td>
<td></td>
<td>3 1,000 0.5, 0.71, 1, 1.41, 2</td>
<td>3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td></td>
<td>1 1,000 3.2, 4.5, 6.25, 7.81</td>
<td>1 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydro-streptomycin</td>
<td></td>
<td>3 1,000 0.64, 0.8, 1, 1.25, 1.56</td>
<td>30 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td>3 1,000 0.64, 0.8, 1, 1.25, 1.56</td>
<td>2 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Flucytosine</td>
<td>DMSO</td>
<td>1,000 5, 7.5, 15, 25, 50, 75, 100</td>
<td>Use same day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>3 1,000 0.064, 0.08, 0.1, 0.125, 0.156</td>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itraconazole DMSO</td>
<td>DMSO</td>
<td>1,000 0.25, 0.75, 2, 6, 15, 25</td>
<td>Use same day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin B</td>
<td></td>
<td>3 1,000 0.64, 0.8, 1, 1.25, 1.56</td>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td></td>
<td>1 1,000 6.4, 8, 10, 12.5, 15.6</td>
<td>4 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nafcillin</td>
<td></td>
<td>1 1,000 1.28, 1.6, 2, 2.5, 3.12</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neomycin</td>
<td></td>
<td>3 1,000 0.64, 0.8, 1, 1.25, 1.56</td>
<td>2 weeks</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If test organism D is used

If test organism A is used

Netilmicin<sup>a</sup> 3 1,000 0.064, 0.08, 0.1, 0.125, 0.156 | 1 week |

Novobiocin or EtOH 3 1,000 0.32, 0.4, 0.5, 0.625 | 5 days |

Nystatin<sup>c</sup> (Use red low actinic glassware) DMF 6 1,000 units 12.8, 16, 20, 25, 31.2 | Use same day |

Oleandomycin ethyl alcohol 3 1,000 3.2, 4, 5, 6.25, 7.81 | 30 days |

Oxacillin 1 1,000 3.2, 4, 5, 6.25, 7.81 | 3 days |

Penicillin G or V 1 units 1,000 0.64, 0.8, 1, 1.25, 1.56 | 4 days |

Polymyxin B<sup>b</sup> 6 units DW 1,000 6.4, 8, 10, 12.5, 15.6 | 2 weeks |

Rifampin MeOH DW (stir) 1,000 3.2, 4, 5, 6.25, 7.81 | 1 day |

Streptomycin 3 1,000 0.64, 0.8, 1, 1.25, 1.56 | 30 days |

Sisomicin<sup>b</sup> 3 1,000 0.064, 0.08, 0.1, 0.125, 0.156 | 2 weeks |

Ticarcillin 1 1,000 3.2, 4, 5, 6.25, 7.81 | 1 day |

Vancomycin DW 1,000 6.4, 8, 10, 12.5, 15.6 | 1 week |

DW = distilled water, HCl = hydrochloric acid, MeOH = methyl alcohol, EtOH = ethyl alcohol, DMSO = dimethylsulfoxide, DMF = dimethylformamide.

<sup>a</sup> Additional suggestions can be found in the current edition of the U.S. Pharmacopeia.

<sup>b</sup> Working standard should be stored below 20°C under an atmosphere of nitrogen. Netilmicin sulfate and sisomicin are hygroscopic and care should be exercised during weighing.

<sup>c</sup> For assay of nystatin pastilles, use 80% aqueous dimethylformamide as the initial solvent and as diluent for all dilutions where dimethylformamide is required.
erratically shaped and sized inhibition zones that invalidate the zone diameters on the plate. The cylinders must be thoroughly decontaminated, cleaned, and rinsed between uses to prevent cross-contamination. Decontaminate and clean cylinders by immersion in water containing a glassware cleaner and autoclave for 15 min. Rinse cylinders thoroughly with tap water followed by distilled water to remove the cleaner. Periodically it may be necessary to clean the cylinders with 6 N nitric acid followed by thorough rinsing to remove protein buildup.

14.3.8 INCUBATION

Incubate assay plates at a temperature suitable for growth of the indicator organism. Use an incubator that maintains a closely controlled, uniformly distributed temperature. Minimize the temperature variation in the incubator because small differences in the incubator temperature can influence zone diameters. A water jacketed incubator maintains a more uniform temperature than does a flowing air incubator and may vibrate less. Vibration during incubation is a particular concern for assays with penicylinders. The duration of incubation depends on the growth rate of the indicator organism. Incubate assay plates in stacks no more than four high to minimize temperature variation within the stack.

14.3.9 ZONE DIAMETER MEASUREMENT

The diameter of zones of inhibition is determined to the nearest 0.1 mm using calipers, a Fisher-Lilly zone reader, or other similar device. Repeated measurements of individual zone diameters on a set of plates using a Fisher Lilly zone reader can be expected to vary by <0.2 mm on average. Consistent variation in excess of 0.2 mm for repeated measurements suggests that the zone edge definition is poor, the zone reader permits slack movement of the measurement scale, or operator variability may be influencing the results. Computerized image analyzers are available that enable the analyst to record images of each assay plate for subsequent analysis. Discrimination in locating the zone edges is a critical variable in computerized image analysis.

14.3.10 CALCULATIONS

14.3.10.1 Self-Contained Assays

There is a linear relationship between the diameter of the zone of inhibition, in millimeters, and the logarithm of the concentration of solutions used to prepare the standard response line. A plot of the resulting data should be a straight line [24,28,29].

Compute the average zone diameter for each standard response line concentration using data from all replicate plates. Compute the average zone diameter for the sample. Using two- or three-cycle semilogarithmic graph paper, plot the logarithm of the standard response line concentrations against the average zone diameter for each concentration. Fit a line to the plotted points using a least squares regression statistical analysis [24,30]. Determine the sample concentration that corresponds to the average zone diameter of the sample. Multiply the determined sample concentration by any specimen dilutions that were made.

14.3.10.2 Internally Normalized Assays

Compute the average zone diameter of each standard response line concentration using data from all replicate plates. Compute the average zone diameter of the common solution on each set of replicate plates. Compute the overall average zone diameter for the common solution on all assay plates in all sets. Compute the average zone diameter for each sample. For each set of replicate plates, compare the computed average response produced by the common solution in that set of plates to the computed overall (all plates) response produced by the common solution. Normalize
the response of the common solution on each set of replicate plates. Normalize the response of the unique solution on each set of replicate plates using the same correction required to normalize the response of the common solution on those plates. For example, if the overall common solution response was 20.0 mm and common solution response for a set of replicate plates was 19.8 mm, add 0.2 mm to the average response of the common solution and add 0.2 mm to the average response of the unique solution on those plates.

Plot the logarithm of the standard response line concentrations against the averaged and normalized zone diameter for each concentration on two- or three-cycle semi-logarithmic graph paper. Fit a line of least squares to the plotted points. Determine the sample concentration that corresponds to the averaged and normalized zone diameter of the sample. Multiply the determined sample concentration by any specimen dilution that was made.

Bioassays frequently are less reproducible and have a greater standard deviation between replicate assays than assays based on physical or chemical parameters. Typical agar diffusion assays have a standard deviation of about 10%; however, bioassays with a standard deviation of 5%–6% are reported [32].

14.3.11 ANTIMICROBIAL AGENTS

The concentration of antimicrobial agents in body fluids can be assayed following the same general principles that apply to bioassay of antibacterial agents. However, the value of determining blood levels for antimicrobial agents other than 5-flucytosine (5-FC) is questionable. The differences between the two types of assays include: the type of indicator organism, inoculum CFU per milliliter, the growth and assay media, temperature, and duration of the incubation, and frequently the solvent used to solubilize the reference standard [33,34].

Bioassays of multiple antimicrobial agents in a single specimen are typically run using intrinsically resistant organisms or organisms that have developed decreased susceptibility during therapy or by selective inactivation. Examples of selective inhibition include heat inactivation of amphotericin B, 90°C for 30–45 min, in the presence of 5-FC, or addition of 0.05% cytosine to the assay medium to suppress the activity of 5-FC in the presence of amphotericin B [1,18,35,42]. Antifungal agent concentrations can also be determined using high performance liquid chromatography (HPLC) [18,35–38].

The clinical implications of specific antifungal agent concentrations have not yet been as clearly established as they have for many antibacterial agents. Interpretation of assay results is best made by laboratory personnel in consultation with local infectious disease and pharmacy staff [40–42].

14.4 ALTERNATIVE ASSAYS

14.4.1 TURBIDIMETRIC BIOASSAY

The term turbidity is used to define any technique that depends upon the change in bacterial mass after incubation in liquid medium and that is used to quantify the amount of antibiotic in a solution. The change in bacterial mass is inversely proportional to the amount of antibiotic present. The change in bacterial mass is measured photometrically after the cells are killed. The cells are killed by heating the culture to at least 80°C for several minutes or by addition of 0.5 mL of 12% formalin for every 10 mL of culture volume [28]. The photometric measurements can be automated and the resulting computer file, if secure, saved as raw data.

The photometric data can be plotted as percent transmission, optical density, or other data algorithm as a function of concentration of antimicrobial, or equivalently, dilution of a reference sample. Turbidimetric assays have the capability of rapidly testing a large number of samples. Using a log phase inoculum, turbidimetric antibacterial assays often require <4 h of incubation [30] (Table 14.7).
Antimicrobial Susceptibility Testing Protocols

The standard response line for a turbidimetric assay typically spans a reduced range of concentrations relative to agar diffusion assays. The plotted standard response line is evaluated point to point without fitting the data to a straight line. The standard response line may or may not be a straight line (Table 14.8). Each rack of assays must contain its own replicated set of standard concentrations and samples. Inclusion of a “known unknown” as the first and last sets of tubes of a turbidimetric assay permits the analyst to monitor and correct for possible changes in indicator organism growth (assay drift) during the assay. Assays can be highly reproducible when a replicated parallel line assay format is employed, incubation temperatures are closely controlled, ±0.1°C, and readings are made using a sensitive photometer. The standard deviation of the replicate assays is typically <4% [24,28,30]. Automated commercially available laboratory instrumentation for running turbidimetric assays following these principles has been developed [28,29]. Use of a validated computer program to analyze the data is recommended because of the large amount of data generated.

Turbidimetric assays are infrequently used to determine the antimicrobial content of clinical specimens; the automated equipment and computer programs are expensive and require highly trained operators. Turbidimetric bioassays are more likely to be used in a quality control or research environment.

14.4.2 ASSAYS BASED ON PHYSICAL OR CHEMICAL METHODS

14.4.2.1 Overview

Historically, about 75%—85% of all antimicrobial concentrations in clinical specimens were determined by microbiological assays. These assays have frequently been replaced by more rapid, sensitive, and accurate nonmicrobiological assays [1,2,9,10]. Currently there are a number of chemical or physical methods available to determine the level of antimicrobial agents in body fluids. The choice of method(s) depends on the antimicrobial agent, type of specimen, and laboratory resources available at the institution where the assay is performed [1,2,4,5,11–18,26] (Table 14.9).
## TABLE 14.8
Microbiological Assay Methods: Examples of Turbidometric Assay Standard Response Lines for Aqueous Specimens

<table>
<thead>
<tr>
<th>Antibiotic (1 mg/mL)</th>
<th>Initial Solvent</th>
<th>Diluent Solvent</th>
<th>Final Antibiotic Concentration (µg/mL)</th>
<th>Storage at 2–8°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>DW</td>
<td>DW</td>
<td>8, 8.9, 10, 11.2, 12.5</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Candicidina</td>
<td>DMSO</td>
<td>DW</td>
<td>0.03, 0.043, 0.06, .08, .085, 0.12</td>
<td>Use same day</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>DW</td>
<td>DW</td>
<td>80, 89, 100, 112, 125</td>
<td>7 days</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>EtOH</td>
<td>DW</td>
<td>2, 2.24, 2.5, 2.8, 3.12</td>
<td>1 month</td>
</tr>
<tr>
<td>Chlor-tetracycline</td>
<td>0.01 N HCl</td>
<td>DW</td>
<td>0.048, 0.054, 0.06, 0.067, 0.075</td>
<td>4 days</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>DW</td>
<td>DW</td>
<td>40, 44.5, 50, 56, 62.5</td>
<td>1 month</td>
</tr>
<tr>
<td>Dihydro-streptomycin</td>
<td>DW</td>
<td>DW</td>
<td>24, 26.8, 30, 33.5, 37.5</td>
<td>30 days</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.1 N HCl</td>
<td>DW</td>
<td>0.08, 0.089, 0.1, 0.112, 0.125</td>
<td>5 days</td>
</tr>
<tr>
<td>Gramicidin</td>
<td>EtOH U.S.P.</td>
<td>EtOH</td>
<td>0.032, 0.0356, 0.04, 0.0448, 0.05</td>
<td>30 days</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>DW</td>
<td>DW</td>
<td>8, 8.9, 10, 11.2, 12.5</td>
<td>1 month</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>DW</td>
<td>DW</td>
<td>0.4, 0.447, 0.5, 0.559, 0.625</td>
<td>1 month</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.1 N HCl</td>
<td>DW</td>
<td>0.192, 0.215, 0.24, 0.268, 0.3</td>
<td>4 days</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>DW</td>
<td>DW</td>
<td>24, 26.8, 30, 33.5, 37.5</td>
<td>1 month</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>DW</td>
<td>DW</td>
<td>24, 26.7, 30, 33.5, 37.5</td>
<td>30 days</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.1 N HCl</td>
<td>DW</td>
<td>0.192, 0.215, 0.24, 0.268, 0.3</td>
<td>1 day</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>DW</td>
<td>DW</td>
<td>2, 2.236, 2.5, 2.795, 3.125</td>
<td>2 weeks</td>
</tr>
</tbody>
</table>

DW = distilled water, HCl = hydrochloric acid, EtOH = ethyl alcohol, DMSO = dimethylsulfoxide.

The gramicidin working standard and the gramicidin standard response line concentrations are used for the assay of tyrothricin.

* Use sterile equipment for all stages of this assay.

## TABLE 14.9
Antimicrobial Assay Methods

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity (µg/mL)</th>
<th>Specificity</th>
<th>Time Required</th>
<th>Sample Size</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>0.1</td>
<td>Metabolite</td>
<td>2–4 h</td>
<td>50–200 µL</td>
<td>Sensitive, specific</td>
<td>Radioactive waste, costly reagents, and equipment</td>
</tr>
<tr>
<td>EMIT</td>
<td>1–2</td>
<td>Highly</td>
<td>10 min</td>
<td>&lt;100 µL</td>
<td>Rapid, specific</td>
<td>Low sensitivity, expensive reagents</td>
</tr>
<tr>
<td>FPI</td>
<td>0.3–2</td>
<td>Highly</td>
<td>10–30 min</td>
<td>50 µL</td>
<td>Automated, sensitive, specific, rapid</td>
<td>Costly reagents and equipment</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.1</td>
<td>Highly</td>
<td>30–60 min</td>
<td>&lt;1 mL</td>
<td>Sensitive, specific, rapid, versatile</td>
<td>Expensive equipment, technically difficult</td>
</tr>
<tr>
<td>Bioassay</td>
<td>0.1–5</td>
<td>Low, other</td>
<td>4–48 h</td>
<td>1–3 mL</td>
<td>Simple, versatile, inexpensive</td>
<td>Poor sensitivity, specificity, speed, and precision</td>
</tr>
</tbody>
</table>

RIA: Radioimmune assay; EMIT: enzyme multiplied immunoassay; FPI fluorometric assay; HPLC: high performance liquid chromatography.
14.4.2.2 High Performance Liquid Chromatography (HPLC)

This technique is specific and sensitive and can be used to assay many classes of antimicrobial agents. HPLC methods have the capability to separate parent drug from metabolites or breakdown products. HPLC is the method of choice for many antimicrobial agents that contain aβ-lactam ring. Separation of the antimicrobial agent from the surrounding biological fluid using organic solvents may be necessary. The disadvantages of HPLC methods include costly equipment, the need for highly trained technologists, and the need to dispose of used solvents. HPLC units are often dedicated to a single function or antimicrobial agent. These factors restrict use of HPLC methods in many laboratories.

14.4.2.3 Fluorometry

Fluorometric methods are accurate, sensitive, highly specific, and can be automated. The disadvantages of fluorometric methods include cost of equipment and specimens are often tested in batches. These tests are frequently used to assay aminoglycoside and glycopeptide concentrations.

14.4.2.4 Radioenzymatic Assay (RIA)

RIA assays have greater sensitivity and specificity, and are more rapidly completed than bioassays. They are infrequently used in clinical laboratories because of the cost of equipment and supplies, and the use of radioisotopes.

14.4.2.5 Enzyme Multiplied Immunoassay Technique (EMIT)

EMIT assays have greater specificity than bioassays; however, they are less sensitive and require expensive reagents. They are not often used in clinical laboratories in the United States.

14.5 INTERPRETATION OF ASSAY RESULTS

The interpretation of the importance of a given antimicrobial concentration is a complex undertaking. Several variables must be considered in addition to the antimicrobial agent and its absolute concentration. The variables include: the type of specimen tested, patient related physiological variables that modify drug disposition or excretion, interactions with concomitant medications, and possible interference caused by metabolic or breakdown products of the antimicrobial. An example of an interference due to drug metabolism is the effect of hydroxyitraconazole on the bioassay for itraconazole.

The concentration of an antimicrobial agent that is acceptable in a urine specimen is unlikely to be acceptable in CSF or serum specimens. The concentration of an antimicrobial agent in serum that has a high probability of producing a therapeutic success can be predicted using the highest minimum inhibitory concentration (MIC) for that antimicrobial that defines a fully susceptible organism of the type causing the infection. Following this rationale, the therapeutic levels of several antimicrobials are presented (Table 14.10).

14.6 GENERIC AGAR DIFFUSION BIOASSAY PROTOCOL

14.6.1 Equipment Needed

1. Flat and level bench top.
2. Analytical balance capable of accurately weighing to at least four decimal places to weigh reference antimicrobial agents.
3. General purpose laboratory weighing scale to weigh chemicals and powdered media.
4. Turbidity standards or photometer to standardize the inoculum.
5. Autoclave to sterilize media and buffers and to decontaminate waste.
6. Appropriate nutritive agar(s) for the inoculum and the assay plates.
7. Appropriate indicator organism(s).
8. Incubators controlled to the desired temperatures.

### TABLE 14.10

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Organism Group</th>
<th>Therapeutic Range a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Lactams</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Enterobacteriaceae</td>
<td>≤8</td>
</tr>
<tr>
<td></td>
<td>Enterococcus spp.</td>
<td>≤8</td>
</tr>
<tr>
<td>Methicillin</td>
<td>Staphylococcus spp.</td>
<td>≤8</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>Staphylococcus aureus</td>
<td>≤2</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>Staphylococcus aureus</td>
<td>≤2</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Staphylococcus spp.</td>
<td>≤0.12</td>
</tr>
<tr>
<td>Most cephalosporins</td>
<td>Staphylococcus aureus</td>
<td>≤8</td>
</tr>
<tr>
<td>Carbapenems</td>
<td>Staphylococcus spp.</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td></td>
<td>≤4</td>
</tr>
<tr>
<td>Meropenem</td>
<td></td>
<td>≤4</td>
</tr>
<tr>
<td>Ertapenem</td>
<td></td>
<td>≤2</td>
</tr>
<tr>
<td><strong>Glycopeptides</strong></td>
<td></td>
<td>≤8</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td></td>
<td>≤8</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>≤8</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
<td>≤16</td>
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<tr>
<td>Amikacin</td>
<td>Enterococcus spp.</td>
<td>≤500</td>
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<tr>
<td>Gentamicin</td>
<td>Other organisms</td>
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<tr>
<td>Kanamycin</td>
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<td>Netilmicin</td>
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<td>Tobramycin</td>
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<td><strong>Macrolides</strong></td>
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<tr>
<td>Azithromycin</td>
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<tr>
<td>Clarithromycin</td>
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<tr>
<td>Erythromycin</td>
<td></td>
<td>≤0.5</td>
</tr>
<tr>
<td><strong>Other Classes</strong></td>
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<td>≤4</td>
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<tr>
<td>Tetracycline</td>
<td></td>
<td>≤1</td>
</tr>
<tr>
<td>Most fluoroquinolones</td>
<td></td>
<td>≤1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>≤1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>≤8</td>
</tr>
<tr>
<td>Clindamycin</td>
<td></td>
<td>≤0.5</td>
</tr>
<tr>
<td>Rifampin</td>
<td></td>
<td>≤1</td>
</tr>
</tbody>
</table>

1. The therapeutic ranges are based upon CLSI document M100-S16 fully susceptible category MIC values. [Clinical and Laboratory Standards Institute (formerly NCCLS). Performance standards for antimicrobial susceptibility testing. document M100-S16, Clinical and Laboratory Standards Institute, Wayne, PA, January 2006.]
10. Appropriate solvent for reference standard and sterile diluent to prepare standard response line concentrations and if necessary to dilute specimens.
11. Sterile petri dishes of the appropriate diameter or sterile Nunc plates.
12. S & S disks of appropriate diameter, well cutter with attached vacuum source, or penicylinders.
13. Water bath controlled to 45°C–48°C; if B. subtilis ATCC 6633 spore suspension is used as the indicator organism, the temperature can be higher, 50°C–55°C.
14. Volumetric flasks of sizes 10, 20, and 25 mL.
15. New disposable 16–18 × 125–175-mm glass tubes for samples.
16. Sterile serological pipettes of assorted sizes.
17. Adjustable micropipette with sterile tips to apply samples or forceps to hold disks while dipping and draining.
18. Calipers or a Fisher-Lilly or equivalent zone reader.
19. Gloves, lab coat, safety glasses, marker pen, paper, semilogarithmic graph paper, pen, and calculator or computer.

14.6.2 PROCEDURE FOR AN INTERNALLY NORMALIZED BIOASSAY

14.6.2.1 Assumptions

1. Use 90-mm petri dishes; 10 mL per plate of nutritive agar seeded with 2 × 10^5 CFU/mL of B. subtilis spores using a B. subtilis spore suspension containing 2 × 10^8 CFU/mL.
2. Use a 5-point standard response line with 4 assay plates (12 values) per concentration.
3. The stock reference solution contains 1.0 mg/mL of drug potency and the standard response line concentrations are 1, 1.5, 2.5, 4, and 9 µg/mL. The midpoint of the standard response line, 2.5 µg/mL, is the internal reference (common) solution. The reference powder has a potency of 90% = 900 µg/mg or mg/g and is soluble in 1% pH 6 phosphate buffer. Apply 20 µL to each 6.3-mm (1/4-in.) S&S 740E disk.
4. Five urine samples require testing. Four urine samples do not require dilution and one has been determined in a screening assay to require a dilution of 1:50 before it is assayed.
5. Each sample is tested on 4 assay plates (12 values). Apply 20 µL of a solution to each 6,3-mm (1/4-in.) S&S 740E disk.

Note: For samples that are screened for antimicrobial activity: the change in zone diameter produced by halving the drug concentration of the standard is the same millimeter change in zone diameter that a 1:2 dilution of the sample will produce. For example, if 2 µg/mL of standard produces an 18-mm zone of inhibition and 1 µg/mL of standard produces a 15-mm zone diameter; a 1:2 dilution of the specimen would reduce the zone diameter by 18 mm – 15 mm = 3 mm. A dilution of 1:8 is mathematically equivalent to three consecutive 1:2 dilutions. A 1:8 dilution of the specimen (1 part specimen + 7 parts diluent) would reduce the zone of inhibition about 3 mm × 3 dilutions = 9 mm. Zones of inhibition whose size is limited by diffusion, typically zones with a diameter exceeding 40 mm, may not closely follow this example. A 1:2 dilution of such a specimen may reduce the zone diameter by less than the calculated number of millimeters.

14.6.2.2 Protocol

1. Inoculate the indicator organism on the surface of an appropriate agar growth medium and incubate. Harvest the resulting bacterial growth using a minimal volume of sterile pH 6–7 phosphate buffer or sterile 0.9% saline in the mid to late log phase of growth. Prolonged incubation, 5–7 days, may be required if spores are required. Standardize the cell density using McFarland barium sulfate turbidity standards or a photometer [3,23].
This step is not necessary if a stock \textit{B. subtilis} spore suspension containing a known number of CFU per milliliter of spores is the indicator and the desired spore concentration in the seeded agar is known. Determine the number of spores in a spore suspension by means of a standard plate count. \textit{Bacillus subtilis} spore suspensions can be kept at 2°C–8°C for several months before use.

2. Determine which bioassay format and size of assay plate will be used (internally normalized assay with 90-mm petri dishes).

3. Determine the number of plates required and prepare a few extras \((20 + 20 + 5 = 45)\).

4. Calculate the amount of agar required \((10 \times 45 = 450 \text{ mL})\).

5. Prepare an amount of suitable nutritive agar for all assay plates following manufacturer’s directions. Include a 10%–15% overage to account for losses during autoclaving and provide a small surplus. If agar is prepared in an Erlenmeyer flask, fill the flask to no more than 65% of capacity \((450 + 50 = 500 \text{ mL}, \text{ prepare the agar in a 1-L flask})\).

6. Autoclave the nutritive agar following manufacturer’s directions. Place the autoclaved agar in the temperature-controlled water bath for at least 1 h before use to permit temperature equilibration of the agar. Do not hold melted and cooled agar in the water bath longer than necessary. Excessive heat can damage the nutrient qualities of media.

7. Add the inoculum to the autoclaved and cooled agar then mix thoroughly without formation of air bubbles \((500 \text{ mL} \times 2 \times 10^5 \text{ CFU/mL} = 1 \times 10^8 \text{ total CFU required/2} \times 10^8 \text{ CFU/mL of spore suspension; use 0.5 mL of spore suspension per 500 mL agar})\).

8. Dispense the desired volume of seeded (inoculated and mixed) agar into each assay plate on a flat and level bench top or in a biosafety hood. Dispense the seeded agar with a Cornwall dispensing syringe or other automated dispenser to minimize plate-to-plate variation in the volume dispensed and to minimize bubble formation. Use of a serological pipette to dispense seeded agar may cause variation in the volume dispensed and increase bubble formation. Bubbles should be avoided; break any bubbles that develop by rapidly sweeping the surface of the agar plate with a small flame or by sweeping to the side using a pipette. Do not overheat the agar as the indicator may be damaged. Bubbles can interfere with zone formation and size determination.

9. Allow the agar to harden in each assay plate; then dry the surface by partially opening the plate lid for 20–30 min. The moist plates may be placed in a biosafety cabinet with the exhaust fan operating to dry. Do not dry plates in an incubator.

10. Prepare reference stock solution at a concentration of 1.0 mg/mL of potency. To prepare a 100 mL volume of reference stock solution: \((1 \text{ mg} \times 100 \text{ mL})/0.9 \text{ potency} = 111.1 \text{ mg of reference powder and 100 mL of solvent are required. Dissolve the powder in the solvent. A low power sonic cleaning bath is a useful aid when dissolving a poorly soluble powder})\).

11. Prepare standard response line concentrations using the reference stock solution as the starting material. Mix all solutions thoroughly before use.

\begin{align*}
10 \mu\text{g/mL} &= 0.25 \text{ mL stock solution at 1,000 } \mu\text{g/mL diluted to volume in a 25-mL volumetric flask with diluent.} \\
9 \mu\text{g/mL} &= 9.0 \text{ mL of 10 } \mu\text{g/mL diluted to volume with diluent in a 10-mL volumetric flask.} \\
4 \mu\text{g/mL} &= 4.0 \text{ mL of 10 } \mu\text{g/mL diluted to volume with diluent in a 10-mL volumetric flask.} \\
1.5 \mu\text{g/mL} &= 1.5 \text{ mL of 10 } \mu\text{g/mL diluted to volume with diluent in a 10-mL volumetric flask.} \\
1.0 \mu\text{g/mL} &= 1.0 \text{ mL of 10 } \mu\text{g/mL diluted to volume with diluent in a 10-mL volumetric flask.}
\end{align*}

12. Prepare the internal reference (common solution).

\begin{align*}
2.5 \mu\text{g/mL} &= 5.0 \text{ mL of 10 } \mu\text{g/mL diluted to volume with diluent in a 20-mL volumetric flask.} \\
\text{Note: Tubes or small flasks may be used instead of volumetric flasks. When using tubes or small flasks, determine the volume of diluent to add by subtracting the volume of reference stock solution used from the total volume required. For example, to prepare the 10-}
\mu\text{g/mL stock solution, add 24.75 mL of diluent to the 0.25 mL of reference stock solution.}
\end{align*}
13. Label each solution as it is prepared. Place all solutions for assay in an ice bath or refrigerator until they are assayed. The reference stock solution should be labeled, dated, assigned an expiration date, and small aliquots stored at the appropriate temperature.

14. Place aliquots of the four urine samples that do not require dilution in separate tubes and label.

15. Place 200 µL of the urine that requires dilution in a tube and add 9.8 mL of the diluent, 1% phosphate buffer pH 6.0, mix thoroughly, and label.

16. Place six disks, equally spaced, approximately 12–14 mm from the edge of an assay plate and place a mark on the edge of the plate to indicate the location of disk number 1. The same disk numbering sequence, either clockwise or counterclockwise from the starting point (disk number 1), should be used on all plates and by all analysts.

17. Place 20 µL of the common solution on disks 1, 3, and 5.

18. Place 20 µL of one standard response line concentration on disks 2, 4, and 6.

19. Replace the lid on the plate and repeat steps 16 to 18 three times.

20. Repeat steps 16 to 19 using each of the remaining standard response line concentrations.

21. For each sample, repeat steps 16 to 19 replacing the standard response line concentration with one of the samples.

22. Place all plates in the temperature controlled incubator with the agar side down in stacks of no more than four plates, for 16–18 h.

23. Remove plates from the incubator.

24. Verify the zero and full scale readings on the caliper or zone reader.

25. Determine the diameter of each zone of inhibition to the nearest 0.1 mm.

26. Compute the average zone diameter for each material on each set on four replicate assay plates.

27. Compute the overall average zone diameter of the common solution on all assay plates; this is the normalized average common solution zone diameter.

28. Normalize the average zone diameter of the common solution on each set of four plates and in like manner normalize the zone diameter of the standard or sample on these plates.

29. Plot the logarithm of the concentration versus linear normalized average zone diameter of all standards and the common solution; fit a standard response line to the data using the method of least squares.

30. Determine the concentration of each sample using the normalized average sample zone diameter and the standard response line. Multiply the resulting concentration by the sample dilution. In this generic assay, the sample dilution of 4 of the urine specimens is 1 and the dilution of the remaining sample is 50.

Note: All calculations may be done manually, using a calculator, or computer. As a rule of thumb, consider excluding as an outlier from the standard response line any single point that is located more than about 0.6–0.7 mm from the line of best fit. A statistical test for outlying data can be used to verify the correctness of the decision to omit the data point.

REFERENCES


15 Molecular Methods for Bacterial Strain Typing

Sophie Michaud and Donna Berg

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15.1 INTRODUCTION

Determining whether two or more isolates of the same species represent the same strain is often needed to understand and control the spread of disease in both hospitals and communities [1]. Typical problems include investigating a putative outbreak of a nosocomial pathogen, such as methicillin-resistant *Staphylococcus aureus* (MRSA), or establishing whether the isolation of a common pathogen such as *Campylobacter jejuni* from two successive episodes of enteritis in a single patient represents a relapse or a reinfection [2].

Biotyping (the pattern of biochemical reactions that can be produced by an isolate) and antimicrobial susceptibility testing are the two phenotypic typing methods performed by the clinical microbiology laboratory during routine assessment of essentially all isolates. These methods are typically characterized by both poor reproducibility and poor discriminatory power. First, most isolates of a species have similar metabolic reactions and therefore represent relatively few biotypes. Second, microorganisms are able to alter unpredictably the expression of many cellular products. Finally, antibiotic resistance is under strong selective pressure in hospitals, is often associated with mobile genetic elements such as transposons and plasmids, or may reflect spontaneous point mutations, for example, resistance to quinolones. Consequently, unrelated isolates may have indistinguishable resistance profiles, and conversely, isolates that are otherwise genetically indistinguishable may show different antimicrobial susceptibilities [1].

Biotyping and antimicrobial susceptibility testing are thus currently most useful as screening techniques. Culturing a set of isolates with a distinctive biotype or antimicrobial susceptibility pattern is often the first indication of an outbreak, which must then be confirmed by clinical and
molecular epidemiological data. The main terms describing genotypic and epidemiological relationships among isolates are defined in Table 15.1.

The primary goal of strain typing studies is to provide laboratory evidence that epidemiologically related isolates are also genetically related and thus represent the same strain. Although molecular typing may contribute substantially to deciphering complex epidemiological processes, it complements, but does not replace basic clinical and epidemiological investigations. The integration of clinical, epidemiological, and typing data are essential for deriving valid and useful conclusions. Bacterial typing is most effective as an aid to outbreak investigations when it is applied to small sets of epidemiologically related isolates in the context of carefully defined questions developed by the clinician or epidemiologist. While typing may indicate that isolates recovered from multiple individuals or from both individuals and the environment represent a single strain, typing alone cannot establish the mode or the direction of transmission [5]. To avoid misinterpretations due to bias or preconceptions, typing should be performed independently and the data should be analyzed according to clear, predefined criteria. The results should then be considered by the infectious diseases or infection control practitioner in collaboration with the microbiology laboratory [1]. Genotypic methods are especially valuable when the epidemiological hypothesis is incorrect; in such instances, analysis of the isolates helps reorient the infection control decisions [5].

Molecular typing may also contribute to understanding the pathophysiology and managing infections in individual patients, based on the assumption that acute infections due to a single species are monoclonal. For example, when multiple isolates of the same species are cultured during distinct episodes of infection in a single patient, bacterial strain typing may help distinguish between a relapse with the same strain, indicating a residual focus or persistent colonization by a virulent organism,

### Table 15.1

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>Pure culture of bacteria obtained by subculture of a single colony from a primary isolation plate and presumed to be derived from a single organism.</td>
</tr>
<tr>
<td>Strain</td>
<td>Isolate or set of isolates that can be differentiated from other isolates of the same genus and species by phenotypic and/or genotypic characteristics. A strain is a relative concept, a descriptive subdivision of a species based on one or more analyses, and therefore should not be used as a synonym for isolate.</td>
</tr>
<tr>
<td>Epidemiologically related isolates</td>
<td>Isolates cultured from specimens taken from patients or the environment during a specific time frame or from a particular area as part of an epidemiological investigation that suggests that the isolates may be derived from a common source.</td>
</tr>
<tr>
<td>Genetically related isolates (clones)</td>
<td>Isolates that are indistinguishable from each other by a variety of genetic tests or that are so similar that they are presumed to originate from a common precursor. Such isolates are often referred to as having a particular “genotype,” representing a specific “genetic lineage” or to be “clonal.”</td>
</tr>
<tr>
<td>Outbreak</td>
<td>Increased incidence of an infectious disease in a specific place during a given period that is above the baseline rate for that place and time frame.</td>
</tr>
<tr>
<td>Outbreak strains</td>
<td>Isolates of the same species that are both genetically and epidemiologically related (e.g., by time, place, and common source); by implication, the outbreak represents a set of isolates that are derived from a common parent.</td>
</tr>
<tr>
<td>Endemic strains</td>
<td>Isolates that are recovered frequently from infected or colonized patients in a particular place and that are indistinguishable or closely related to each other by typing methods, but for which no direct or epidemiological relationship can be identified.</td>
</tr>
</tbody>
</table>

and a reinfection with a different strain. Studying the clonality of acute and chronic infections may also help to define the portal of entry of systemic infection when isolates of the same species are recovered from multiple samples in individual patients. For example, when multiple isolates of *Staphylococcus epidermidis* are recovered from independent cultures of patients with implanted foreign devices (e.g., prosthetic heart valves or joints), isolates representing a single strain are presumed to indicate the presence of an active infection, whereas the identification of multiple different strains is most compatible with contamination with the patient’s own colonizing flora [5].

Molecular strain typing performed with standardized protocols and computer-assisted normalized databases has also been applied successfully to national and international surveys of the emergence, molecular evolution, and geographical spread of epidemic clones of MRSA [6,7], *Streptococcus pneumoniae* [8–10], *Salmonella typhimurium* [11], *Pseudomonas aeruginosa* [12], *Neisseria meningitidis* [13], and *Mycobacterium tuberculosis* [14]. PulseNet, coordinated by the Centers for Disease Control (CDC), enables standardized pulsed-field gel electrophoresis (PFGE) typing of foodborne pathogens (*Escherichia coli* O157:H7, typhoidal and nontyphoidal Salmonella serotypes, *Listeria monocytogenes*, *Campylobacter*, *Vibrio cholerae*, and *Shigella* spp.) to be used by a network of public health laboratories; data can be compared directly in the central database after transfer on the Internet (www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm) [15]. Similar procedures are now being piloted in Europe, for example, the Enter-Net (the human enteric pathogen surveillance network; www.phls.co.uk/International/Enter-Net/enter-net.htm) and the HARMONY networks (harmonization of antibiotic resistant measurement, methods of typing organisms and ways of using these and other tools to increase the effectiveness of nosocomial infection control; www.phls.co.uk/international/harmony.htm).

Several DNA-based methods have been used for molecular strain typing of bacteria, including analysis of plasmid DNA, restriction endonuclease analysis of chromosomal DNA, Southern blot analysis of restriction fragment length polymorphisms (RFLP) of ribosomal RNA (ribotyping), macrorestriction fragment polymorphisms resolved by PFGE, polymerase chain reaction (PCR), and amplified fragment length polymorphism analysis (AFLP). A detailed description of the type-ability, reproducibility, discriminatory power, ease of interpretation, ease of performance, and extent of use of these genotypic methods has been published elsewhere [16,17]. This chapter particularly focuses on PFGE, which is considered the reference typing method for most bacterial species [18].

15.1.1 PFGE

Developed by Schwartz and Cantor in 1984, PFGE is a variation of agarose gel electrophoresis in which the electric field across the gel periodically changes direction and/or intensity (“pulsed”) rather than being kept constant as in conventional agarose gel electrophoresis. The pulsed field allows clear separation of very large molecular length DNA fragments ranging from 10 to 800 kbase [19], in contrast to unidirectional electrophoresis, which cannot separate DNA molecules larger than about 50 kbase in size because of molecular trapping in the gel [20].

Macrorestriction analysis of microbial genomes consists in PFGE separation of a limited number (<30) of large (>50 kbase) fragments of a bacterial chromosome digested with infrequently cutting restriction enzymes. This technique has emerged during the 1990s as the method of choice for studying the molecular epidemiology of bacterial pathogens [17,18,20–22]. It has been proven to be highly discriminatory (95%–100%) and reproducible for most bacterial species so far evaluated with large collections of unrelated isolates [20,23–25], and its performance has been found comparable or superior to that of other available techniques [18,23,25–39]. The high resolving power of PFGE is related to the fact that macrorestriction patterns scan >90% of the chromosome for large rearrangements (affecting >5% of fragment length) and approximately 0.05% of the genome for point mutations affecting restriction sites [20]. Of note, DNA prepared in agarose contains both chromosomal and plasmid DNA [40].
15.1.1.1 Performing PFGE

Conventional DNA extraction methods in solution are unsuitable for macrorestriction analysis because mechanical shearing degrades genomic DNA into fragments of several hundred kilobases that are inadequate for megabase-size restriction digestions [20]. Suitable chromosomal DNA is obtained by incorporating bacteria into agarose plugs. The cell preparation depends on the particular organism. Nonfastidious organisms are normally grown overnight in appropriate broth medium with orbital shaking, whereas fastidious organisms are preferably harvested from colonies grown on an appropriate solid medium [20].

Bacteria are suspended in an appropriate buffer, recovered by centrifugation, and then resuspended to a density of $1 \times 10^9$ CFU/mL, depending on the organism. The standardization of the DNA concentration in the agarose plugs is essential to obtaining comparable and informative fragment patterns [20]: Insufficient DNA will produce faint or incomplete patterns, whereas excess DNA will generate smears and result in retarded mobility of fragments during PFGE. The DNA concentration can be estimated from the bacterial cell density measured with a spectrophotometer or a densitometer, and adjusted to a standard value for a given species.

The embedded bacteria are then subjected to in situ detergent–enzyme lysis with a solution typically containing N-lauryl sarcosine, ethylenediaminetetraacetic acid (EDTA), proteinase K [2,41,42], RNAse, lysozyme, lysostaphin (for staphylococci [43]), and/or mutanolysin (for enterococci [44]). The detergent and proteolytic enzyme act together to remove cellular constituents, while the high EDTA concentration inhibits nuclease activity. To reduce the incubation time, researchers mix these cell-wall lytic enzymes with the cell suspension immediately prior to incorporation in the agarose [2,42,44–46].

After cellular and protein lysis, the agarose plugs are extensively washed with water and Tris-EDTA (TE) to remove proteolytic activity, detergent, and excess EDTA. The embedded high molecular size DNA is then digested with a restriction enzyme selected on the basis of the rarity of its recognition sequence in the target genome [20]. Hexamer endonucleases with G + C recognition sequences are rare cutters of A + T rich genomes, and vice versa [47]. Examples of restriction endonucleases commonly used for macrorestriction analysis of important human pathogens are shown in Table 15.2. In general, the enzyme Smal is used for PFGE typing of S. aureus [46], S. epidermidis, S. pneumoniae [48], and Enterococcus spp. [44], and XbaI for enterobacteriaceae, P. aeruginosa (some authors rather recommend SpeI [40]), and Stenotrophomonas maltophilia [41]. The application of multiple enzymes can be used to dictate especially stringent criteria for strain identity.

Slices of agarose containing the chromosomal DNA fragments are inserted into the wells of an agarose gel, and the electrophoresis resolves chromosomal restriction profiles typically composed of 5 to 20 distinct fragments ranging from approximately 10 to 800 kbase. The relative simplicity of the restriction profiles greatly facilitates the analysis and comparison of multiple isolates [18].

The optimal running parameters used for separation of DNA digests depend on the organism, enzyme, and PFGE apparatus. The most popular PFGE system used for bacterial typing is the contour-clamped homogeneous electric field (CHEF) apparatus, in which an hexagonal array of electrodes periodically alternates uniform fields with an angle of reorientation of 120°. Large DNA fragments migrate through the agarose in a zig-zagging motion, in response to changes in the direction of the electric field; the larger the molecule, the longer the time required for the migration. Thus, the time interval between changes of field direction (or pulse time) will primarily determine the size window for separation of DNA molecules by PFGE [49]. A typical ramp time from 5 to 40 s enables optimal separation of DNA fragments from 50 to 600 kbase, while increasing the pulse time to 75 s extends the separation to 1 Mbase with standard CHEF conditions [20].

The mobility of the DNA fragments in an agarose gel further depends on DNA concentration, the type and concentration of the agarose, voltage, ionic strength of the electrophoresis buffer, electrophoresis time and temperature [50]. Changes in any of these factors can lead to alterations
in the profiles, which then influence the comparability of patterns both within a single gel as well as between different gels.

PFGE gels are normally run at a temperature of 14°C, which is maintained with the use of a buffer cooling and recirculating system to prevent the generation of temperature gradients within the gel during the run [20]. Low ionic strength buffers (e.g., 0.25–0.5 × Tris-Acetic acid EDTA (TAE) or Tris-Boric acid EDTA (TBE)) are preferred to reduce heat generation and shorten the run times [49]. A voltage of 6 V/cm is used for the separation of molecules from 50 kbase to 1.5 Mbase in size. An agarose concentration of 1% is normally used for the separation of DNA fragments ranging from 50 kbase to 1 Mbase. Special PFGE-grade agarose with a high physical strength and low electro-endosmosis allows for a more rapid electrophoresis without loss of resolution [41].

Gels are stained with a fluorescent dye such as ethidium bromide [22], and the electrophoretic patterns are visualized by photography or digital image capture under UV light. The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness. With the aid of the computerized gel scanning and analysis software, it is possible to create data banks of PFGE patterns for all organisms, enabling the creation of reference databases to which any new strain can be compared for identifying its phylogenetic relationship to other similar strains [22].

### TABLE 15.2
**Suitable Restriction Enzymes for PFGE Typing of Selected Bacteria**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>SmaI, SsrII, CspI, BamH1</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>SmaI, SsrII, KpnI</td>
</tr>
<tr>
<td><em>Enterococcus spp.</em></td>
<td>SmaI, ApaI</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>SmaI, ApaI</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>ApaI, SmaI, NotI</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>XbaI, NotI, SfiI</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>XbaI, ApaI</td>
</tr>
<tr>
<td><em>Enterobacter spp.</em></td>
<td>XbaI, SpeI</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>XbaI</td>
</tr>
<tr>
<td><em>Citrobacter spp.</em></td>
<td>XbaI</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>XbaI</td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>XbaI, SfiI</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>SfiI, NotI</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>XbaI, SsrII, DraI</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>NotI, BglII, SpeI, SfiI</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>SpeI, DraI, SspI, XbaI</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>XbaI</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>SpeI</td>
</tr>
<tr>
<td><em>Acinetobacter baumanii</em></td>
<td>SmaI, ApaI</td>
</tr>
<tr>
<td><em>Burkholderia spp.</em></td>
<td>SpeI</td>
</tr>
<tr>
<td><em>Campylobacter spp.</em></td>
<td>KpnI, SmaI</td>
</tr>
<tr>
<td><em>Bacteroides spp.</em></td>
<td>NotI</td>
</tr>
<tr>
<td><em>Mycobacterium spp.</em></td>
<td>SfiI, NotI</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>SfiI, NotI</td>
</tr>
</tbody>
</table>

A detailed PFGE protocol suitable for both gram-positive and gram-negative organisms is described in the Appendix. Rapid PFGE protocols have been published for *E. coli* O157:H7 and other gram-negative organisms [41], *C. jejuni* [2, 42], *Enterococcus* spp. [44], *S. pneumoniae* [51], and MRSA [46].

### 15.1.1.2 Troubleshooting

The most common problems encountered in preparing DNA for PFGE are inadequate cell lysis, DNA degradation, incomplete protein digestion, partial restriction digests, and migration difficulties (Figure 15.1).

When the lysis step is insufficient, intact cells remain in the agarose after proteinase K digestion and very little DNA migrates out of the plug during the electrophoresis. This results in intense staining of the material remaining in the well, the migration of a single, very large DNA fragment, and very faint chromosome bands [40] (Figure 15.1, lanes 2–3). Agarose plugs containing incompletely lysed bacteria can be reprocessed by using the TE washout procedure to remove the proteinase K and then recycling the plugs through the entire procedure beginning with lysis solution [40].

Faint bands or a broad smear at the lower half of the gel may indicate DNA degradation by endogenous nucleases, which most commonly results from the failure to chill and wash the bacteria promptly when harvesting the broth culture [20,40] (Figure 15.1, lane 4). DNA degradation frequently occurs with isolates of *Clostridium* spp., and occasionally with *C. jejuni* or *P. aeruginosa*. Some *C. jejuni* PFGE protocols recommend treatment of the bacterial suspension in 10% formaldehyde for 15 min to inhibit DNase activity [2,52].

![FIGURE 15.1 Composite of seven PFGE agarose gels demonstrating frequently encountered problems. Lanes 1, 7, 10, and 15 contain the molecular size standard *S. aureus* NCTC 8325 digested with *Sma*I. Lanes 2 and 3 contain two *C. jejuni* isolates with inadequate lysis, shown by the migration of a single, very large DNA fragment and faint chromosome bands. Lane 4 contains degraded DNA prepared from an isolate of *C. jejuni*; the broad smear observed reflects DNA that has been spontaneously and randomly fragmented by endogenous endonucleases. Lane 5 contains a complete *Sma*I digest of a clinical isolate of MRSA and lane 6, a partial *Sma*I digest of the same clinical isolate; typical characteristics of the partial digest demonstrated here include an unusually large fragment and intermediate-sized fragments of variable intensity. Lanes 7 to 9 illustrate distorted lanes due to an unleveled PFGE chamber. Lane 11 illustrates a partial *Kpn*I digest of a clinical isolate of *C. jejuni* that migrated inadequately due to an artifact in the gel. Lanes 12 and 13 contain the same clinical *Sma*I digest of MRSA, but the agarose plug was damaged during its insertion in the well of lane 12. In lane 14, only a small part of the agarose plug was inserted in the gel.](image-url)
Incomplete protein digestion typically results in smearing proximal to the highest DNA band as well as between the DNA fragments. This can be rectified by increasing the amount of proteinase K or the length of digestion. Partial restriction digests are characterized by an unusually large fragment and intermediate-sized fragments of variable intensity between the DNA fragments (Figure 15.1, lane 6) [40]. All the reagents used to prepare the DNA (proteinase K, EDTA, detergent) will inhibit the activity of restriction enzymes if not completely removed; extensive washes in water and TE are thus required to remove all traces of these agents from the samples [53]. If the cell lysis is believed to be adequate but the restriction enzyme is suspect, the plugs can be redigested with fresh restriction enzyme after a TE washout [40].

The most common cause of curved lanes is heterogeneity in either the electric fields (e.g., because of the failure of several electrodes) or the temperature across the gel [49]. If the gel or the chamber are not level, the gel is covered by different depths of buffer and the current varies across the gel. Therefore, temperature gradients can form, leading to DNA moving faster in the warmer regions of the gel (Figure 15.1, lanes 7–9). Lanes can also become distorted if the gel is not fastened securely during electrophoresis or if air bubbles are trapped underneath and generate localized heating in the gel. Finally, bands can become distorted if the plugs are unevenly cut, only partially inserted into the gel, or damaged during insertion (Figure 15.1, lanes 11, 12, and 14).

15.1.1.3 Analyzing Typing Data

15.1.1.3.1 Visual Analysis of the Restriction Patterns

The use of molecular epidemiology data for infection control decisions is based on the assumption that isolates representing the outbreak strain have recently derived from a single (or common) precursor; this implies that outbreak isolates will have the same genotype, and that epidemiologically unrelated isolates will have different genotypes [3]. Demonstrating that isolates represent distinctly different strains indicates that they are not derived from a common source, but the final assignment of isolates with related genotypes requires careful consideration. Even for isolates with indistinguishable genotypes, the discriminatory power of the typing method and the genetic diversity within the species influence the likelihood that the result actually reflects epidemiological relatedness [5]. For example, some epidemiologically unrelated isolates may, by chance, have similar or indistinguishable genotypes, particularly if there is limited genetic diversity within a species. This is especially true for MRSA, a common nosocomial pathogen for which most strains originate from a small number of ancestral clones [54]. Thus, when MRSA is endemic in a hospital, it can be difficult to confirm the occurrence of an MRSA outbreak, particularly if the endemic strain is responsible.

The visual analysis of the restriction patterns for a discrete set of epidemiologically related isolates consists of three steps: (a) the examination of the patterns to identify the most common band profile among the isolates, which is presumed to represent the outbreak strain pattern; (b) the comparison of the size and number of the fragments in the outbreak pattern with the fragments that make up the patterns of the other isolates; and (c) the classification of each isolate’s pattern for its relatedness to the outbreak pattern, on the basis of pairwise, fragment-for-fragment comparisons [4]. The absence of a common pattern indicates that the isolates are most likely unrelated and therefore refutes the idea that they represent an outbreak.

Tenover et al. [3] have developed stringent criteria for interpreting chromosomal DNA restriction patterns produced by PFGE. These interpretative criteria are intended for use in analyzing discrete sets of isolates (typically 40 or less) obtained during epidemiological studies of putative outbreaks in hospitals or communities spanning relatively short periods (1 to 3 months); they are not appropriate for studies of large populations of organisms collected over extended periods of 1 year or longer [18]. They can be used when PFGE resolves at least 10 fragments; when fewer bands are detected, the robustness and discriminatory power of the criteria are unknown.
Patterns are classified into four categories: indistinguishable, closely related, possibly related, and unrelated to the outbreak pattern. Isolates are designated genetically indistinguishable if their restriction patterns have the same numbers of bands and if the corresponding bands are the same apparent size. Such isolates are considered to represent the same strain and to be part of the outbreak. However, PFGE patterns are often altered by random genetic events (e.g., point mutations, insertions and deletions of DNA), during the course of an outbreak or even during the course of a single infection when strains are cultured repeatedly over time or isolated multiple times from the same patient [3]. Therefore, patterns that differ from the outbreak pattern by two to three bands (i.e., changes consistent with a single genetic event such as simple insertions or deletions of DNA or the gain or loss of restriction sites) are considered to be closely related to the outbreak pattern. Isolates that differ from the outbreak strain by four to six bands (i.e., changes consistent with two independent genetic events) are considered to be possibly related to the outbreak strain. Although such isolates may have derived from the same genetic precursor as the outbreak strain, they are not as closely related genetically and are likely to differ by other typing techniques; consequently, they are less likely to be related epidemiologically. Such variation has been observed among isolates collected over long periods (≥6 months) or taken from large numbers of patients involved in extended outbreaks. Finally, isolates are considered to be unrelated to the outbreak strain if their PFGE profile differs from the outbreak pattern by seven or more bands (i.e., changes consistent with three or more independent genetic events).

In reporting the typing results for a discrete set of isolates, the outbreak profile is typically reported as type A and all isolates whose restriction patterns are indistinguishable from this pattern are considered as representing the outbreak strain. Patterns that are closely or possibly related to the outbreak pattern are considered as subtypes of A (e.g., A1, A2, etc.), and are considered to be probably or possibly epidemiologically related, respectively. The final determination of whether to include indistinguishable, probably, and possibly related isolates in the epidemiological case definition of the outbreak requires the integration of both molecular and clinical analyses [4]. Finally, patterns that differ substantially from the outbreak pattern are designated as different types (e.g., B, C, etc.) and are considered to be unrelated epidemiologically.

15.1.1.3.2 Analyzing Electrophoresis Gels by Software
Numerization of PFGE profiles, together with computer-assisted normalization and introduction of data into a relational database, is necessary to analyze patterns from large series of isolates compared on multiple gels. Computer-assisted analysis allows systematic quality control of DNA fingerprints, rapid identification of identical or closely related patterns, and quantitative analysis of pattern similarity in large databases of several hundred profiles assembled following multicenter surveys or long term surveillance programs [20]. Several specialized software packages are commercially available, including GelCompar and BioNumerics (Applied Maths, Kortrijk, Belgium), BioImage (BioImage, Ann Arbor, MI, USA), and Taxotron (Taxolab, Institut Pasteur, Paris, France).

The process of analyzing electrophoresis gels by software consists of five sequential steps: (1) creation of a digitized image; (2) importation of this image into a computer analysis software; (3) recognition of the different lanes containing the images of the DNA fingerprints; (4) normalization of the different lanes to remove distortions; and (5) analysis and comparison of the electrophoretic profiles [50].

The gel image is digitized either by direct image capture with a digital camera or by scanning a gel photograph with a flatbed document scanner. Digitized images are saved in TIFF files (tagged image file format) with 8-bit optical density (OD) depth (256 gray values). Once imported into a fingerprint analysis software, the lanes of the gel are defined by determining the overall borderlines of the desired parts of the gel and of the different “raw” DNA fingerprints. At the end of this step, each selected DNA pattern has its own individual entry in the computer database and is thereby separated from the rest of the gel [50].

The normalization phase allows the alignment of patterns by associating bands to an external reference pattern and by subsequent interpolation of the intermediate values. Molecular size stan-
standards should be loaded on each side and in every fifth well of the gel so that when normalizing the patterns no isolate should be more than two lanes from a standard. The other (nonreference) tracks are aligned gradually according to the closest neighboring reference tracks on either side [50]. Since the normalized band positions are calculated through extrapolation, the external reference standard should cover at least the whole molecular size range of the fragments to be analyzed; if this condition is not respected, the results of the normalization phase will be unpredictable for those bands that reflect fragments with a higher or lower molecular size than the highest or lowest bands of the external reference standard. If a pattern has only one neighboring external reference standard, the normalization process will also lead to unpredictable results [50]. Finally, gels should have the same reference system to be compatible.

Restriction patterns are compared through a cluster analysis followed by the calculation of similarity coefficients. The result of this process is usually shown as a phylogenetic tree (dendrogram), in which the sequential union of clusters, together with the similarity value leading to this union, is depicted (Figure 15.2).

Cluster analysis involves a stepwise reduction in the number of isolates by placing them into groups with similar DNA patterns [50]. The most neutral and commonly used algorithm for clustering DNA patterns is the unweighted pair group method using arithmetic averages (UPGMA) [24]. Similarity coefficients (SCs) are then calculated for all patterns; each SC value is a number between 0 (no similarity at all) and 1 (100% identical patterns). The Dice coefficient is the most reliable coefficient for measuring genetic distances between fragment analysis patterns and is the preferred measure for comparing PFGE patterns [24]. The Dice coefficient is calculated as the number of matching size fragments multiplied by two and divided by the total number of fragments in a pair of patterns. It is also known as the coefficient of Nei and Li when it is applied to RFLP data [20].

\[
\text{Dice} = \frac{2 \times N_{\text{common}}}{N_1 + N_2}
\]

Isolates whose PFGE patterns are highly similar, as indicated by a Dice coefficient ≥0.90, are likely to be related genetically and epidemiologically. Typically, such putative matches must be confirmed by visual examination of the original patterns or direct side-by-side comparisons. In general, the correlation of PFGE pattern similarity with genomic relatedness decreases rapidly below 70% similarity values [20]. Still, the loss of one band or the addition of an extra band does not have a dramatic effect. For example, when applying the Dice coefficient in the case of a profile with 13 bands being compared to a pattern where one band is missing, the Dice SC is SC = \(2 \times 12/(12 + 13) = 0.96\). Even in a large database containing many thousands of patterns, these two patterns will group in a similar branch of the dendrogram [50].

Even in the best laboratory situation, the different steps will almost always lead to disturbances or misalignments, thereby influencing the reproducibility of the DNA patterns. Optimization and position tolerance are two software parameters that allow correction of these misalignments to a certain degree. The position tolerance is the maximal shift (in percentage of the pattern length) between two bands allowed to consider the bands as matching. The optimization is the shift allowed between any two patterns and within which the program will look for the best possible matching; it allows track-to-track corrections to compensate for the remaining misalignments by shifting one or both tracks with respect to the other until they reach their maximum correlation [55]. Both settings can be adjusted by the user, but can also be defined automatically by the program [50].

15.1.1.4 Molecular Size Standards, Reproducibility Isolate, and Controls

To ensure accurate fragment size estimates (and adequate normalization of patterns when analyzing gels by software), researchers should include suitable molecular size markers in at least every fifth
FIGURE 15.2 Example of a dendrogram generated by BioNumerics version 2.0 (Applied Maths, Kortrijk, Belgium) representing relatedness among PFGE profiles of KpnI digests of 27 isolates of C. jejuni. Optimization and a position tolerance of 1.0% were applied. The overall Dice similarity coefficient (SC) among the isolates was 31%. The SC among the reproducibility isolate (153B-80) was 100%. The software allows analysis of both genotypic and clinical epidemiological data together by displaying both the band profile and the pertinent epidemiological information of each isolate.
Figure 15.3 Example of a 15-lane PFGE gel of Smal digests of MRSA. Lanes 2, 8, and 14 include Smal digests of S. aureus NCTC 8325 as a reference standard (shown as * in the gel). Lane 13 contains the Smal digest of an MRSA strain used as a reproducibility control (R). Lanes 1 and 15 were left blank; the remaining lanes were used for study isolates. These isolates were typed without suspecting a nosocomial outbreak of MRSA; the similarity of the patterns illustrates the low discriminatory power of PFGE for MRSA due to the limited genetic diversity within this species, and the importance of interpreting the typing results in the light of clinical epidemiological data and of a specific question defined by the epidemiologist.

When multiple gels are analyzed by software, a well-characterized reproducibility isolate should also be processed along with the unknown isolates being tested [56]. Obtaining expected results with the reproducibility organism affirms that the procedure, including the cell lysis, washing, and endonuclease digestion steps, the gel, and the electrophoretic conditions have been appropriate [3]. In addition, since both optimization and position tolerance settings can influence the results of the clustering analysis, they should be validated by comparing the DNA profiles for a given set of gels: optimal settings should result in a Dice SC of 100% among the reproducibility isolates, being a measure for the analysis of the clinical isolates fingerprints [50]. The reproducibility isolate also allows quality control of the reproducibility of the gel: Any gel with reproducibility values lower than 100%, using the Dice coefficient, should not be included in the database [20].

Finally, when typing a set of isolates suspected of being part of an outbreak, it is helpful to include a sample of epidemiologically unrelated isolates as well to ensure that endemic strains can be differentiated from outbreak strains. This is particularly important for analyzing outbreaks of MRSA in which the overall number of PFGE patterns is limited [3].

lane, that is, in the outside lanes and in the middle of the gel (Figure 15.3) [3,20]. Examples of molecular size standards are phage lambda concatemers, referred to as “lambda ladder,” preparations containing restriction endonuclease-digested Saccharomyces cerevisiae DNA, and DNA restriction fragment patterns of several well-characterized organisms in which the size of each chromosomal fragment has been determined, such as S. aureus NCTC 8325, Enterococcus faecium ATCC 51558, E. coli ATCC 47076, and Enterococcus faecalis ATCC 47077[3].
15.1.1.5 Limitations of PFGE

PFGE requires relatively expensive, specialized equipment, costing from $15,000 to $20,000 [16,18]. Although technical protocols have been simplified over recent years and the duration of some of the incubation periods has been shortened, the technique remains technically demanding and still requires 24–72 h turnaround time [2,20,41,46,48]. This effort is partially offset because DNA in agarose is stable for years at 4°C and can be easily released into solution for use in other protocols [18].

In practice, a technique is statistically useful when the most commonly detected types represent less than 5% of random unrelated isolates [5]. This may be difficult to achieve for some pathogens such as MRSA, *Haemophilus influenzae* type b, and *E. coli* O157:H7, which represent genetically restricted subsets of strains within a species [54,57,58]; consequently, epidemiologically unrelated isolates may have very similar genotypes and may be indistinguishable by PFGE, as well as by other typing methods [18,23,59]. Some isolates of a given species may be untypeable by PFGE and must be resolved by using other genotypic techniques such as PCR. In addition, certain organisms such as *Clostridium difficile* and *Aspergillus* spp. may not be typeable by PFGE because their DNA cannot be extracted intact [16].

The level of reproducibility achievable between PFGE patterns obtained within and between gels is a limitation that can be only partially offset by normalization of the gels. PFGE is not suited for population genetics studies of bacterial species because the distance between two isolates in the dendrogram does not represent the genetic distance between them; for these purposes, techniques such as multilocus enzyme electrophoresis (MLEE), or most recently, multilocus sequence typing (MLST), are preferable.

15.2 RIBOTYPING

In the early 1980s researchers began examining ribosomal RNA sequences as a means of understanding relationships between organisms. Practical requirements, time, and technical skill limited the method’s use. In 1995 the DuPont Company (Wilmington, DE) introduced an automated ribotyping instrument, the RiboPrinter Microbial Characterization System [60].

The RiboPrinter consists of a characterization unit (Figure 15.4) and a computer workstation. The technician picks bacterial cells from an agar plate, suspends them in buffer, and transfers them to a sample carrier. A heat treatment station inactivates the samples. The technician adds two lysing agents and places the sample carrier in the RiboPrinter. For most isolates, the system uses *EcoRI* to digest the DNA, but the user can substitute other restriction enzymes. Electrophoresis separates the DNA fragments by size on a precast agarose gel. The result is transferred to a nylon membrane. The RiboPrinter exposes the membrane to a series of chemical and enzymatic treatments, causing the DNA fragments to chemiluminesce. The RiboPrinter captures the resulting band pattern image using a digitizing camera and stores the pattern in the computer’s memory. This digital representation of the ribosomal RNA pattern is the RiboPrint pattern.

The RiboPrinter system includes built-in analysis software and a database of RiboPrint patterns. Each sample can be identified (given a genus, species, or serovar name) by comparing the generated RiboPrint pattern with the identification patterns in the database. Each sample is also compared with previously processed samples and grouped into dynamic RiboGroups, groups of patterns within a specific similarity range, that reflect the genetic relatedness of the samples. At the end of each run, the RiboPrinter generates a Microbial Characterization System Report (Figure 15.5). Sophisticated data analysis tools enable the operator to further process this information and share it with a network of users [61].
FIGURE 15.4 RiboPrinter Characterization Unit. Illustrates the loading of disposables for the processing of a batch of isolates.

FIGURE 15.5 RiboPrinter Microbial Characterization System Report. The system report prints automatically at the end of each run. Details of run conditions and image data for the batch are printed on the top half of the report. Identification, the digital representation of the ribosomal RNA pattern (the RiboPrint pattern), and the assigned Ribogroup are shown on the bottom half of the report.
Potential uses for automated ribotyping are growing. It can provide quality assurance in the food, agricultural, and pharmaceutical industries. Hospital infection control practitioners will benefit from timely epidemiological information [60,62].

The main limitation to expanding usage is the system’s cost. The unit, its disposables, and maintenance may be prohibitively expensive. It is also not approved by the U.S. Food and Drug Administration at this time. However, the system’s many benefits may outweigh its costs. Results are available in 8 h, and new batches of eight organisms can be loaded every 2 h. Automation minimizes errors due to technique, and computerized data analysis eliminates subjective interpretation of results [61].

APPENDIX 15.1 — PREPARATION PROTOCOL FOR PFGE BUFFERS
AND REAGENTS

Adapted from Maslow et al. [40].

0.5 M EDTA (pH 7.6 and 8.0):
Dissolve 10–15 g of NaOH pellets into approximately 700 mL of distilled water and then add 186.1 g of solid sodium EDTA. When completely dissolved, bring pH to exactly 7.6 or 8.0 with concentrated HCl and make up to a final volume of 1,000 mL with distilled water. Autoclave and store at room temperature.

1 M Tris (pH 7.6):
Dissolve 60.5 g of Tris base into approximately 300 mL distilled water and carefully bring to pH 7.6 with concentrated HCl. Then make up to a final volume of 500 mL with distilled water. Autoclave and store at room temperature.

PIV (Phage buffer IV) (10 mM Tris, pH 7.6; 1 M NaCl):
Dilute 5 mL of 1 M Tris (pH 7.6) and 29.25 g of NaCl to 500 mL with distilled water. Autoclave and store at 4°C.

Lysis buffer (6 mM Tris, pH 7.6; 1 M NaCl; 100 mM EDTA, pH 7.6; 0.5% Brij-35; 0.2% sodium deoxycholate; 0.5% sodium lauroyl sarcosine):
Dilute 6.0 mL of 1 M Tris (pH 7.6), 58.0 g of NaCl, 200 mL of 0.5 M EDTA (pH 7.6), 5 mL of Brij-35, 2.0 g of sodium deoxycholate acid, and 5.0 g of sodium lauroyl sarcosine to 1,000 mL with distilled water. Filter sterilize and store at 4°C.

RNase (10 mg/ml; stock solution):
A 50-mg stock vial of RNase has 5,400 U/ml enzyme. To get a concentration of 10 mg/ml, dilute the stock vial in 5 mL of sterile water. Aliquot 0.3 mL into –16 Eppendorf tubes. Store at –20°C.

Lysozyme (50 mg/ml; stock solution):
Dilute 0.5 g lysozyme in 10 mL of sterile water. Aliquot 0.5 mL into 20 Eppendorf tubes. Store at –20°C.

Lysostaphin buffer (50 mM Tris, pH 7.6; 0.15 M NaCl):
Dilute 1.5 mL of 1 M Tris (pH 7.6) and 0.26 g of NaCl to 30 mL with distilled water. Filter sterilize and store at 4°C.

Lysostaphin stock solution (for Staphylococcus spp.):
Five milligrams of stock lysostaphin has 5,150 U enzyme. To get a concentration of 500 U/mL, dilute the stock vial to 10.30 mL with lysostaphin buffer. Aliquot 0.5 mL into 20 Eppendorf tubes. Store at –20°C.

Lysis solution (20 µg RNase/mL, 1 mg lysozyme/mL, and for Staphylococcus spp., 5 U lysostaphin/mL):
When ready to use, add to each milliliter of lysis buffer stock solution 2 µl of RNase
(10 μg/mL), 20 μL* of lysozyme (50 mg/mL), and for Staphylococcus spp., 10 μL of lysostaphin (500 U/mL).

*For Enterococcus spp., use 60 μL of lysozyme (for a final concentration of 3 mg/mL).

ES buffer (0.5 M EDTA, pH 8.0; 0.1% sodium lauroyl sarcosine):
Dilute 10 g of N-lauroyl sarcosine in 1,000 mL of 0.5 M EDTA (pH 8.0). Filter sterilize and store at 4°C.

ESP stock solution (2 mg/mL):
Dilute 100 mg of proteinase K (1 vial) in 50 mL of ES buffer. Shake well and then separate solution in half into two 50-mL centrifuge tubes (so that all 50 mL is submerged in the water bath). Place in a floatie and incubate in a 50°C water bath for approximately 1 h to dissolve the powder. After incubation, mix back together into one 50-mL centrifuge tube and store at 4°C.

ESP working solution (100 μg/mL):
Add 5 mL of ESP stock solution to 95 mL of ES buffer. Store at 4°C.

TE buffer (10 mM Tris, 0.1 mM EDTA; pH 7.6):
Dilute 5.0 mL of 1 M Tris (pH 7.6) and 0.1 mL of 0.5 M EDTA (pH 7.6) to a total volume of 500 mL with distilled water. Autoclave and store at room temperature.

5X TBE (stock solution) (0.45 M Tris, 0.45 M boric acid, 0.01 M EDTA; pH 8.0):
Dissolve 163.5 g Tris base, 83.4 g boric acid, and 60.0 mL of 0.5 M EDTA (pH 8.0) to a total volume of 3,000 mL with distilled water. Store at room temperature in a 3-L amber bottle.

0.5X TBE (working solution):
Dilute 300 mL of 5X TBE solution to a total volume of 3,000 mL with distilled water. Store at room temperature in a 3-L amber bottle.

**APPENDIX 15.2 — GENERAL PROTOCOL FOR PFGE**

Adapted from Maslow et al. [40] and Gautom [41].

**SAMPLE PREPARATION**

**Day –2**

1. For each isolate, inoculate a single colony from the clinical plate or glycerol into 0.5 mL trypticase soy (TS) or brain heart infusion (BHI) broth.
2. Grow for 2 h or until turbid.
3. Streak out onto 5% sheep blood TS agar and incubate overnight (ON).

**Day –1**

Pick a single colony, inoculate 5 mL of TS broth, and incubate ON on a roller at 37°C. To prevent overgrowth of culture and release of nucleases, inoculate broth just before leaving at night and put on ice in the morning, provided there is sufficient growth.

**PLUG PREPARATION**

**Day 1**

**Material Preparation**

1. For each isolate, label one 15-mL snap-top tube, one 5-mL snap-top tube, one 7-mL scintillation vial, one 40-mL flat-bottom tube, and two 2-mL freezer tubes that have been filled with 0.4 mL of glycerol and sterilized.
2. Label one plug mold per isolate. Tape the bottom of the plug molds, place them in a metal tray, put this tray in a glass tray filled with ice, and place it in the refrigerator for at least 30 min. Cold molds are essential to the rapid gelling of the plugs.
3. Turn on a heat block to 50°C. Place one 5-mL snap-top tube per isolate in the heat block to warm up.
4. Take the cultures out of the warm room and place them on ice.

**Preparation of Bacterial Suspension and Agarose**

1. Put 5 mL of cold PIV into each 15-mL snap-top tube and place on ice.
2. Dispense 1.5 mL of each ON culture into the appropriately labeled 15-mL snap-top tube containing cold PIV.
3. Centrifuge the 15-mL snap-top tubes at 500 × g for 15 min at 4°C.
4. While the cells are centrifuging, dispense 1.4 mL of the ON culture into each of the two freezer tubes. Cap the tubes securely and vortex them vigorously. Store them at –70°C. Preparing frozen stocks from the ON broth ensures that the exact colony being analyzed by PFGE has been saved.
5. Decant the PIV buffer from the cell pellet, resuspend the cells in 1.5 mL of fresh cold PIV, and place on ice.
6. Mix InCert agarose (1.3% [wt/vol]) in PIV buffer in a 15-mL tube. Prepare 1 mL per isolate (plus 1 mL extra), vortex, and place into boiling water to dissolve. Revortex prior to aliquoting.
7. Carefully dispense 1 mL of dissolved agarose into each 5-mL snap-top tube, and keep the tubes in the 50°C heat block.
8. Take the chilled plug molds placed in the ice-filled glass tray out of the refrigerator. Do not let chilled trays sit on the bench for a long time.
9. Add 1 mL of cells in PIV to the 5-mL snap-top tube with 1 mL of molten InCert agarose, pipet up and down a few times, and promptly aliquot the mixture down the side of each well of the plug molds, being careful not to trap any bubbles in the plugs. Mix cells with agarose one sample at a time. Do not prepare all isolates at once before aliquoting, or the organisms will start to produce nucleases while waiting in hot agarose. Keeping the bacteria and agarose plugs cold during the above procedures is critical to preparing high-quality plugs with intact DNA. The cold decreases premature cell lysis, decreases the activity of endonucleases, and facilitates homogeneous gelling of the agarose. The final concentration of organisms is ∼1 × 10⁹ CFU/ml. Since 1 CFU = 1 × 10⁻¹⁴ g of DNA, each plug has ∼1 µg of DNA.
10. Place the molds (in their trays) at 4°C for 30 min to solidify.

**Lysis**

1. While the plugs are solidifying, thaw the RNAse and lysozyme (and lysostaphin if needed) on ice for approximately 20 min.
2. Make fresh lysis solution and keep on ice. Use 4 mL of lysis solution per isolate. For each milliliter of lysis solution, add 2 µL of RNAse (10 mg/mL), 20 µL of lysozyme* (50 mg/mL), and, if needed, 10 µl of lysostaphin (500 U/mL).
   * For *Enterococcus* spp., use 60 µL of lysozyme.
3. Dispense 4 mL of lysis solution into each labeled scintillation vial.
4. Wearing gloves, remove the tape gently from the plug molds and place it in contaminated waste (the tape has live organisms on it). Using a spatula, push out all plugs into the appropriate scintillation vial with lysis solution. Disinfect the trays and rinse with water, and place the contaminated plug molds in 15% bleach solution ON.
5. Incubate the scintillation vials at 37°C ON on a roller.
6. Turn on the 50°C water bath to have it ready early on day 2.

Day 2. Protease Digestion

1. Carefully aspirate the lysis solution using a bulb syringe and Pasteur pipette.
2. Dispense 4 mL of ESP solution into each tube and incubate ON at 50°C, shaking gently.

Day 3

Restriction Enzyme Digestion

1. Turn on a water bath at the temperature appropriate for the restriction enzyme.
2. Chill the scintillation vials with plugs on ice for about 10 min. The plugs can now be used for restriction enzyme digestion as outlined below or can be placed in fresh ESP solution and stored at 4°C. DNA in the plugs is stable at 4°C in ESP for at least 2 years.
3. Using a spatula, carefully take out one plug and put it in its respective 40-mL flat-bottom tube.
4. Wash the plugs six times, 10 min each wash, in a 50°C water bath with constant agitation (150 rpm), twice with 15 mL of preheated (50°C) sterile water, and four times with 10 mL of preheated (50°C) TE.
5. Label one 1.5-mL Eppendorf tube for each isolate.
6. Thaw on ice bovine sterile albumin (BSA) and the enzyme buffer.
7. Aliquot into each Eppendorf tube, in order, 267 µL of sterile water, 3.0 µL of BSA, 30 µL of enzyme buffer, and the washed plug.
8. Add restriction enzyme, mix gently, and incubate for 2–4 h at the appropriate temperature.
9. Chill the tubes before handling the inserts.
10. Carefully remove the digestion mixture from each Eppendorf tube.
11. Add 1 mL of ESP into each Eppendorf tube. Plugs can be stored in ESP buffer at 4°C indefinitely (>1 year).

Gel Preparation and Loading

1. Make the gel with SeaKem high-melting-temperature agarose (1% [wt/vol]) dissolved in 0.5X TBE (for a 15-lane gel, dissolve 0.8 mg Seakem agarose in 80 mL of TBE). Boil on hot plate, then cool to 50°C in a water bath.
2. Pour agarose slowly into the gel mold; avoid air bubbles. Let the gel solidify at room temperature for 15–30 min.
3. Using a surgical blade, cut a slice ∼1–1.5 mm thick off the end of the plug and load it into a well of the gel using the narrow end of a Teflon-coated spatula. Make sure the slice is at bottom–front of the well and is not twisted.
4. Place the plugs in the gel in the following order (when using a 15-lane gel): lanes 1 and 15: no plug; lanes 2, 8, and 14: *S. aureus* NCTC 8325 or molecular weight standard; lanes 3–7 and 9–12: isolates to be tested; and lane 13: reproducibility isolate.
5. Return the remainder of the plug to the Eppendorf tube and store at 4°C.
6. Fill all the wells with agarose and place the gel in a PFGE gel box with recirculating 0.5X TBE cooled to 14°C. Run at 200 V (6 V/cm), with linear ramping (from 1 to 40 s), for 22 h.

Day 4. Gel Visualization

Stain the gel for 20 min in 1 L of sterile water containing 1 µg/mL ethidium bromide. Destain the gel by 2 washes of 30 min each in 1 L of sterile water, and photograph it under UV light. Since
it is a powerful mutagen, always wear gloves when handling solutions containing ethidium bromide and dispose these solutions into an appropriate dangerous waste container.

REFERENCES


16 Pharmacy and Microbiology: Interactive Synergy

Beulah Perdue Sabundayo and Cassandra B. Calderón

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16.1 INTRODUCTION

Traditionally, pharmacists and microbiologists have been allocated to the basement of most hospitals, having little or no contact with each other except when passing in the hallways. Today, managed care has forced these two departments to work together as part of a team. Many of the decisions concerning patient care including safety, efficacy, and cost containment are a cooperative effort among physicians, pharmacists, and microbiologists, especially in the field of infectious diseases. The work these individuals perform and their specialized view on matters make each member invaluable, and together, a winning team. This chapter discusses the pharmacy and therapeutics committee, the antimicrobial subcommittee, and the role pharmacists and microbiologists play on such committees. It emphasizes the cooperative and sometimes dependent relationship between the two departments. This chapter also discusses the tools used in managed care, such as the drug formulary, antimicrobial streamlining, medication usage evaluations, and critical pathways as they pertain to the interaction between pharmacists and microbiologists.

16.2 PHARMACY AND THERAPEUTICS COMMITTEE

The continued success of many large companies and institutions depends upon work performed in committees, subcommittees, and task forces. Hospitals are no different. Even though the committee structure of institutions may differ slightly, most hospitals have established a pharmacy and therapeutics (P&T) committee. First established in the 1930s, this committee was responsible for developing a formal list of medications available in the hospital, now known as the formulary [1]. Then in 1959, the American Society of Hospital Pharmacists and the American Hospital Association officially described the role of the P&T committee in maintaining the formulary [2]. Today, most hospitals have created a P&T committee, as stipulated in their medical staff’s bylaws, as an advisory committee to the medical executive committee [1]. They act as the primary governing body to
ensure the appropriate use of medications within the hospital and as the formal communication link between the medical staff and the department of pharmacy.

The P&T committee is responsible for all matters related to medication use in the hospital. Their basic objectives are to specify drugs of choice and alternatives, to minimize therapeutic redundancies, and to maximize cost-effectiveness [3]. These objectives are accomplished through a variety of programs overseen by the committee, including maintaining the hospital’s formulary, overseeing medication usage evaluations, and approving clinical pathways. In addition, the P&T committee reviews adverse drug reactions and drug-use policies and procedures, and discusses continuous quality improvement projects. They also serve in an educational capacity by establishing policies and procedures to help ensure safe, effective, and cost-effective drug therapy. In addition, they arrange educational programs for the staff on matters related to drug use and disseminate information regarding the actions approved by the committee [4]. Other committees, subcommittees, and task forces usually facilitate the work done by this committee. For example, the antimicrobial subcommittee and the infection control committee usually present information and recommendations to the P&T committee.

The infrastructure, membership, and chairperson of the P&T committee can vary among hospitals and the roles played by these members are specific to the needs of the individual hospital. Most P&T committees are multidisciplinary groups of members, primarily elected from the medical staff, pharmacy department, and nursing. Among the physicians, these members are usually practicing physicians and represent different medical and surgical subspecialties. Members from the department of pharmacy usually include the director of pharmacy, a clinical pharmacist, and possibly other representatives from the department. Some hospitals have a hospital administrator sit on the committee. The chairperson of the committee is usually a physician, but may be a pharmacist or hospital administrator. Microbiologists may or may not be members of the P&T committee, but play a vital role on the antimicrobial surveillance and infection control committees, which usually report to this committee. Representatives from ancillary departments or committees are invited, as needed. A survey of 267 teaching hospitals showed that these committees are very large, averaging almost 20 members, with the majority being physicians. It also revealed that each P&T committee had at least one pharmacist, with an average of 3.2 pharmacist members. Two to three members were nurses, and a hospital administrator may have been elected to the committee. In 98.9% of the hospitals, the chair of the committee was a physician [5].

16.3 ANTIMICROBIAL SUBCOMMITTEE OF THE P&T COMMITTEE

The antimicrobial or antibiotic subcommittee of the P&T committee is a multidisciplinary group responsible for reviewing each classification of antimicrobials. Members of this subcommittee may also be members of the P&T committee, but often times include additional health professionals from a variety of departments. The chair is typically a well-respected physician from the infectious disease department. Other members of the subcommittee include a clinical pharmacist, a clinical microbiologist, physicians representing medical, surgical, obstetrics and gynecology, oncology, and pediatric subspecialties. These physicians generally have an interest in infectious diseases or may be trained specifically in infectious diseases [6].

Having an antimicrobial subcommittee allows for more involvement by the medical, pharmacy, and microbiology staff in drug policy decisions specific to their hospital. The subcommittee extensively reviews each antimicrobial class and makes formal recommendations to the P&T committee on the preferred drugs for that class. Usually, the pharmacist reviews the literature, compares the drugs to each other, and presents the data to the group. The microbiologist presents data on resistance patterns seen throughout the hospital and in specific units. Decisions are based on in vitro activity, safety, efficacy, and cost resulting in minimal duplication of therapeutically equivalent drugs. A
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subcommittee allows for more extensive education of the medical staff through one-on-one consultations with its members or through formal educational programs developed by the subcommittee and endorsed by the P&T committee. The subcommittee provides continuous data about the use and misuse of antimicrobials within the institution through the review and publication of the antibiogram and medication use evaluations [6,7].

16.4 THE DRUG FORMULARY SYSTEM

As previously described, one of the major responsibilities of the P&T committee is to develop and maintain the hospital’s drug formulary. The antimicrobial subcommittee is usually responsible for the antimicrobial section of the formulary. The formulary is a continually revised list of drugs with information pertaining to the appropriate and safe use of each medication [8]. The first formulary was introduced in a private hospital in 1816 [9]. Since then, its utility has grown more important and complex. The formulary should represent the clinical judgment of the P&T committee members in the diagnosis, management, and prevention of disease. There are three types of drug formularies: open, closed (restrictive), and managed. An open formulary allows for the use of all drugs and drug products currently available on the market. Drugs are used without a formal, objective review and approval process. In a closed or restrictive formulary, there is a limited list of drugs that are available for use. The drugs on this list have undergone a rigorous review process by members of the P&T committee before being included on the formulary. A managed formulary is an open formulary with preferred products that have guidelines for use. Most institutions use a closed formulary, which allows the department of pharmacy to maintain a manageable inventory by only having the medications that are on formulary readily available for use. When extenuating circumstances arise, these institutions usually have a method for obtaining drugs that are not on the formulary upon the request of the physician. Future use of that drug will depend on the review and approval by the P&T committee so that it may then be included on the formulary, if deemed necessary [8,10].

The drug formulary system is the process by which the P&T committee maintains policies for drug use [11]. The goal of the formulary system is to optimize pharmaceutical care for patients in terms of efficacy, safety, and cost. Information in the medical and pharmacy literature and economic concerns are coupled to form a comprehensive, unbiased profile of the drug. Initially a drug is evaluated in terms of efficacy and safety. The side effects of drugs are always considered as well as patient adherence to the specific drug regimen. After a drug is deemed acceptable in terms of safety and efficacy, then economic factors are evaluated. The drug must be evaluated for how it will affect total health care costs for the institution. Through this comprehensive process, drugs may either be added, deleted, or have restricted use within the formulary system [8,10–14].

The formulary system is responsible for the maintenance of the drug formulary. This entails medication usage reviews to assure appropriate use, evaluation, and analysis of treatment protocols; procedures such as critical pathways, as well as monitoring, analyzing, and reporting adverse events. The formulary system should provide educational programs to the medical staff about institution specific policies and procedures about the appropriate use of the drugs. This is accomplished by providing in-services, newsletters, and publications within the institution. The formulary system should also proactively inform the medical staff of changes to the formulary and be able to provide rationales for specific formulary decisions when asked. A well-defined process should be in place so that physicians can request drugs be added or removed from the formulary. The formulary system should also provide a process to educate patients about their medications by interacting with medical, nursing, and/or pharmacy staff based on the guidelines within the institution. Depending on the type of formulary system used, a well defined process enables the department of pharmacy to maintain a manageable inventory and therefore control costs by only having drugs that are on the formulary readily available within the institution [8,10,11,13].
16.5 ANTIMICROBIAL STREAMLINING

Antimicrobial medications often make up the majority of a hospital’s formulary, accounting for 20%–40% of total drug expenditures [15,16]. For this reason, along with the propensity for the development of drug resistance with overuse and inappropriate use, antimicrobials are often the target of drug monitoring and control programs. In general, the initial choice of an antibiotic is empiric, based on the physician’s knowledge of the type of infection and the microorganism believed to inhabit the institution (obtained from the antibiogram). Often times with empiric therapy the belief is that therapy must be broad and intravenous in the hospital setting. Review of empiric therapy has demonstrated that without intervention from other sources (i.e., integrated monitoring programs), many physicians will continue the agents that were originally chosen for empiric therapy even if less expensive, easier to administer (i.e., oral) agents are available. Many clinical pharmacy programs have been developed to affect empiric antibiotic usage (the first 2–3 days of therapy).

Data on the individual patient’s infecting organism is often available from the microbiology lab within 2–3 days of initiation of therapy, allowing the physician to modify therapy if needed. Cultures may show susceptibility to a less expensive agent than what was originally chosen. It is at this point that streamlining of therapy comes into play [17,18].

Streamlining of drugs involves changing to a less expensive agent or discontinuing therapy when appropriate [17,19]. Intervention programs are generally targeted at one or more of the following: dosage adjustment, parenteral regimen change, change from intravenous to oral, early discontinuation of therapy, or change to management by a clinical pharmacokinetic service (i.e., aminoglycoside dosage adjustments based on serum concentrations) [17,19,20]. The key to the success of such a program is making sure that selected patients have an organism with susceptibilities identified and the patient is showing signs of improvement prior to intervention. Antimicrobial streamlining may be the most crucial place where physicians, clinical pharmacists, and microbiologists interact to improve quality of care for the patient and control the costs of antimicrobial agents. While a major goal of streamlining therapy is to contain costs, it is imperative that patient outcomes be well-documented [17,20].

Many programs have been developed to implement streamlining. The most successful programs have resulted from strong leadership from the infectious disease division, a clinical pharmacy service responsible for the daily running of the program, and computer software that easily interacts with the microbiology laboratory. Support from the antimicrobial subcommittee and hence the P&T committee is essential in the form of review and approval of all policies and procedures [17,20].

16.6 MEDICATION USAGE EVALUATIONS

A medication usage evaluation (MUE), previously referred to as a drug usage evaluation (DUE), is a method to evaluate and improve the appropriate, safe, and cost-effective use of medications within the hospital or clinic setting. They can be performed on an individual medication (i.e., ciprofloxacin or imipenem/cilastatin), a therapeutic class (i.e., fluoroquinolones or β-lactam antibiotics) or a specific disease state or condition (i.e., treatment of urinary tract infections or drug causes for Clostridium difficile diarrhea). The goal of an MUE is to improve an individual patient’s quality of life through achievement of predefined, medication-related outcomes [21]. MUEs are usually developed for high-volume, high-cost, and/or high-risk medication, diagnoses or procedures.

Hospital specific guidelines or criteria are written based on current medical and pharmacy literature and are then approved by the P&T committee. These guidelines incorporate the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) medication-use indicators emphasizing all aspects of the medication experience. Items reviewed include prescribing, dispensing, and administering the medication, as well as monitoring the patient’s response. Within each category there are processes listed that help the institution identify areas for specific improvement. For example, the dispensing indicator requires the reviewer to determine if the order was reviewed
and processed correctly, and the medication prepared correctly and dispensed in a timely manner [22]. The proper use of medications is a cooperative goal among the prescribing physician, the dispensing pharmacist, the administering nurse, and all those who monitor the patient for appropriate outcomes. Through this process, we can improve the medication experience of the patient by identifying and resolving actual or potential medication-related problems [23].

Many institutions have an ongoing medication usage evaluation program solely dedicated to antibiotics primarily because unlike all other medications, the overuse or misuse of antibiotics carries the risk of selecting resistance to certain microorganisms. The goal of this program is similar to the MUE described previously, which is to promote the rational, safe, and cost-conscious use of antimicrobials within the hospital. For example, with the aid of a medication usage evaluation, the Veterans Affairs Medical Center affiliated with the University of Arizona determined that clindamycin usage was associated with an epidemic of *C. difficile* infections in their institution [24]. This is where input from microbiology lab personnel alerting physicians to increasing resistance to certain antimicrobials is important in identifying which agents require more careful monitoring.

### 16.7 CRITICAL PATHWAYS

With the rapid growth of managed care, many health care organizations are looking to critical pathways as a means of maintaining high quality health care while at the same time reducing costs. Critical pathways, also known as clinical pathways, were first implemented in the health care arena in the 1980s, when hospital reimbursement became competitive and capitated [25]. Today they are comprehensive medical management plans for patient care. Pathways cover the entire patient experience from admission, diagnosis, throughout the hospital stay, and after discharge. Unlike MUEs, which focus only on pharmacologic interventions, a critical pathway encompasses all aspects of patient care, including pharmacologic and nonpharmacological therapies, interventions, activities, and outcomes. They optimize the sequence and timing of different interventions performed by physicians, nurses, and other health professionals, to minimize delays and resources while maximizing quality of care. Similar to MUEs, critical pathways are usually developed based on the JCAHO indicator criteria for high-volume, high-cost, and/or high-risk diagnoses or procedures within a given institution [26]. Some goals of critical pathways are to improve patient care, reduce the variation in medical practice, increase continuity of care, and improve the utilization of resources. They are also used as a guide for other members of the health care team as well as patients and family members on what to expect from the hospital experience.

Although potentially beneficial, no controlled study has proven that critical pathways decrease resource use or improve patient satisfaction or outcomes [27]. Nonetheless, many health care organizations are actively developing critical pathways. A directory listing over 2,000 critical pathways developed in various institutions was published in 1995 [28]. The experiences of some hospitals have been published in the literature on various disease states including lumbar laminectomy, stroke, and cesarean section [29,30].

### 16.8 CONCLUSION

This chapter illustrates a few ways in which pharmacy and microbiology may interact. Primarily through a cooperative effort on the antimicrobial subcommittee, the antibiotic formulary is maintained and infectious disease-related critical pathways are reviewed and approved. The department of microbiology also provides information that assists in the antibiotic streamlining and MUEs being performed efficiently. However, many of the more interesting interactions are on a daily basis, involving the diagnosis and management of patients. The treatment of patients with infectious diseases is dependent on the department of microbiology. Physicians rely on the microbiology department to provide accurate and timely information on the microorganisms that are isolated.
from clinical specimens. It is the role of the clinical pharmacist to help the physicians interpret those data and decide on the best and most cost-effective treatment for that specific patient. The complete acceptance of policies, procedures, and monitoring programs is based on the multidisciplinary work of both the pharmacy and microbiology departments in conjunction with the medical staff. This work is displayed through the P&T committee, in particular the antibiotic subcommittee, and through the publication of policies and procedures for the appropriate use of antimicrobials. Ongoing information is gathered through the process of MUEs, antibiotic streamlining, and critical pathways. Through this interdisciplinary work, patients benefit from the knowledge and expertise of all those involved in their care.

REFERENCES

17 Interactions between Clinicians and the Microbiology Laboratory

John H. Powers

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17.1 INTRODUCTION

The treatment and prevention of infectious diseases is unique. Symptomatic illness in infectious diseases is the result of the host response to invading microorganisms. In the treatment of most human diseases, clinicians administer drugs that bind to some receptor site that is part of the human host. However, in most infectious diseases, other than vaccines and immunological therapies, therapy is directed at the invading organism rather than the human host. Inhibition or killing of the microorganisms will aid the human immune system in eradicating the invading pathogens with a subsequent resolution in the signs and symptoms of the disease. We have learned that some antimicrobials also may have a direct effect in modulating the host immune system as well [1,2]. The ultimate goal, of course, is the cure of the patient’s signs and symptoms, and not merely the elimination of the pathogens. The microbiology laboratory provides information to clinicians that allows them to treat patients most appropriately. The microbiology laboratory interacts with clinicians in other important ways as well, including providing information related to infection control, educating clinicians and house staff, and participating in clinical research. The efficient functioning of the microbiology laboratory is dependent upon good communication between clinicians and their colleagues in the microbiology laboratory [3].

17.2 DIRECT PATIENT CARE

The most important interactions between clinicians and the microbiology laboratory are in the day-to-day context of direct patient care [4]. The information that clinicians obtain from the microbiology laboratory serves several important purposes. First, the results from appropriately obtained specimens sent to the microbiology laboratory allow clinicians to confirm that the patient’s illness indeed is due to an infection and not some other noninfectious cause [5]. An erroneous diagnosis of
infection may result in unnecessary exposure of patients to antimicrobial drugs. This may result in unnecessary adverse events related to antimicrobials, or eliminating the patient’s protective normal flora with resulting colonization with antimicrobial-resistant organisms [6]. Most importantly, however, is that clinicians may wait two to three days to observe a potential response to antimicrobial therapy, thereby delaying finding the correct diagnosis. This delay in diagnosis may result in a delay in the appropriate therapy for the patient’s illness, which may in turn result in increased morbidity or even mortality for some infections [7].

The information from the microbiology laboratory is one factor which helps clinicians to choose the most appropriate antimicrobial therapy for a given patient. In some cases clinicians may wait for the results of microbiological testing before initiating treatment. For instance, in a child who presents with less severe disease such as pharyngitis, the clinician may await the results of a throat culture before starting treatment. In a severe or serious illness such as acute bacterial meningitis, clinicians empirically initiate antimicrobial therapy based on the most likely pathogens implicated in that type of infection. In both cases the results from the microbiology laboratory obtained in a timely fashion aid the clinician in choosing the most appropriate therapy. Data indicate that in some infections, especially more serious diseases, a delay in the initiation of appropriate therapy may result in worse outcomes for patients [7]. Inappropriately broad therapy may also result in increased adverse events for patients or the selection of resistant organisms [6].

The results of microbiologic testing can also help clinicians determine which antimicrobials may be potentially effective against the pathogenic microorganisms by evaluating the ability of the antimicrobial to inhibit or kill the microorganisms in vitro [8]. While the most relevant information on drug efficacy comes from clinical outcomes in clinical trials establishing the safety and efficacy of that drug for a given infection in a specific patient population, the microbiological results also are helpful. The causative pathogen may be resistant in vitro to drugs commonly used to treat that infection, in some cases making clinical cure less likely. Also, clinicians may encounter patients infected with organisms that are sufficiently rare that there is little information from clinical trials on treatment. In the absence of clinical trial data, in vitro testing may provide clinicians with useful information about the potential for antimicrobial efficacy.

The microbiology laboratory provides a useful service for clinicians by interpreting the results of in vitro testing [9]. Many clinicians, other than infectious disease specialists, may not be familiar with how in vitro testing translates into susceptibility or resistance of an organism for a given antimicrobial. The microbiology laboratory compares the amount of drug necessary to inhibit growth of the organisms in vitro (the minimum inhibitory concentration or MIC) for an antimicrobial to standard breakpoints put forth for that antimicrobial–organism combination. The U.S. Food and Drug Administration includes susceptibility breakpoints in drug labeling. This allows the laboratory to determine whether the organism is considered susceptible or resistant in vitro to the antimicrobial. Laboratories, in turn, relay the information about susceptibility or resistance to clinicians, greatly simplifying the clinician’s task in interpreting such data. In some situations, however, it is still useful to know the actual MIC value. It is important to keep in mind that susceptibility testing is one factor among many used in making treatment decisions for patients. In some cases, the definition of “resistance” may not accurately reflect clinical outcomes in patients [11].

The usefulness of microbiological testing in patient care depends upon adequate communication between clinicians and the microbiology laboratory [3]. Clinicians and microbiologists must also ensure adequate quality control in their respective parts of the testing process. First, clinicians must know when it is useful to obtain microbiological data and when it is not. For instance, in most young, healthy women with an uncomplicated urinary tract infection, the results of microbiological testing of urine cultures are sufficiently predictable that obtaining culture data adds little to clinical management [12]. Obtaining culture data in such cases results in unnecessary work for the microbiologists and increases the cost of treating such infections. Similarly, clinicians should evaluate carefully the utility of repeating microbiological testing in selected patients. For instance, daily sputum cultures on patients receiving mechanical ventilation add little useful information for clinical
decision making. On the other hand, clinicians often repeat blood cultures in bacteremic patients, especially those with endocarditis, to document clearing of the organisms [13].

When clinicians decide that microbiological information will be helpful in clinical decision making, they should ensure that they obtain specimens properly [4,14]. This entails obtaining an adequate amount of specimen. For instance, the yield of blood cultures is directly related to the amount of specimen obtained. The source of the specimen is also important. For instance, in patients with pneumonia sputum specimens obtained by deep cough are more helpful than saliva. Adequate labeling of specimens may also help in determining their clinical importance. For instance, cultures from existing drains may not be helpful and may be potentially misleading in that such drains may be colonized with commensal flora.

Clinicians and microbiologists should communicate when deciding whether susceptibility testing is warranted in specific situations. The decision to perform susceptibility testing is based on the source of the specimen, the types of organisms isolated, whether the organisms are present in pure or mixed culture, and the predictability of the susceptibility of the isolated organisms and host factors in the infected person. For instance, isolation of viridans streptococci from a throat culture could be considered “normal oral flora” and require no further testing, but isolation of the same organisms from a blood culture in a patient with a presumptive diagnosis of endocarditis may be an indication for more extensive testing. Isolation of coagulase-negative staphylococci may be a contaminant in otherwise healthy persons, but may be clinically significant in immuno compromised persons or patients with medical devices in place. These examples also illustrates that it is helpful to provide the microbiology laboratory with a differential diagnosis for the patient. This may cause the lab to continue to incubate the cultures for a longer time looking for fastidious organisms, or in other cases to use selective media. A differential diagnosis of “fever” is sufficiently nonspecific as to add little useful information to clinical decision making. When the differential diagnosis contains diseases that may be potentially transmitted to laboratory staff, such as tularemia, the clinicians should make sure to communicate this information to the laboratory staff.

When there are questions about how to properly obtain a specimen, it is helpful to communicate with the microbiology laboratory in advance. This is especially important when specimens are difficult to obtain, such as those obtained intra-operatively. Some institutions have manuals distributed throughout patient care areas or computer access to such manuals to aid clinicians in properly obtaining and labeling specimens.

The microbiology laboratory should provide most routine microbiological testing, including susceptibility testing for most bacteria. Occasionally, clinicians may require more specialized testing, such as susceptibility testing for anaerobic organisms. Laboratory personnel can aid clinicians in determining when such testing is appropriate. Other specialized testing may be sent out to reference laboratories. When testing is not performed on site, laboratory personnel should ensure that specimens are transported to the outside laboratory as soon as possible.

Microbiology laboratory personnel should ensure that results are provided to clinicians in a timely manner regardless of whether testing is performed on site or sent out to a reference laboratory. When laboratory personnel obtain critically important information, such as positive results from blood cultures, it is often helpful for the microbiologist to communicate this information directly to clinicians or other patient caregivers. This ensures that clinicians can act on this information as soon as possible. These results may require immediate changes in the patient’s antimicrobial therapy and may have an important impact on the outcome of the patient’s illness.

In conjunction with clinicians, laboratory personnel should decide which antimicrobials are appropriate to include in routine susceptibility testing and which results to report to clinicians [15]. This decision entails considerations of the site of infection, local antimicrobial resistance patterns, and the cost of the antimicrobials. In some cases, laboratory staff and clinicians may decide to test the susceptibility of organisms to certain antimicrobials but not routinely report the results. This is usually done in an effort to encourage use of narrower spectrum or less expensive but similarly efficacious alternatives for therapy [16]. Lab staff should provide unreported results to clinicians upon request because special circumstances such as patient allergies may necessitate the use of these antimicrobials.
Laboratory personnel and clinicians should also collaborate on how to report results. In some institutions, clinicians prefer to only receive the interpretation of results, that is, designating an organism as susceptible or resistant to an antimicrobial based on standardized breakpoints. In other cases, clinicians wish to receive the actual MIC values in addition to the interpretation of the results. Results may be reported as soon as they are received in the microbiology laboratory in institutions where results are provided through a computerized database. When a computer database is not available, the laboratory may need to designate staff to reply to phone inquiries from clinicians.

17.3 INFECTION CONTROL

The microbiology laboratory can provide important information related to infection control concerns in both the hospital and outpatient setting [17,18]. The susceptibility patterns of organisms vary greatly by geographic location and from institution to institution. Microbiology laboratories can provide an antibiogram of the susceptibility patterns of common organisms that can aid clinicians in empiric therapy in their practice location. The laboratory should ensure that the antibiogram does not contain duplicate organisms cultured multiple times from the same patient. This may give a spurious indication of the susceptibility pattern for an organism in that institution. Often, laboratories only tabulate the results of susceptibility testing on inpatient isolates or combine results of isolates on inpatients and outpatients. If possible, it is helpful to provide clinicians with separate results on inpatient and outpatient isolates. The widespread use of antimicrobials in the inpatient setting may result in resistance patterns that do not reflect those in the outpatient setting. On the other hand, increasing reports of antimicrobial resistance in outpatient infections, such as the description of methicillin-resistant Staphylococcus aureus–related skin infections, should prompt surveillance for such organisms. Clinicians need to know if such organisms are prevalent enough in their practice area to warrant a change in the empiric therapy of these common infections when data from clinical trials supports such a change.

Microbiology laboratory personnel should have a close working relationship with hospital infection control staff. As the hub of microbiological information in an institution, the microbiology laboratory may detect increases in infections with specific organisms in a given unit in the hospital, or may note changes in resistance patterns in certain organisms that may not be apparent to individual clinicians [19]. Laboratory personnel also can alert infection control practitioners to the presence of patients in the hospital who are colonized or infected with organisms that may require various forms of isolation, such as tuberculosis, or specific types of antimicrobial-resistant organisms.

17.4 EDUCATION

The microbiology laboratory can serve as a source of education about microbiological issues for clinicians and house staff. Seminars, grand rounds, and regularly scheduled tutorials can help keep clinicians informed about such topics as resistance patterns in the hospital, new antimicrobials on susceptibility panels, and new microbiological diagnostic tests. While aiding clinicians in the use of these tests for patient care, educational efforts can also help to decrease unnecessary microbiologic testing and ease the workload on laboratory staff [4]. For instance, microbiology staff can help clinicians in deciding when susceptibility testing is sufficiently predictable that it is not necessary, as with penicillin susceptibility testing for group A streptococci. Educational efforts can also give clinicians and house staff appropriate expectations about the timing of results. For instance, if house staff are not aware that a test is run only on specific days of the week, they may unnecessarily call the laboratory daily in anticipation of pending results.

17.5 RESEARCH

The microbiology laboratory is also an important participant in clinical research. The laboratory may participate in the evaluation of new diagnostic microbiological methods to ascertain their accuracy
and to determine if those tests are cost-efficient for their particular institution [20]. The microbiology laboratory may also participate in evaluating new methods for susceptibility testing. Also, the microbiology laboratory is an important partner in clinical research, testing the safety and efficacy of new antimicrobials. The microbiology laboratory is often responsible for the initial testing of isolates from subjects participating in a clinical trial. The laboratory is often responsible for storing and shipping such isolates to a central laboratory as well. Finally, the laboratory is an important partner in epidemiological research on infection control related issues in the hospital and outpatient setting [19].

17.6 CONCLUSIONS

Infectious disease physicians have the ability to make a specific diagnosis of infection and gauge the most appropriate treatments for patients based on the results of microbiological testing performed in the laboratory. Perhaps in no other subspecialty in medicine is there such a symbiotic relationship between caregiver and laboratory personnel. The efficient functioning of the microbiology laboratory and a good relationship of the laboratory with patient caregivers are dependent upon good communication between the various groups. Good communication between the laboratory and clinicians results in the most appropriate use of microbiological testing, well-informed clinicians with realistic expectations of the laboratory, and the most cost-efficient use of resources. In the final analysis, smooth interactions between the microbiology laboratory and patient caregivers result in better care for patients, the common goal of all those involved in the treatment of infectious diseases.

REFERENCES


# 18 Clinical Microbiology in the Development of New Antimicrobial Agents

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18.1 INTRODUCTION

The insidious, unrelenting ability of microorganisms to develop resistance poses a constant challenge for the pharmaceutical industry to devise new antimicrobial agents with improved spectrum and potency. This challenge is underscored by the vast array of currently available antibacterial agents ranging from penicillin and the extended-spectrum penicillins to the various generations of tetracyclines, macrolides, aminoglycosides, cephalosporins, carbapenems, and quinolones.

The microbiological procedures necessary for approval of a new antibiotic by regulatory agencies in the United States are rigorous and extensive, involving a wide variety of preclinical and clinical studies by numerous investigators and laboratories. In this chapter, we discuss the various types of information required for the microbiological section of new drug applications (NDAs). The requirements are essentially similar for all antibiotic classes. However, for purposes of illustration, most of our discussion centers on the second- and third-generation quinolones.

18.2 MECHANISMS OF ACTION

An in-depth description of the mechanism of action of the antibiotic is one of the first requirements. Details also are provided regarding chemical structure and any structural or other similarities with marketed antimicrobial drugs. Such details can provide information that is important in the drug’s potential for resistance or scope of antimicrobial spectrum. For example, the major mechanism of bacterial killing by the quinolones involves inhibition of the activities of DNA gyrase, a Type II topoisomerase enzyme found in bacterial cells. By binding to a complex created by gyrase and DNA, quinolones prevent bacterial DNA synthesis and transcription. This unique interaction of quinolones with the gyrase–DNA complex explains their relative lack of cross-resistance with other classes of antimicrobial agents.

In addition to inhibiting DNA gyrase, newer quinolones like gatifloxacin, moxifloxacin, and trovafloxacin also inhibit the in vitro activity of topoisomerase IV. This bacterial cell enzyme plays a key role in chromosome partitioning during cell division and appears to be the primary target of quinolone activity in gram-positive bacteria (most notably *Staphylococcus aureus* and *Streptococcus pneumoniae*). In contrast, DNA gyrase is the main quinolone target in gram-negative bacteria like *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.

18.3 STRUCTURE-ACTIVITY RELATIONSHIPS

Structural changes within members of an antibiotic class can significantly alter activity, spectrum, and propensity for development of resistance.

Figure 18.1 illustrates the chemical structures of representative first-, second-, and third-generation quinolones. All of these molecules are synthetic analogs of nalidixic acid, the first member of the quinolone class. Chemical modification of nalidixic acid, which has a limited spectrum and rapid development of resistance, began with carbon-for-nitrogen substitution at position 8 and other side-chain modifications. The resultant second-generation quinolones like ciprofloxacin and ofloxacin displayed greater potency, increased spectrum, and less tendency toward resistance. The striking superiority of ciprofloxacin over older quinolones paved the way for use of these agents in a variety of infections caused by a wide range of organisms. Substitutions based on the 6-fluoro, 7 piperazinyl molecule led to development of the other second-generation quinolones, sparfloxacin and clinafloxacin.

The third-generation quinolone, trovafloxacin, was created by a 7-azabicyclo modification of the ciprofloxacin molecule while the newer third-generation drugs, gatifloxacin and moxifloxacin, are characterized by an 8-methoxy side-chain modification at the C-8 position. These features maintain or exceed the potent antibacterial activity of ciprofloxacin against most entrobacteriaceae,
while improving activity against gram-positive organisms, anaerobes, and atypical pathogens like *Mycoplasma pneumoniae*, *Chlamydia pneumonia*, and *Legionella* spp.

### 18.4 SPECTRUM OF IN VITRO ACTIVITY

Studies determining the activity spectrum and relative potency of the new antibiotic form a lengthy part of NDAs. The initial step in the investigative process is to determine the minimal inhibitory concentration (MIC) of the drug against pathogenic bacteria that are within the spectrum of marketed members of the antibiotic class. Here, the MIC is defined as the lowest concentration of drug that inhibits the visible growth of an organism in microbroth dilution tests run under standard conditions recommended by the Clinical and Laboratory Standards Institute (CLSI). Some laboratories may perform macrobroth or agar dilution tests rather than the microbroth dilution. In the case of quinolones, pathogenic organisms tested would include gram-positive and gram-negative species, both aerobic and anaerobic, atypical organisms, and acid-fast mycobacteria (Table 18.1).

In addition to type strains obtained from the American Type Culture Collection (ATCC), the bacterial panel includes isolates with known mechanisms of resistance to ciprofloxacin and other second-generation quinolones, for example, methicillin-resistant *S. aureus*. The panel would also

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Antimicrobial Susceptibility Testing Protocols

**TABLE 18.1**
Major Organisms Tested for *In Vitro* Activity of Third-Generation Quinolones

<table>
<thead>
<tr>
<th>Gram-positive organisms</th>
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<tr>
<td><em>Streptococcus pneumoniae</em></td>
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<tr>
<td><em>Streptococcus pyogenes</em></td>
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<tr>
<td><em>Staphylococcus aureus, S. epidermidis</em></td>
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<tr>
<td>MRSA/CRSA</td>
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<td>Enterococci</td>
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<thead>
<tr>
<th>Gram-negative organisms</th>
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<tbody>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td></td>
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<tr>
<td><em>Moraxella catarrhalis</em></td>
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</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa, other nonfermentative gram-negative bacilli</em></td>
<td></td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
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<tr>
<th>Anaerobes</th>
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<tbody>
<tr>
<td><em>Bacteroides spp.</em></td>
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<tr>
<td><em>Fusobacterium</em></td>
<td></td>
</tr>
<tr>
<td><em>Peptostreptococcus</em></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium spp.</em></td>
<td></td>
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<tr>
<td><em>Prevotella</em></td>
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<th>Atypical bacteria</th>
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<tbody>
<tr>
<td><em>Chlamydia spp.</em></td>
<td></td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
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<tr>
<td><em>Legionella spp.</em></td>
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<tr>
<th>Acid-fast bacteria</th>
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<tr>
<td><em>Mycobacterium tuberculosis</em></td>
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</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td></td>
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<tr>
<td><em>Mycobacterium kansasii</em></td>
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* Methicillin- and ciprofloxacin-resistant *S. aureus*.

include organisms against which older quinolones are only minimally or moderately effective, for example, *S. pneumoniae* and anaerobes including *Bacteroides fragilis*.

**18.4.1 Clinical Isolates**

If the early type–strain studies are promising, the activity of the new antibiotic is evaluated against recent isolates from a variety of clinical environments such as outpatient and inpatient settings and community, teaching, and federal hospitals. In these studies, the new antibiotic is directly compared with currently approved drugs, especially those within the same class. In the case of quinolones, typical comparative agents would include ciprofloxacin, gatifloxacin, levofloxacin, and moxifloxacin. Against certain organisms, for example, *S. pneumoniae* or *M. pneumoniae*, the comparison might also include nonquinolone drugs approved for treatment of these infections, such as certain cephalosporins, amoxicillin and clavulanate, and macrolides. Comparative nonquinolone drugs in the evaluation of anaerobic activity would include metronidazole or the combination of piperacillin and tazobactam.

**18.4.2 Microbiological Criteria**

Comparative *in vitro* activity data submitted to regulatory agencies are presented as a compilation of all available data from carefully conducted trials by the pharmaceutical company and independent
investigators. Examples of microbiological criteria for study inclusion are use of quality control strains recommended by the CLSI, susceptibility testing by the latest methods approved by CLSI, and standardization of all assay techniques. To be deemed effective in vitro, a minimum of 100 clinical isolates tested by both diffusion and dilution techniques is required for the common causative organisms. Fewer isolates are acceptable only for fastidious bacteria, infrequently isolated pathogens, Chlamydia spp., or other organisms with difficult growth or testing methodology issues.

The results of in vitro activity tests are compiled for all proposed clinical indications. Data for each species are reported as the concentration of drug necessary to inhibit 50% and 90% of strains (MIC\(_{50}\) and MIC\(_{90}\), respectively), the range of MIC, and median MIC\(_{90}\) of common organisms associated with these conditions. Because of the broad spectrum of activity of third-generation quinolones, data on in vitro activity may include pathogens found in about 14 infections, ranging from acute sinusitis, community and nosocomial pneumonias, and skin and soft tissue infections, to complicated intra-abdominal or pelvic infections (Table 18.2).

Since antibiotic susceptibility varies among geographical areas, the majority of studies are conducted on isolates from patients at a variety of locations across the United States. Any data derived from other countries must be demonstrated as microbiologically relevant, that is, similar antimicrobial activity, to the treatment of patients in the United States.

### 18.4.3 Prototype In Vitro Studies

The following are examples of the types of in vitro studies that would be incorporated into the database of an application for a new third-generation quinolone.

**Gram-positive activity.** A multicenter study [1] comparing the activity of trovafloxacin, gatifloxacin, and sparflloxacin against over 1,600 ciprofloxacin-resistant gram-positive cocci isolated from patients with significant bloodstream, respiratory tract, wound, and urinary tract infections. The majority of the organisms were *S. aureus*, enterococci, and coagulase-negative staphylococci.

**Anaerobic activity.** A study [2] comparing the MICs of gatifloxacin, trovafloxacin, ciprofloxacin, sparflloxacin, ampicillin, ampicillin and sulbactam, clindamycin, and metronidazole against 351 recent clinical isolates of *B. fragilis*, *Fusobacterium*, and other anaerobes.

**Gram-negative activity.** A study [3] comparing the activity of gatifloxacin, ciprofloxacin, and ofloxacin against *Enterobacteriaceae* and nonfermenting gram-negative bacteria. MICs were determined for 450 clinical isolates representing 25 species.

18.5 PHARMACOKINETIC EVALUATION

Like in vitro activity, pharmacokinetic (PK) evaluation is started early in the development of a new antibiotic. After all, in vitro activity alone is useless when an antibiotic is not sufficiently concentrated in serum or at the infection site.

The first PK studies are performed in animal models from which appropriate dosage for humans is calculated following administration of multiple dosing regimens. Afterward, single and multiple oral and intravenous dosage regimens are administered to small groups of healthy human volunteers to obtain more precise information on the absorption, bioavailability, distribution, extent of protein binding, metabolism, and excretion of the drug. PK parameters include peak serum concentrations (C_{max}), terminal elimination half-life (T_{1/2}), and area under the serum concentration-time curve (AUC). C_{max} and AUC data collected from these preclinical phase I studies are especially useful in helping to establish proposed breakpoints for MIC and disk-diffusion testing. Tentative breakpoints are, of course, essential before the start of clinical trials in patients with various infections.

Other studies include investigation of the effects of food on drug absorption and any drug interactions that might alter the PK behavior of the new antibiotic. For example, ciprofloxacin and other second-generation quinolones undergo metabolism via the hepatic cytochrome P450 system, and drugs that compete for this pathway, such as theophylline, are known to accumulate when administered with these quinolones. In contrast, trovafloxacin and gatifloxacin undergo less oxidative metabolism and do not interact with P450.

Data on the volume of distribution, which reflects tissue penetration, are supplemented with those obtained using representative models of tissue penetration such as inflammatory blister fluid and cells obtained by bronchial lavage. The NDA may also include tissue distribution studies showing that the antibiotic diffuses into infection sites and maintains concentrations equal to or greater than the MIC_{90} of a pathogen for an adequate period.

On oral administration, ciprofloxacin is highly bioavailable with a high volume of distribution and low protein binding. Thus, its concentrations in body tissues and fluids are higher or similar to blood concentration. Compared with ciprofloxacin, the newer quinolones have equal or greater bioavailability, higher peak plasma concentrations, and greater volume of distribution, leading to even better tissue penetration. Longer serum half-lives permit once-daily dosage with most of these drugs.

18.5.1 PHARMACODYNAMICS

Pharmacodynamic (PD) factors are critical considerations because they help establish the relationship between antibiotic dosage regimens and antimicrobial activity. Such evaluations can be performed using in vitro or animal models of infection or, less often, during clinical trials. A combined PK/PD evaluation includes relating drug concentrations in plasma to the in vitro susceptibility of the target organism. Such studies have shown that for some antibiotics, for example, β-lactams, the most significant PK parameter in estimating clinical and microbiological efficacy is the time that antibiotic serum levels exceed the MIC for the pathogen. In contrast, the activity of quinolones is concentration rather than time dependent, so that the ratio of peak serum concentrations or area under the curve (AUC) to MIC become the most predictive parameters of potential clinical and microbiological efficacy.

This concept is illustrated in Figure 18.2, which shows the relationship of the AUC/MIC ratio (AUIC) to bacteriologic eradication in 94 hospitalized patients treated with intravenous ciprofloxacin
for serious acute infections. At an AUIC < 125, the probability of microbiological cure was only 26% while at an AUIC > 125, it was 80% [5].

18.6 QUALITATIVE ASPECTS OF ANTIBACTERIAL ACTIVITY

Qualitative microbiological aspects such as determination of minimal bactericidal concentration (MBC), the rate of bacterial killing, intracellular activity, and culture conditions that might alter in vitro activity are all evaluated. Similar to in vitro spectrum studies, these evaluations are conducted using comparative quinolones or other classes of antibiotics and all assays are standardized as required by regulatory agencies.

18.6.1 BACTERICIDAL ACTIVITY

Bactericidal activity is usually assessed by assays measuring the MBC in single time point susceptibility studies performed in broth. In these studies, the MBC is defined as the lowest concentration of drug that kills 99.9% (>3 log_{10}) of the initial inoculum in 24 h. This may be supplemented by another method that samples for viability at timed intervals over 24 h. The ideal MBC is identical to or only 1 to 3 log above the MIC concentrations for the representative pathogen. With the third-generation quinolones, MBCs are usually within one dilution of the MIC for representative strains of S. aureus, S. pneumoniae, E. coli, Enterobacter cloacae, and P. aeruginosa as well as anaerobes like B. fragilis and Peptostreptococcus. MBCs for penicillin-resistant S. pneumoniae range from one to two dilutions above the MIC.

18.6.2 KILLING KINETICS

The rate of bacterial killing is yet another important NDA requirement. With some organisms and infections, for example, bacterial endocarditis, this may be a more important determinant of clinical outcome than are MBCs. In vitro time–kill studies of clinical isolates most frequently use a macrodilution technique to evaluate the speed of bacterial killing. The evaluation includes representative
organisms with high and reduced susceptibility to available quinolones. The increments of time are spaced to allow quantification of both speed and duration of bactericidal effect of the antibiotics, usually at their MIC and at concentrations 4 and 8 times their MICs. The time–kill curve shown in Figure 18.3 plots the killing rate of trovafloxacin, ciprofloxacin, and sparfloxacin against a strain of quinolone–methicillin-susceptible Staphylococcus aureus (MSSA).

At its MIC of 0.5 µg/mL, ciprofloxacin exhibited only a bacteriostatic effect and was not highly bactericidal even at 4 times its MIC. In contrast, trovafloxacin and sparfloxacin at their respective MICs of 0.25 µg/mL and 0.5 µg/mL produced a bactericidal effect at 24 h [6]. Clinically, ciprofloxacin is effective against MSSA.

More recently, techniques called in vitro pharmacokinetic models have also been used. These methods simulate human pharmacokinetics so that the projected peak drug dose achieved in human serum can be evaluated for its time to killing. In a study [7] using this method, the third-generation quinolone, moxifloxacin, was found to kill S. pneumoniae at a significantly faster rate than sparfloxacin or levofloxacin. A similar study [8] of gatifloxacin found that the projected human dose was effective in killing strains of penicillin and erythromycin susceptible and resistant S. pneumoniae within 6 to 10 h.

18.6.3 Antibiotic Synergy

Standard time-kill methods and in vitro dynamic models are also used to evaluate antibiotic synergy [9]. In standard time-kill studies, synergy is defined as a decrease of 2 log units in colony-forming units (CFU) for the combination of antibiotics versus the bactericidal activity of the most active single agent. Synergy studies are most often performed on organisms that are known to rapidly develop resistance to a class of antibiotics. With ciprofloxacin, for example, it has been demonstrated that P. aeruginosa is less likely to develop resistance when the quinolone is administered with azlocillin.

18.6.4 Intracellular Uptake

Quinolones are known to accumulate in phagocytic cells at levels from 10- to 28-fold higher than extracellular concentrations. Thus, the degree of intracellular antibiotic uptake must be evaluated to verify activity of the new antibiotic against intracellular pathogens such as Legionella and Mycobacterium. Since these organisms replicate exclusively within phagocyte cells, their eradication depends
Clinical Microbiology in the Development of New Antimicrobial Agents

on intracellular levels of antibiotic above the MICs rather than serum or tissue antibiotic levels. Studies measuring the speed and extent of antibiotic accumulation in guinea pig macrophages, human neutrophils, and tissue cultured epithelial cells may be presented in the NDA. In such studies, the intracellular activity of the new quinolone at various concentrations is compared with that of erythromycin or other marketed drugs effective against infections caused by intracellular pathogens.

18.6.5 DRUG METABOLITES

The in vitro activity of any drug’s metabolites detected during the early pharmacokinetic studies in humans may also be included in NDAs. Such activity, if greater than the parent compound, may be important in the treatment of urinary tract infections and pyelonephritis when limited levels of the parent compound are recovered in urine.

18.6.6 POSTANTIBIOTIC EFFECT (PAE)

Quantification of the PAE (defined as the continued suppression of bacterial growth after short exposure to an antibiotic) is recommended by regulatory agencies. Either in vitro methods or in vivo animal models may be used to evaluate this effect.

Although the clinical significance of PAE is uncertain, the phenomenon may be important in devising dosage regimens and reducing development of resistance due to subinhibitory concentrations of antibiotic between doses. Most antibiotics display some PAE against gram-positive bacteria but quinolones also display a PAE against gram-negative organisms. The PAEs of third-generation quinolones vary with the pathogen; overall, the effect appears to range from 1 to 6 h.

18.6.7 CULTURE CONDITIONS

The in vitro activity of ciprofloxacin and other marketed quinolones is affected by a variety of culture conditions. Acidic pH diminishes their activity for gram-negative organisms, especially at a pH of 6 or less. Likewise, significant MIC increases in certain organisms are observed with inocula of 10^7 CFU or more.

Since susceptibility testing methods must be standardized, a new quinolone is evaluated to determine if its MICs for various selected bacterial species are affected by these and other culture conditions. Determination of the inoculum size effect is accomplished by exposing the antibiotic to inocula of a clinical isolate from 10- to 100-fold less to 10- to 100-fold greater than the standard inoculum for broth and agar dilution. Differences in MIC values signify that inoculum size can interfere with test accuracy. MIC values are also compared using microbroth and agar dilution test methods, at varying pH levels and with the addition of cations. All of these variables can interfere with test accuracy and must be clarified before the appropriate growth medium for susceptibility testing can be determined.

18.7 ASSESSMENT OF BACTERIAL RESISTANCE

Unlike β-lactam antibiotics, plasmid-encoded resistance and enzyme inactivation have not been observed with quinolones. Acquired resistance to quinolones is tied to their mechanism of action and involves spontaneous mutations in the bacterial chromosomal genes. Most often, resistance arises from mutations in bacterial DNA gyrase that impair the ability of these drugs to bind with the gyrase–DNA complex. The other mechanism involves mutations of chromosomally encoded drug influx and efflux systems resulting in prevention of quinolone entry into the bacterial cell or expulsion of drug from the cell. Single mutations of genes result in a stepwise selection of resistance. Over time, mutations in both DNA gyrase and topoisomerase IV or double DNA gyrase mutations may occur. Such multiple site mutations seem to have an additive effect in raising the level of resistance.
18.7.1 Selection Potential

The stability of a new quinolone against strains of various species is usually assessed by means of in vitro cell population analyses. These include measurements of MIC on daily passage of strains in media containing increasing concentrations of drug or plating of large inocula into media containing high antibiotic concentrations. Such studies have shown that, even with second-generation quinolones, resistance is rarely encountered in organisms like *E. coli* and other *Enterobacteriaceae* that are innately highly susceptible to quinolones. Resistant mutants are more often found among *P. aeruginosa* and *S. aureus*, organisms against which these antibiotics are less active.

The frequency of resistant mutants among gram-negative bacteria exposed to newer quinolones reportedly ranges from $10^6$ at twice the MIC to $10^{10}$ at 16 to 32 times the MIC. Mutants with *P. aeruginosa* and *E. coli* appear to occur at rates similar to those of ciprofloxacin. However, resistant mutants of gram-positive bacteria appear to occur less often with newer quinolones than with ciprofloxacin and other second-generation agents.

For example, one study [10] found that selection for less susceptible mutants of MRSA and *S. pneumoniae* was 10- to 100-fold higher with ciprofloxacin and ofloxacin than with the third-generation quinolone, gatifloxacin. A lower selection tendency for these organisms has also been observed with trovafloxacin and moxifloxacin in comparison with ciprofloxacin. The reduced potential for resistance appears to be related to the fact that substantially more mutational steps are required before gram-positive organisms become resistant to third-generation quinolones. In most cases, the early step mutants remain susceptible to these newer agents.

18.7.2 Cross Resistance

Yet another aspect of study is to determine whether the new quinolone antibiotic displays cross resistance with strains resistant to marketed quinolones or other classes of antibiotics. This involves determination and comparison of the MICs of the various test antibiotics.

The third-generation quinolones have not demonstrated any consistent advantage over ciprofloxacin for gram-negative strains with high-level ciprofloxacin resistance or multidrug-resistant enterococci. They are, however, active against ciprofloxacin-resistant pneumococci and *S. aureus*, and potent against some strains of MRSA with high-level resistance to ciprofloxacin. In addition, the new quinolones are more active than ceftriaxone or erythromycin against penicillin-resistant pneumococci, and they are highly active against multiresistant *Enterobacteriaceae* susceptible to quinolones but resistant to β-lactams and aminoglycosides.

18.8 Clinical Laboratory Test Methods

Several more laboratory procedures are required before a new antibiotic is evaluated for efficacy in clinical trials. Most of these tests revolve around defining standards and quality control parameters for disk diffusion tests. Regulatory agencies recommend that both MIC dilution and disk diffusion techniques be used for testing during clinical trials.

18.8.1 Disk Potency

The first step is to determine the appropriate antibiotic potency for the disks used in diffusion tests. This is done by comparing various concentrations of the new antibiotic with the disks of marketed quinolones with similar spectra and MICs. Generally, about 100 strains of bacteria with known and different MICs are tested with disks containing at least three different concentrations of the new antibiotic, for example, 5 µg, 10 µg, and 20 µg.

Once an antibiotic disk concentration is selected, it must be correlated with the MIC values already obtained by macrobroth dilution. Usually, this involves constructing regression lines of a zone of inhibition versus MIC for about 50 isolates covering the spectrum of the new antibiotic.
Regression analyses ordinarily include one or more comparative agents of the same class as the test agents. As with *in vitro* susceptibility testing, the bacterial strains used are obtained from various geographical areas in the United States and represent the major routine isolates that are within the spectrum of the new antibiotic. According to the CLSI, ideal disk concentrations will give zone diameters of 30–35 mm for highly susceptible strains and zones no larger than 15 mm for resistant strains.

### 18.8.2 MIC AND DISK ZONE BREAKPOINTS

When there is good correlation between disk concentration, zone diameter, and MICs, it is possible to establish interpretive breakpoint criteria for the dilution and diffusion techniques. MIC breakpoints are set first because these are derived from pharmacokinetic and pharmacodynamic properties of the antibiotic following a recommended dosage schedule. The highest MIC value for susceptibility must be lower than the drug concentration attained in blood or tissue with dosages and routes of administration. In addition, the MIC breakpoint should fall within the limits of clusters of bacteria with comparable susceptibilities. Such distribution patterns are often apparent on scattergrams devised from zone diameters as they relate to MICs. The population of bacterial strains within the susceptible or resistant category must correlate with clinical outcome. Thus, these preclinical breakpoints are considered tentative and may later be modified following the results of phase II and phase III clinical trials.

The CLSI [11] recommended approach for disk susceptibility testing involves the selection of two zone breakpoints that define strains as resistant, susceptible, and intermediate. Strains in the susceptible category attain MICs that are generally under serum or tissue levels of the antibiotic using usual dosage regimens. Intermediate strains have MICs in the middle of the overall range of MIC distribution, so response rates may be lower than for susceptible strains. Strains in the resistant category are not inhibited by the usual systemic concentrations of the drug.

Figure 18.4 [14] shows a gatifloxacin scattergram for rapidly growing bacteria, and Table 18.3 shows the resultant preclinical breakpoints.

The intermethod error rate is acceptable according to CLSI guidelines.

---

**FIGURE 18.4** Scattergram comparing gatifloxacin MIC results by the broth microdilution method with the zones of inhibition around 5-µg gatifloxacin disks. Regression equation: \( y = 2.2x + 42.0, r = 0.80 \). Reprinted from Girard, A., Girard, D., Gootz, T. et al. *AAC*, 39, 2210, 1995, with permission.
18.8.3 Quality Control (QC) Limits and Disk Stability

QC limits for representative bacteria must be established for both MIC and zone diameters. QC standards using the selected antibiotic disk concentration are derived from studies involving 7 to 10 laboratories. Such studies must use at least five lots of medium from two manufacturers as well as a reference lot of Mueller-Hinton agar medium. To minimize major errors in interpretation, appropriate ATCC quality control strains are tested along with clinical isolates. In addition, the filter paper disk containing the antibiotic reagent is evaluated for stability over time and under appropriate storage conditions. The NDA requirement is that two to three lots of disks be tested by the disk manufacturers. Zone diameters must fall within QC limits, and separate standardization studies must be performed for rapidly growing aerobic bacteria, fastidious organisms, and anaerobes.

18.9 Animal Models of Infection

Animal models are an integral part of the evaluation of new antimicrobial agents because they establish an in vivo link between in vitro testing and proposed human clinical trials. As mentioned earlier, such models are used to assess numerous antibiotic properties ranging from toxicity, pharmacokinetics, and postantibiotic effect to intracellular killing and antibiotic synergy. In this discussion, we highlight some of the models used to evaluate the potential role of new quinolones in the treatment of a variety of infections. All animal model studies are conducted with controls and appropriate comparative antibiotics.

18.9.1 Mouse Protection Studies

These are basic screening tests in which acute systemic infections are induced by intraperitoneal injection of a lethal bacterial dose in mice. Varying doses of the test antibiotics are administered to the treatment group and, at a set time interval, the dose of each antibiotic required to protect 50% of mice from death (PD₅₀) is determined. If in vivo efficacy is substantially lower than would be expected from in vitro activity tests, peritoneal specimens are tested for antibiotic susceptibility to assess possible emergence of resistance.

The simple technique is most useful in comparing in vitro with in vivo antibiotic activity and is used to evaluate many organisms from gram-positive S. aureus and S. pneumoniae to gram-negative K. pneumoniae, E. coli, E. cloacae, and P. aeruginosa.

18.9.2 Thigh Muscle Infections

The thigh muscle test allows for evaluation of drug–organism interaction in neutropenic mouse models without intervening host-defense mechanisms.
In such models, mice are made neutropenic with cyclophosphamide. Then the animals are inoculated with the infecting organism into each thigh and later with subcutaneous injection of the test antibiotic. Thigh muscles are removed, homogenized, and analyzed for number of CFU by plating selected samples on agar. This test is most often used to assess compatibility and verify the efficacy of antibiotic combinations shown to possess additive or synergistic effects in in vitro studies. Thigh muscle infections are also used to study antibiotic efficacy against bacteria with varying degrees of pathogenicity and susceptibility [12].

**18.9.3 Mixed Aerobic–Anaerobic Infection**

A frequently used technique to evaluate mixed infection mimics the two-phase response that occurs in postsurgical infections. After intraperitoneal implantation of capsules containing either intestinal contents or *E. coli* and *B. fragilis*, there is an initial peritonitis phase in which *E. coli* and other gram-negative pathogens predominate. The few untreated animals who survive this onslaught develop abscesses in which the major pathogens are *B. fragilis* and other anaerobes. Table 18.4 shows the organisms isolated following treatment of these simulated mixed infections with either trovafloxacin or clindamycin or gentamicin [13].

As can be seen, the antibiotics dramatically reduced the death rate from peritonitis as well as the likelihood of anaerobic abscess formation.

**18.9.4 Lung Infections**

Respiratory tract infections are a major indication for second- and third-generation quinolones. Thus, murine models of pneumonia are commonly used to evaluate the potential efficacy of a new drug of this class. In one such model, the organisms are instilled intranasally. This is later followed by a 3-day course of oral antibiotic therapy after which the PD₅₀ is calculated. In such a study [14] comparing the efficacy of ciprofloxacin and trovafloxacin against penicillin-susceptible

**TABLE 18.4** Incidence of Mortality and Abscess Formation in Rats Infected with a Cecal Inoculum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality (No. of Deaths/Total)</th>
<th>Abscesses (No. of Abscesses/No. of Survivors)</th>
<th>Organisms Recovered from Abscess</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trovafloxacin</td>
<td>1/18 (5.6%)</td>
<td>2/17</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> = 0.0008</td>
<td><em>Lactobacillus</em> spp</td>
</tr>
<tr>
<td>Clindamycin + gentamicin</td>
<td>0/19 (0%)</td>
<td>3/19</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> = 0.0013</td>
<td>Enterococci</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Lactobacillus</em> spp</td>
</tr>
<tr>
<td>Control</td>
<td>13/19 (68%)</td>
<td>6/6</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enterococci</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Lactobacillus</em> spp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. perfringens</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>B. fragilis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Fusobacterium</em> spp</td>
</tr>
</tbody>
</table>

Male Wister rats were infected with a cecal inoculum and treated for 7 days with either 20 mg trovafloxacin every 8 h or a combination of 15 mg clindamycin and 2 mg gentamicin every 8 h. Control animals were not treated. Abscess formation was assessed in surviving animals 7 days after infection. The organisms in the abscesses of surviving animals were identified. A chi-square test was used to compare treated groups with the untreated control group.

S. pneumoniae, trovafloxacin protected 90%–100% while ciprofloxacin protected only 20%–30% of the challenged animals. These superior *in vivo* results with trovafloxacin are in keeping with the greater *in vitro* potency of newer quinolones against pneumococci as well as their improved pharmacokinetics such as higher \( C_{\text{max}} \), half-life, and AUC values.

### 18.10 *IN VITRO* STUDIES DURING CLINICAL TRIALS

Preclinical *in vitro* and animal studies pave the way for the definitive test of antibiotic efficacy — the results of randomized, blinded, well-controlled clinical trials in patients with various infections. The initial phase II clinical studies consist of a limited number of infections and limited groups of patients. If efficacy and safety are confirmed, these early trials are followed by larger, often comparative, phase III trials. Such studies include a variety of potential drug indications and patients of various ages with and without associated diseases. By the time an NDA is completed, the number of patients participating in clinical trials may be as great as 10,000.

All clinical trials must define techniques for collecting and transporting culture specimens for each type of infection. Uniform clinical microbiology laboratory procedures are mandatory and, as in preclinical susceptibility testing, appropriate ATCC quality control strains are tested along with clinical isolates.

An important objective of the *in vitro* studies is to reevaluate the estimated preclinical breakpoints to ensure that they accurately predict clinical response. This is accomplished by analyzing new data on the following: (1) zone diameter and MIC ranges from isolates obtained at the infection site, (2) expanded PK and PD studies of infected patients, (3) clinical and bacteriological efficacy in treated patients.

The zone diameters and MICs obtained for isolated organisms are related to both microbiological and clinical responses in each patient and for each potential indication. Afterward, paired disk and MIC susceptibility tests for each isolated organism are evaluated to establish the correlation between disk zone diameters and their corresponding MICs. As with preclinical studies, the final susceptibility breakpoints are determined using regression analyses and Metzler-DeHaan statistical analysis of scattergrams for nonfastidious aerobic organisms like *S. aureus* and *Enterobacteriaceae*. Likewise, separate breakpoints are required for *S. pneumoniae*, *H. influenzae*, and other fastidious pathogens, as well as anaerobes.

### 18.11 CONCLUSIONS

Clinical microbiological procedures play a pivotal role in the development of new antimicrobial agents. The microbiological studies described in this chapter provide some insight into the numerous preclinical and clinical analyses that must be included in NDAs for new quinolones. The culmination of these diverse tasks is the setting of susceptibility guidelines to help clinicians select those antibiotics most likely to produce clinical and microbiological cures for their patients.

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Antimicrobial Susceptibility Testing Protocols

The clinical microbiology laboratory is often a sentinel for the detection of drug-resistant strains of microorganisms. It is vital that microbiology laboratories stay current with standard and emerging methods and have a clear definition of their role in integrated patient care.

Unique in its scope, Antimicrobial Susceptibility Testing Protocols gives laboratory personnel an integrated resource for updated lab-based techniques and charts, and presents the role of clinical microbiology within the context of modern medicine. The authors provide a comprehensive, up-to-date procedural manual including protocols for bioassay methods and molecular methods for bacterial strain typing. Divided into three sections, the text begins by introducing basic susceptibility disciplines including disk diffusion, macro and microbroth dilution, agar dilution, and the gradient method. It covers step-by-step protocols with an emphasis on the detection of resistant microorganisms. This section also introduces currently available automated systems, as well as recent laboratory evaluations and algorithms. The second section describes specialized susceptibility protocols such as surveillance procedures for detection of antibiotic-resistant bacteria, serum bactericidal assays, time-kill curves, population analysis, and synergy testing. The final section is designed to be used as a reference resource. Chapters cover antibiotic development; design and use of an antibiogram; and the interactions of the clinical microbiology laboratory with the hospital pharmacy; and infectious disease and control. The text includes extensive references at the end of each chapter as well as a table of antibiotic classes and bug-drug susceptibilities.