

Manual of Antimicrobial Susceptibility Testing

Marie B. Coyle, Coordinating Editor



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Manual of Antimicrobial Susceptibility Testing

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Preface

The goal of this manual is to help clinical and public health laboratorians understand the principles and practices of antibiotic susceptibility testing. It closely follows the content and design of the excellent CD-ROM released in 2002 by the Centers for Disease Control and Prevention in Atlanta, Georgia. The CD-ROM is entitled *Antimicrobial Susceptibility Testing—A Self-study Program* authored by F. C. Tenover, J. F. Hindler and E. Rosner.

In addition to providing accurate susceptibility reports for guiding patient care, a major goal of this manual is to have laboratories from different hospitals, regions and nations follow exactly the same procedures and quality control practices. In this way susceptibility patterns from throughout the Americas can be reliably compared. With more reliable results on antimicrobial resistance, infectious disease specialists, epidemiologists and public health leaders will be able to recognize emerging resistance and novel resistance patterns. Early recognition will enable the health care system to provide optimal patient therapy and to minimize the spread of resistant organisms within institutions and across communities.

The major mechanisms of antibiotic resistance are explained. The background information in each chapter is designed to help the reader understand the principles and pitfalls of susceptibility testing methods. Emphasis is placed on disk diffusion testing because this method has proven to be accurate, reproducible, technically simple and relatively inexpensive. The importance of quality control and quality assurance is stressed so that laboratories and clinicians can be assured that their susceptibility reports are accurate.

Scientists continue to develop more effective drugs to combat infectious diseases; however, this combination of new drugs and new resistance mechanisms has increased the complexity of antimicrobial susceptibility testing. These tests often must be modified to ensure that the laboratory can detect organisms with novel resistance patterns.

The target audience for this manual includes microbiologists in the laboratory including supervisors and technologists who perform and interpret the results of antimicrobial susceptibility tests in clinical or public health laboratories. Readers who have experience working in a clinical microbiology laboratory will find the information in this manual to have direct application for their susceptibility testing methods. Laboratory directors should find this information useful as they design new procedures and update policies for susceptibility testing. This manual also could be a resource for senior students in medical technology or medical microbiology programs.

To have the most benefit from studying this manual it is important that the reader has access to recent NCCLS publications that are described in Chapter 3.

Because of the potential for aerosols when handling suspensions of organisms for susceptibility tests we have included a few of the more basic biohazard rules in the Appendix.

Marie B. Coyle
Coordinating Editor

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We could not have produced this manual without the cooperation of Fred Tenover, who allowed us to adapt a superb course developed for CDC by him, Janet Fick Hindler and Eunice Rosner. Authors Stephen J. Cavalieri, Ronald J. Harbeck, Yvette S. McCarter, José H. Ortez, Ivonne D. Rankin, Robert L. Sautter, Susan E. Sharp and Carol A. Spiegel generated a Spanish version suitable for the needs of clinical microbiologists in the Americas. We also thank Janet Fick Hindler for her generosity in providing figures and for her valuable advice; Kimberly Smith of CDC and Steve Moseley of the University of Washington also made figures available to us. Ivonne Rankin consistently responded to our urgent requests for translations of Spanish documents. The technical and linguistic skills of Jorge Garza provided essential support for completion of this manual. Jean-Marc Gabastou served as the smoothly running hub for all the communications between volunteers for PAHO and the ASM.

The group of authors was assembled and dazzlingly led by Marie B. Coyle, who was appointed to this task by the ASM International Microbiology Education Committee. Draft materials were submitted to a number of expert colleagues, who helped improve it with their comments and constructive criticism. Special thanks are also due Luis Actis, Octavio Martinez, Stephen Lerner (former Chair of the International Committee), José Ramiro Cruz (PAHO Regional Advisor for Laboratory and Blood Services), and Lily Schuermann (ASM's Director of International Affairs) for their assistance at different stages in the preparation of this Manual. We are deeply grateful to Dan Lissit of ASM who has skillfully and patiently guided the development of this manual from its inception. On behalf of the ASM International Microbiology Education Committee, I express my gratitude to all the authors and contributors to this fine piece of work. Finally, we gratefully acknowledge the support of the Pan American Health Organization and the American Society for Microbiology who generously provided funding for this publication.

Daniel Sordelli
Chair, International Microbiology Education Committee

I

Modes and Mechanisms

1

Antimicrobial Modes of Action

OBJECTIVES

After completing this chapter the reader should be able to:

- Compare and contrast the basic structure of gram-positive and gram-negative bacteria.
- Explain how each group of antimicrobial agents targets key structures or metabolic pathways in bacteria.
- List the mechanisms of resistance in bacteria.
- Describe how bacteria acquire resistance to antimicrobial agents.

BACKGROUND

Each class of antimicrobial agents has a unique mode of action. In order to understand how antimicrobial agents work it is necessary to understand some basic features of bacterial cell structure and how target sites of antimicrobial agents function in the bacterial cell.

Although the structures of gram-positive and gram-negative bacteria are similar, there are several key differences. These differences account for the ability of an antimicrobial agent to inhibit the growth of either gram-positive or gram-negative bacteria. However, some agents are active on both types of bacteria and these often are referred to as broad-spectrum agents.

STRUCTURE OF GRAM-NEGATIVE BACTERIA

As seen in Figure 1.1 the outermost structure of a gram-negative cell has many parts.

Cell Wall

- The **outer membrane** serves as the primary permeability barrier of the cell and helps to retain proteins in the periplasmic space. (Some authors do not consider this membrane to be part of the cell wall.)
- **Porins** are water-filled channels in the outer membrane that facilitate transport of nutrients and low molecular weight substances, including antimicrobial agents, into the cell. Bacteria vary in the number and types of porins they contain.

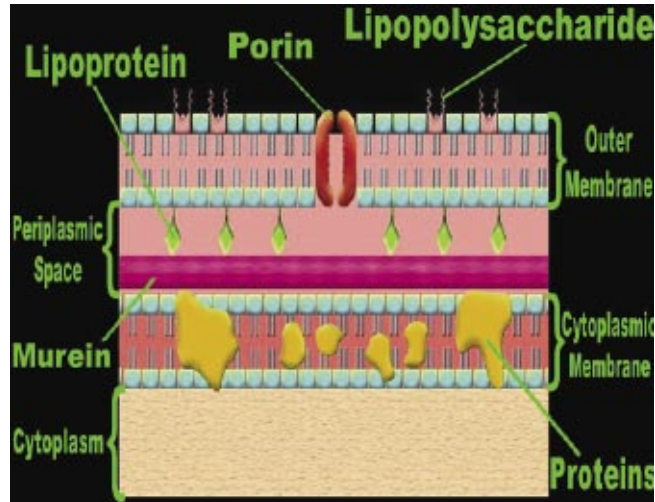


Figure 1.1—Cell wall of gram-negative bacteria

- **Lipopolysaccharides** are found on the surface of the cell and are the major component of endotoxin. They contribute to the bacterium's ability to cause disease and they give gram-negative bacteria their net negative charge.
- **Lipoproteins** attach the outer membrane to the murein layer.
- The **peptidoglycan layer** of gram-negative bacteria is a relatively thin polymer consisting of cross-linked N-acetylmuramic acid and N-acetylglucosamine. It is often referred to as the murein layer or cell wall and is responsible for maintaining the shape of the organism. It is located within the periplasmic space.
- The **periplasmic space** lies between the outer membrane and the cytoplasmic membrane. Periplasmic proteins include binding proteins for specific substrates, hydrolytic enzymes and detoxifying enzymes.

Cytoplasmic Membrane

The cytoplasmic membrane surrounds the cytoplasm of the cell and contains proteins and phospholipids. Many of the proteins contained in the cell membrane are enzymes responsible for cellular metabolism. The cytoplasmic membrane also serves as a permeability barrier and a permeability link for substances entering the cell.

Cytoplasm and Other Internal Components

The cell cytoplasm contains the chromosome, ribosomes and other internal structures. The vast majority of bacteria have a single chromosome but a few, such as *Vibrio cholera*, have two chromosomes.

STRUCTURE OF GRAM-POSITIVE BACTERIA

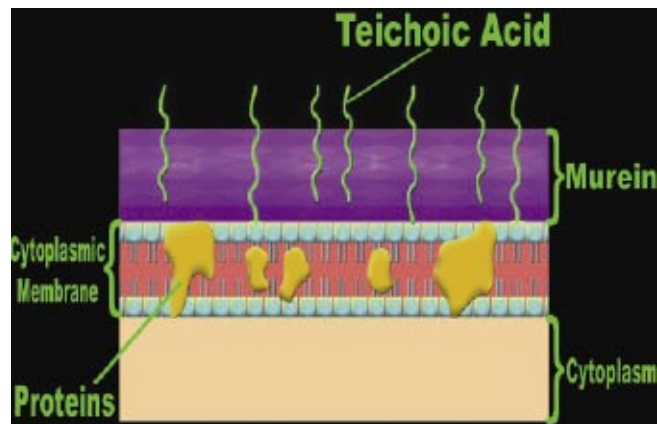


Figure 1.2—Cell wall of gram-positive bacteria

Cell Wall

Since the gram-positive cell wall contains only two major components it is much less complicated than the gram-negative cell wall.

- **Teichoic acids** are polymers that are interwoven in the peptidoglycan layer and extend as hair-like projections beyond the surface of the gram-positive cell. They also are major surface antigens in those organisms that possess them.
- The **peptidoglycan layer**, or murein layer, of gram-positive bacteria is much thicker than that of gram-negative bacteria. It is responsible for maintaining the shape of the organism and often is referred to as the cell wall.

The Cytoplasmic Membrane, Cytoplasm, and Other Internal Components

These structures are very similar in both gram-positive and gram-negative bacteria.

ANTIMICROBIAL AGENTS

Bacteriostatic vs. Bactericidal Agents

Bacteriostatic agents, such as tetracycline, inhibit the growth and multiplication of bacteria. Upon exposure to a bacteriostatic agent, cells in a susceptible population stop dividing. However if the agent is removed, the cells once again multiply.

Bactericidal agents, such as fluoroquinolones, not only inhibit the growth of cells but also trigger pathways within the cell that lead to cell death. The actions of bactericidal drugs are irreversible so once susceptible cells are exposed to a bactericidal agent, they die.

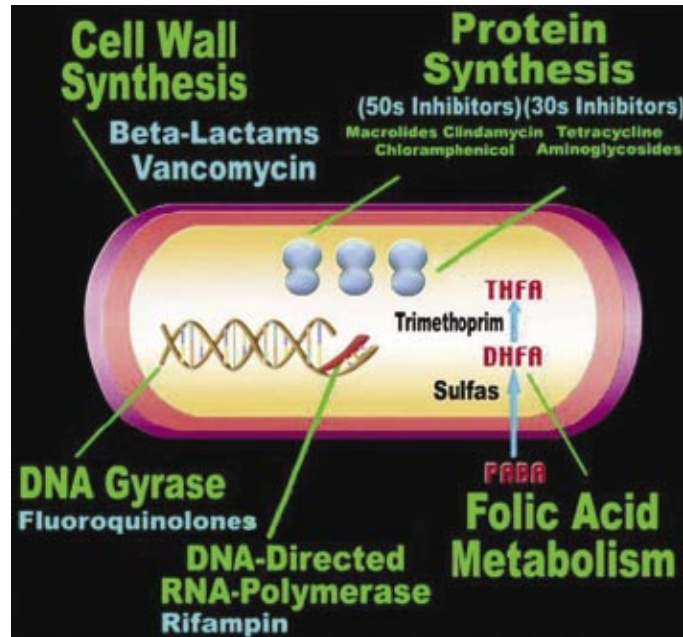


Figure 1.3—Target sites of some antimicrobial agents

Modes of Antimicrobial Action

Antimicrobial agents are classified by their specific modes of action against bacterial cells. These agents may interfere with cell wall synthesis, inhibit protein synthesis, interfere with nucleic acid synthesis or inhibit a metabolic pathway. The modes of action of antimicrobial agents against gram-positive and gram-negative bacteria are very similar.

- **Interference with cell wall synthesis:** Antimicrobial agents that interfere with cell wall synthesis block peptidoglycan synthesis and thus are active against growing bacteria. Antimicrobial agents that interfere with cell wall synthesis are bactericidal.
- Activity of beta lactams on **gram-negative bacteria:** In gram-negative bacteria, beta lactam antimicrobials enter the cell through porin channels in the outer membrane. In susceptible cells, beta-lactam molecules bind to penicillin binding proteins (PBPs) that are enzymes required for cell wall synthesis. The attachment of the beta-lactam molecules to the PBPs, located on the surface of the cytoplasmic membrane, blocks their function. This causes weakened or defective cell walls and leads to cell lysis and death.
- Activity of beta lactams on **gram-positive bacteria:** Since gram-positive bacteria do not possess an outer membrane, beta lactam antimicrobials diffuse through

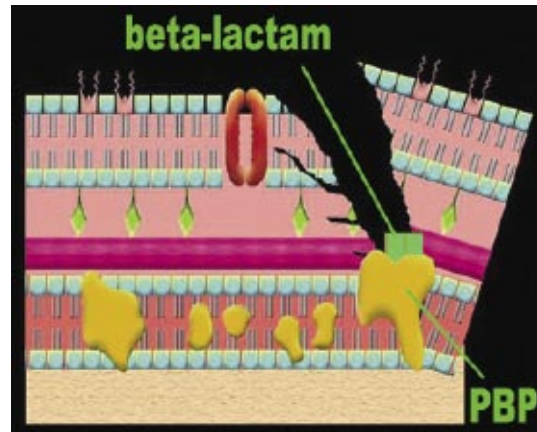


Figure 1.4—Effect of beta lactams on gram-negative bacteria

the cell wall. The next steps are similar to those in gram-negative bacteria. In susceptible cells the beta lactam molecules bind to PBPs, which results in weakened cell walls and cell lysis.

- **Interference with the cytoplasmic membrane:** Polymyxin molecules diffuse through the outer membrane and cell wall of susceptible cells to the cytoplasmic membrane. They bind to the cytoplasmic membrane and disrupt and destabilize it. This causes the cytoplasm to leak out of the cell resulting in cell death. Antimicrobial agents that interfere with the cytoplasmic membrane are bactericidal.
- **Interference with protein synthesis by binding to the 30S ribosomal subunit.**
 - Tetracyclines (e.g. tetracycline, minocycline and doxycycline) bind to the 30S subunit of the ribosome and block the attachment of transfer RNA (tRNA). Since new amino acids cannot be added to the growing protein chain, synthesis of protein is inhibited. The action of tetracyclines is bacteriostatic.
 - Aminoglycosides (e.g. gentamicin, tobramycin, amikacin, and streptomycin) also bind to the 30S ribosomal subunit and can block protein synthesis in two different ways. First they can attach to the 30S subunit of the ribosome and prevent the 30S subunit from attaching to messenger RNA (mRNA). Second, the presence of the aminoglycoside on the ribosome may cause misreading of the mRNA. This leads to the insertion of the wrong amino acid into the protein or interference with the ability of amino acids to connect with one another. These activities often occur simultaneously and the overall effect is bactericidal.
- **Inhibition of protein synthesis by binding to the 50S ribosomal subunit**
 - Macrolides (e.g. erythromycin, azithromycin and clarithromycin) and lincosamides (e.g. clindamycin) attach to the 50S ribosomal subunit causing termination of the growing protein chain and inhibition of protein synthesis. They are primarily bacteriostatic.
 - Chloramphenicol also binds to the 50S subunit of the ribosome and interferes with binding of amino acids to the growing protein. Antimicrobial agents that inhibit protein synthesis in this manner are bacteriostatic.

- **Inhibition of protein synthesis by inhibition of the 70S initiation complex**
 - Linezolid (an oxazolidinone) is a new potent inhibitor of protein synthesis. It binds to a site on the bacterial 23S ribosomal RNA of the 50S subunit and prevents the formation of a functional 70S initiation complex, which is necessary for bacterial protein synthesis. It is active against a wide variety of gram positive bacteria but has no clinically helpful activity against gram-negative bacteria.
- **Interference with nucleic acid synthesis is caused by two classes of drugs**
 - Fluoroquinolones (e.g. nalidixic acid, ciprofloxacin, levofloxacin and gemifloxacin) interfere with DNA synthesis by blocking the enzyme DNA gyrase. DNA gyrase helps to wind and unwind DNA during DNA replication. The enzyme binds to DNA and introduces double stranded breaks that allow the DNA to unwind. Fluoroquinolones bind to the DNA gyrase-DNA complex and allow the broken DNA strands to be released into the cell, which leads to cell death.
 - Rifampin binds to DNA-dependent RNA polymerase, which blocks the synthesis of RNA and results in cell death.
- **Inhibition of the metabolic pathway for folic acid synthesis is caused by the sulfonamides and trimethoprim:** For many organisms para-aminobenzoic acid (PABA) is an essential metabolite and is involved in the synthesis of folic acid, an important precursor to the synthesis of nucleic acids. Sulfonamides are structural analogs of PABA and compete with PABA for the enzyme dihydropteroate synthetase. Trimethoprim acts on the folic acid synthesis pathway at a point after the sulfonamides. It inhibits the enzyme dihydrofolate reductase. Trimethoprim and sulfonamides can be used separately or together. When used together they produce a sequential blocking of the folic acid synthesis pathway and have a synergistic effect. Both trimethoprim and the sulfonamides are bacteriostatic.

MECHANISMS OF ANTIMICROBIAL RESISTANCE

There are a number of ways by which microorganisms are resistant to antimicrobial agents. These include: 1) the bacteria produce enzymes that either destroy the antimicrobial agent before it reaches its target or modify the drug so that it no longer is recognized by the target; 2) the cell wall becomes impermeable to the antimicrobial agent; 3) the target site is altered by mutation so that it no longer binds the antimicrobial agent; 4) the bacteria possess an efflux pump that expels the antimicrobial agent from the cell before it can reach its target; and 5) specific metabolic pathways in the bacteria are genetically altered so that the antimicrobial agent cannot exert an effect.

Production of Enzymes

- **Beta-lactamases** are enzymes that hydrolyze beta-lactam drugs. As a result the cell is resistant to the action of the beta lactam drugs.
 - In gram-negative bacteria the beta lactam drugs enter the cell through the porin channels and encounter beta-lactamases in the periplasmic space. The beta-lactamases destroy the beta-lactam molecules before they have a chance to reach their PBP targets.
 - In gram-positive bacteria the beta-lactamases are secreted extracellularly into the surrounding medium and destroy the beta-lactam molecules before they have a chance to enter the cell.
- **Aminoglycoside-modifying enzymes:** Gram-negative bacteria may produce adenylating, phosphorylating or acetylating enzymes that modify an aminoglycoside so that it is no longer active.

- **Chloramphenicol acetyl transferase:** Gram-negative bacteria may produce an acetyl transferase that modifies chloramphenicol so that it is no longer active.

Bacterial Outer Membrane Impermeability

- **Alteration of porins in gram-negative bacteria:**
 - Gram-negative bacteria may become resistant to beta lactam antibiotics by developing permeability barriers. This usually is caused by altered porin channels in the outer membrane that no longer allow the entrance and passage of antibiotic molecules into the cell. When beta-lactams cannot reach the PBPs, the cell is resistant.

Alteration of Targets

- PBPs in both gram-positive and gram-negative bacteria may be altered through mutation so that beta lactams can no longer bind to them; thus the cell is resistant to these antibiotics.
- Ribosomes. Methylation of ribosomal RNA confers macrolide resistance.
- DNA gyrase and topoisomerase IV. Mutations in the chromosomal genes for DNA gyrase and topoisomerase IV confer quinolone resistance.

Efflux Pumps

- A wide variety of efflux pumps provide antimicrobial resistance in both gram-positive and gram-negative bacteria. Active efflux of antibiotics is mediated by trans-membrane proteins inserted in the cytoplasmic membrane and, in the case of gram-negative organisms, in the outer membrane and the periplasm. These proteins form channels that actively export an antimicrobial agent out of the cell as fast as it enters.

Alteration of Metabolic Pathways

- Some microorganisms develop an altered metabolic pathway that bypasses the reaction inhibited by the antimicrobial. Mutations that inactivate thymidylate synthetase block the conversion of deoxyuridylate to thymidylate. These mutants require exogenous thymine or thymidine for DNA synthesis and therefore are resistant to antagonists of the folate pathway such as the sulfonamides and trimethoprim.

INTRINSIC VS. ACQUIRED RESISTANCE

In some species antimicrobial resistance is an **intrinsic or innate** property. This intrinsic resistance may be due to one or more of the resistance mechanisms previously described. For example, *E. coli* is intrinsically resistant to vancomycin because vancomycin is too large to pass through porin channels in their outer membrane. Gram-positive bacteria, on the other hand, do not possess an outer membrane and thus are not intrinsically resistant to vancomycin.

Bacteria also can **acquire resistance** to antimicrobial agents by genetic events such as mutation, conjugation, transformation, transduction and transposition.

- **Mutation.** Chromosomal resistance develops as a result of spontaneous mutation in a locus that controls susceptibility to a given antimicrobial agent. Spontaneous mutation occurs at a relatively low frequency but, when the bacteria are exposed to the antibiotic, only the mutant cell survives. It then multiplies and gives rise to

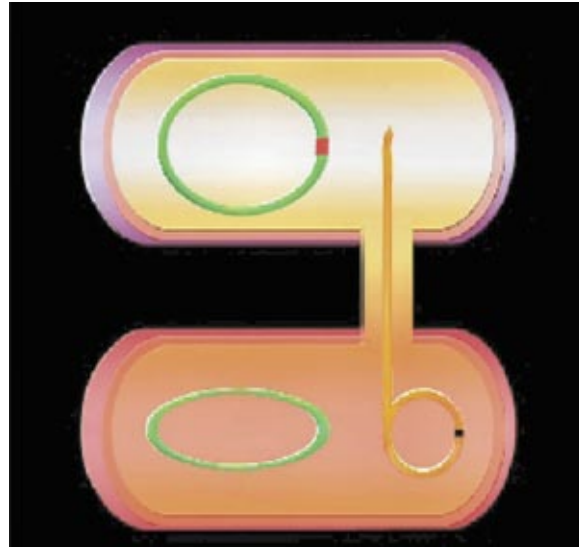


Figure 1.5—Plasmid entering a cell

a resistant population. Spontaneous mutations may also occur in plasmids. For example, mutations in plasmids containing genes for beta-lactamase enzymes can result in altered beta-lactamases often with extended activity.

- **Conjugation.** Bacteria often contain extrachromosomal genetic elements called plasmids, many of which carry genes for antimicrobial resistance. When two bacterial cells are in close proximity, a bridge-like structure known as a pilus forms between them. This allows a copy of the plasmid as it is replicated, to be transferred to another cell. The result is a bacterium that expresses the antimicrobial resistance encoded in the plasmid.
- **Transformation.** Bacteria may encounter naked fragments of DNA that carry antimicrobial resistance genes. These fragments are taken into the cell by a process called transformation. The DNA fragment is incorporated into the host cell chromosome by recombination and the resulting cell is resistant.
- **Transduction.** When bacterial viruses (bacteriophage) are multiplying in the cytoplasm of a bacterium, fragments of DNA from plasmids or chromosomes may by chance be packaged in a viral coat and enter another host cell. When the fragments contain genes for resistance to an antimicrobial agent they can confer resistance in the new host cell.
- **Transposition.** Specialized genetic sequences known as transposons are “mobile” sequences that have the capability of moving from one area of the bacterial chromosome to another or between the chromosome and plasmid or bacteriophage DNA. Since transposon DNA can carry genes for antimicrobial resistance they have contributed to the development of plasmids encoding genes for multiple antibiotic resistance. Some transposons are capable of moving from one bacterium to another without becoming incorporated into a chromosome, a plasmid or a bacteriophage.

REVIEW

The reader should now understand how specific groups of antimicrobial agents inhibit bacterial growth and the mechanisms by which bacteria develop resistance to these antimicrobial agents.

Remember that:

- Not every antimicrobial agent is equally effective against both gram-positive and gram-negative bacteria. Differences in the structures of the two groups account for the differences in their susceptibility patterns.
- Antimicrobial agents within the same class typically have similar modes of action.
- The modes of action of antimicrobial agents include inhibition of cell wall synthesis, DNA replication, protein synthesis and metabolic pathways.
- Genetic information may be passed from one bacterium to another by four mechanisms: conjugation, transformation, transduction and transposition.

CASE STUDY

Presentation

A 27-year-old female presents to her physician with increased urinary frequency and pain on urination. Based on her symptoms her physician diagnosis a urinary tract infection (UTI) and prescribes a 3-day course of trimethoprim-sulfamethoxazole. This is the third urinary tract infection that she has had in the past 12 months, all treated with the same antimicrobial. The following day she was feeling much better and decided not to continue the antibiotic. One week later she presents to the Emergency Department (ED) with flank pain, fever, chills and increased urinary frequency. A urine culture collected in the ED was positive with >100,000 colonies/mL of *E. coli*. Susceptibility testing revealed that the isolate was resistant to trimethoprim-sulfamethoxazole. What are the possible explanations for this woman's continued UTIs and worsening of symptoms?

Discussion

Initially this patient most likely presented with cystitis (infection of the bladder). *E. coli* is the most common cause of uncomplicated UTIs especially in women. It does not appear that a culture was performed in the past. While cultures may not be performed in the first instance of an uncomplicated UTI, repeat infections should have prompted a culture to determine the identity of the pathogen and its susceptibility pattern. This patient went on to develop pyelonephritis (infection of the kidney) as evidenced by her fever, chills and flank pain. It is important to distinguish cystitis from pyelonephritis since pyelonephritis is more serious and requires prolonged therapy.

This patient had previous UTIs for which she repeatedly received trimethoprim-sulfamethoxazole. One of the deleterious effects associated with the use of antimicrobial agents is the development of resistance. This may have been a result of plasmid transfer among intestinal organisms in response to antimicrobial pressure exerted by her repeated treatments with the same antimicrobial or the persistence of organisms in her urinary tract due to her noncompliance with therapy.

The urine culture and susceptibility testing in this instance revealed the pathogen as well as appropriate antimicrobials for therapy.

SELF-ASSESSMENT QUESTIONS

1. TRUE or FALSE
The cell wall is a good target for antimicrobial action because of the difference in structure of bacterial versus mammalian cells.
2. Which example best describes *the concept* of emerging antimicrobial resistance?
 - A. The transfer of bacterial plasmids containing resistance genes through conjugation.
 - B. The increasing incidence of resistance to a variety of antimicrobial agents in a variety of bacterial species.
 - C. The increase in numbers of MRSA infections.
3. Which of the following statements describe bacterial plasmids? Select all that apply.
 - A. They result from chromosomal mutations.
 - B. They are extrachromosomal DNA.
 - C. They may contain resistance genes.
 - D. They may be readily transferred among bacteria.
 - E. They are frequently present in bacteria that cause infections.
4. Answer the following as True or False.
 - A. The cell wall of gram-positive bacteria is thicker than the cell wall of gram-negative bacteria.
 - B. The fluoroquinolone class of antimicrobials acts by inhibiting cell wall synthesis.
 - C. Efflux is associated with pumping antimicrobials out of the cell.
 - D. Changes in penicillin binding proteins lead to hydrolysis or inactivation of beta-lactam agents.
 - E. Changes in porins often limit the amount of drug that can enter the cell.
 - F. Beta-lactamases may be transported out of the cell and act extracellularly in some organisms.
5. Which of the following selective pressures contribute to emerging antimicrobial resistance? Select all that apply.
 - A. Taking penicillin for viral infections.
 - B. Taking only 3 days of a 7-day course of ciprofloxacin.
 - C. Increasing consumption of vitamin C.
6. Match the following antimicrobials with their mode of action.

A. Inhibition of protein synthesis	(1) Cephalosporins
B. Inhibition of DNA synthesis	(2) Aminoglycosides
C. Inhibition of folic acid pathway	(3) Quinolones
D. Inhibition of cell wall synthesis	(4) Trimethoprim
7. Indicate whether the following antimicrobials are (1) bacteriostatic or (2) bactericidal:
 - A. Ciprofloxacin
 - B. Tetracycline

- C. Gentamicin
 - D. Sulfamethoxazole
8. Match the following antimicrobials with the bacterial mechanism of resistance
- | | |
|------------------------------------|---------------------|
| A. Altered porins | (1) Sulfonamides |
| B. Alteration of ribosomes | (2) Aminoglycosides |
| C. Altered metabolic pathway | (3) Macrolides |
| D. Production of modifying enzymes | (4) Beta-lactams |

2

Beta-Lactamases

OBJECTIVES

At the completion of this chapter the reader should be able to:

- Discuss the classification of beta-lactamases.
- Describe the differences between inducible and constitutive beta-lactamase production.
- List the organisms that should be tested routinely for beta-lactamase production.

BETA-LACTAMASES—GENERAL

Beta-lactamases are enzymes produced by bacteria that inactivate beta-lactam drugs by hydrolyzing the beta-lactam ring of the beta-lactam molecules. Most beta-lactamases inactivate either penicillins or cephalosporins, but some can inactivate both classes of drugs.

Most gram-positive bacteria secrete their beta-lactamases so that beta-lactam drugs are inactivated extracellularly, i.e., in the surrounding medium. By contrast, the beta-lactamases of gram-negative bacteria remain inside the cell and inactivate beta-lactam drugs in the periplasmic space, i.e., the space between the outer membrane and cytoplasmic membrane.

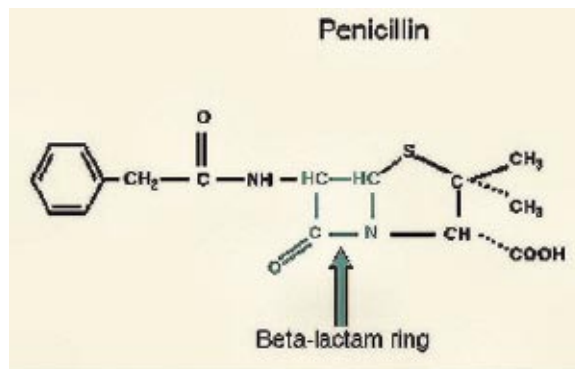


Figure 2.1—Penicillin molecule with the beta-lactam. Ring highlighted

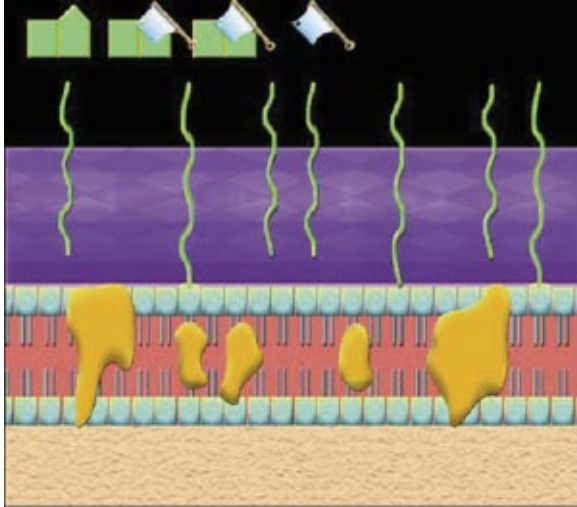


Figure 2.2—Beta-lactamases (hatchets) in gram-positive organisms

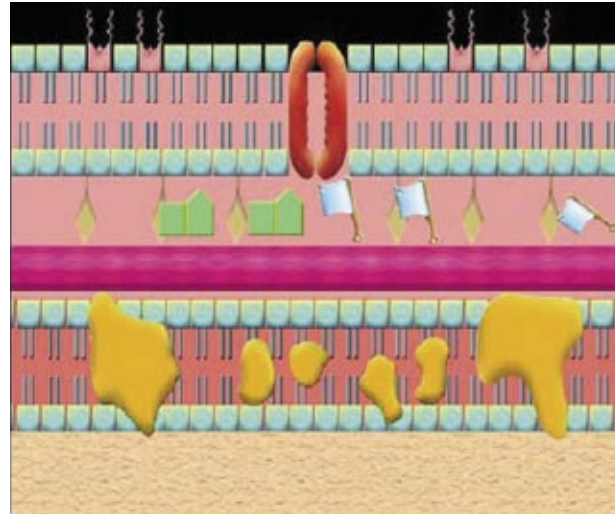


Figure 2.3—Beta-lactamases in gram-negative organisms

Genes that encode beta-lactamases can be located on the bacterial chromosome, plasmids, or transposable elements. For example:

- The beta-lactamase gene that mediates penicillin resistance in *Staphylococcus aureus* is typically located on a plasmid.
- The beta-lactamase gene that mediates ampicillin and ticarcillin resistance in *Klebsiella pneumoniae* is located on a chromosome.
- Plasmids and transposable elements enhance the spread of beta-lactamase genes among a variety of bacterial species.

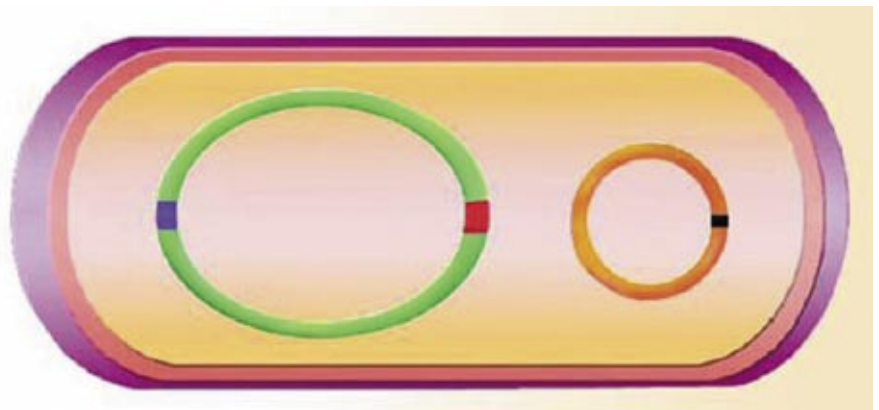


Figure 2.4—Bacterium with circular chromosome and plasmid

CLASSIFICATION OF BETA-LACTAMASES

Several classification schemes for beta-lactamases have been proposed according to their hydrolytic spectrum, susceptibility to inhibitors, gene location (plasmid or chromosome), and gene sequence or protein sequence.

There are two major classification systems:

The Ambler system is based on the molecular structure of the beta-lactamase molecule and its amino acid sequence. This classification, which initially was introduced by Ambler in 1980, recognizes four molecular classes designated A to D. Classes A, C, and D include evolutionarily related groups of enzymes with serine at their active site. Class B beta-lactamases have one or two zinc molecules at their active site and they are inhibited by EDTA.

The Bush classification is based on the substrates that a beta-lactamase hydrolyzes and on inhibition of its activity by compounds such as clavulanic acid, EDTA, and aztreonam or oxacillin. This functional classification scheme of β -lactamases, proposed by Bush, Jacoby and Medeiros in 1995, defines the four following groups according to their substrate and inhibitor profiles.

- Group 1—cephalosporinases that are not well inhibited by clavulanic acid
- Group 2—penicillinases, cephalosporinases, and carbapenemases that generally are inhibited by beta-lactamase inhibitors clavulanic acid, sulbactam and tazobactam. Subgroups also are defined according to rates of hydrolysis of carbenicillin or cloxacillin (oxacillin) by the Group 2 penicillinases
- Group 3—metallo-beta-lactamases that hydrolyze penicillins, cephalosporins, and carbapenems and that are inhibited by EDTA and not by inhibitors structurally related to the beta-lactamases (clavulanic acid, sulbactam and tazobactam).
- Group 4—penicillinases that are not well inhibited by clavulanic acid

Beta-lactamases—Inducible

Inducible beta-lactamase production is initiated, or induced, when bacteria harboring a beta-lactamase gene are exposed to a beta-lactam agent. The action of the antibiotic on the cell wall activates a genetic cascade mechanism that initiates beta-lactamase production. Beta-lactamase production is turned off when no antibiotic is present in or around the cell.

Beta-lactamases—Constitutive

Constitutive beta-lactamases are those that are produced continually by bacteria. An example of the constitutive beta-lactamase production is the SHV-1 chromosomal enzyme of *K. pneumoniae* that mediates ampicillin and ticarcillin resistance.

Beta-lactamases—Bush Group 2

Many beta-lactamases are clustered in subgroups of Bush Group 2.

Examples of **broad-spectrum beta-lactamases** include the plasmid-mediated TEM-1, TEM-2 and SHV-1 enzymes that confer resistance to ampicillin and 1st generation cephalosporins in *Enterobacteriaceae*. The name TEM was derived

Bush, Jacoby-Medeiros Functional Group		Ambler Molecular Class	Attributes of Beta-Lactamases in Functional Group
Group	Sub-group		
1		C	AmpC beta-lactamases in gram—negative bacteria. Genes often are chromosomal but may be plasmid-encoded. Confer resistance to all classes of beta-lactams, except carbapenems (unless combined with porin changes). Not inhibited by clavulanic acid.
2		A, D	Most Group 2 enzymes are inhibited by clavulanic acid (unless otherwise noted).
	2a	A	Staphylococcal and enterococcal penicillinases included. Confer high resistance to penicillins.
	2b	A	Broad-spectrum beta-lactamases, including TEM-1 and SHV-1, primarily from gram—negative bacteria.
	2be	A	Extended-spectrum beta-lactamases (ESBLs) conferring resistance to penicillins, oxyimino-cephalosporins and monobactams.
	2br	A	TEM (IRT) beta-lactamases and one SHV type that are inhibitor-resistant.
	2c	A	Carbenicillin-hydrolyzing enzymes.
	2d	D	Cloxacillin-(oxacillin)-hydrolyzing enzymes; modestly inhibited by clavulanic acid.
	2e	A	Cephalosporinases.
	2f	A	Carbapenem-hydrolyzing enzymes with active site serine.
3	3a, 3b, 3c	B	Metallo-beta-lactamases conferring resistance to carbapenems and all beta-lactam classes except monobactams. Not inhibited by clavulanic acid.
4		?	Miscellaneous penicillinases that do not fit into other groups. Not inhibited by clavulanic acid.

^a Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A Functional Classification Scheme for Beta-lactamases and Its Correlation to Molecular Structure. *Antimicrob. Agents Chemother.* 39:1211–1233.

from the initials of the first patient from whom a beta-lactamase-producing *E. coli* was isolated in 1965. SHV is considered a distant relative of TEM; SHV is derived from its early classification as the “sulfhydryl variant.”

The $bla_{\text{TEM-1}}$ gene (i.e. the beta-lactamase gene that encodes the TEM-1 beta-lactamase) is responsible for:

- Ampicillin resistance in *Enterobacteriaceae* and *Haemophilus influenzae*
- Penicillin resistance in *Neisseria gonorrhoeae*

The $bla_{\text{SHV-1}}$ gene that encodes SHV-1 type beta-lactamases also is responsible for ampicillin resistance in *Enterobacteriaceae*.

The **extended spectrum beta-lactamases (ESBLs)** are enzymes that hydrolyze and cause resistance to newer beta-lactams especially oxyimino-cephalosporins and aztreonam. The majority of the ESBLs are derivatives of the widespread beta-lactamases TEM-1 and SHV-1. Today at least 160 ESBLs have been recognized with the majority assigned to a TEM or SHV group in sequential numbers.

A major characteristic of Group 2 beta-lactamases is their inhibition by clavulanic acid which binds to the beta-lactamases and interferes with their hydrolysis of beta-lactam antibiotics.

METHODS—BETA-LACTAMASE TESTING

Beta-lactamase tests determine if narrow-spectrum beta-lactam agents (e.g. penicillin and ampicillin) can be used to treat a few key species of bacteria.

Beta-lactamase tests **MUST NOT** be used to detect extended-spectrum beta-lactamases (ESBLs), AmpC beta-lactamases, metallo-beta-lactamases, or carbapenemases.

The species for which beta-lactamase testing is useful include:

Enterococcus species
Haemophilus influenzae
Moraxella catarrhalis
Neisseria gonorrhoeae
Staphylococcus species
Some anaerobic bacteria

(More details are presented in later chapters.)

In order to detect an inducible beta-lactamase in vitro, such as the one present in most *Staphylococcus aureus* strains, the *S. aureus* isolate is inoculated onto an agar plate. Then an oxacillin disk is placed on the plate and the plate is incubated overnight. The following day, a sample is taken from the growth located on the periphery of the zone of inhibition around the disk (i.e., where the beta-lactamase production is induced) and used in the beta-lactamase test. The most common beta-lactamase test uses a chromogenic beta-lactam substrate (such as a cephalosporin) that changes color when its beta-lactam ring is hydrolyzed:

Procedure:

1. Inoculate a large quantity of the test organisms onto filter paper impregnated with a chromogenic beta-lactam substrate (use inoculating loop).
2. Incubate for the length of time and at the temperature specified by the manufacturer.
 - Different species may require different incubation times.
 - Many reactions occur instantly.
3. Examine for a color change.
 - Negative = no color change
 - Positive = color change, usually from colorless or yellow → red

Interpretation: A positive test indicates resistance to:

Amoxicillin
Ampicillin
Carbenicillin
Mezlocillin
Penicillin
Piperacillin
Ticarcillin

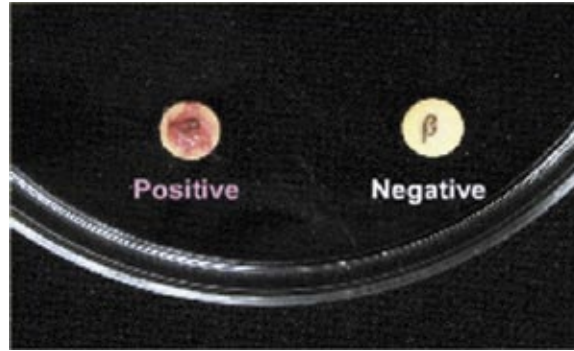


Figure 2.5—Positive and negative beta-lactamase tests

The above beta-lactams are hydrolyzed by the types of beta-lactamases detected with the routine beta-lactamase test.

A negative reaction does not always mean that the organism is susceptible to the agents mentioned earlier. Some bacteria have multiple mechanisms of resistance to beta-lactam agents. For example, *Neisseria gonorrhoeae* may be resistant to penicillin due to production of beta-lactamase or to alterations of penicillin binding proteins (PBPs). Conventional antimicrobial susceptibility tests are needed to confirm penicillin resistance due to altered PBPs.

REVIEW

The reader should now understand the action of beta-lactamases and the methods for routine beta-lactamase testing in the clinical microbiology laboratory.

Remember:

There are many different types of beta-lactamases and their spectra of activities are quite variable. The chromogenic cephalosporin assay for beta-lactamases, often used in the clinical microbiology laboratory, is useful only for *Enterococcus* species, *H. influenzae*, *M. catarrhalis*, *N. gonorrhoeae*, and *Staphylococcus* species. It is not helpful for determining beta-lactamase activity in isolates other than these species.

CASE STUDY

Presentation

A woman brings her 2½-year-old child to the physician with complaints that the child is suffering ear pain and drainage, fever, lethargy and irritability. After examining the child, the doctor explains to the mother that the child has an ear infection. The physician prescribes a 7-day course of amoxicillin for the child. After 5 days, the mother and the child return to the physician's office. The child's condition has not improved and she still has pain and fever. The physician switches the infant to a 7-day prescription of amoxicillin/clavulanate (Augmentin) and the symptoms clear. What are possible explanations for this child's initial lack of response to therapy and subsequent recovery?

Discussion

Bacterial causes of otitis media in infants, children and adults include primarily *S. pneumoniae* (40–50%), *H. influenzae* (20–25%) and *M. catarrhalis* (10–15%). Amoxicillin remains the drug of choice for initial treatment because of its 25-year record of clinical success, acceptability, limited side effects and relatively low cost. However, the drug is ineffective against *M. catarrhalis* and beta-lactamase producing strains of *H. influenzae*. The current incidence of ampicillin-resistant *H. influenzae* and that of *M. catarrhalis* as a cause of otitis media is not high enough to require a drug other than amoxicillin for initial therapy. However, parents should contact the physician if the child does not respond to therapy.

This child's infection probably was due to a beta-lactamase producing strain of *H. influenzae* or *M. catarrhalis*. This would account for the child's failure to respond to initial therapy with amoxicillin and its response to amoxicillin + clavulanate. Clavulanate is a strong inhibitor of the beta-lactamase enzyme produced by *H. influenzae* and *M. catarrhalis*, neutralizing its effects and allowing the amoxicillin to kill the organisms.

If an appropriate specimen from this patient had been cultured and yielded *H. influenzae* or *M. catarrhalis*, a chromogenic cephalosporin assay could have been performed to rapidly detect the production of the beta-lactamase enzyme.

SELF-ASSESSMENT QUESTIONS

1. Answer the following statements as True or False.
 - A. Gram-positive bacteria produce beta-lactamases that destroy beta-lactam drugs outside of the cell.
 - B. TEM beta-lactamases are only produced by staphylococci.
 - C. TEM beta-lactamases are produced by gram-negative species, including *Enterobacteriaceae*, *H. influenzae* and *N. gonorrhoeae*.
 - D. Constitutive beta-lactamases are produced at the same levels regardless of exposure of the bacterium to beta-lactam agents.
 - E. The Ambler classification of beta-lactamases is based on the degree to which a beta-lactamase hydrolyzes or inactivates penicillin.
2. A beta-lactamase positive isolate of *H. influenzae* is resistant to which of the following antimicrobial agents? Select all that apply.
 - A. Amoxicillin
 - B. Ampicillin
 - C. Cefotaxime
 - D. Ciprofloxacin
 - E. Imipenem
 - F. Penicillin

II

Test Methods

3

A Guide to Using the NCCLS Documents

OBJECTIVES

At the completion of this chapter, the reader should be able to:

- Be familiar with the NCCLS organization and its mission.
- Select appropriate antimicrobial agents for testing and reporting after taking into consideration the organism, hospital formulary, and site of infection.
- Use the interpretive criteria found in the NCCLS standards in order to report susceptibility results.

To understand the information in this chapter it is essential that the reader have in hand the NCCLS documents M2, M7 and M100.



The Clinical and Laboratory Standards Institute (CLSI), formerly known as “The National Committee for Clinical Laboratory Standards (NCCLS),” is a non-profit organization with members representing multiple disciplines. Its mission is that of promoting the development and use of voluntary laboratory consensus standards and guidelines.

NCCLS ANTIMICROBIAL SUSCEPTIBILITY TESTING DOCUMENTS

The NCCLS produces documents addressing various topics in clinical laboratory science, such as analysis of glucose in serum samples and protection of laboratory workers from blood-borne pathogens.

Documents for routine antimicrobial susceptibility testing and reporting are developed by a subcommittee that includes experts in infectious diseases, pharmaceuticals, and clinical laboratory practices.

- M2 Performance Standards for Antimicrobial Disk Susceptibility Tests
- M6 Protocols for Evaluating Dehydrated Mueller-Hinton agar
- M7 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically
- M11 Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria
- M23 Development of *In Vitro* Susceptibility testing Criteria and Quality Control Parameters
- M39 Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data
- M100 Performance Standards for Antimicrobial Susceptibility Testing

Other documents for more specialized types of antimicrobial susceptibility tests are managed by separate subcommittees.

- M21 Methodology for the Serum Bactericidal Test
- M24 Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes
- M26 Methods for Determining Bactericidal Activity of Antimicrobial Agents
- M27 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts
- M31 Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals
- M37 Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents
- M38 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi

NCCLS Numbering Scheme

Everything the reader needs to know about performing a routine antimicrobial susceptibility test, from media selection to quality control, can be found in the NCCLS standards.

NOTE: Throughout this manual we will discuss many issues related to antimicrobial resistance and susceptibility testing, including use of the NCCLS standards. To obtain maximum benefit while proceeding through this chapter, the reader should refer to the most recent NCCLS standards. Excerpts from the NCCLS documents are included as examples only. For information on obtaining the NCCLS documents, visit their website at <http://www.nccls.org>.

The NCCLS uses specific schemes to number documents. For example, in the document M2-A7:

M– denotes a Microbiology document

2– is the number assigned by the NCCLS for the specific document for disk diffusion testing

A– means it is an approved document

7– indicates that it is the 7th edition of the M2 document

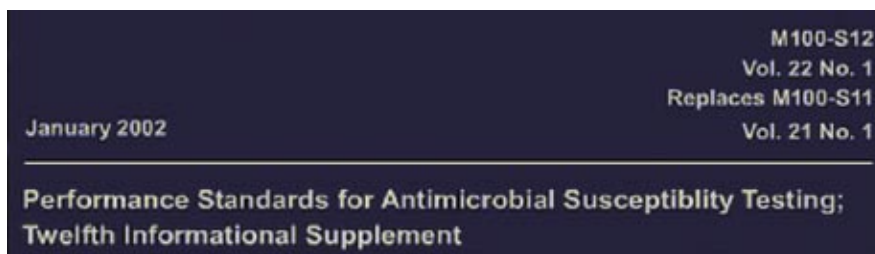


Figure 3.1—NCCLS M100 Document

M2 & M7 Standards

Document M2 describes how to perform the disk diffusion test and M7 describes how to perform the minimal inhibitory concentration (MIC) test for aerobic bacteria. Both M2 and M7 have a contents page to guide the reader to specific information. For example, to see the M2 instructions for reading plates and interpreting results, the reader would go to section 5.4.

If a document has been revised, changes from the previous edition are listed at the beginning of the document.

In the United States, any commercial diagnostic device used for patient testing in a licensed clinical laboratory, must be cleared by the Food and Drug Administration (FDA). For an antimicrobial susceptibility testing device to receive clearance, the manufacturer must demonstrate that its product generates results comparable to those produced by the reference NCCLS method. When using a commercial system, the recommendations of the manufacturer must be followed precisely.

NCCLS Document M100 contains tables for interpreting the results of disk diffusion and MIC tests. NCCLS M2 and M7 can not be used without the tables in M100 for interpreting the test results. Because these tables have very similar designs, the reader must make certain that the correct tables are used. The headings at the top of the tables are labeled “M2–Disk Diffusion” or “M7–MIC.”

In this chapter we will focus on the disk diffusion tables, but we will point out key differences between disk diffusion and MIC tables as we progress through the various topics. The introduction at the beginning of the tables explains some of the terminology and describes how to best use them.

Document Revisions

M2 and M7 are revised every three years. However, because new drugs and break-points may be introduced frequently, the NCCLS updates the tables in M100 every January. To insure that they have the most recent M2 and M7 standards, readers should check the NCCLS website (<http://www.nccls.org>).

Contents of Tables in Document M100

There are three types of tables:

Table 1 and Table 1A suggest drugs for testing and reporting

Tables 2A–2J contain criteria used to interpret results

Table 3 and Table 3A specify acceptable ranges for QC organisms

Glossary I lists individual antimicrobial agents within drug classes
Glossary II provides agents' abbreviations and routes of administration.

Tables 1 & 1A

Table 1 suggests drugs for testing and reporting on “nonfastidious” bacteria. Notice that the drugs listed for the *Enterobacteriaceae* and *Pseudomonas aeruginosa* are different.

Drugs are divided into Groups A, B, and C according to whether they should be tested and reported routinely or selectively. Group U drugs are only for isolates from urine.

Table 1A is similar to Table 1, but addresses “fastidious” organisms. Overall, Tables 1 and 1A are very similar for disk diffusion and MIC testing. However, always refer to the tables intended for the method you are using. For some drug/organism combinations, such as cefotaxime and *Streptococcus pneumoniae* only MIC testing is appropriate because disk diffusion testing is not reliable for this combination.

All tables have important essential footnotes and comments.

Tables 2A through 2H

Tables 2A through 2H contain the susceptible, intermediate, and resistant zone diameter interpretive criteria for the eight major organism groups previously seen in Tables 1 and 1A. These include:

Nonfastidious organisms	Fastidious organisms
2A. <i>Enterobacteriaceae</i>	2E. <i>Haemophilus</i> spp.
2B. <i>P. aeruginosa</i> ^a	2F. <i>Neisseria gonorrhoeae</i>
2C. <i>Staphylococcus</i> spp	2G. <i>Streptococcus pneumoniae</i>
2D. <i>Enterococcus</i> spp.	2H. <i>Streptococcus</i> spp.

^a and other non-Enterobacteriaceae

Tables with interpretive criteria for organisms not included in Table 1 or 1A:

- 2I. *Vibrio cholera*
- 2J. *Helicobacter pylori* [MIC Interpretive Standards only]
- 2K. Potential Agents of Bioterrorism

Table 2A is exclusively for *Enterobacteriaceae* and lists:

- Testing conditions
- Minimal QC recommendations
- General comments

Headings in M100 Table 2A

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter, Nearest Whole mm			Equiv. MIC Breakpoints (mcg/mL)		Comments
			R	I	S	R	S	

Zone diameters and equivalent MIC breakpoints for each antimicrobial agent are listed along with additional information. Note that interpretative criteria vary depending on the antimicrobial agent.

Interpretive Criteria (R, I, and S)

Scattergrams (also known as scatterplots) are used to establish MIC and disk diffusion interpretive criteria that also are called breakpoints. The scattergram represents results from MIC tests and disk diffusion tests of many strains with a hypothetical antimicrobial “X.”

- Breakpoints are established by taking the following steps:
 - Several hundred isolates are tested by the standard NCCLS disk diffusion and MIC methods. The MIC and corresponding zone diameter is plotted for each isolate. In this scatterplot, each dot represents results from testing one or more isolates.
- Next, **MIC breakpoints** are established following analysis of:
 - The distribution of MICs
 - Pharmacokinetic and pharmacodynamic properties of the antimicrobial agent (basically, how the antimicrobial agent is distributed and works in the patient)
 - Clinical data correlating individual MIC results with patient outcomes
- Then the **Disk Diffusion breakpoints** are established by:
 - Examining the scattergram to determine the zone measurements that best correlate with the resistant, intermediate, and susceptible MIC breakpoints
 - The number of “outliers” (red dots) is counted to calculate the percent of isolates that demonstrate disagreement between the disk diffusion and the MIC interpretations. For the interpretive criteria to be acceptable, the percentage of errors cannot exceed preset limits established by the FDA and NCCLS.

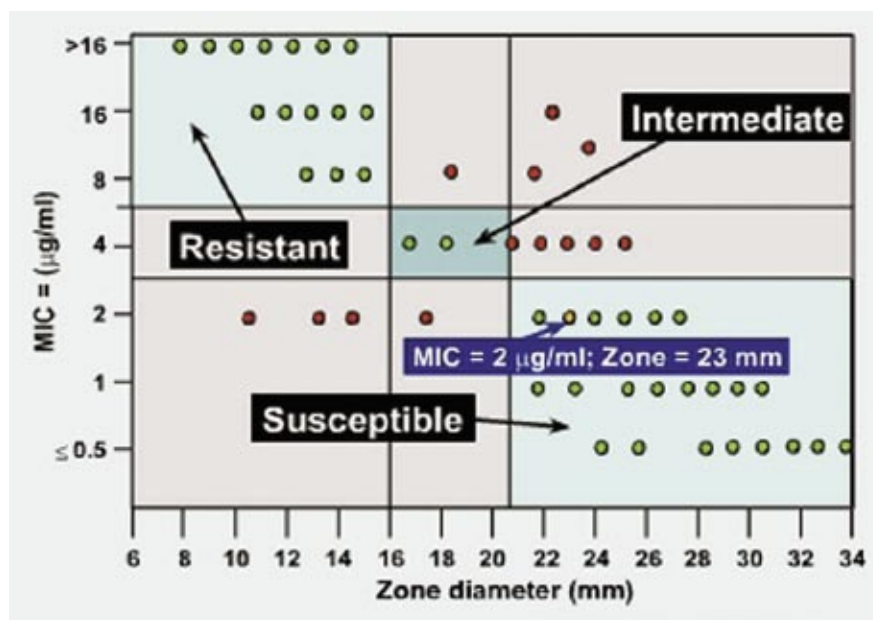


Figure 3.2—Scattergram

Interpretive Errors

The interpretive errors with our hypothetical disk diffusion test are categorized as follows:

Error Category	MIC	Disk Diffusion
Very Major (false susceptible)	R	S
Major (false resistant)	S	R
Minor	S or R	I
Minor	I	S or R

For antimicrobial agent “X,” the following interpretive criteria were derived:

Method	Susceptible	Intermediate	Resistant
Disk Diffusion (mm)	≥21	17–20	≤16
MIC (mcg/mL)	≤2	4	≥8

Tables 2I & 2J

Table 2I provides information about *Vibrio cholera*, which is encountered infrequently in the United States. This table helps microbiologists in other countries where *V. cholera* is more common.

Table 2J addresses *Helicobacter pylori* which can be reliably tested only by the MIC method.

Table 2K

Table 2K provides the MIC interpretive standards for potential agents of bioterrorism: *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, and *Burkholderia pseudomallei*.

Other Bacteria

NCCLS does not have recommendations for every species that might warrant susceptibility testing in the clinical laboratory. It is inappropriate to use specific recommendations for one organism group to test a species that is not part of that group.

Tables 3 & 3A

Table 3 contains acceptable QC limits for nonfastidious bacteria. Table 3A is very similar to Table 3, but addresses fastidious bacteria. The QC limits for ATCC QC strains are listed for each antimicrobial agent. Testing these strains in the same way that patient isolates are tested can verify that the system is working properly. If results for the QC strain fall within the limits listed in Tables 3 and 3A, the reader

can be certain the test system is working properly. The frequency and specifics of testing the QC strains can be found in the QC section of document M100.

NCCLS EXERCISE

Below is an exercise to help the reader use the NCCLS standards and the supplemental tables. In order to work through the exercise, you should have on hand the most up-to-date version of the M100 document.

This exercise was developed by people who have experience performing antimicrobial susceptibility tests using the disk diffusion method. We will discuss details of performing both a disk diffusion test and an MIC test in other chapters.

Assume that you are the supervisor of a microbiology laboratory that serves a community hospital and outpatient clinics. You are asked by the Pharmacy and Therapeutics (P&T) committee to develop a panel of antimicrobial agents to be used specifically for testing gram-negative bacteria isolated from urine. The P&T committee is composed of members representing the medical, surgical, and pharmacy services with input from the infectious disease service, the laboratory and possibly others.

Construct a panel of 8–10 antimicrobial agents that would be appropriate to test by disk diffusion against isolates of *Enterobacteriaceae* from urine.

Remember, it is important to test the antimicrobial agents that are used in your institution. This list is referred to as the pharmacy's formulary and can be provided by your pharmacy. The agents on the formulary are selected by the P&T committee

Selecting the Panel

You now have three lists to assist the reader:

Your institution's Formulary (see below)

NCCLS M100 Table 1

NCCLS M100 Glossary

Pharmacy's Formulary

Aminoglycosides

gentamicin, tobramycin

Beta-lactams

amoxicillin/clavulanic acid, ampicillin, cefotaxime, ceftazidime, cephalexin, cephalothin, imipenem, oxacillin, penicillin, piperacillin, piperacillin/tazobactam

Fluoroquinolones

Ciprofloxacin

Macrolides

clarithromycin, erythromycin

Tetracyclines

doxycycline, tetracycline

Other

chloramphenicol, clindamycin, metronidazole, nitrofurantoin, trimethoprim/sulfamethoxazole, vancomycin

Narrowing Down the List

In M100 Table 1, find the *Enterobacteriaceae* column. The agents are divided into Groups A, B, C, or U and subdivided into small boxes.

Group A

Positioning drugs in groups and boxes will guide you in the drug selection process. Refer to “Introduction to the Tables” in NCCLS M100 for more information.

Group A includes primary agents that are tested and reported routinely for *Enterobacteriaceae*. You can see three boxes, one of which contains both cefazolin and cephalothin because they have a comparable spectrum of activity. You usually need to test only one drug per box, typically the one on your institution’s formulary.

Which of these will you include on your panel?

- A. Ampicillin, Cefazolin, Gentamicin
- B. Ampicillin, Cephalothin, Gentamicin

(Choice B is correct. These agents are in Group A and are on the formulary.)

Group B

Group B offers additional choices of drugs to test and report selectively if needed. Some laboratories follow a selective reporting protocol and report results of the secondary agents (Group B) tested only if the isolate is resistant to the primary agents (Group A) of the same drug family.

What does the “or” in some boxes of Table 1 mean?

Under *Enterobacteriaceae* in Group B, note the box that contains cefotaxime, ceftizoxime, and ceftriaxone. The three drugs, listed alphabetically, are connected by an “or” because the cross-resistance and susceptibility profiles are nearly identical. Therefore, the results (susceptible, intermediate, or resistant) of one can be used to predict the results of the other two.

This listing is different from the box with cephalothin and cefazolin for Group A which does not contain an “or.” Therefore, if the box does not have an “or,” we cannot deduce results and must test each drug separately, if needed.

From Group B select agents appropriate for treatment of urinary tract infections (UTIs) and prepare a panel that would be useful to physicians to treat outpatients as well as hospitalized patients. If needed, here are instructions for getting started.

1. Use the NCCLS Glossary (in M100) to see which agents belong to each of the four groups posed in the question.
2. Determine which of these agents are on the formulary.
3. Determine which of these agents are appropriate for testing and reporting for *Enterobacteriaceae* isolated from urine.

Based on the information in NCCLS document M100 Table 1 Group B, which specific antimicrobial agent would you select from each of the four groups listed below?

- A. An extended-spectrum cephalosporin or cephalosporin III (parenteral cephem)

- B. An expanded-spectrum penicillin or ureidopenicillin.
- C. A beta-lactam/beta-lactamase inhibitor combination (orally administered)
- D. A fluoroquinolone

Answers:

A. cefotaxime; B. piperacillin; C. amoxicillin-clavulanic acid; D. ciprofloxacin

Rounding Out the List

Group C agents usually are tested only in special circumstances, so we will refrain from adding any of these to our panel.

Group U agents are used to treat only lower urinary tract infections and must not be reported on isolates from other body sites.

Which group U agent would you add?

- A. Gatifloxacin
- B. Nitrofurantoin
- C. Trimethoprim

Answer:

B. Nitrofurantoin is on the hospital formulary and is appropriate for reporting on urine isolates.

Group U agents should be tested only if they are appropriate for your institution or patient population.

The Panel

This is the panel selected by the authors:

Amoxicillin-clavulanic acid
Ampicillin
Cefotaxime
Cephalothin
Ciprofloxacin
Gentamicin
Nitrofurantoin
Piperacillin
Trimethoprim-sulfamethoxazole

Following review of the panel, the P&T committee suggests a meeting to discuss the list. Now they have a few questions for you...

Physician Inquiry # 1

A physician who is treating a patient with a *Citrobacter freundii* urinary tract infection requests results for levofloxacin. What would you do? See the following information:

Check M100 Table 1, Group B, and agents grouped within the boxes. Because levofloxacin is in the same box as ciprofloxacin and connected with an “or” we can extrapolate the levofloxacin result from the ciprofloxacin result because cross susceptibility and resistance between the drugs are nearly identical.

So how would you report the results? See Report Example below.

Always *report the result for the drug actually tested*, and add a comment, regarding comparability with other drugs. Any exceptions to this rule will be covered in the chapter relating to the specific organism group.

Lab Report

Specimen Source: Urine

Results: *Citrobacter freundii*

Amoxicillin-clavulanic acid	R
Ampicillin	R
Cefotaxime	R
Cephalothin	R
Ciprofloxacin	S
Gentamicin	S
Nitrofurantoin	S
Piperacillin	R
Trimethoprim-sulfamethoxazole	R

Comment: Ciprofloxacin-susceptible *Citrobacter freundii* are levofloxacin susceptible.

Physician Inquiry # 2

How would you respond to the physician if he requests that the panel include mostly agents that are administered orally? Most acute, uncomplicated urinary tract infections occur in outpatients, so orally administered agents are desirable.

What resource in document M100 would help you answer the question?

- A. NCCLS Table 1
- B. NCCLS Glossary I
- C. NCCLS Glossary II

Answer:

- C. Glossary II allows you to determine how drugs are administered: (PO per OS, oral; IM, intramuscular; IV, intravenous)

Referring to Glossary II, how would ampicillin be administered? Select all that apply.

- A. PO (oral)
- B. IM (intramuscular)
- C. IV (intravenous)

Answer:

A, B, and C. Ampicillin exists in all three forms, so the physician can select the route and dosing based on the type of infection.

Now check nitrofurantoin; how is it administered?

- A. PO (oral)
- B. IM (intramuscular)
- C. IV (intravenous)

Answer:

- A. Nitrofurantoin can be administered only by mouth.

Looking at the list of selected drugs, you can see that five of the nine have an oral route of administration.

Physician Inquiry # 3

The physician now wants to know if the panel can include an orally administered cephalosporin.

Cephalothin is a narrow spectrum, or first-generation, cephalosporin, but it is only available intravenously (IV). Cephalexin does have an oral (PO) form. Cephalothin results can be used to predict results for oral cephalosporins, such as cephalexin. (See M100 Table 1, Footnote a.)

Why are some drugs listed in Table 2A but not in Table 1? Select all that apply. Refer to “Introduction to the Tables” in NCCLS M100 for more information.

- A. This is probably a mistake.
- B. Some drugs may have an indication for the organism group but generally are not appropriate for routine testing and reporting in the United States.
- C. Some drugs are investigational and have not yet been approved by the FDA

Answer:

- B. and C. These drugs on Table 2 are listed as test/report group “O” for “Other” or “Inv” for “Investigational.”

Drugs Selected for Table 1

The criteria that guide positioning of a drug in table 1 include:

- Proven clinical efficacy
- Consensus recommendations for use
- Acceptable *in vitro* activity
- Prevalence of resistance (utility of drug in context of resistance to other agents)
- Minimizing emergence of resistance
- Cost

Physician Inquiry # 4

How would you describe to a physician your selective reporting criteria for drugs on this new panel? Please see above for the panel selected by the authors

Remember that Group B lists agents that might be selectively reported if the organism is resistant to agents of the same family in Group A.

Applying this algorithm, we would report:

Amoxicillin-clavulanic acid—only if the isolate is resistant to ampicillin

Cefotaxime—only if the isolate is resistant to cephalothin

The committee agrees that selective reporting may encourage clinicians to use narrower spectrum agents. Of course, there may be clinical circumstances where this is not appropriate.

Look at Lab Report A below:

Lab Report A

Specimen Source: Urine

Results: *E. coli*

Amoxicillin-clavulanic acid	S
Ampicillin	S
Cefotaxime	S
Cephalothin	S
Ciprofloxacin	S
Gentamicin	S
Nitrofurantoin	S
Piperacillin	S
Trimethoprim-sulfamethoxazole	S

Are the antimicrobial agents correct based on the selective reporting algorithm we decided to use?

- A. Yes
- B. No

Answer:

B (No). Because *E. coli* is susceptible to ampicillin and cephalothin, neither amoxicillin/clavulanic acid nor cefotaxime should be reported.

Look at Lab Report B below:

Lab Report B

Specimen Source: Urine

Results: *E. coli*

Amoxicillin-clavulanic acid	S
Ampicillin	R
Cefotaxime	S
Cephalothin	R
Ciprofloxacin	S
Gentamicin	S
Nitrofurantoin	S
Piperacillin	S
Trimethoprim-sulfamethoxazole	R

Are the antimicrobial agents correct based on the selective reporting algorithm we decided to use?

- A. Yes
- B. No

Answer:

A (Yes). This *E. coli* is resistant to the primary agents ampicillin and cephalothin and therefore the secondary agents, amoxicillin/clavulanic acid and cefotaxime should be reported.

Summary of how this list of drugs was selected for inclusion in your panel:

1. Reviewed NCCLS Table 1 to select drugs appropriate for testing on urine isolates of *Enterobacteriaceae*.
2. Reviewed the agents on the institution's formulary.
3. Used the NCCLS Glossary to select drugs that can be administered orally.
4. Worked with the P&T committee to develop a selective reporting strategy of the panel of drugs selected.

SELF-ASSESSMENT QUESTIONS

1. What is the NCCLS?
 - A. A government agency that accredits laboratories.
 - B. A committee of university professors who write documents for laboratory practice.
 - C. An organization that promotes the development and use of voluntary laboratory standards and guidelines.
2. The primary document that should be used as a guideline in performing disk diffusion susceptibility tests is the
 - A. Federal Register
 - B. NCCLS protocol on disk testing
 - C. Bailey and Scott's textbook
 - D. ASM Manual of Clinical Microbiology
 - E. Cumitech 6 publication
3. Should you follow the exact instructions in M2 or M7 if you use a commercial susceptibility testing device?
 - A. Yes
 - B. No
4. Which of the following drugs has separate interpretive criteria for disk diffusion and MIC test depending on whether the drug is administered orally or parenterally?
 - A. cefaclor
 - B. ceftazidime
 - C. cefuroxime
 - D. cefazolin
 - E. ceftazidime

5. For an *E. coli* with a resistant zone diameter of 13 mm or less for ampicillin. What would be the equivalent MIC (in $\mu\text{g/ml}$) breakpoint for resistant? Use NCCLS M100 Table 2A to answer this question.
- A. ≤ 8
 B. 16
 C. ≥ 32

6. When testing *E. coli* ATCC 25922, we obtained the following zone diameter readings:

Drug	Zone (mm)
Amoxicillin/clavulanic acid	22
Ampicillin	13
Cefazolin	25

By using the appropriate QC table in NCCLS M100 (Table 3) would it be acceptable to report all three of these agents on *Enterobacteriaceae* from your patients?

- A. Yes
 B. No
7. A physician asks you to test an *E. coli* for penicillin because the patient has been receiving penicillin for a strep throat. Would you test it?
- A. Yes
 B. No
8. An antibiogram can best be defined as:
- A. The aminoglycoside and penicillin susceptibility pattern of a bacterium
 B. A Gram stain of bacteria exposed to antibiotics
 C. The overall susceptibility profile of a bacterium
 D. The antibiotic susceptibility and biochemical characteristics of a bacterium
 E. The antibiotics to which a bacterium is resistant
9. (T or F) When determining which antimicrobial agents to routinely test and report, the laboratory should obtain assistance from the infectious disease service, pharmacy, and infection control.
10. (T or F) If there is no NCCLS guideline for interpreting susceptibility results, it is appropriate to use specific recommendations for one organism group to test a species that is not part of that group .
11. (T or F) For some drug/organism combinations, such as cefotaxime and *Streptococcus pneumoniae*, only MIC testing is appropriate because disk diffusion testing is not reliable for this combination.

4

Disk Diffusion Testing

OBJECTIVES

At the completion of this chapter, the reader should be able to:

- List the steps required to perform a disk diffusion test.
- Name the variables that must be controlled when performing the test.
- Recognize problems that may occur if test variables are not properly controlled.
- Discuss the two basic methods of inoculum preparation and the application of each.
- Interpret specific organism/antimicrobial agent zone diameter as susceptible intermediate, or resistant based on NCCLS recommendations.

BACKGROUND

The principle of disk diffusion testing has been used in microbiology laboratories for over 70 years. Alexander Fleming used a variant of this technique when working with penicillin in the 1950s. At that time, there were as many different procedures in use as there were microbiologists.

Drs. Bauer, Kirby, Sherris, and Turck painstakingly tested all of the variables used in the procedure, such as the media, temperature, and depth of agar. In 1966, they published their landmark paper describing the test that is used today.

NCCLS adopted the basic procedural steps in the Bauer paper as the disk diffusion reference method. These steps must be followed precisely to obtain accurate results.

PERFORMING THE TEST—OVERVIEW

Once isolated colonies are available from an organism that has been identified as a potential pathogen, it is necessary to proceed as follows to perform the susceptibility test.

1. Select colonies
2. Prepare inoculum suspension
3. Standardize inoculum suspension
4. Inoculate plate
5. Add antimicrobial disks
6. Incubate plate
7. Measure inhibition zones
8. Interpret results



Figure 4.1—Selecting well-isolated colonies for the inoculum

Select Colonies

One of the most important steps in the testing process is preparing the inoculum. This involves selecting appropriate colonies for testing, suspending them in broth, and standardizing the suspension.

First, select several colonies of the organism you are testing. If you select 3–5 colonies, rather than just one, your chances of detecting resistance are higher.

NOTE: Using an inoculating loop or a cotton swab pick only well-isolated colonies from the plate to avoid testing mixed cultures. If you do not have well-isolated colonies, subculture the organism to a fresh plate.

Preparing and Standardizing Inoculum Suspension

There are two methods for inoculum preparation: direct colony suspension and log phase growth. Only the direct colony suspension method will provide accurate results for some organisms. For both methods, the turbidity of the test suspension must be standardized to match that of a 0.5 McFarland standard (corresponds to approximately 1.5×10^8 CFU/ml). The adjusted suspensions should be used as inocula within 15 minutes.

NOTE: McFarland standards are made of either barium sulfate or latex particles, if using barium sulfate, vortex prior to using, if using latex, invert to mix. A recipe for the 0.5 McFarland standard is in the Appendix.

Direct Colony Suspension

For the direct colony suspension method, colonies must not be older than 18–24 hours. Standardize the inoculum at the same time you prepare the suspension.

Suspend the colonies in saline or broth (e.g. Mueller-Hinton or tryptic soy). Then, adjust the inoculum to a turbidity equivalent to a 0.5 McFarland standard. You can compare the turbidity of the suspensions by placing the tubes in front of a white paper or file card with black lines.

Use direct colony suspension for the following organisms:

- All staphylococci
- Fastidious bacteria that grow unpredictably in broth: e.g., streptococci



Figure 4.2—Standardizing the inoculum

Log Phase Method

The log phase method is used for most organisms that grow rapidly except staphylococci. Once you have inoculated the colonies into a broth, incubate to log phase growth. A growth curve for a typical bacterium is shown below. Log phase growth occurs after 2–8 hours incubation.

Following incubation, adjust the turbidity to match that of a 0.5 McFarland standard. Make sure you know how to adjust and standardize the inoculum.

- What would you do if the suspension of organisms is too turbid?
- What would you do if the suspension is too light for direct colony suspension?
- What would you do if the suspension is too light for log phase method?

Correct answers:

- Add more broth or saline to match the turbidity of a 0.5 McFarland standard.
- Pick more colonies and suspend in broth.
- Re-incubate the suspension.

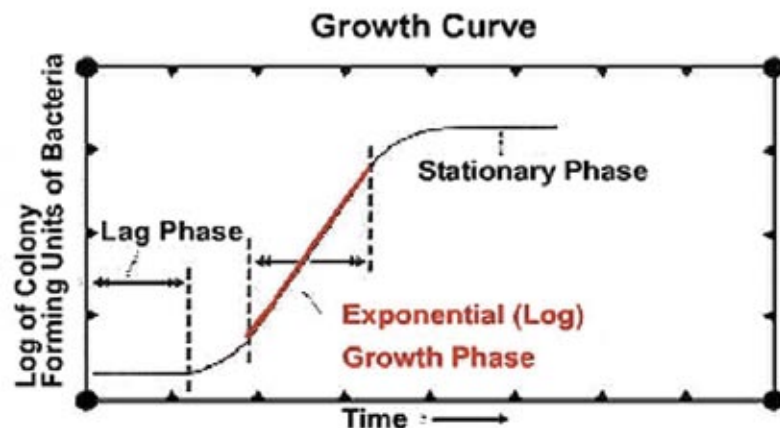


Figure 4.3—Plot of log phase growth in broth



Figure 4.4—Removing excess liquid from the swab

NOTE: Do not use over-night cultures in liquid media nor other non-standardized inocula to inoculate the plates.

Preparing for Plate Inoculation

Remove the container of disks from the freezer or refrigerator. *Before opening the container*, allow the disks to equilibrate to room temperature for one to two hours to minimize condensation and reduce the possibility of moisture affecting the concentration of antimicrobial agents.

Allow a Mueller-Hinton Agar (MHA) plate to warm to room temperature so that any excess moisture will be absorbed into the medium. You can expedite this step by placing the plates in the incubator with their lids ajar for 10–15 minutes. Ensure the MHA plate has the proper depth of 4 mm.

Vortex the organism suspension to make sure it is well-mixed. Then, dip a fresh, sterile cotton-tipped swab into the suspension. Remove the excess liquid from the swab by pressing it against the side of the tube.

Inoculating the Plate

Starting at the top of the MHA plate inoculate the surface with the swab. Cover the entire plate by streaking back and forth from edge to edge. Rotate the plate approximately 60° and repeat the swabbing procedure. Rotate the plate 60° again and swab the entire plate a third time. This will ensure that the inoculum is evenly distributed.

Technical tip: Incubate the plate within 15 minutes of standardizing the inoculum suspension.



Figure 4.5—Inoculation of the plate

Applying the Antimicrobial Disks

Apply the disks containing the antimicrobial agents within 15 minutes of inoculating the MHA plate. Disks may be placed on the plate one at a time or with a multi-channel disk dispenser as seen in Figure 4.6.

Typically, up to 12 disks can be applied to a 150 mm diameter plate or up to 5 disks on a 100 mm plate. Press each disk down firmly to ensure complete, level contact with the agar. Don't forget this step or disks may end up in the lid of the plate after incubation.

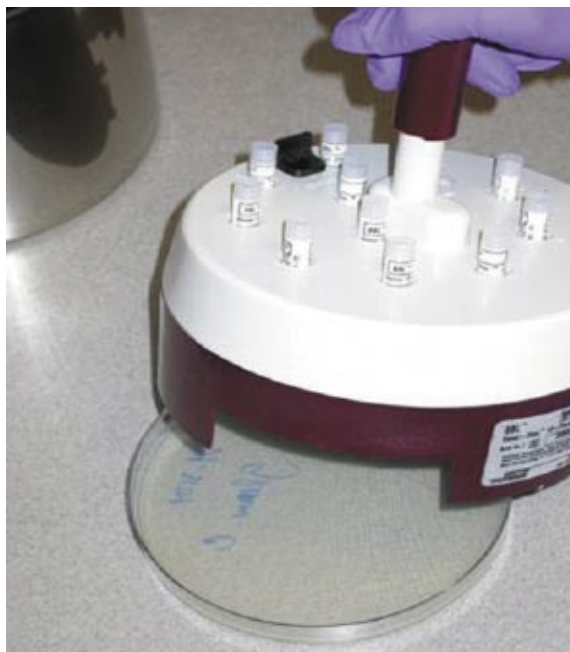


Figure 4.6—Applying disks with dispenser



Figure 4.7—Tapping disks to ensure contact

Points to remember about antimicrobial disks:

- Do not use disks beyond their expiration date.
- Do not store disks in a frost-free freezer.
- Use FDA cleared products.
- Use disks with the content specified in NCCLS standards.
- Do not relocate a disk once it has touched the agar surface.

Incubating the Plate

- Invert and incubate plates with agar side up.
- For nonfastidious bacteria, incubate in ambient air at 35°C for 16–18 hours.
- For disk diffusion testing of fastidious bacteria, use NCCLS-recommended incubation conditions, as shown in the table below.

Bacteria	Medium	Incubation	
		Time (h)	Atmosphere
<i>Haemophilus</i> spp.	Haemophilus Test Medium	16–18	CO ₂ (5%)
<i>Neisseria gonorrhoeae</i>	GC agar base + 1% sup.	20–24	CO ₂ (5%)
<i>Streptococcus pneumoniae</i>	MHA-5% sheep blood	20–24	CO ₂ (5%)
Other <i>Streptococcus</i> spp.	MHA-5% sheep blood	20–24	CO ₂ (5%)

Measuring Zones—Reflected Light

Following removal of the plate from the incubator:

- Examine closely to make certain the lawn of growth is even and confluent so you can see unobstructed zones.

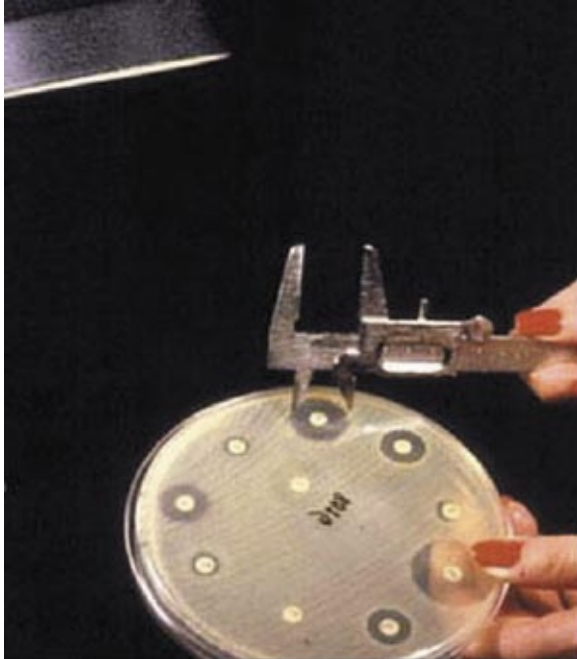


Figure 4.8—Reflected light is used to measure zones from back of plate

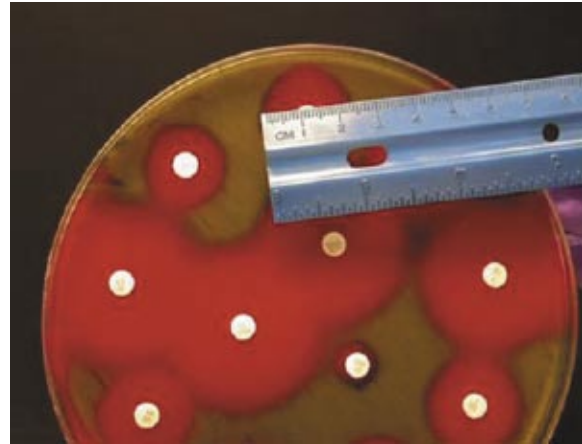


Figure 4.9—Measuring zones on a blood agar plate with lid off

To measure zones from the back of the plate using reflected light:

- Hold the plate a few inches above a black nonreflecting surface.
- Measure to the nearest millimeter with a ruler or calipers.
- Reflected light is used for *Enterobacteriaceae*, such as *E. coli*, other gram-negative bacilli, staphylococci, and enterococci (except for oxacillin and vancomycin).
- Reflected light also is used when measuring zones on blood MHA (BMHA).

Technical tip: When testing streptococci on MHA with 5% sheep blood (BMHA), remove the lid and measure zones from the top of the plate.

Measuring Zones—Transmitted Light

Use transmitted light, rather than reflected light, when measuring zones for:

- Staphylococci with oxacillin
- Enterococci with vancomycin

INTERPRETING RESULTS

Measuring Unusual Zones

Some zones may be difficult to measure. Figs. 4.10, 4.11 and 4.12 present two examples of unusual zones.

Double zone: Measure the innermost zone.

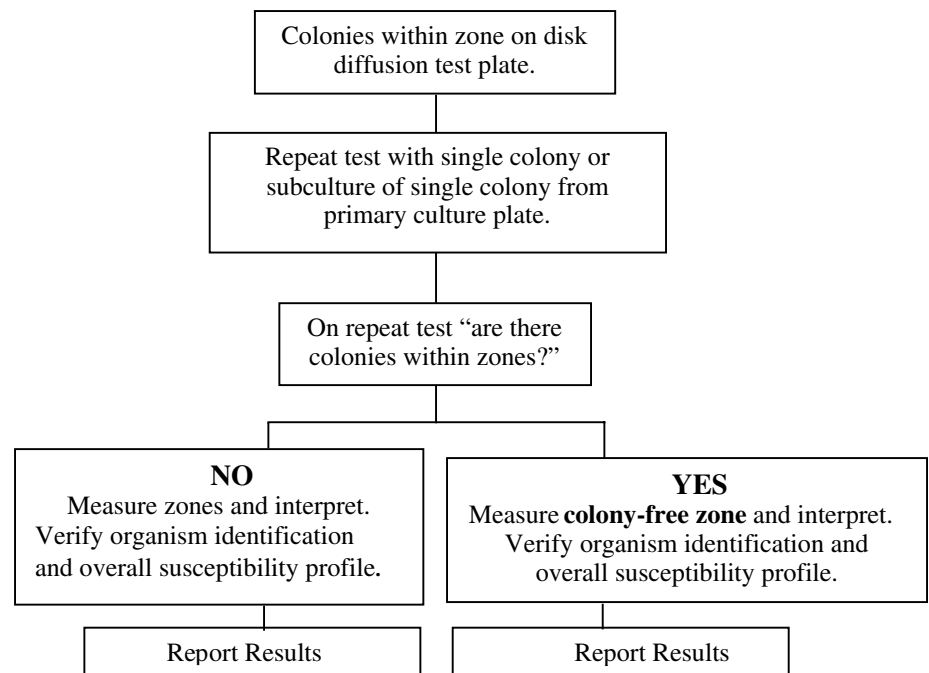


Figure 4.10–Double zone of inhibition



Figure 4.11–(zone with inner colonies)

Colonies within the zone: This can be due to either a mixed culture, which usually is obvious, or a resistant subpopulation of the test bacterium.



Interpreting Colonies within Zones

Sometimes it is difficult to tell a mixed culture from a resistant subpopulation.

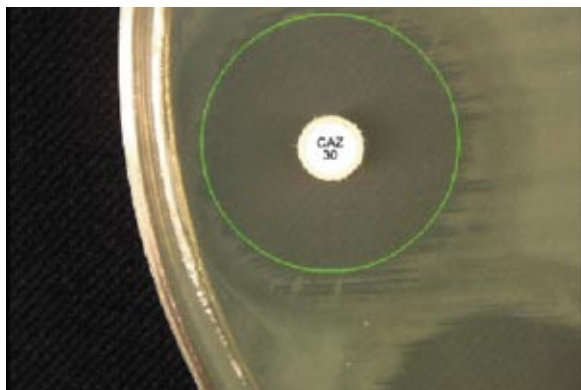


Figure 4.12—Feathered zone around CAZ disk

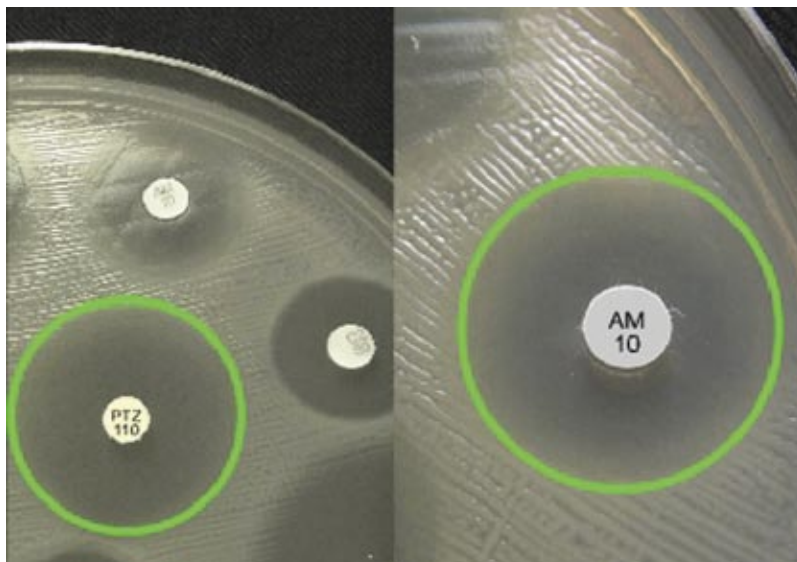


Figure 4.13—Zones with swarming *P. mirabilis*

Interpreting Zone with Feathered Edge

Measure the point at which you can see an obvious demarcation between growth and no growth. Avoid straining to see the tiniest colonies.

Swarming due to *Proteus mirabilis*

Measure the obvious zone. Ignore the swarm even if it covers the zone.

Trimethoprim-Sulfamethoxazole

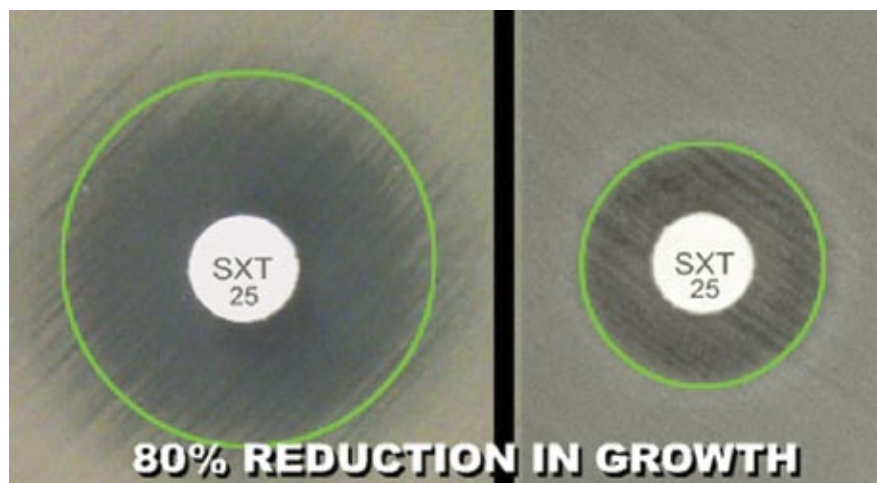


Figure 4.14—Zones with sulfamethoxazole

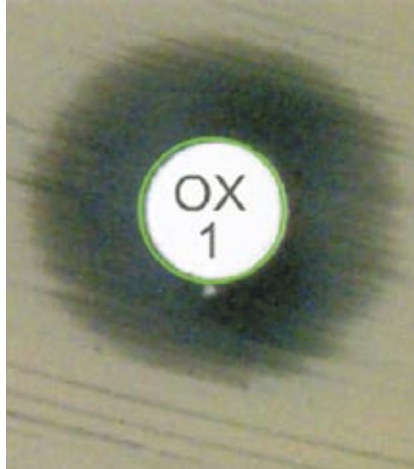


Figure 4.15—Heterogeneous resistance to oxacillin

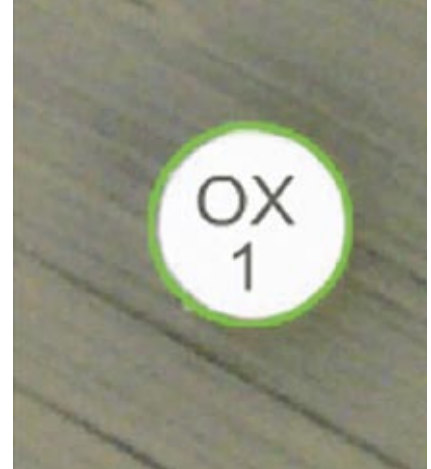


Figure 4.16—Homogeneous resistance to oxacillin

Zones with trimethoprim-sulfamethoxazole (and also sulfonamides and trimethoprim alone) may be difficult to read because this agent may not inhibit bacteria from multiplying until the bacteria have gone through several generations of growth. You may see a light haze of growth within the zone. Measure the zone at the point where there is an 80% reduction in growth.

Interpreting Heterogeneous and Homogeneous Resistance

Heterogeneous Resistance in *S. aureus*

Although we ignore the hazy growth often observed around disks within zones of inhibition for swarming *Proteus* spp. and around trimethoprim-sulfamethoxazole disks, the haze around an oxacillin disk when testing *S. aureus* is significant and should not be ignored.

Homogenous Resistance in *S. aureus*

S. aureus with homogenous resistance show confluent growth up to the disk. See the Staphylococcus chapter for additional information.

Controlling Test Variables

Review the variables listed below that must be controlled in performance of the disk diffusion test.

- Media composition
- Media pH
- Agar depth
- Concentration of inoculum
- Inoculation procedure
- Antimicrobial concentration in disk
- Disk storage

- Number of disks on plate
- Incubation temperature
- Incubation atmosphere
- Incubation time
- Endpoint measurement

Chapter 6 in this manual describes quality control practices that provide confidence that all variables are properly controlled. In addition, refer to the troubleshooting guide in the Appendix.

Drug	Culture on 11/2	Culture on 11/5	Culture on 11/9
Amikacin	S	S	S
Ceftazidime	S	S	S
Ciprofloxacin	S	S	S
Gentamicin	S	S	R
Piperacillin	S	S	S
Tobramycin	S	S	R

CASE STUDY

Pseudomonas aeruginosa was isolated for the third time from a patient's tracheal aspirate. The first two isolates were susceptible to gentamicin and tobramycin; however, the third isolate was resistant to these two agents. As shown below, the results from the other four drugs were the same on all three days.

Case Study Commentary

Why might the results on 11/9 be different from those on the previous two days? More than one answer may be correct.

- The isolate acquired resistance
- The patient acquired a new strain of *P. aeruginosa*
- There were changes or problems in the test system

QC test results with the recommended *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were as follows:

Date	MHA Lot #	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853
11/2	A	OK	OK
11/5	A	OK	OK
11/9	B	OK	Amikacin 15 mm Gentamicin 14 mm Tobramycin 16 mm
11/10	B	OK	Amikacin 13 mm Gentamicin 14 mm Tobramycin 17 mm
11/10	C	OK	OK

Acceptable QC Ranges

<i>P. aeruginosa</i> ATCC 27853	
Amikacin	18–26 mm
Gentamicin	16–21 mm
Tobramycin	19–25 mm

Answer: All three choices are correct.

- A. There always is a possibility that the isolate acquired resistance; however, it should be confirmed by repeating the test.
- B. The patient could have acquired a new strain of *P. aeruginosa*, particularly if the patient was in the hospital for a long period of time.
- C. There were changes or problems in the test system. However, quality control testing should detect problems with a test system.

Corrective action:

11/9

Due to a backorder of MHA, QC on MHA lot B could not be done prior to testing patient's isolates.

Because QC results were outside the acceptable range, all aminoglycoside results on patient's isolates were verified and isolates were retested on MHA lot C.

11/10

QC was acceptable on MHA lot C and results from the patient's isolates tested on this medium were reported. MHA lot B was returned to the manufacturer.

When the patient's isolate from the culture on 11/9 was retested on MHA lot C, it had the same profile as his previous isolates.

What is the most likely problem with MHA lot B?

- A. The agar depth is too shallow
- B. The cation content is too high
- C. The media pH is too high

Answer:

- A. Is incorrect because only the aminoglycoside results were out of control.
- B. Is correct. Since only aminoglycosides and *P. aeruginosa* ATCC 27853 are out of control, this is the likely explanation. An unacceptably high concentration of the divalent cations, calcium, and magnesium can cause decreased susceptibility of *P. aeruginosa* to the aminoglycosides.
- C. Is incorrect. Aminoglycosides may show less activity at an acidic or a low pH, but at a higher pH this should not occur.

REVIEW

Now the reader should be familiar with disk diffusion testing of nonfastidious bacteria and know a little about testing fastidious bacteria.

Remember to:

- Use the most current NCCLS standards that provide instructions for performing disk diffusion test (the M2 standard) and interpreting and reporting results (the M100 tables).
- Follow all the recommended steps precisely.
- Become familiar with exceptions to the standard method for testing certain organism/antimicrobial agent combinations. This will be covered in subsequent chapters including more information on testing fastidious bacteria.

SELF-ASSESSMENT QUESTIONS

1. (T or F) If you select 3–5 colonies, rather than just one, when preparing the inoculum suspension, your chances of detecting resistance are higher.
2. (T or F) Any zone around an oxacillin, methicillin, or nafcillin disk should be examined using transmitted light; other zones should be examined using reflected light.
3. (T or F) The direct suspension standardization method for inoculum preparation should be used when testing staphylococci and the penicillinase-resistant penicillins.
4. (T or F) It is acceptable to store antimicrobial solutions and disks in frost-free freezers.
5. Fill in the blanks.

Direct Colony Suspension Method

Select colonies ___ hours old to avoid testing nonviable cells. Standardize the turbidity of the inoculum to that of a ___ McFarland standard.

Log Phase Method

Collect colonies using a swab or inoculating loop or needle, inoculate to broth, and incubate at 35°C for ___ hours to reach log phase growth. Standardize the turbidity of the inoculum to that of a ___ McFarland standard.

6. Would you use the direct colony suspension, log phase method, or both for each of the following? Match method with bacteria.

A. Log phase method	Enterobacteriaceae	_____
B. Direct colony suspension	Staphylococci	_____
C. Both methods are acceptable	Fastidious bacteria	_____
7. The standard Kirby-Bauer disk diffusion test which utilizes unsupplemented Mueller-Hinton agar can be used reliably to test
 - A. All clinical isolates
 - B. Rapidly growing non-fastidious aerobic organisms
 - C. Rapidly growing aerobic and anaerobic organisms
 - D. The enterics and *Staphylococcus* sp. only
 - E. All clinical isolates except those that are considered “normal flora”

-
8. If antimicrobial disks are not placed on a Mueller-Hinton agar plate within 15 minutes following inoculation, what would most likely occur?
 - A. Zones may become too large
 - B. Zones may become too small
 - C. Some zones may become too large and others too small
 - D. No effect on zones
 - E. Colonies may appear within zones of inhibition
 9. You are reading zone sizes on a Mueller-Hinton plate. The lawn of growth appears acceptable, but you notice colonies within a zone. What might cause this growth pattern? More than one answer may be correct.
 - A. The colonies within the zone likely represent a mixed culture
 - B. The colonies represent a subpopulation of resistant bacteria
 - C. The inoculum is too light
 10. Continuing from question 9, what would you do next?
 - A. Ignore colonies within zone
 - B. Consider isolate resistant
 - C. Repeat the test using colonies from the primary plates or a subculture of these

5

MIC Testing

OBJECTIVES

When readers have completed this chapter, they should be able to:

- List the steps required to perform a broth microdilution Minimal Inhibitory Concentration (MIC) susceptibility test.
- Name the variables that must be controlled when performing the test.
- Recognize the problems that may occur if test variables are not properly controlled.
- Determine the number of viable organisms in the inoculum to ensure that the correct number of organisms is inoculated into each well.
- Interpret specific organism/antimicrobial agent MIC results as susceptible, intermediate, or resistant based on NCCLS recommendations.

SCOPE

This chapter reviews the reference broth microdilution MIC test as described in NCCLS document M7. Other MIC methods are not discussed in detail but can be found in NCCLS document M7. Commercial systems for MIC testing are not reviewed in this manual.

BACKGROUND OF THE TEST

The minimal inhibitory concentration (MIC) of an antimicrobial agent is the lowest (i.e. minimal) concentration of the antimicrobial agent that inhibits a given bacterial isolate from multiplying and producing visible growth in the test system. We determine the concentration in the laboratory by incubating a known quantity of bacteria with specified dilutions of the antimicrobial agent. Using NCCLS interpretive criteria the results are interpreted as susceptible, intermediate, or resistant.

MIC tests can be performed using broth or agar media, but broth microdilution is the most widely used method in clinical laboratories.

Several companies manufacture MIC panels that contain dilutions of one or multiple antimicrobial agents in a broth microdilution format. Before a commercial product can be used for clinical isolates in the United States, it must be cleared for use by the FDA (Food and Drug Administration).

Every commercial test has unique procedural steps. For example, the inoculum preparation procedures vary considerably from method to method. The reliability

of any susceptibility test for detecting antimicrobial-resistant bacteria depends on following the instructions precisely.

MATERIALS

Broth Microdilution MIC Panel

Broth microdilution MIC testing is performed in a polystyrene panel containing approximately 96 wells. A panel may contain 7–8 dilutions of 12 different antimicrobial agents. One well serves as a positive growth control (broth plus inoculum), and one serves as a negative control (broth only). Most systems have a volume of 0.1 mL in each well.

To facilitate testing appropriate antimicrobial agents against specific isolates, a laboratory usually has one type of panel for gram-positive bacteria and another for gram-negative bacteria. For testing urine isolates some laboratories may have a different type of panel that contains drugs appropriate for treating lower urinary tract infections. Panels containing special media are required for testing fastidious bacteria.

Mueller-Hinton broth is recommended as the medium of choice for susceptibility testing of commonly isolated, rapidly growing aerobic, or facultative organisms. The broth must have the appropriate divalent cation content provided by the manufacturer (Ca⁺⁺ and Mg⁺⁺). Each batch must be tested with a pH meter after the medium is prepared. The pH should be between 7.2 and 7.4 at room temperature (25°C). For fastidious organisms Mueller-Hinton broth may be supplemented with 2–5% lysed horse blood.

The performance of each batch of broth is evaluated by using a standard set of quality control organisms. If a new lot of broth does not yield the expected results, the cation content of the broth as well as each step of the test should be investigated. Meanwhile a different lot should be evaluated.

Agar Dilution MIC Testing

In the agar dilution method, the antimicrobial agent is incorporated into the agar medium with each plate containing a different concentration of the agent. The inocula can be applied rapidly and simultaneously to the agar surfaces using an inoculum-replicating apparatus. Most available replicators transfer 32–36 inocula to each plate.

Mueller-Hinton agar is prepared from a dehydrated base. The pH of the agar must be between 7.2 and 7.4 at room temperature. Supplemental cations must not be added to the agar. It may be supplemented with 5% defibrinated sheep blood or lysed horse blood.

The advantages of agar dilution testing include the reproducible results and satisfactory growth of most nonfastidious organisms. However, its disadvantages include the labor required to prepare the agar dilution plates and their relatively short shelf life. Agar dilution testing generally is not performed in routine clinical laboratories but can be ideal for regional reference laboratories or research laboratories that must test large numbers of isolates.

Procedure for Broth Microdilution MIC Testing

NOTE: The processes of selecting colonies for testing, preparing and standardizing the inoculum suspension for MIC tests are the same as those described in Chapter 4 for disk diffusion.

It is important to prepare all materials before beginning the MIC test.

1. Cells for the inoculum
Isolated colonies must be selected from an 18- to 24-hour culture on an agar plate. A non-selective medium, such as blood agar, should be used.
2. Prepare the inoculum suspension.

- a. Growth method

- Select three to five well-isolated colonies of the same morphological type from the agar plate. The top of each colony is touched with a wire loop and the growth is transferred to a tube containing 4–5 mL of suitable broth medium, such as tryptic soy broth.
- The broth culture is incubated at 35°C until it achieves the turbidity of a 0.5 McFarland standard (usually 2–6 hours).
- The turbidity of the actively growing broth culture is adjusted with sterile saline or broth. The resulting suspension contains approximately 1 to 2×10^8 CFU/mL. To perform this step properly, either a photometric device should be used or, if done visually, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines. See Figure 4.2 in Chapter 4.

- b. Direct colony suspension method

- As an alternative method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies and adjusting it to match a 0.5 McFarland standard. This method is recommended for testing fastidious organisms such as *Haemophilus* spp., *N. gonorrhoeae*, streptococci and for testing potential methicillin or oxacillin resistance in staphylococci.

3. Mix the inoculum suspension prior to diluting.

Within 15 minutes of adjusting the inoculum to the 0.5 McFarland turbidity standard, mix the suspension and dilute it so that the final concentration in each well is 5×10^5 CFU/mL. Deliver 2.0 mL of the original suspension into 38 mL of water (1:20 dilution). The prongs of the inoculator will deliver 0.01 mL (1:10 dilution) into each well. Inoculate MIC panel carefully to avoid splashing from one well to another. (See figure 5.1.)

Failure to adjust the inoculum and to dilute it within 15 minutes may adversely affect the concentration of organisms and test results.

4. For a purity check of the inoculum

Remove a loopful of the suspension from the growth control well or inoculum reservoir, and subculture to a blood agar plate. Incubate overnight at 37°C in 3–5% CO₂, an atmosphere that enhances detection of a larger array of contaminants. Examine for contamination prior to reading the MIC panel.

5. To prevent the panels from dehydrating during incubation, do one of the following:

Place a plastic seal over the panel, or

Place the panel in a plastic bag, or

Place the panel in another type of container, or

Place a plastic lid or empty microdilution plate on top of the panel

Dilution of Standardized Inoculum for MIC Tests

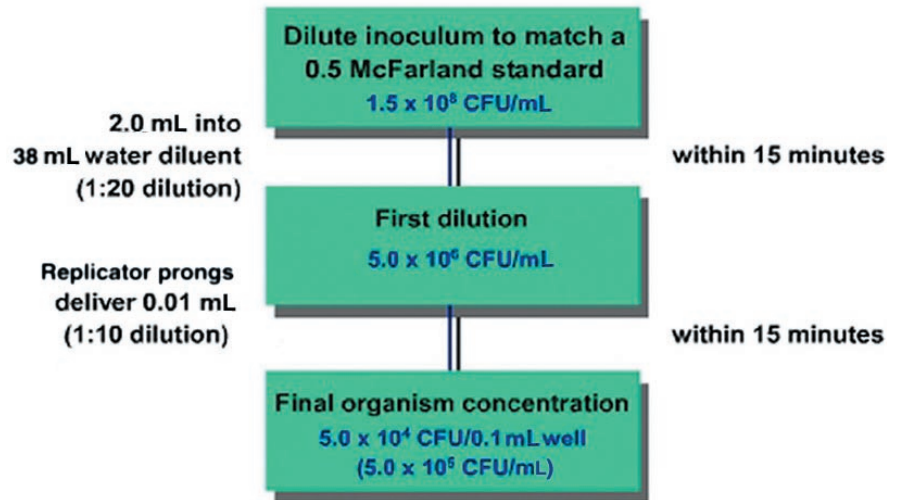


Figure 5.1—Dilution scheme for preparing a standardized inoculum for MIC tests

6. Incubate panels containing non-fastidious bacteria in ambient air at 35°C from 16–20 hours; do not stack more than four panels together.
7. Special incubation concerns:

Results for nonfastidious bacteria and most antimicrobial agents can be read and reported after 16–20 hours. However, in order to detect resistance to some antimicrobial agents, the procedure must be modified for:

 - staphylococci—incubate oxacillin and vancomycin for 24 hours.
 - enterococci—incubate vancomycin, high level gentamicin resistance (synergy test) and high level streptomycin resistance (synergy test) for 24 hours. If results are negative for streptomycin at 24 hours, reincubate for an additional 24 hours before reporting results (a total of 48 hours). Resistant results can be reported when detected.

For more information, see Chapters 8 and 9 in this manual.

Media and Incubation Conditions

Organism	Medium	Incubation	
		Time (hrs)	Atmosphere ^b
<i>Haemophilus</i> spp	HTM ^a	20–24	Ambient air
<i>S. pneumoniae</i>	CAMHB ^c + 2–5% LHB ^d	20–24	Ambient air
Other Strep. spp	CAMHB + 2–5% LHB	20–24	Ambient air

^a Haemophilus Test medium

^b As mentioned above, purity check plates are incubated in 3–5% CO₂

^c Cation-adjusted Mueller Hinton broth

^d Lysed horse blood.

For testing of fastidious bacteria by broth microdilution, use NCCLS-recommended media and incubation conditions as shown in the table below:

Reading MIC Panels

1. Remove the MIC panel and the purity check plate from the incubators.
2. Examine the purity plate by using reflected light and then using transmitted light. Sometimes contamination is subtle. If the purity plate shows contaminants, the MIC test cannot be interpreted and should be repeated.
3. Check the positive control well for growth. Turbidity or a button of growth >2 mm should be present, indicating adequate growth in the MIC panel.
4. Check the negative control well. It should be clear.
5. Read the MIC endpoint as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye. See Figure 5.2.
6. For trimethoprim, sulfonamides, and trimethoprim-sulfamethoxazole, read the endpoint at the concentration where there is a >80% reduction in growth compared to the positive control.
7. Some agents, particularly bacteriostatic agents, may demonstrate a very light haze or small buttons (<2 mm) of growth in several successive well. This is often referred to as “trailing.” Generally, this faint growth can be ignored unless it occurs with the following:
 - Oxacillin or vancomycin when testing staphylococci
 - Vancomycin when testing enterococci

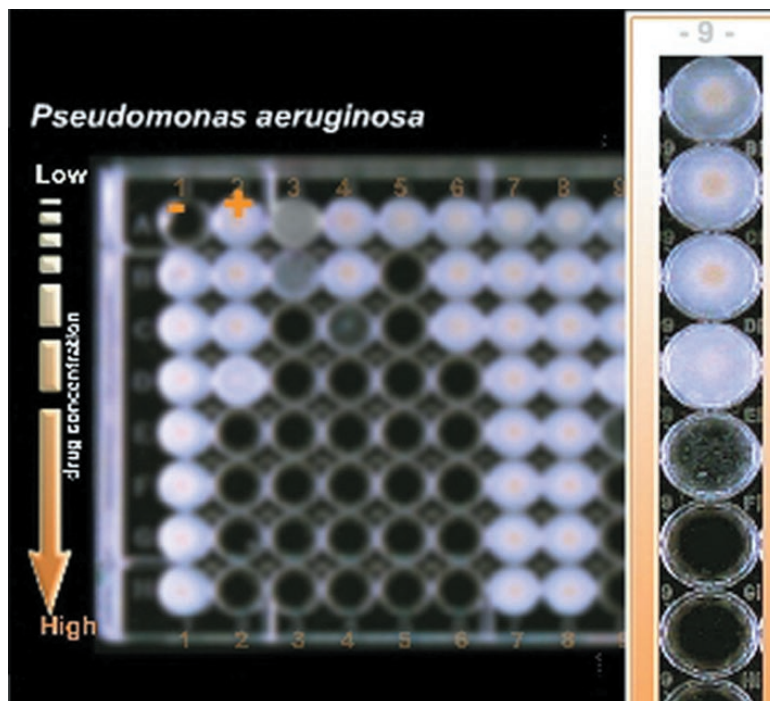


Figure 5.2—An MIC microtiter plate

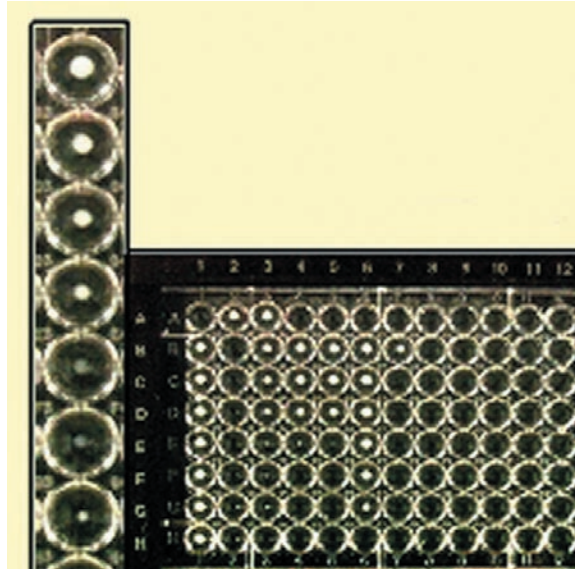


Figure 5.3—MIC with trailing.

Interpreting MIC Results

NCCLS document M7 (Tables 2A–2J) contains the MIC interpretive criteria (otherwise known as “breakpoints”) that you will need to interpret your MIC test results. If you are not familiar with the M7 document see Chapter 3 of this manual.

Controlling Test Variables

The following variables **MUST** be controlled in performing the broth microdilution MIC test:

- Concentration of the inoculum
- Endpoint measurement
- Incubation atmosphere
- Incubation temperature
- Incubation time
- Media composition¹
- Media pH

1. Important components of Mueller-Hinton broth (MHB) used for nonfastidious bacteria are listed below:

Cations:

MHB is supplemented with calcium and magnesium to attain concentrations that approximate physiologic concentrations. These correspond to 25 mg/L calcium and 12.5 mg/L magnesium. When supplemented the broth is referred to a cation-adjusted Mueller-Hinton broth or CAMHB. If the concentration of these cations is too high, false resistance may occur for *Pseudomonas aeruginosa* and aminoglycosides. A low cation concentration has the opposite effect (possible false susceptibility). High concentrations of cations bind the tetracyclines and render them less active against all bacteria (low concentrations have the opposite effect).

Thymidine:

Use CAMHB that contains no or very low concentrations of thymidine. Excessive concentrations can result in false resistance to sulfonamides, trimethoprim and trimethoprim-sulfamethoxazole.

To make certain that each lot of CAMHB has acceptable thymidine concentrations, test the quality control strain *Enterococcus faecalis* ATCC 29212 and trimethoprim-sulfamethoxazole. The MIC should be <0.5/9.5 mcg/mL for the medium to be considered acceptable.

Other:

Test oxacillin and other penicillinase-stable penicillins in CAMHB containing 2% NaCl.

Perform high-level aminoglycoside or synergy screen tests for enterococci in brain heart infusion broth or dextrose phosphate broth.

Antimicrobial Concentrations Tested

The number of dilutions and range of concentrations tested may vary among broth microdilution MIC panels for different antimicrobial agents.

The range of concentrations tested should encompass the interpretive breakpoints and the anticipated MIC of the quality control organism.

Generally 6–8 dilutions are tested for a “full range” MIC test.

Panels that include only those concentrations that define the breakpoint (typically only 2 or 3 dilutions) are called breakpoint panels. Breakpoint panels are often difficult to quality control because the QC results are typically above or below the concentrations on the panel.

The table below shows the interpretive categories for ampicillin. For the breakpoint panel, only three concentrations are tested and these represent susceptible, intermediate and resistant interpretations.

Full range versus breakpoint MIC panels

Full Range MIC (mcg/mL)	Breakpoint MIC (mcg/mL)	Interpretation
0.5	–	Susceptible
1.0	–	
2.0	–	
4.0	–	
8.0	8.0	
16.0	16.0	Intermediate
32.0	32.0	Resistant

Quality Control

QC Strains:

Quality control of MIC tests involves testing the NCCLS-recommended ATCC reference strains. This is explained in detail in the QA/QC chapter.

Inoculum Concentration:

Since the concentration of organisms in the inoculum is critical to achieving accurate results, it is suggested that this variable be monitored periodically by using the procedure outlined below to determine the number of organisms in the growth control well of an MIC panel.

1. Immediately following inoculation of a broth microdilution test panel, transfer 0.01 mL from the growth control well to 10 mL of saline. Vortex.
2. Transfer 0.1 mL of this dilution to an agar medium and spread to thoroughly disperse. Repeat on a second agar plate.
3. Following overnight incubation (16–20 hours), count the number of colonies present on the agar and determine the average number of colonies on the two plates.
4. An acceptable count is an average between 30–100 colonies. When multiplied by the dilution factor of 10^4 , this indicates an inoculum concentration of approximately 5×10^5 CFU/mL.

Other MIC Methods

Agar Dilution

NCCLS document M7 includes recommendations for agar dilution MIC testing and provides recommended testing conditions for the following:

Organism	Medium	Incubation	
		Time (hrs)	Atmosphere
Nonfastidious bacteria	MHA ^a	20–24	Ambient air
Other Strep. spp.	MHA & 5% sheep blood	20–24	Ambient air
<i>Neisseria gonorrhoeae</i>	GC ^b agar base	20–24	CO ₂ (5%)

^a Mueller-Hinton agar

^b Gonococcal

Because of the labor required and shelf life constraints, agar dilution testing is generally not performed in routine clinical laboratories.

Broth Macrodilution

In this method, the final twofold dilutions of antibiotic are prepared volumetrically in the broth. A minimum final volume of 1 mL of each dilution is needed for the test. Within 15 minutes after the inoculum is standardized, as described previously, 1 mL of the adjusted inoculum is added to each tube containing the antimicrobial agent. Each tube is mixed and incubated at 35°C for 16 to 20 hours in an ambient air incubator. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes as detected by the unaided eye. Generally, microdilution MICs for gram-negative bacilli are the same or a twofold dilution lower than the comparable macrodilution MICs.

Performing MIC vs. Disk Diffusion Tests

MIC tests are required for some organisms/antimicrobial combinations for which disk diffusion testing has proven to be unreliable. These include:

Streptococcus pneumoniae

Penicillin—perform penicillin MIC test when isolates show zones of inhibition <20 mm around oxacillin disks (a screening test for penicillin resistance).

Cefotaxime or ceftriaxone—use an MIC test because breakpoints for disk diffusion testing have not been established for these agents.

Viridans streptococci

Penicillin—determine MICs when isolates are from normally sterile body sites.

Staphylococcus species

Vancomycin—perform vancomycin MIC tests to detect decreased susceptibility to vancomycin since this cannot be determined using the disk diffusion test.

CASE STUDY

A 35-year-old man suffered severe injuries to his torso and legs in a motorcycle accident. The injuries made it necessary to amputate his left leg. One week after he was admitted to the surgical intensive care unit, the amputation site became red and swollen. Cultures of the site grew *P. aeruginosa*. The physician contacted the laboratory to obtain the antimicrobial susceptibility test results and the technologist gave him a verbal report of the qualitative results (susceptible, intermediate, and resistant) only. The physician asked for the MIC results for gentamicin and tobramycin, even though both of them were reported as susceptible.

A technologist should understand the reason for the physician's interest in obtaining MIC results as well as the susceptible, intermediate and resistant results from testing the *P. aeruginosa* isolate from this patient.

The verbal lab report included the following:

Specimen source: Wound drainage

Results: *Pseudomonas aeruginosa*

Drug	Susceptibility
Ceftazidime	S
Ciprofloxacin	R
Gentamicin	S
Imipenem	S
Piperacillin	S
Tobramycin	S

Do you see any difference between gentamicin and tobramycin?

Now view the MIC report, do you see any difference between gentamicin and tobramycin?

Drug	Interpretation	MIC
Ceftazidime	S	<0.5
Ciprofloxacin	R	>4
Gentamicin	S	4
Imipenem	S	<0.5
Piperacillin	S	<8
Tobramycin	S	0.5

The MIC results for gentamicin and tobramycin are both in the susceptible range; however, the MIC of tobramycin is 8 times higher than the MIC of gentamicin and is on the borderline between Susceptible and Intermediate. Thus the MIC information suggests that tobramycin is a better agent than gentamicin for this infection.

Serious *P. aeruginosa* infections frequently are treated with a combination of an aminoglycoside and a beta-lactam agent. When the physician obtained the MIC results, he prescribed a combination of tobramycin and ceftazidime. The patient's infection cleared and, following 2 months of hospitalization, he was moved to a rehabilitation unit.

REVIEW

Remember to:

- Use the most current NCCLS M7 standard that provides instructions performing an MIC test on aerobic bacteria. In addition, use the most current NCCLS M100 tables for guidance in interpreting and reporting results.
 - If using a commercial test system follow the manufacturer's recommendations.
 - Become familiar with exceptions to the standard method for testing certain organism/antimicrobial combinations.
-

SELF-ASSESSMENT QUESTIONS

1. What NCCLS document provides standard instructions for performing an MIC test on aerobic bacteria?
2. What NCCLS document provides tables for guidance in interpreting and reporting disk diffusion and MIC test results?
3. What are the exceptions to the standard method for testing certain organism/antimicrobial combinations?
4. Is it essential to obtain MIC results versus qualitative (susceptible, intermediate and resistant) results on all blood isolates?
 - A. Yes
 - B. No

6

Quality Assurance/ Quality Control (QA/QC)

OBJECTIVES

At the completion of this chapter the reader should be able to:

- List the components of a quality assurance program for antimicrobial susceptibility testing.
- Identify the specific NCCLS-recommended quality control strains for disk diffusion and MIC tests and describe how the strains should be maintained.
- Given an acceptable antimicrobial agent disk diffusion range for a quality control strain, determine if a specified test result is within range.
- Discuss corrective action to be taken when a daily or weekly quality control result is out of range.
- Describe a strategy for verifying antimicrobial susceptibility test results on patient' isolates.

DEFINITIONS

QC—Quality Control: A process in the laboratory designed to monitor the analytical phase of testing procedures to ensure that tests are working properly.

QA—Quality Assurance: A program designed to monitor and evaluate the ongoing and overall quality of the total testing process, including the pre-analytical, analytical, and post-analytical phases of testing.

QS—Quality Systems: An institution-wide program to ensure that the entire “path of workflow” related to any test is working according to pre-established criteria.

Corrective action: The necessary response when any problem is identified through QC, QA, or QS.

QA PROGRAM FOR ANTIBIOTIC SUSCEPTIBILITY TESTING (AST)

The components of a comprehensive Quality Assurance Program for AST are:

- Test reference QC strains (15%)
- Technical competency (15%)
- Organism antibiogram verification (15%)
- Clinically relevant testing strategies (15%)

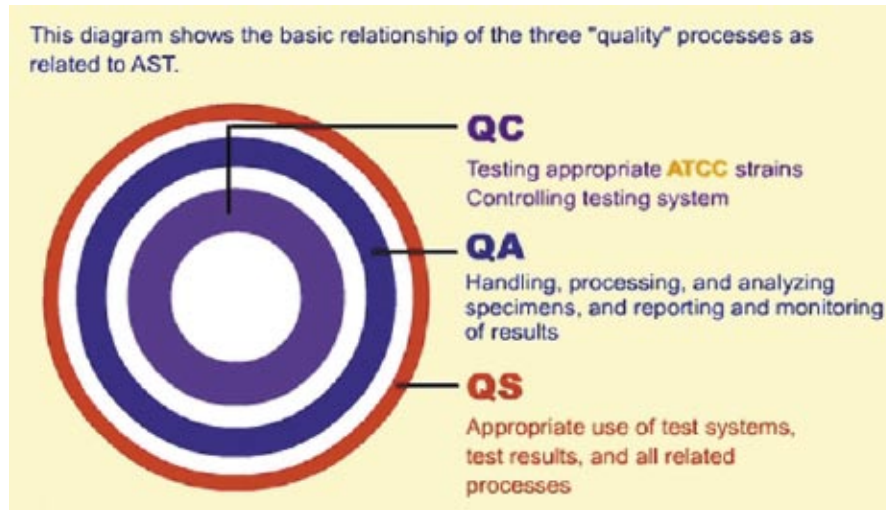


Figure 6.1—A “bull’s eye” diagram of QA/QC/QS

- Supervisor review of results (15%)
- Procedure manual (10%)
- Cumulative antibiogram (5%)
- Proficiency surveys (5%)
- Other (5%)

The percentages reflect the relative amount of effort required in a clinical laboratory for each component.

“Organism antibiogram verification” refers to reviewing the results on each patient isolate to make sure that the antibiogram is consistent with the identification of the isolate.

Specific QC requirements for AST, in addition to the general requirements for QA and QC under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), include:

- Checking each new batch of media and each lot of antimicrobial disks before (or concurrent with) initial use, using approved reference strains.
- Verifying that the disk diffusion zone sizes or MIC values for reference strains are within established limits before reporting AST results.
- Using the appropriate control strains to check the accuracy of the procedure each day that tests are performed.
- Testing the control strains on a weekly basis, provided NCCLS standards for daily AST are met; or, performing daily quality control as outlined in NCCLS standards.

You can view the CLIA website at <http://www.cms.hss.gov/clia>.

QC Strains—General

NCCLS recommends use of ATCC strains for QC of antimicrobial susceptibility tests. QC is performed to ensure the test system is working properly. In this chapter we will focus on the disk diffusion method to illustrate key points. Recommendations for QC of MIC tests are similar.

QC Strains—Acceptable Ranges

The acceptable ranges for the QC strains are listed in the NCCLS M 100 Table 3 or Table 3A.

Technical tip: NCCLS M100 table are updated annually; QC ranges may be added or modified with each update. New data are always listed in boldface type within M100

QC Strains—Selection for Testing

When selecting QC strains for testing:

- Start with the QC strain(s) that most closely resembles the patients' isolates being tested.
- Focus on QC strains that have defined QC ranges for the drugs that will be tested as listed in Table 3 and Table 3A.
- NCCLS-defined QC ranges are valid only when using NCCLS reference methods or methods that have been shown to perform comparably to these.
- Refer to NCCLS Tables 2A-2H, which list the minimal QC recommendations for a specific organism group. Here you can see the strains recommended for QC when testing isolates of *Enterobacteriaceae*.

Remember you must use the recommended testing conditions for the respective organism group. For example, testing *Enterobacteriaceae*:

Medium: Mueller Hinton agar

Inoculum: Growth method or direct colony suspension

Incubation: 35°C ambient air; 16–18 hours

Technical tip: Test QC strains in a manner identical to that used for testing patients' isolates

QC Strains—Description

NCCLS-recommended QC strains have been selected based on their susceptibility or resistance to particular antimicrobial agents and their reliable performance when tested using NCCLS reference methods.

Some strains are used only for QC of disk diffusion tests, others only for MIC tests; some are used for both.

Some strains are used only for specific resistance tests (e.g., *S. aureus* ATCC 43300 is used only for the oxacillin-salt agar screen test).

Technical tip: If using a commercial test system, use QC strains and procedures recommended by the manufacturer

Gram-positive QC Strains

Below are the basic characteristics and primary usage of gram-positive QC strains as described in NCCLS standards:

Enterococcus faecalis ATCC 29212

Susceptible to ampicillin, vancomycin, and high level aminoglycosides

Use for QC of:

- Antimicrobial agents tested against gram-positive bacteria (MIC)
- Vancomycin agar screen plate (negative control)
- High-level aminoglycoside resistance screening (negative control)
- Media to ensure it is acceptable for testing sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole.

Enterococcus faecalis ATCC 51299

Resistant to vancomycin (*vanB*-containing strain) and high levels of aminoglycosides

Use for QC of:

- Vancomycin agar screen plate (positive control)
- High-level aminoglycoside resistance screening (positive control)

Staphylococcus aureus ATCC 25923

Beta-lactamase negative.

Use for QC of:

- Antimicrobial agents tested against gram-positive bacteria (DD)

Staphylococcus aureus ATCC 29213

Beta-lactamase positive

Use for QC of:

- Antimicrobial agents tested against gram-positive bacteria (MIC)

Staphylococcus aureus ATCC 43300

Methicillin/oxacillin resistant *S. aureus* (MRSA)

Use for QC of:

- Oxacillin salt-agar screen plate

Streptococcus pneumoniae ATCC 49619

Penicillin intermediate

Use for QC of:

- Pneumococcal testing (DD/MIC)

Gram-negative QC Strains

Below are the basic characteristics and primary usage of gram-negative QC strains as described in NCCLS standards:

Escherichia coli ATCC 25922

Beta-lactamase negative

Use for QC of:

- Antimicrobial agents tested against gram-negative bacteria (DD/MIC)

Escherichia coli ATCC 35218

Beta-lactamase positive

Use for QC of:

- Beta-lactam/beta-lactamase inhibitor combination agents (DD/MIC)

Technical tips to prevent the loss of resistance plasmid:

- Stock culture should be stored at -60°C.
- Avoid repeated subculture of the stock because cells that have lost the plasmid can grow more rapidly than the resistant cells.

Haemophilus influenzae ATCC 49247

Beta-lactamase negative, ampicillin resistant (BLNAR)

Use for QC of:

- *Haemophilus* spp. (DD/MIC)

Haemophilus influenzae ATCC 49766

Ampicillin-susceptible

Use for QC of:

- Selected cephalosporins and *Haemophilus* spp. (DD/MIC)

Klebsiella pneumoniae ATCC 700603

Extended spectrum beta-lactamase (ESBL) producer

Use for QC of:

- ESBL screening and confirmatory tests
- Should be stored at -60°C to prevent loss of the resistance plasmid

Neisseria gonorrhoeae ATCC 49226

Chromosomally mediated penicillin-resistant

Use for QC of:

- *Neisseria gonorrhoeae* (DD/MIC)

Pseudomonas aeruginosa ATCC 27853

Typical strain susceptible to anti-pseudomonal agents

Use for QC of:

- Antimicrobial agents tested against gram negative bacteria (DD/MIC)
- Media to ensure the cation content and pH are satisfactory, particularly for testing aminoglycosides

QC Strains—Maintenance

Each laboratory must maintain a collection of QC strains appropriate for its needs. These strains must be stored properly to preserve their antimicrobial susceptibility characteristics.

For long-term storage of stock cultures, use one of the following:

- Store in a suitable stabilizing medium, such as Trypticase soy broth with 10–15% glycerol or defibrinated sheep blood (preferably at -60°C).
- Store lyophilized (freeze-dried).
- Obtain commercial freeze-dried stock cultures.

For working stock cultures once each month (or more frequently, if necessary):

- Subculture from the permanent stock culture (frozen or lyophilized) to plated media.
- Subculture 4–5 isolated colonies from plated media to an agar slant and incubate overnight, to prepare working stock culture.
- Store non-fastidious strains on Trypticase soy agar slants and fastidious strains on chocolate agar slants, at $2-8^{\circ}\text{C}$.

Two days prior to QC testing:

- Subculture growth from the agar slant to plated media and incubate overnight.
- Select 4–5 isolated colonies from the plate for QC testing and test with the same method used for patients' isolates.

QC Results—Documentation

Results from all disk diffusion QC test should be documented on a QC log sheet. Note the description of the information required on this log sheet.

Technical tip: Results for QC strains must fall within NCCLS-specified ranges for testing to be acceptable.

QC log sheet^a

Antimicrobial	AM	CZ	CIP	CAZ	GM	PIP					
Disc Potency	10	30	5	30	10	100					
Disc Lot #											
Date Opened											
Date Expired											
Std. Range (mm)	16–22	23–29	30–40	25–32	19–26	24–30					
Setup Date	Tech.	Tech. Rev. ^b						Manufacturer			C.A. ^c
								Media	Lot #	Expir. Date	

^a In the laboratory this table would be printed on the long axis of the page so there would be adequate space for recording information.

^b A second technologist (or supervisor) who reviews the work and outcome.

^c C.A. Corrective action.

QC Strains—Frequency of Testing

Appropriate QC strains should be tested each day susceptibility tests are performed on clinical isolates from patients. Corrective action must be taken if more than 1 in 20 daily QC results for a given drug /organism combination is out of range.

However, if you can establish satisfactory performance of daily disk diffusion or MIC QC test by following NCCLS standards, and the results are within NCCLS ranges and clearly documented in your laboratory QC records, you can switch to a weekly QC schedule and test your QC strains once per week.

Test QC strains for screen tests (e.g. VRE, MRSA) daily.

Proceeding from Daily to Weekly QC

To demonstrate established proficiency in daily QC testing:

- Test appropriate QC strains and antimicrobial agents for 30 consecutive tests (not calendar) days. Record the results for each agent, each day it is tested.
- For each drug/organism combination, no more than 3 of 30 zone diameters (or MIC results) may be out of the acceptable NCCLS-defined QC range. However, if any drug/organism result is out-of-range on two successive days, this must be investigated.
- If the above conditions are met, you may begin weekly QC, if desired.

Weekly QC Schedule

Test QC strains once per week.

Always test QC strains prior to (or concurrent with) using any new lot of materials for testing patient isolates.

Test QC strains for 30 days if:

- A new test system is introduced
- A new antimicrobial agent is added to your test battery
- A major change in testing method is adopted, such as:

Conversion from manual zone measurements to an automated zone reader (for disk diffusion testing)

Use of different broth manufacturer for panels or a different method for reading MIC test

A new panel with different concentrations of drugs is introduced

Quiz #1

Why do we test QC strains with known antimicrobial susceptibility and resistance profiles? Select all that apply

- A. To ensure media performs satisfactorily.
- B. To ensure antimicrobial (disk or MIC tube) potency is accurate.
- C. To ensure the inoculum is prepared and standardized properly.
- D. To ensure test materials are not contaminated.
- E. To ensure that resistant subpopulations of bacterial will always be detected.
- F. To ensure endpoints are measured reliably.
- G. To verify technologists' competency in test performance.

Answer:

All except E are correct. QC strains do not contain low-level resistant sub-populations; however, low-level resistance might be encountered among patients' isolates. Consequently, satisfactory performance of QC strains does not guarantee that all types of low-level resistance will always be detected among isolates with all test systems.

Quiz #2

You are working in a student health office that primarily cultures wound specimens and specimens from uncomplicated urinary tract infections. *Staphylococcus aureus* isolated from skin infections are tested by disk diffusion.

Which gram-positive QC strains will you test? Select from the following list of NCCLS-recommended ATCC QC strains:

- A. *Enterococcus faecalis* ATCC 29212
- B. *Enterococcus faecalis* ATCC 51299
- C. *Staphylococcus aureus* ATCC 25923
- D. *Staphylococcus aureus* ATCC 29213
- E. *Staphylococcus aureus* ATCC 43300
- F. *Streptococcus pneumoniae* ATCC 49619

Answer:

- A. Incorrect. There is no NCCLS disk diffusion QC range for *E. faecalis* 29212.
- B. Incorrect.
- C. Correct.
- D. Incorrect. This *S. aureus* strain is for QC of MIC tests.
- E. Incorrect. This *S. aureus* strain is for QC of the exacillin salt-agar screen test.
- F. Incorrect.

Quiz #3

Scenario: Your MIC panel for testing *Enterobacteriaceae* includes ciprofloxacin at concentrations of 0.12–4.0 mcg/mL. Which of the following strains would be best for QC of ciprofloxacin?

- A. *Escherichia coli* ATCC 25922
- B. *Escherichia coli* ATCC 35218
- C. *Pseudomonas aeruginosa* ATCC 27853

Answer:

- A. Incorrect. Acceptable range is 0.004–0.016 mcg/mL. The acceptable QC range for *E. coli* ATCC 25922 is below the lowest concentration included in the panel. An acceptable result would be ≤ 0.12 mcg/mL; however this off-scale result would not ensure that the test is working properly.
- B. Incorrect. There is no QC range for ciprofloxacin and *E. coli* ATCC 35218.
- C. Correct. Acceptable range is 0.25–1.0 mcg/mL. The QC range for *P. aeruginosa* ATCC 27853 falls within the range of concentrations of ciprofloxacin tested on this panel, i.e., has an on-scale endpoint.

Quiz #4

When testing ampicillin-sulbactam, why must we include *E. coli* ATCC 35218 in addition to *E. coli* ATCC 25922?

- A. *E. coli* ATCC 35218 is ampicillin resistant due to beta-lactamase production and monitors the sulbactam (beta-lactamase inhibitor component) in the disk.
- B. *E. coli* ATCC 35218 is ampicillin resistant due to altered penicillin-binding proteins and monitors the sulbactam (beta-lactamase inhibitor component) in the disk.
- C. *E. coli* ATCC 35218 is resistant to sulbactam whereas *E. coli* ATCC 25922 is not.
- D. *E. coli* ATCC 35218 does not grow as well as *E. coli* ATCC 25922 and is needed to monitor the growth-supporting potential of the medium.

Answer:

- A. Correct.
- B. Incorrect.
- C. Incorrect
- D. Incorrect

Quiz #5

A laboratory wants to change from daily to weekly QC for disk diffusion testing. Look at the results from 30 days of testing ampicillin and *E. coli* ATCC 25922 for which the acceptable QC range is 16–22 mm.

QC Record (30 Days)	
<i>E. coli</i> ATCC 25922—ampicillin	
Day	Zone (mm)
1	16
2	17
3	15
4	20
5	17
6	20
7	19
8	16
9	18
10	17
11*	
*	
*	
30*	

* Results on days 11–30 are in control.

Can the laboratory now perform QC for ampicillin and *E. coli* disk diffusion tests weekly?

- A. Yes
- B. No

Answer:

A. Correct. Only 1 of 30 results is outside the acceptable range of 16–22 mm.

Quiz #6

Now look at the cefazolin QC results for days 21–30. During the first 20 days, only one result was outside the acceptable range of 21–27 mm.

QC Record (30 Days)	
<i>E. coli</i> ATCC 25922 – cefazolin	
Day	Zone (mm)
1	*
*	*
16	20
*	*
20	*
21	24
22	25
23	23
24	25
25	25
26	19
27	25
28	20
29	23
30	20

* Except for day 16, all results on days 1–20 are in control.

Can the laboratory now perform QC for cefazolin and *E. coli* disk diffusion tests weekly?

- A. Yes
- B. No

Answer:

B. No is the correct answer. There are four results out of range during the 30 days of testing. The laboratory cannot change to weekly QC testing with cefazolin.

Corrective Action

The laboratory should take the following corrective actions before it begins weekly QC testing with cefazolin:

- The supervisor should review the test method as it is performed and look for deviations from the standard protocol (e.g. any shortcuts taken by the technologist).
- If the disk diffusion test for cefazolin is being used for patient's isolates, cefazolin results should be withheld because they may be erroneous.

- An alternative testing method for cefazolin might be explored.
- Different lot number of materials and reagents should be used to perform another 30 days of tests for cefazolin and *E. coli* ATCC 25922.
- Old and new lots of disks and MHA should be tested in parallel.

IF a weekly QC result is out of range and the reason is obviously due to:

- Contamination
- Testing the incorrect QC strain
- Testing the incorrect drugs
- Using incorrect testing conditions (e.g. incubation length or atmosphere was inappropriate)

THEN retest the problematic agent and appropriate QC strain the day the problem is noted; if results are acceptable, corrective action has been completed.

IF a weekly result is out of range and the reason and resolution for the problem IS NOT obvious:

THEN initiate corrective action:

- Retest the problematic agent and QC strain the day the problem is noted and for four additional days.
- If all five results are in control, revert to weekly QC testing.
- If any of the five results are out of control, continue with corrective action to identify the source of the problem and correct it.
- Once the problem is corrected, test for an additional 30 consecutive test days.

Technical tip: Record all results, including those out of range, in the QC log book

Quiz #7

You have been on a weekly QC schedule. Which of the following would now require 30 days of daily QC testing? Select all that apply:

- A. You switch from ciprofloxacin to levofloxacin on your gram-negative urine panel.
- B. You begin testing a new lot number of ampicillin disks.
- C. You have a new technologist performing disk diffusion testing.
- D. You had a weekly QC problem with gentamicin only. Follow-up daily testing for 5 days demonstrated one result of five out of control.

Answer:

- A. Correct.
- B. Incorrect. It is not necessary to perform 30 days of QC testing each time a new lot of materials is used.
- C. Incorrect.
- D. Correct. Only gentamicin requires 30 days of testing; other drugs can remain on a weekly schedule.

Quiz #8

When would daily QC testing be appropriate? Select all that apply.

- A. If results of QC strains are out of range on three or more days during the 30 day period.
- B. For drugs that are infrequently tested.
- C. For laboratories that perform antimicrobial susceptibility tests infrequently.

Answer:

- A. Correct. QC strains should be tested daily and corrective action should be performed to resolve the problem.
- B. Correct.
- C. Correct.

Quiz #9

Klebsiella pneumoniae ATCC 700603 is used only for QC of ESBL screen and confirmatory tests.

What is the most likely explanation if the results for this strain (when used for ESBL screening and confirmatory QC tests) are out of control on two successive days?

- A. The test is contaminated.
- B. The isolate, which happened to be stored at -20°C, lost its resistance plasmid.
- C. The test was incubated at too low a temperature.

Answer:

- A. Incorrect.
- B. Correct.
- C. Incorrect.

Corrective Action—Checklist

As part of the corrective actions, make certain that you check all of the following:

- ✓ Zone diameters or MIC endpoints were measured correctly.
- ✓ Turbidity standard is homogeneous and free of clumps.
- ✓ Inoculum suspension was prepared properly.
- ✓ Test materials were stored properly.
- ✓ Test materials have not reached or passed their expiration dates.
- ✓ Incubator temperature and atmosphere are within the specified range and other equipment is properly maintained.
- ✓ QC strain used for testing is acceptable. Follow recommendations for maintenance of QC strains.
- ✓ The person performing testing has been certified as competent to perform the testing.
- ✓ The pH of the medium is in the established range.
- ✓ The depth of the agar in the plates is 4 mm.

Random QC Problems

QC results may be out of range because of a random technical error or by chance. These random QC problems may be due to:

- Contamination of the QC test.
- Selecting the incorrect QC strain for testing (e.g., organism mix-up).
- Using inappropriate testing conditions.

It is unlikely that random QC problems would have an impact on patient's testing results:

- Random technical errors may occur by chance alone in one out of 20 QC tests.
- When a QC result is out of range because of random problem, retest on the day the problem is observed. If the problem is truly random, retesting will resolve the problem and the result can be reported the following day.
- The corrective action for reporting patients' results when a random QC problem occurs is relatively simple. The results from patients' isolates can be reported if:

The reason for the out-of-range QC result is obvious and relates to only one of the QC strains, (i.e., other results are in control).

It is likely that the out-of-range QC result will be correct upon repeating testing.

System QC Problems

QC results may be out of range because of a performance problem with the testing system or method.

- System problems may involve multiple components of the test system.
- System problems often contribute to erroneous patient results if not resolved.
- Corrective action must be performed immediately and documented.
- The corrective action procedure for laboratories on a daily QC schedule is slightly different from the corrective action procedure for labs on a weekly QC schedule.

Corrective Action—Patients' Results

Daily QC Schedule

IF one agent is implicated, THEN withhold results for that drug.

IF more than one agent is implicated, THEN withhold all patients' results. To avoid substantial delays in reporting, consider one or more of the following:

- Use an alternate test method.
- Use different lots of materials; however, if the problem is not related to test materials, this may delay reports further.
- Send patients' isolates to a reference laboratory for testing.

Weekly QC Schedule

IF a system problem is suspected and identified by weekly QC testing, THEN:

- Retrospectively review susceptibility results on patients' isolates generated since the last acceptable QC results were obtained (even if daily supervisory review did not reveal any problem).
- Retest patients' isolates, if necessary.
- Call physicians and send amended reports if necessary.

Frequently, system problems are identified when verifying individual patient isolate results.

Corrective Action—Exercise #1

For each of the following, is the problem more likely to be random or system?

- A. A single antimicrobial agent is out of range for one QC strain.
- B. A single antimicrobial agent is out of range for more than one QC strain.
- C. A single antimicrobial agent is out of range on more than one test day.
- D. QC test appears grossly contaminated as indicated by multiple colony types within a zone of inhibition.
- E. Results of several drugs are out-of-range; however, the same lots of materials have been in use for two weeks without any problems.
- F. Several antimicrobial agents are out-of-range.

Answer:

- A. Random.
- B. System.
- C. System.
- D. Random.
- E. Random. Possibly organism mix-up for that day only.
- F. System. Unless the wrong organism was picked for testing.

User's Responsibilities

The user must ensure that:

- Products are stored according to manufacturer's specifications.
- Individuals performing testing have demonstrated competency in test performance.
- Individuals performing testing follow manufacturer's instructions precisely and accurately perform the recommended Quality Control test.

Manufacturer's Responsibilities

The manufacturer must ensure that the:

- Antimicrobial agent potency is accurate.
- Antimicrobial agent potency is stable.

- Antimicrobial agent in the disk, well, or strip is properly identified and contains the appropriate chemical form of the agent (i.e., sodium, salt, free acid, etc.).
- Product was prepared in compliance with good manufacturing processes.

Manufacturers must maintain accountability for the manufacturing process and products.

Corrective Action—Exercise #2

Scenario: Your laboratory performs weekly QC tests for disk diffusion testing and has been using the same lot numbers of materials for the past three weeks. The gentamicin results are shown below.

Day 1:

Gentamicin result		
QC strain	Acceptable range (mm)	Today's results (mm)
<i>E. coli</i> ATCC 25922	19–26	14

What should you do?

- Repeat the testing of *E. coli* ATCC 25922 with the same materials.
- Use a new lot of MHA plates.
- Use a new cartridge and new lot (if available) of gentamicin disks.
- Subculture a fresh isolate of *E. coli* ATCC 25922 from a frozen stock culture.

Answer:

- Correct. Since the problem is only with *E. coli* ATCC25922 (but not with *P. aeruginosa* ATCC 27853) and appears to be due to the positioning of the disk, it is highly likely that this is a random problem due to an identifiable error.
- Incorrect.
- Incorrect.
- Incorrect.

What will you do with results from the patients' isolates?

- Withhold results for gentamicin and *Enterobacteriaceae* but release them for *P. aeruginosa*.
- Report gentamicin according to routine policies.

Answer:

- Incorrect.
- Correct.

Day 2:

Gentamicin result

Gentamicin result

QC strain	Acceptable range (mm)	Today's results (mm)
<i>E. coli</i> ATCC 25922	19–26	20

What will you do now? Select all that apply.

- A. Record all results in the QC log and conclude this was a random problem.
- B. Resume weekly QC testing and test *E. coli* ATCC 25922 again next week.
- C. Continue testing of *E. coli* ATCC 25922 and gentamicin with the same materials for four more test days.

Answer:

- A. Correct.
- B. Correct.
- C. Incorrect.

Corrective Action—Exercise #3

Scenario: Your laboratory performs weekly QC of disk diffusion tests. You opened a new cartridge of imipenem disks yesterday and tested them for the first time. The results are shown below.

Day 1:

Imipenem results

QC strain	Acceptable range (mm)	Today's results (mm)
<i>E. coli</i> ATCC 25922	26–32	22
<i>P. aeruginosa</i> ATCC 27853	20–28	17

What will you do with QC testing? Select all that apply.

- A. Repeat testing with the same materials.
- B. Use a new lot of MHA plates.
- C. Use a new cartridge and new lot (if available) of imipenem disks.
- D. Subculture a fresh isolate of each QC strain from frozen stock cultures.

Answer:

- A. Incorrect.
- B. Incorrect.
- C. Correct. Imipenem is among the least stable drugs that are tested routinely in clinical laboratories. If disks were exposed to increased temperatures for any length of time (e.g. box with disks stayed on loading dock for several summer days), deterioration may have occurred. If available, use a new cartridge and new lot of imipenem disks and test in parallel with the old lot.
- D. Incorrect.

What will you do with results from testing the patients' isolates?

- A. Withhold all results
- B. Withhold all imipenem results on gram-negative bacteria tested with the same cartridge of disks.
- C. Release all results from today that are clearly susceptible or clearly resistant, but withhold imipenem results that are around the breakpoint and retest.
- D. Send all isolates that need imipenem test results to a reference lab.

Answer:

- A. Incorrect.
- B. Correct.
- C. Incorrect.
- D. Incorrect.

Day 2:

Imipenem results

QC strain	Acceptable range (mm)	Today's results (mm)	
		Old lot	New lot
<i>E. coli</i> ATCC 25922	26–32	20	28
<i>P. aeruginosa</i> ATCC 27853	20–28	18	24

What will you do now? Select all that apply.

- A. Record results in QC log.
- B. Resume weekly testing and test both QC strains again next week.
- C. Continue testing both QC strains with imipenem (new lot only) for four more test days.
- D. Report imipenem results from testing patients' isolates today with the new lot of disks.

Answer:

- A. Correct.
- B. Incorrect.
- C. Correct.
- D. Correct.

Patient Results—Accuracy Checklist

To ensure that antimicrobial susceptibility test results on patients' isolates are accurate, make certain:

- ✓ Results with QC strains are within the acceptable range.
- ✓ Growth is satisfactory.
- ✓ Test is not contaminated (mixed).
- ✓ The overall susceptibility profile or antibiogram is consistent with expected results for the agents tested and the identification of the isolate; check results for all antimicrobial agents tested and not just those that will be reported.
- ✓ Atypical resistance, if present, is confirmed.

Verify inconsistent results and atypical resistance by repeating:

- ✓ Identification tests and/or
- ✓ Susceptibility tests

QUIZ #10

Which of the following may contribute to erroneous AST results on a patient's isolate even when results for the QC strains are within the acceptable range? Select all that apply.

- A. Contaminated inoculum
- B. Sporadic instrument malfunction while reading results
- C. Difficult to measure endpoints (e.g., trailing with trimethoprim-sulfamethoxazole)
- D. Low frequency resistant subpopulation
- E. Transcription/technical errors

Answer:

- A. Correct.
- B. Correct.
- C. Correct.
- D. Correct.
- E. Correct.

Verification Guidelines

Verification—Typical Antibigrams

Because many bacterial species are associated with a typical antibiogram, knowledge of typical antibigrams can help confirm results of patients' isolates. However, with emerging resistance this may not always be true.

Listings of the more common antibigrams for various species can be found in the following publications:

- Isenberg, HD (ed). 1998. *Essential Procedures for Clinical Microbiology*. American Society for Microbiology, Washington, DC.
- Lorian, V. 2005. *Antibiotics in Laboratory Medicine*, 5th ed. Williams & Wilkins, Baltimore, MD.
- Murray, PR, EJ Baron, JH Jorgensen, MA Tenover, and RH Tenover (eds.). 2003. *Manual of Clinical Microbiology*, 8th ed. American Society for Microbiology, Washington, DC.

Verification—Expert Systems

“Expert” systems (i.e. software systems that check resistance patterns of bacterial isolates for inconsistencies or atypical results) often are available with automated AST instruments.

Some expert systems use an institution's cumulative susceptibility statistics to flag unusual isolates. So, if a certain species is typically susceptible (S) or resistant

(R) to an antimicrobial agent >90% of the time, the system would flag an isolate that was reported with the opposite result.

Example: If 98% of *E. coli* isolates are susceptible to ciprofloxacin in an institution, isolates that are resistant are flagged for follow up.

Verification—Relatedness of Antimicrobial Agents

Generally, drugs within a class exhibit a hierarchy of activity against specific organism groups. For example, against *Enterobacteriaceae*, 1st generation cephalosporins are less active than 2nd, 3rd, or 4th generation cephalosporins.

Knowledge of the various activity levels of antimicrobial agents within a class should be used to assess the overall profile of results obtained on patients' isolates.

When reviewing the antibiograms of bacterial isolates, investigate results when the typically "more active" drug appears less effective than the drug which is typically "less active."

Verification—Relatedness of Cephalosporins

The typical hierarchy of activities for 1st, 2nd, 3rd, and 4th generation cephalosporins for gram-negative organism groups is:

(> means activity is greater than and = means activity is comparable to)

Enterobacteriaceae

4th generation cephalosporins > 3rd generation cephalosporins
>2nd generation cephalosporins > 1st generation cephalosporins

P. aeruginosa (only 3rd and 4th generation cephalosporins are active)

cefepime = ceftazidime > cefoperazone > ceftizoxime = ceftriaxone = cefotaxime

Verification—Relatedness of Penicillins

Enterobacteriaceae

Piperacillin-tazobactam > piperacillin = mezlocillin > ticarcillin = carbenicillin > ampicillin

P. aeruginosa

Piperacillin-tazobactam = piperacillin > mezlocillin = ticarcillin > carbenicillin

Verification—Results to Verify

Appendix A in NCCLS M39-A "Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data," lists "Some atypical findings suggesting verification of susceptibility results and confirmation of organism identification."

Appendix A lists examples of resistance patterns that have yet to be reported or are infrequently identified in a hospital.

Some results that may be verified by repeat testing unless the patient had the isolate previously:

- *Staphylococcus aureus* intermediate or resistant to vancomycin
- *Enterococcus faecalis* resistant to ampicillin or penicillin
- *Enterococcus faecium* resistant to quinupristin-dalfopristin or
- *Enterococcus faecalis* susceptible to quinupristin-dalfopristin

- Beta-hemolytic streptococci “nonsusceptible” to penicillin
- *Enterobacteriaceae* resistant to amikacin
- *Enterobacteriaceae* resistant or intermediate to imipenem
- *Stenotrophomonas maltophilia* resistant to trimethoprim-sulfamethoxazole
- *Haemophilus influenzae* resistant to ampicillin and beta-lactamase negative or resistant to amoxicillin-clavulanic acid or 3rd generation cephalosporins
- Any other isolate demonstrating “nonsusceptible” results for organism/antimicrobial combinations for which only susceptible category criteria are defined in NCCLS M100.

Other results may be verified by re-examination of the test or repeat testing.

- *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *Acinetobacter baumannii*, or *P. aeruginosa* susceptible to ampicillin, cefazolin, or cephalothin
- *Klebsiella* spp., *Providencia* spp., or indole-positive *Proteus* spp. susceptible to ampicillin

Certain results may be unusual at some institution but not others.

If resistance at a specific institution is common, repeat testing may not be necessary to verify individual patient isolates.

Whether a result is unusual depends on global and local patterns of resistance and might well vary from institution to institution or from year to year.

- *Staphylococcus aureus* resistant to oxacillin
- *Enterococcus* spp. from sterile body site with high-level resistance to gentamicin
- *Streptococcus pneumoniae* resistant to penicillin or third-generation cephalosporins
- Viridans group *Streptococcus* spp. intermediate or resistant to penicillin
- *Klebsiella* spp. or *Escherichia coli* with extended-spectrum-beta-lactamase
- *Enterobacteriaceae* resistant to ciprofloxacin

If an isolate is resistant to all relevant drugs; obtain guidance for testing additional agents

Verification Policies

In developing specific verification policies for results that may be unusual in your setting, consider:

- Your confidence in the test system used
- Competency of technologists in AST, including their ability to recognize problems
- Incidence of “atypical” resistance
- Patient population (patients receiving long-term antimicrobial therapy tend to have more resistant organisms)
- Possible clinical impact of results (e.g. reporting MRSA leads to patient isolation and possible therapy with vancomycin)

Verification—Exercise #1

Lab Report

Escherichia coli

Antimicrobial agent	Susceptibility
Amikacin	R
Ampicillin	S
Cefazolin	S
Cefotaxime	S
Ciprofloxacin	S
Gentamicin	R
Tobramycin	R

Verify this isolate?

- A. Yes
- B. No

Answer:

- A. Yes. It is very uncommon for *Enterobacteriaceae* to be resistant to gentamicin, tobramycin, and amikacin. This test was subsequently shown to be contaminated with an *Enterococcus* spp.

Verification—Exercise #2

Lab Report

Escherichia coli

Antimicrobial agent	Susceptibility
Ampicillin	R
Amox-Clavulanic acid	S
Cefazolin	S
Cefotaxime	S
Ciprofloxacin	S
Gentamicin	S
Trimeth/sulfa	R

Verify this result?

- A. Yes
- B. No

Answer:

- B. No. Many *E. coli* are resistant to ampicillin and trimethoprim-sulfamethoxazole.

Verification—Exercise #3

Lab Report

Citrobacter freundii

Antimicrobial agent	Susceptibility
Amikacin	S
Ampicillin	R
Cefazolin	R
Cefotaxime	S
Ciprofloxacin	S
Gentamicin	S
Imipenem	R
Tobramycin	S
Trimeth/Sulfa	S

Verify this result?

- A. Yes
- B. No

Answer:

- A. Yes. It is very uncommon for *Enterobacteriaceae* to be resistant to imipenem. Imipenem is very temperature labile, which may account for this result.

Verification—Exercise #4

Lab Report

Enterobacter cloacae

Antimicrobial agent	Susceptibility
Amikacin	S
Ampicillin	R
Cefazolin	R
Cefotaxime	R
Cefuroxime	R
Ciprofloxacin	S
Gentamicin	S
Piperacillin	R
Tobramycin	S
Trimeth/Sulfa	S

Verify this isolate?

- A. Yes
- B. No

Answer:

- B. No. *E. cloacae* that produce large quantities of AmpC beta-lactamase have this profile.

Verification—Exercise #5

Lab Report

Klebsiella pneumoniae

Antimicrobial agent	Susceptibility
Amikacin	S
Ampicillin	R
Cefazolin	S
Cefotaxime	R
Ciprofloxacin	S
Gentamicin	S
Imipenem	S
Tobramycin	S
Trimeth/Sulfa	S

Verify this isolate?

- A. Yes
- B. No

Answer:

- A. Yes. It would be uncommon to encounter *Enterobacteriaceae* that are resistant to cefotaxime (a 3rd-generation cephalosporin) but susceptible to cefazolin (a 1st generation cephalosporin). This may happen with ESBL-producing strains on rare occasions; however, the cefazolin result would be reported as resistant.

Verification—Exercise #6

Lab Report

Pseudomonas aeruginosa

Antimicrobial	Susceptibility
Amikacin	R
Ceftazidime	R
Ciprofloxacin	R
Gentamicin	R
Piperacillin	R
Tobramycin	R

Verify this isolate?

- A. Yes
- B. No

Answer:

- A. Yes. When an isolate is resistant to all agents routinely reported, verification will confirm the limited therapeutic choices for treating infections caused by the isolate. In these cases, it might be helpful to consult with the physician

(and possibly infectious diseases specialists) to determine if testing of additional agents may be appropriate.

Verification—Exercise #7

Lab Report

Enterococcus faecalis

Antimicrobial agent	Susceptibility
Ampicillin	R
Vancomycin	R
Gentamicin synergy	R
Streptomycin synergy	R

Verify this isolate?

- A. Yes
- B. No

Answer:

- A. Yes. Ampicillin resistance is very uncommon in *E. faecalis*, but frequently seen in *E. faecium*. Most VRE are *E. faecium* and it is likely that the identification of the isolate is incorrect in this example.

Verification—Exercise #8

Lab Report

Staphylococcus aureus

Antimicrobial agent	Susceptibility
Clindamycin	S
Erythromycin	S
Gentamicin	S
Oxacillin	R
Penicillin	R
Vancomycin	S

Verify this isolate?

- A. Yes
- B. No

Answer:

- A. Yes. Historically, nearly all MRSA were multiply resistant. More recently, however, MRSA that are not multiply resistant have been recovered with increasing frequency from patients with community-acquired infections. Because this profile is uncommon, it should be verified.

Verification—Exercise #9

Lab Report

Streptococcus pneumoniae

Antimicrobial agent	MIC	Susceptibility
Cefotaxime	0.5	S
Erythromycin	>1	R
Levofloxacin	≤0.5	S
Penicillin	4	R
Trimeth/Sulfa	>4/76	R
Vancomycin	>1	R

Verify this isolate?

- A. Yes
- B. No

Answer:

- A. Yes. To date, vancomycin resistance to *S. pneumoniae* has not been reported and there are only susceptible interpretive criteria for vancomycin and *S. pneumoniae*

Verification—Exercise #10

Lab Report

Streptococcus pneumoniae

Antimicrobial agent	MIC	Susceptibility
Cefotaxime	≤0.03	S
Erythromycin	≤0.25	S
Levofloxacin	≤0.5	S
Penicillin	≤0.03	S
Trimeth/Sulfa	≤0.5/9.5	S
Vancomycin	≤0.5	S

Verify this isolate?

- A. Yes
- B. No

Answer:

- B. No. This is a typical profile for *S. pneumoniae*

Quality Systems (QS)

Having reviewed various aspects of the QA program for AST, which includes the QC program, it is time to take a step back and look at the QS approach which views the QA program as part of the workflow of the entire microbiology laboratory and beyond.

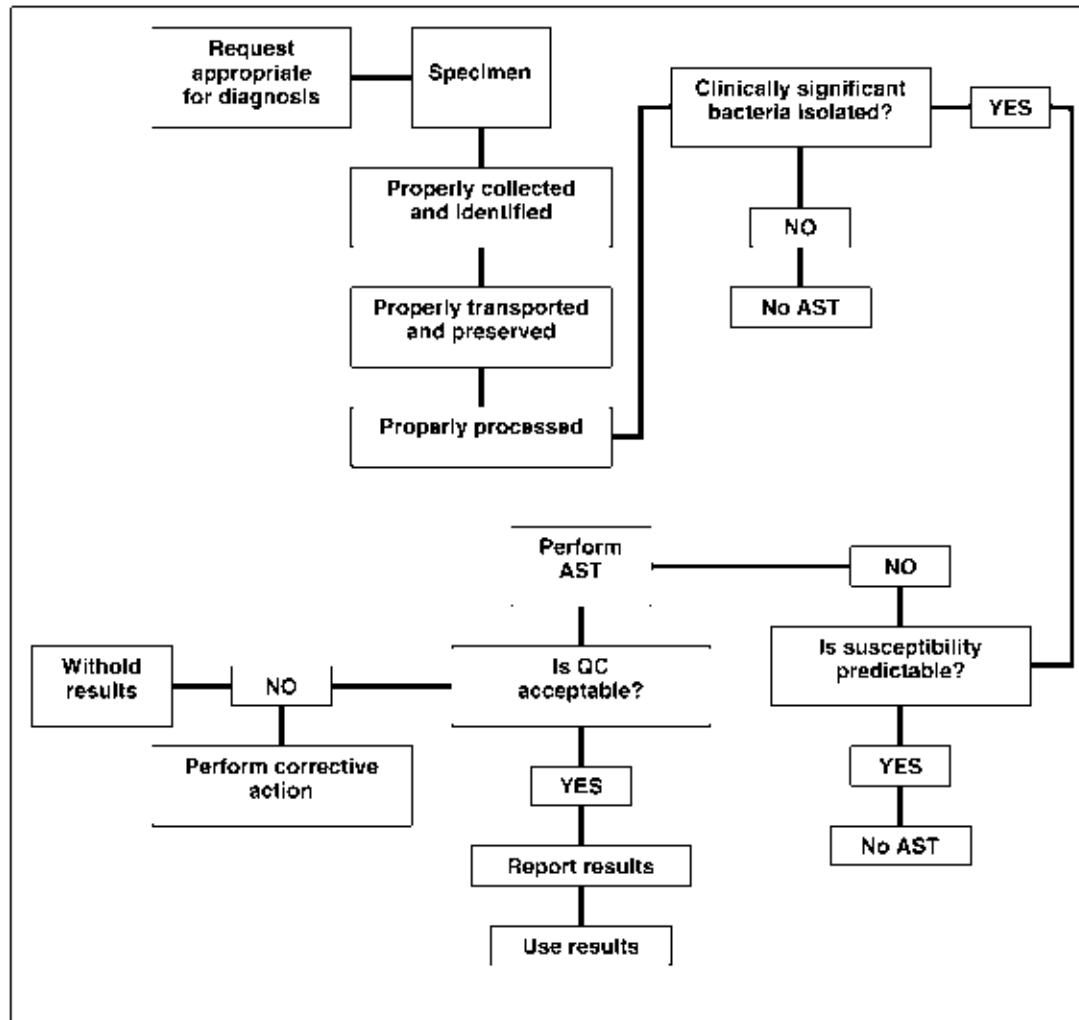


Figure 6.2—A sample workflow chart for laboratories

As specified in NCCLS GP26-A-V, “Champions for Change: Creating a Quality System Model,” QS essentials include the organization, personnel, equipment, purchasing/inventory, process control, documents/records, occurrence management, internal assessment, process improvement, service, and satisfaction as they relate to the path of the workflow.

Laboratory Path of Workflow

NCCLS recommends that each healthcare service develop a flowchart for antimicrobial susceptibility tests, such as that shown in Figure 6.2.

REVIEW

The reader should now understand the steps that are necessary to ensure results reported from your laboratory are accurate and reproducible.

Remember to:

- Use NCCLS-recommended ATCC quality control strains for QC testing.
- Review each isolate's results before reporting them to ensure that they are consistent with the organism's identification and that the results of all antimicrobial agents tested make sense. Errors may occur when testing patients' isolates even if results with QC strains are acceptable.
- Record any out-of-range result or QA problem and thoroughly document all corrective action taken.
- Continually monitor the testing process as part of your quality assurance program. In addition to the accurate testing and reporting, appropriate test selection, specimen collection, and transport and appropriate use of test results are essential to optimize patient care.

SELF-ASSESSMENT QUESTIONS

Because this chapter follows the design of a workbook, the questions with their informative answers are located throughout the text where the author and editor believe that they will be most educational for the reader.

7

Commercial Systems

OBJECTIVES

At the completion of this chapter the reader should be able to:

- Discuss the importance of reading the package insert for a commercial system prior to use and whenever updates are issued.
- Discuss the importance of reading the additional literature that is supplied with the product.
- Discuss what is meant by “limitations” for use of the commercial system.
- Describe how to determine the appropriate quality control measures.
- Discuss a strategy for verification when a new commercial system is introduced into your laboratory.

CASE STUDY

Your laboratory has been using the disk diffusion method for routine antimicrobial susceptibility testing for several years. When MICs are required, isolates are sent to a reference laboratory for testing. Now the laboratory director wants to purchase an automated antimicrobial susceptibility test system that will provide MIC results for most organisms the same day the test is set up.

Your job is to obtain as much information as possible on the proposed MIC system to determine if it is appropriate for your institution.

How will you approach this task? After working through this chapter you will be prepared to answer this question.

NCCLS METHODS VS. COMMERCIAL TEST SYSTEMS

When testing a patient’s isolates, a clinical laboratory can use either:

- One of the NCCLS reference methods (e.g. disk diffusion, broth microdilution, or agar dilution) as described in the NCCLS M2 or M7 standards and/or
- A commercial antibiotic susceptibility test (AST) system cleared for use by the Food and Drug Administration (FDA)

FDA CLEARANCE

The FDA publishes “Guidance and Review Criteria for Assessment of Antimicrobial Susceptibility Devices.” This document describes the information that manufacturers must submit to obtain “FDA-cleared” status for their antimicrobial susceptibility test system. You can access this document at <http://www.fda.gov/cdrh/ode/631.html>.

For an antimicrobial susceptibility test instrument or device to be cleared by FDA, each individual antimicrobial agent in the test must be cleared. The manufacturer must demonstrate that:

- Results obtained with the commercial system are comparable to results obtained with an NCCLS reference method.
- Overall performance of the commercial system meets FDA specifications.
- The performance of the commercial system can be reliably monitored in the clinical laboratory by following the manufacturer’s recommended quality control procedures.

THE PACKAGE INSERT

The manufacturers of FDA-cleared commercial systems must include instructions for using the system with each product distributed. The package insert must contain specific information on the following subjects:

- Intended use
Explains the purpose of using the product; emphasizes that reliable performance is guaranteed only when the product is used specifically for its intended purpose (e.g. testing of specific bacteria).
- Summary and principles
- Precautions
- Storage
- Product deterioration
Educates the user regarding proper storage, maintenance, expiration date, and acceptable appearance of product and packaging (e.g., if packaging is torn, or used beyond its expiration date, the product may be compromised).
- Specimen collection and preparation
- Materials provided
- Materials required but not provided
- Procedural outline
Contains the step-by-step instructions for performing the test; emphasizes that each step must be followed exactly as described.
- Quality control
Describes the step-by-step instructions for performing the quality control tests required to ensure the system is working properly; emphasizes following the manufacturer’s recommendations, even when different from NCCLS quality control protocol.
- Limitations of the procedure
Describes specific situations that limit the system from performing reliably; emphasizes the importance of using the product only for its intended purpose, in order to attain reliable results.
- Expected values

- Misleading results
Defines situations in which the in vitro results correlate poorly with clinical outcome.
- Performance characteristics
Lists data generated from various studies that the manufacturer conducted during extensive testing; data include the percent essential and category agreement when compared with reference methods and results of reproducibility studies.
- Reproducibility
- References

QUALITY CONTROL

Manufacturer Responsibility

The manufacturer of any commercial system for antimicrobial susceptibility testing has distinct responsibilities for ensuring quality.

The manufacturer must ensure that:

- Potency of the antimicrobial agents is accurate and stable.
- Antimicrobial agent in the disk, well, or strip is properly identified and contains the agent specified.
- Product complies with the proper manufacturing regulations.

The manufacturer must:

- Define the appropriate quality control measures that will ensure that the system performs according to specifications, thereby producing accurate results.
- Perform extensive quality control during product development and evaluation.
- Submit the quality control data with an application requesting clearance to the FDA.
- Continue extensive quality control testing of each lot during manufacturing.

User Responsibility

The user also has responsibilities for ensuring quality.

The user must ensure that:

- Products are stored according to the manufacturer's specifications.
- Individuals performing testing have demonstrated competency in test performance.
- Individuals performing testing follow the manufacturer's instructions for quality control precisely and accurately.

The user must:

- Test the NCCLS-recommended quality control strains, and any additional manufacturer-selected strains.
- Perform all quality control procedures defined by the manufacturer in the package insert.

VERIFICATION

According to the Clinical Laboratory Improvement Amendments of 1988 (CLIA), before implementing a new antimicrobial susceptibility test system, laboratory personnel must verify that the system performs according to the manufacturer's specifications.

Verification of FDA-cleared products is a one-time process that must be completed before a new test or modification of an existing test can be used for patient testing. This ensures that results are accurate and reproducible.

You can access CLIA at <http://www.cms.hhs.gov/clia>

Although CLIA regulations do not specify how verification of a new antimicrobial susceptibility test system must be performed, the process should include:

- Reviewing current literature
- Reviewing information available from the manufacturer
- Speaking with others who have used the product
- Performing a limited evaluation in your laboratory by testing control and clinical isolates

For ideal verification, conduct in-house parallel testing of the new system with an NCCLS reference method, such as disk diffusion or a broth microdilution MIC. To verify testing of nonfastidious bacteria, test a minimum of 100–200 randomly selected fresh clinical isolates representing various species including (if available):

- 5–10 isolates of gram-positive bacteria with known resistance characteristics
 - Oxacillin-resistant *Staphylococcus aureus* (ORSA)
 - Oxacillin-resistant coagulase-negative staphylococci;
 - Vancomycin-resistant *Enterococcus faecium* and/or *Enterococcus faecalis* (VRE).
 - Enterococcus* spp. with high-level gentamicin and streptomycin resistance.
- 5–10 isolates of gram-negative bacteria with known resistance characteristics
 - ESBL-producing *Escherichia coli* and *Klebsiella* spp.
 - E. coli* and *Klebsiella* spp. that are non-ESBLs, but resistant to extended-spectrum cephalosporins
 - 3rd generation cephalosporin-resistant *Enterobacteriaceae*, other than *E. coli* and *Klebsiella* spp.
 - Ceftazidime-and/or cefepime-resistant *Pseudomonas aeruginosa*
 - Imipenem-resistant *P. aeruginosa*
 - Gentamicin-and/or tobramycin-resistant *Enterobacteriaceae*
 - Gentamicin-, tobramycin- and amikacin-resistant *P. aeruginosa*
 - Fluoroquinolone-resistant *Enterobacteriaceae*
 - Fluoroquinolone-resistant *P. aeruginosa*

Perform and monitor QC daily as recommended by the manufacturer. To assess reproducibility with the QC or other strains, examine the results from 30 consecutive runs.

COMMERCIAL SYSTEMS

Manual

E-test

The E test or PDM Epsilon meter method has been used successfully to test anaerobes and other aerobic organisms. The term “epsilometer” refers to a thin, 5×50 mm, inert, nonporous strip with a continuous exponential gradient of antimicrobial agent immobilized on one side and an interpretive scale printed on the other. The antimicrobial agent gradient covers a broad concentration range, corresponding to approximately 20 twofold dilutions. The slopes and concentration ranges are optimally designed to correspond to clinically relevant MIC ranges and breakpoints are selected for categorizing susceptibility groups.

An agar plate containing a suitable test medium is inoculated with a test organism according to the manufacturer’s instructions. Test strips are then applied in an optimal pattern so that the maximum concentration on each strip is nearest to the outer edge of the Petri dish. The plate is immediately incubated aerobically or anaerobically for the prescribed time period.

When applied to an inoculated agar plate, the antimicrobial gradient is immediately released from the test strip into the agar, creating a continuous and exponential gradient of antimicrobial agent concentrations beneath the linear axis of the carrier. After incubation, an inhibition ellipse is seen. The zone edge intersects the carrier strip at the antimicrobial concentration no longer inhibitory to the growth. The point of intersection gives the “inhibitory concentration” (IC) in mcg/mL—a direct measure of the susceptibility of the microorganism to the tested antimicrobial agent. ICs are read directly from the scale on the carrier strip.

Automated Systems

Vitek

The Vitek system is an automated, walkway system manufactured by bioMérieux, Inc., Hazelwood, MO. It is based on the basic principle of photometry. The bacteria utilize a substrate which results in a color and density change. These changes are detected by light-emitting diodes and phototransistor detectors.

It is made up of a filter-sealer module, a reader incubator, a computer module, a data terminal and a printer. It is capable of identifying gram-negative and gram-positive bacteria, anaerobes and yeast cells. It also performs antimicrobial susceptibility testing. It is capable of performing urine screens, with enumeration and identification.

The system will identify *Enterobacteriaceae* in 4–6 hrs and nonfermenting bacilli in 6 to 18 hrs.

MicroScan Automated Microbiology Systems

These instruments are based on a principle of photometry-fluorometry. Three systems are available:

TouchSCAN-SR: a semi automated panel reader with data management system.

The reader will read the panel manually and the system will automatically provide an interpretation.

AutoSCAN-4: an automated panel reader with data management system. The user loads the panel and the system will read and interpret the panel automatically.

AutoSCAN-WA: fully automated system with walk-away automation and data management system.

The components vary with the system, but each system comes with computerized data management system with mainframe interface options.

The MicroScan system can process frozen or dehydrated panels. The conventional chromogenic panels are used for the identification of gram-negative and gram-positive bacteria in 18–24 hours. Antimicrobial susceptibility testing is available for the determination of MICs for aerobic and anaerobic organisms. Rapid chromogenic panels are available for the identification of *Haemophilus/Neisseria*, yeast and anaerobes in 4 hours. Rapid fluorogenic panels also are available for the identification of gram-negative and gram-positive bacteria in 2 hours, as well as susceptibility testing.

REVIEW

You now should be aware of important issues related to use of commercial system for antimicrobial susceptibility testing and reporting.

Remember:

The FDA is responsible for clearing commercial antimicrobial susceptibility testing systems for use in the United States. However, for a commercial system to obtain FDA clearance, the manufacturer must demonstrate that results are comparable to those produced by an NCCLS reference method.

Prior to using a new commercial system in your laboratory, verify that the results generated are accurate and reproducible.

When using any commercial system read the package insert thoroughly and follow the manufacturer's instructions precisely.

Be aware of the limitations described in the package insert of the commercial system.

SELF-ASSESSMENT QUESTIONS

1. Which NCCLS document contains information about commercial antimicrobial susceptibility test systems?
 - A. M2
 - B. M7
 - C. M100
 - D. None of the above
2. Based on NCCLS M2 and M7, which of the following would NOT be considered a reference method?
 - A. Disk diffusion
 - B. Broth microdilution
 - C. Agar dilution
 - D. E-test

3. You are using a commercial system that has a limitation for testing *Burkholderia cepacia*. How would you test this species?
 - A. Test the isolate using the commercial system and qualify results with the comment that “Results are presumptive.”
 - B. Test the isolate using the commercial system and report results if they are typical for *B. cepacia*.
 - C. Use an alternative MIC system for testing the isolate.
4. Which of the following should you consider when selecting isolates for in-house parallel testing of a new commercial antimicrobial susceptibility test system? Select all that apply.
 - A. Include isolates that have clinically significant resistance.
 - B. Include isolates that represent the types of species encountered in your institution.
 - C. Include equal numbers of each species.
 - D. Include isolates that have on-scale MICs whenever possible.
5. In order to determine if the proposed commercial MIC system is appropriate for your institution, what information will you collect for your laboratory director? Select all that apply.
 - A. Published articles
 - B. Information from the manufacturer
 - C. Information from other users
 - D. NCCLS publications
6. Your laboratory director is pleased with the information that you have assembled and asks you to verify performance of the commercial system in-house. How would you do this?
 - A. Test the recommended QC strains alone for 30 consecutive test days.
 - B. Test 200 clinical isolates with the new system in parallel with a reference method.
 - C. Test the recommended QC strains for 30 consecutive test days and test 20–30 randomly selected clinical isolates.
 - D. Test the recommended QC strains for 30 consecutive test days and test 150–200 random clinical isolates and isolates with select resistance characteristics.
7. Data from an in-house verification study show that the very major error rate for oxacillin and *S. aureus* is 10% (1 out of 10 isolates are falsely susceptible). What will you do? Select all that apply.
 - A. Closely examine all of the in-house and published performance data for oxacillin and *S. aureus*.
 - B. Retest the implicated isolate.
 - C. Test additional ORSA
 - D. Since it was only one isolate disregard the data.
8. Who is responsible for clearing commercial antimicrobial susceptibility testing systems for use in the US?
9. How will the manufacturer demonstrate that results are comparable to those produced by an NCCLS reference method?
10. Prior to using the system, what is the laboratory’s responsibility?

11. What is the package insert? Why should it be read thoroughly before the system is put into place?
12. What is meant by “limitations of the procedure” as described in the package insert?
13. Which of the AST methods would NOT be considered a reference method, based on NCCLS M2 and M7?
14. What is the principle of the E-test procedure?

III

Gram-Positive Organisms

8

Staphylococci

OBJECTIVES

After completion of this chapter the reader should be able to:

- Discuss the mechanisms of resistance to penicillin, oxacillin, erythromycin, clindamycin and vancomycin in *Staphylococcus* species
- Select the antimicrobial agents appropriate for routine testing and reporting against *Staphylococcus* species
- Name three testing methods that can be used to detect oxacillin resistance in *Staphylococcus* species
- Compare and contrast true oxacillin-resistant *Staphylococcus aureus* (ORSA) with borderline oxacillin-resistant *S. aureus* (BORSA)
- Use oxacillin and penicillin test results to predict susceptibility to other beta-lactam agents against staphylococci
- Describe reliable methods for detecting vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA)

BACKGROUND

S. aureus is a major human pathogen that can cause a wide variety of infections in both healthy and immunocompromised individuals. It also can colonize the skin and nares facilitating transmission particularly in healthcare settings unless proper infection control practices are followed. Resistance to commonly used antibiotics is increasing worldwide. Over 90% of *S. aureus* are resistant to penicillin. Oxacillin resistance is found in over 50% of isolates from intensive care units in the US. Increasingly, oxacillin resistance is being observed in community-acquired isolates of *S. aureus* as well. Vancomycin is commonly prescribed to treat infections caused by multiply resistant *S. aureus*. However, isolates with decreased susceptibility to vancomycin have been reported in a variety of countries including the United States. In 2002 the first fully vancomycin resistant isolate (MIC = 1024 µg/mL) was reported from a dialysis patient in Michigan. This organism had the *vanA* vancomycin resistance gene. Two more strains have subsequently been isolated from Pennsylvania and New York.

Coagulase-negative staphylococci (CoNS) are common skin and genitourinary tract inhabitants. Their isolation from culture often indicates contamination. Nevertheless they can be significant pathogens in immunocompromised patients and in those with intravenous catheters and medical devices. Although there are many species of CoNS, *S. epidermidis* is the most common isolate recovered from clinical specimens. *S. saprophyticus* is a major cause of acute, uncomplicated urinary

tract infections. CoNS isolates typically are more resistant to antimicrobial agents than *S. aureus* with the prevalence of resistance to beta-lactams as high as 60–70%. As a result, vancomycin frequently is used to treat CoNS infections.

CASE STUDY

A 35-year-old man was admitted to the hospital following a gunshot wound to the abdomen. Five days post-surgery he developed an infection at the wound site. Purulent material from his wound yielded a pure culture of *S. aureus*. Two days later, the final report of the culture and antimicrobial susceptibility testing studies was issued. The only susceptible result was for vancomycin. After receiving the report, the physician called the laboratory and requested that additional agents be tested since the patient had a history of adverse reactions to vancomycin. Several other agents had been tested but not reported, based on the laboratory's selective reporting protocol. The internal laboratory report seen below shows results for all of the antimicrobial agents tested.

Ciprofloxacin	R
Clindamycin	R
Erythromycin	R
Gentamicin	S
Linezolid	S
Oxacillin	R
Penicillin	R
Tetracycline	R
Trimethoprim/sulfamethoxazole	S
Vancomycin	S

After working through this chapter the reader will know how to respond to the physician.

ANTIMICROBIAL RESISTANCE

Penicillin Resistant *S. aureus*

In 1944, two years after the introduction of penicillin, the first penicillin resistant *S. aureus* was reported. It was found that this isolate produced a penicillinase enzyme (a type of β -lactamase) that hydrolyzed the beta-lactam ring of penicillin. As stated above, in many geographic regions today the incidence of penicillin resistance due to beta-lactamase production exceeds 90%

Oxacillin Resistant *S. aureus* (ORSA)

Oxacillin and methicillin are semisynthetic penicillins that are stable to staphylococcal β -lactamase by virtue of the strategic placement of certain side chains on the molecule. These drugs were developed specifically for the treatment of infections

caused by beta-lactamase producing *S. aureus*. However, resistance to methicillin type drugs soon developed by the acquisition of the *mecA* gene. This gene codes for a novel penicillin binding protein PBP2a. This protein participates in cell wall synthesis despite the presence of methicillin type drugs. These isolates were initially termed methicillin resistant *S. aureus* or MRSA although they are more appropriately termed oxacillin resistant *S. aureus* or ORSA since oxacillin is the drug that is generally tested in the laboratory. ORSA are considered resistant to all penicillinase-stable penicillins including oxacillin, methicillin, nafcillin, cloxacillin, and dicloxacillin. In addition, all ORSA are resistant to all other beta-lactam agents. ORSA usually are resistant to multiple classes of agents including macrolides, lincosamides and tetracyclines. They also can be resistant to fluoroquinolones and aminoglycosides.

ORSA can demonstrate homogenous or heterogeneous resistance to oxacillin. In homogeneously resistant strain, all daughter strains have *mecA* and are oxacillin resistant. In heterogeneously resistant strains, all daughter strains have *mecA* but many do not express resistance to oxacillin.

Borderline Oxacillin Resistance

Infrequently, *S. aureus* have oxacillin MICs that are near the interpretative breakpoint for resistance and are referred to as BORSA for borderline oxacillin resistant *S. aureus*. Unlike ORSA these strains may be treatable with beta-lactam/beta-lactamase inhibitor combinations. They do not contain *mecA*, usually are not multiply resistant, and usually do not grow on oxacillin-salt agar. BORSA can be due to one of several mechanisms. Some strains are hyperproducers of β -lactamase that partially inactivates oxacillin and other beta-lactams. In other rare strains, there are modifications of PBP 1, 2, and 4 that do not bind oxacillin efficiently. Finally, some strains with oxacillin MICs near the susceptible breakpoint actually contain *mecA*, exhibit heteroresistance and generally are not referred to as BORSA.

MLS Resistance

Resistance to macrolides such as erythromycin, and lincosamides such as clindamycin, usually is due to an *erm* gene. These *erm* genes code for production of an RNA methylase enzyme that modifies the ribosomal binding site of macrolides, lincosamides, and streptogramins B. This is known as MLS resistance. Strains possessing *ermA*, *ermB* or *ermC* typically are erythromycin resistant but, when initially tested, may appear clindamycin susceptible (especially *ermC* strains). In such isolates clindamycin resistance is expressed after induction with erythromycin.

msrA-mediated Resistance

A second mechanism of resistance to erythromycin is mediated by the *msrA* gene. This gene encodes an efflux pump that pumps erythromycin out of the cell. Strains with *msrA* are susceptible to clindamycin but they are resistant to streptogramins B.

Vancomycin Resistance

Vancomycin-intermediate *S. aureus* (VISA)

Recently strains with reduced susceptibility to vancomycin (MICs 8–16 µg/mL) and fully resistant (MICs ≥32 µg/mL) have been described. The former strains are termed vancomycin-intermediate *S. aureus* (VISA) or glycopeptide-intermediate *S. aureus* (GISA). The mechanism of reduced susceptibility to vancomycin is unclear but is due in part to a thickened cell wall containing precursors capable of binding vancomycin extracellularly. Most VISAs are ORSA containing *mecA* but some have lost the *mecA* gene and have become oxacillin susceptible. Most VISAs have been detected in patients with a history of vancomycin use and ORSA infection. Fewer than 50 clinical cases of VISA infection have been reported worldwide. However, dozens of strains with MICs of 4 µg/mL, which is the upper end of susceptible, have been reported. These strains should be considered potential VISA and retesting is recommended.

Full Resistance to Vancomycin

S. aureus strains with full resistance to vancomycin are termed VRSA. As mentioned earlier they are extremely rare. Only three strains have been detected as of mid-2004.

Resistance in CoNS

Antimicrobial resistance in CoNS is similar to that seen in *S. aureus*, although they generally are more resistant. As in *S. aureus*, *mecA* is responsible for most oxacillin resistance. Reduced vancomycin susceptibility is more frequent in *S. haemolyticus* and *S. epidermidis* than in *S. aureus*.

METHODS

Consult Table 1 and Glossary I in the NCCLS M-100 for guidance on the selection of antimicrobial agents to test against staphylococci.

Disk Diffusion Testing

The disk diffusion method can be used for antimicrobial susceptibility testing of staphylococci with some modifications. The inoculum should be prepared using the direct colony suspension method. This method is preferred over the broth culture methods because the slowly growing, heteroresistant subpopulations may be overwhelmed by the more rapidly growing susceptible cells. For the same reasons, a full 24-hour incubation should be allowed before determining oxacillin susceptibility. Finally, oxacillin zones of inhibition should be measured with transmitted rather than reflected light (Figure 8.1). Use of transmitted light allows for better detection of heteroresistant ORSA since these subpopulations may only be visible as a slight haze of growth (Figure 8.2). Homogeneously ORSA exhibit confluent growth around oxacillin disk (Figure 8.3).

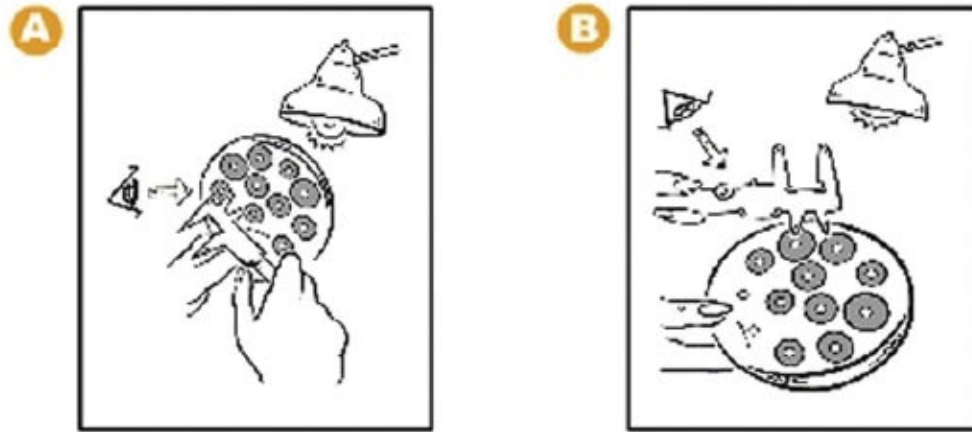


Figure 8.1—Transmitted (A) vs reflected light (B)

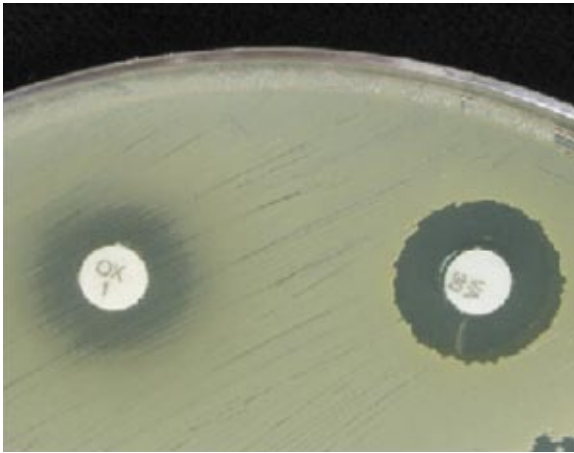


Figure 8.2—A slight haze around the oxacillin disk indicates heteroresistance



Figure 8.3—Heavy growth up to the oxacillin disk reflects homogeneous resistance

Oxacillin Interpretative Criteria

As seen in the table below, oxacillin interpretative criteria for *S. aureus* are different from those for CoNS. For *S. aureus* against oxacillin, a zone of inhibition ≥ 13 mm is susceptible, and ≤ 10 mm is resistant. The same disk diffusion method is used for all *Staphylococcus* spp. but the oxacillin interpretative criteria for CoNS are larger; a zone ≥ 18 mm is susceptible and ≤ 17 mm is resistant.

Oxacillin Interpretive Criteria for Disk Diffusion (mm)

Organism	Susceptible	Intermediate	Resistant
<i>S. aureus</i>	≥13	11–12	≤10
CoNS	≥18		≤17

Use of Cefoxitin Disk Test to Predict Oxacillin Resistance

Recently the NCCLS has recommended a cefoxitin disk susceptibility test using alternative breakpoints as an accurate method to predict *mecA*-mediated oxacillin resistance in staphylococci. The standard disk diffusion method with a cefoxitin 30 µg disk is used with the following interpretative breakpoints:

Cefoxitin Disk Interpretive Criteria for Oxacillin Susceptibility

Organism	Cefoxitin Disk Zone Diameter (mm)	
	Oxacillin Susceptible	Oxacillin Resistant
<i>S. aureus</i>	≤20	≥19
CoNS	≤25	≥24

Staphylococcal isolates with cefoxitin zone diameter sizes greater than or equal the appropriate breakpoint should be reported as oxacillin susceptible whereas isolates with cefoxitin zone diameter sizes less than or equal the appropriate breakpoint should be reported as oxacillin resistant.

Broth Microdilution MIC Testing

The broth microdilution method can be used for antimicrobial susceptibility testing of staphylococci with some modifications. The inoculum should be prepared using the direct colony suspension method.

For staphylococcal isolates that yield intermediate penicillin MICs of 0.06–0.12 µg/mL, susceptibility should be confirmed after induction with oxacillin. Some of these strains may produce small quantities of beta-lactamase that can affect the patient's response to therapy. Induction can be accomplished by inoculating the isolate onto a blood agar and adding an oxacillin disk. It is not necessary to use a standardized suspension for the inoculation. After overnight incubation, growth from the periphery of the inhibition zone should be selected for a conventional beta-lactamase test (Figure 8.4). Review Chapter 2 for details. If the result is positive the isolate is reported as penicillin resistant. See algorithm in Figure 8.5.

Cation-adjusted Mueller-Hinton broth plus 2% NaCl should be used as the test medium for oxacillin testing and a full 24-hour incubation should be allowed before determining oxacillin and vancomycin susceptibility. Oxacillin interpretative criteria for *S. aureus* are different from those for CoNS. For *S. aureus* against oxacillin, an MIC of ≤2.0 µg/mL is susceptible and ≥4 µg/mL is resistant. For CoNS an oxacillin MIC of ≤0.25 µg/mL is susceptible and ≥0.5 µg/mL is resistant.

The interpretative criteria developed by NCCLS for testing CoNS against oxacillin were based on the presence of *mecA* in resistant strains. The breakpoints are applied to all CoNS although they work best with *S. epidermidis*. For serious infec-



Figure 8.4—Selecting cells that have been induced for beta-lactamase production

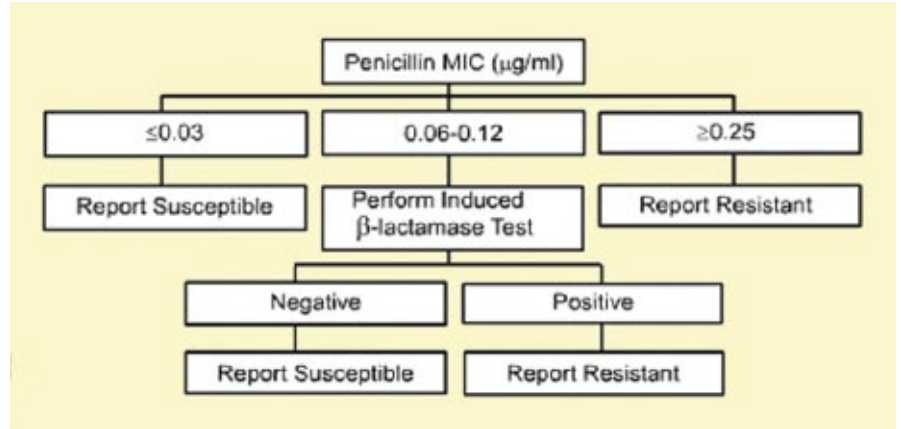


Figure 8.5—Strategy for testing staphylococci with intermediate penicillin MICs

Oxacillin Interpretive Criteria for MIC (µg/mL)

Organism	Susceptible	Resistant
<i>S. aureus</i>	≤2.0	≥4.0
CoNS	≤0.25	≥0.5

tions caused by other CoNS with oxacillin MICs of 0.5–2 µg/mL, additional tests for *mecA* or PBP2a may be appropriate. Strains that are *mecA* negative or do not produce PBP2a should be reported as oxacillin susceptible.

Occasionally, *mecA* isolates of *S. aureus* have an oxacillin MIC of 4 µg/mL which is the resistance breakpoint. These heteroresistant strains must be distinguished from *mecA*-negative strains that have borderline MIC results because of a different resistance mechanism. See algorithm in Figure 8.6. After confirming that the culture is pure and the identification is correct the isolate should be tested by a rapid method for *mecA* or PBP 2a. If either test is positive, the strain should be reported as ORSA. If they are negative, the MIC should be repeated. If the MIC is ≤2 µg/mL the strain should be reported as oxacillin susceptible. If the MIC is ≥4 µg/mL, the strain should be reported as resistant with an unusual resistance mechanism.

Inducible Clindamycin Resistance Testing

As mentioned above, staphylococci can be resistant to erythromycin via either the *erm* or *msrA* genes. Strains with *erm*-mediated erythromycin resistance may possess inducible clindamycin resistance and may appear susceptible to clindamycin by disk diffusion. Such strains should be tested for this attribute by placing erythromycin and clindamycin disks 20 mm apart on a MHA plate inoculated with the strain in question. After overnight incubation, the plate should be observed for a blunted clindamycin zone of inhibition (Figure 8.7). If the zone is not blunted, the isolate should be reported as susceptible. If the zone is blunted, the isolate should be reported either as resistant or as susceptible with a comment that resistance may develop during clindamycin therapy.

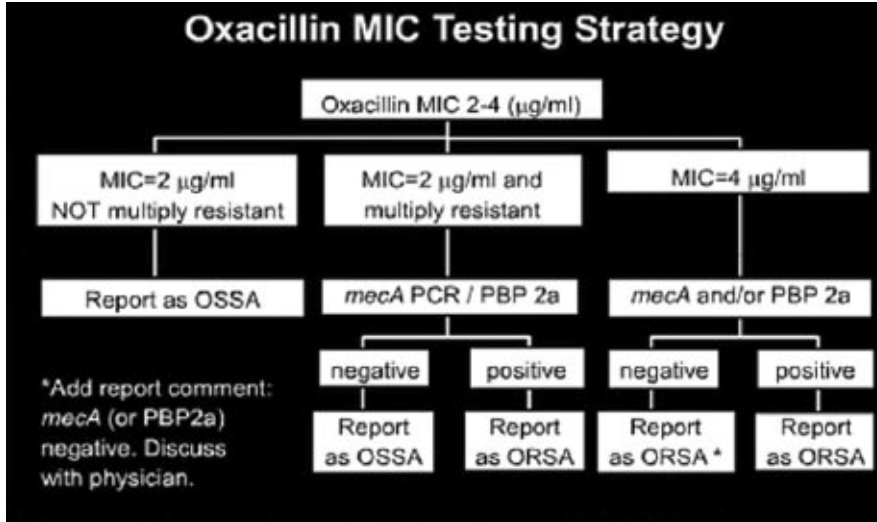


Figure 8.6—Strategy for testing isolates with an oxacillin MIC 2 to 4 µg/ml



Figure 8.7—Blunting of clindamycin zone due to inducible resistance.

Summary of erythromycin and clindamycin profiles in *S. aureus*

Erythromycin	Clindamycin	Genetic determinant	Inducible clindamycin-R?
S	S	–	No
R	R	<i>ermB</i>	No, already R
R	S	<i>ermC</i>	Yes
R	S	<i>msrA</i>	No

VISA/VRSA Detection

The NCCLS reference broth microdilution is reliable for the detection of VISA (vancomycin MIC 8–16 µg/mL) and VRSA (vancomycin MIC > 16µg/mL). The E-test also performs well for detection of these organisms. Disk diffusion and some automated commercial methods are unreliable for the detection of VISA or VRSA. If these methods are used, supplemental testing using growth on BHI-vancomycin (6 µ/mL) agar should be performed at least on all ORSA strains. For strains that grow on this medium or if the patient is failing vancomycin therapy, a vancomycin MIC should be performed. VISA grow slower than typical *S. aureus* and may take two days to develop visible colonies. Colony morphology is variable on the same plate with large white-to-cream colonies and small grayish colonies (Figure 8.8). For suspected VISA, (MIC 4–16 µg/mL) the identification and susceptibility should be repeated. In addition, the institution's infection control department, the local health department, as well as the CDC (SEARCH@cdc.gov) should be contacted. The isolate should be sent to the CDC for confirmation of the identification and vancomycin susceptibility results. Finally, the isolate should be saved.

CDC confirmation criteria for VISA are:

- Vancomycin broth microdilution MIC = 8–16 µg/mL
- Vancomycin E test MIC = 6–16 µg/mL
- Growth on BHI-vancomycin (6 µg/mL) agar in 24 hours



Figure 8.8—Variable colonial morphology of VISA after 48 hours of incubation

As with *S. aureus*, disk diffusion and some commercial systems have not been reliable in detecting CoNS with decreased susceptibility to vancomycin. Species that are most likely to appear less susceptible to vancomycin include *S. epidermidis* and *S. haemolyticus*. Clinical response may be poor in some patients infected with CoNS species that have decreased susceptibility to vancomycin. Therefore, if CoNS from normally sterile body sites have vancomycin MICs ≥ 4 $\mu\text{g/mL}$, it may be useful to determine the species identification.

Agar Dilution MIC Testing for All Agents

When testing staphylococci by the agar dilution MIC method the direct colony suspension inoculum preparation should be used and incubation should be for a full 24-hours. For oxacillin the test medium should be Mueller-Hinton agar supplemented with 2% NaCl

Oxacillin-salt Agar Screen Test for *S. aureus*

The oxacillin-salt agar screen test is useful as a screen for ORSA or to definitively confirm oxacillin results that are equivocal by other methods. It is not reliable for CoNS. It should be inoculated using the direct colony suspension that matches a 0.5 McFarland standard. It should be spot inoculated with a 1 μL loop or a swab dipped into the inoculum suspension and spread over an area the size of a dime. The plate is then incubated for a full 24 hours. The appearance of > 1 colony or film of growth (including a light film) indicates oxacillin resistance (Figure 8.9). Quality control is accomplished using *S. aureus* ATCC 29213 (susceptible) and *S. aureus* ATCC 43300 (resistant). Although this test is called a “screen” the results can be considered definitive for assessing oxacillin resistance in *S. aureus*.

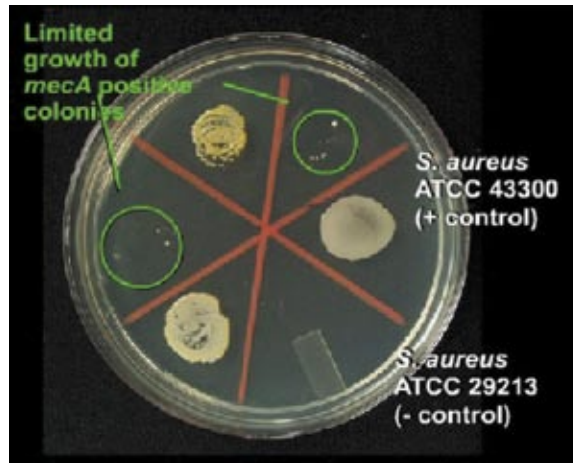


Figure 8.9—Oxacillin salt agar screen for *mecA* positive strains

Molecular Testing for the *mecA* Gene or Its Product, PBP2a

Molecular testing for the *mecA* gene or its product, PBP2a, can also be performed to determine the oxacillin resistant phenotype. Molecular tests for *mecA* include a cycling probe reaction (available as a FDA-cleared kit) and an in-house polymerase chain reaction (PCR). PBP2a can be detected by a commercially available latex agglutination test.

REPORTING RESULTS—BETA-LACTAMS

Results from testing oxacillin and penicillin can be used to predict the susceptibility to other beta-lactam antibiotics. Therefore, penicillin and oxacillin are the only beta-lactams that need to be tested. If a given strain is susceptible to both antibiotics, it is also susceptible to other penicillins, plus cephems and carbapenems. If it is resistant to penicillin but susceptible to oxacillin, it is resistant to beta-lactamase labile penicillins but susceptible to beta-lactamase stable penicillins, and beta-lactam/beta-lactamase inhibitor combinations as well as cephems and carbapenems. If the strain is resistant to both penicillin and oxacillin, it is resistant to *all* beta-lactams.

Results with other beta-lactam antibiotics may be reported to the physician by way of a comment. For example if a strain is oxacillin susceptible but penicillin resistant, the comment may state “cefazolin and other beta-lactams (except amoxicillin, ampicillin, and penicillins) are active against oxacillin susceptible and penicillin resistant staphylococci.” Oxacillin resistant staphylococci, regardless of in vitro susceptibility results, should be reported as resistant to all beta-lactams, including carbapenems and combinations of beta-lactam/beta-lactamase inhibitors.

S. saprophyticus

S. saprophyticus is a CoNS that causes urinary tract infections. These infections usually are successfully treated with common urinary tract antimicrobial agents. A comment to this effect can be added to the report. Although the oxacillin MICs of *S. saprophyticus* typically range between 0.5–2.0 µg/mL (in the resistant category) this species usually lacks the *mecA* gene. Thus susceptibility testing is not recommended for urinary tract isolates.

Quality Control

Refer to Chapter 6 of this manual for specific instructions on quality control of testing staphylococci. NCCLS-recommended QC strains are:

- Disk diffusion—*S. aureus* ATCC 25923 (penicillin susceptible strain)
- MIC tests—*S. aureus* ATCC 29213 (penicillin resistant, beta-lactamase positive strain)
- Oxacillin-salt screen agar—*S. aureus* ATCC 29213 (oxacillin susceptible) & 43300 (oxacillin resistant)

CASE STUDY COMMENTARY

Review the Case Study in the Introduction to this Chapter and the antibiotic susceptibilities shown below. Now you should be able to explain the rationale behind your routine reporting protocol and provide appropriate supplemental results as requested by the physician. Recall that vancomycin is not appropriate for this patient.

Results Reported

Specimen source: Wound

Results: Many *Staphylococcus aureus*

Clindamycin	R
Erythromycin	R
Oxacillin	R
Penicillin	R
Vancomycin	S

Results from all Drugs Tested

Specimen source: Wound

Results: Many *Staphylococcus aureus*

Ciprofloxacin	R
Clindamycin	R
Erythromycin	R
Gentamicin	S
Linezolid	S
Oxacillin	R
Penicillin	R

Tetracycline	R
Trimethoprim-Sulfamethoxazole	S
Vancomycin	S

An appropriate response to the physician would be an explanation of the rationale for (or against) testing and reporting of the supplemental antimicrobial agents for the patient's ORSA.

- Gentamicin. Although the isolate is susceptible to gentamicin, this drug is not reported routinely because aminoglycosides are not considered first-line agents against staphylococci. Sometimes they are used in combination with a cell wall active agent (e.g. vancomycin) to treat serious staphylococcal infections.
- Fluoroquinolones. The patient's isolate is resistant to ciprofloxacin, which is a fluoroquinolone. This is not uncommon for ORSA. Staphylococci that are resistant to one fluoroquinolone are typically resistant to other fluoroquinolones.
- Trimethoprim/sulfamethoxazole. The isolate is susceptible to this drug. Some laboratories routinely report trimethoprim/sulfamethoxazole on ORSA although it is not considered a first-line agent for most ORSA infections.
- Beta-lactams other than oxacillin. The isolate is an ORSA and is therefore resistant to all beta-lactams.
- Tetracyclines. The isolate is resistant to tetracycline. However, it is possible that other tetracyclines (e.g. doxycycline, minocycline) would be more active than tetracycline against staphylococci, and might warrant testing if this were a consideration for treatment based on the patient's condition.
- Linezolid. The new oxazolidinone agent, linezolid, is not on the laboratory's routine *Staphylococcus* panel, but linezolid is being used for treating some types of ORSA infections. Although resistance among staphylococci is very uncommon, linezolid should be tested if linezolid therapy is being considered.
- Others. Chloramphenicol, quinupristin-dalfopristin, and rifampin are not on the laboratory's routine *Staphylococcus* panel; however, if any of these are considered for therapy, they should be tested.
- Other Macrolides besides erythromycin. Because the isolate is erythromycin resistant, it can be considered resistant to azithromycin and clarithromycin. These three drugs are listed together in NCCLS Table 1 with an "or", which denotes that they have essentially the same activity against staphylococci.

Since ORSA often are multiply resistant, there are limited therapeutic options for treating infections caused by ORSA. If vancomycin, the primary agent prescribed, is not appropriate for a particular patient with an ORSA infection, it would be best for the physician to confer with an infectious disease physician or clinical pharmacist when developing a treatment plan. This plan would be based on the clinical condition of the patient and results from testing supplemental agents.

The infectious diseases physician may ask the laboratory to test agents not on the laboratory's routine panel. Each laboratory should develop a strategy for handling agents not on the routine panel. This might involve a backup test system or identification of a reference laboratory that can test supplemental agents in a timely manner. In this case, the physician treated the patient successfully with linezolid.

REVIEW

Remember to:

- Use the most current NCCLS standards (M2 and M7) for testing staphylococci.
- Used the oxacillin-salt agar screen test only for *S. aureus* and not for CoNS.
- For confirmed ORSA and oxacillin-resistant CoNS report all beta-lactams, including carbapenems and beta-lactamase inhibitor combinations, as resistant, despite in vitro results.
- Use results from testing oxacillin and penicillin to deduce results for other beta-lactams for staphylococci.
- Note special testing concerns for detecting staphylococci with decreased susceptibility to vancomycin.

SELF-ASSESSMENT QUESTIONS

1. For each of the following characteristics, indicate those that relate to ORSA and those that relate to BORSA
 - A. Contains *mecA*
 - B. Contains a new penicillin-binding protein, PBP 2a
 - C. Contains modified PBPs 1, 2, and 4
 - D. Is multiply resistant
 - E. Is infrequently encountered in clinical specimens
2. Which of the following beta-lactams should be included in a test panel for *Staphylococcus* spp.? Select all that apply.
 - A. Penicillin
 - B. Oxacillin
 - C. Cephalosporin
3. Which of the following additional classes of antimicrobial agents should be included in a test panel for staphylococci?
 - A. Aminoglycosides
 - B. Clindamycin
 - C. Fluoroquinolones
 - D. Glycopeptides
 - E. Macrolides
 - F. Tetracyclines
 - G. Trimethoprim-sulfamethoxazole
4. If you place disk diffusion tests in the incubator at 3PM, will you detect all ORSA if you read the plates at 7AM the next morning?
 - A. Yes
 - B. No
5. Which of the following are correct for disk diffusion or broth microdilution MIC testing of staphylococci? Select all that apply.
 - A. Direct colony suspension inoculum
 - B. Log or stationary phase inoculum
 - C. Addition of 2% NaCl to cation-adjusted Mueller-Hinton broth for broth microdilution testing

- D. 16–18 hours incubation for all drugs
 - E. 24 hours of incubation for oxacillin
 - F. 24 hours of incubation for vancomycin
6. Indicate if each of the following profiles is typical of ORSA.

Drug	A	B	C
Clindamycin	R	S	R
Erythromycin	R	S	R
Oxacillin	R	R	R
Penicillin	R	R	S
Trimethoprim-sulfamethoxazole	R	S	S
Vancomycin	S	S	S

7. Which of the following would be correct for an erythromycin-resistant and clindamycin-susceptible *S. aureus*? Select all that apply.
- A. Report clindamycin S
 - B. Report clindamycin R
 - C. Perform clindamycin induction test routinely, and if positive, report R, if negative report S.
 - D. Perform clindamycin induction test only on request. If positive, report S with a comment that resistance may develop during clindamycin therapy. If negative, report S.
8. Answer the following as True or False
- A. Disk diffusion methods reliably detect VISA.
 - B. It is important to inform infection control and local public health authorities about patients with probable VISA.
 - C. VISA isolates should be saved for further studies, including submission to public health authorities.
 - D. *S. aureus* with a vancomycin MIC of 4 µg/mL should be considered suspicious for VISA
 - E. Growth of *S. aureus* on BHI-vancomycin (6 µg/mL) agar screen plate after 24 hours of incubation is suggestive of VISA
 - F. VISA isolates are commonly encountered.
9. For a *S. aureus* that is penicillin resistant and oxacillin susceptible, which of the following would be considered susceptible? Select all that apply.
- A. Amoxicillin-clavulanate
 - B. Ampicillin
 - C. Cephalothin
 - D. Piperacillin
10. What should you do?
- Scenario: You isolate *S. saprophyticus* from two blood cultures and a urine culture. *S. saprophyticus* in blood is uncommon but may occur. The physician is interested in susceptibility results, particularly for oxacillin.
- A. Perform an oxacillin disk diffusion test.
 - B. Perform an oxacillin MIC test and use *S. aureus* interpretative criteria.
 - C. Perform an oxacillin MIC test and use CoNS interpretative criteria.
 - D. Perform a *mecA* or PBP2a analysis

-
- E. Inform the physician that there are no reliable susceptibility tests for *S. saprophyticus*.
11. Answer the following as True or False
- A. Either the direct colony suspension or the log phase growth method can be used for preparing inocula for disk diffusion or MIC tests with staphylococci.
 - B. The oxacillin-salt agar screen plate can be used to test for oxacillin resistance in both *S. aureus* and CoNS
 - C. Results of testing oxacillin should be used to predict the activity of cepheems against staphylococci.
 - D. The vancomycin disk diffusion test is unreliable for *S. aureus*.

9

Enterococci

OBJECTIVES

After completing this chapter the reader should be able to:

- Discuss a practical protocol for antibiotic susceptibility testing (AST) of *Enterococcus* spp. in a clinical laboratory.
- List the testing conditions for disk diffusion and MIC tests of *Enterococcus* spp. including test medium, inoculum preparation and atmosphere of incubation.
- Explain vancomycin resistance in enterococci including intrinsic low-level resistance versus acquired higher-level resistance.
- Describe aminoglycoside resistance in enterococci and methods to detect high-level resistance.
- Define laboratory reporting strategies for limiting vancomycin usage.

BACKGROUND

Enterococcus spp. are common inhabitants of the gastrointestinal tract. In most immunocompetent individuals the organism does not cause serious infections, unless it invades heart valves and causes endocarditis. This syndrome causes a life-threatening disease requiring therapy with an aminoglycoside and a cell wall-active agent. The organism also causes urinary tract infections, wound infections and septicemia, particularly in a debilitated host. *Enterococcus* spp. are intrinsically resistant to many antimicrobial agents, including clindamycin, oxacillin, and cephalosporins. Treatment of serious enterococcal infections such as endocarditis requires a cell-wall active agent (e.g., ampicillin, penicillin or vancomycin) plus an aminoglycoside such as gentamicin or streptomycin. Less serious infections such as urinary tract infections can be treated with a single agent, such as ampicillin or nitrofurantoin.

Increasing numbers of *Enterococcus* spp. have developed resistance to ampicillin, vancomycin and exhibit high-level resistance to aminoglycosides. Newer agents such as linezolid and quinupristin-dalfopristin may be used to treat strains of vancomycin-resistant enterococci (VRE).

CASE STUDY

A 54-year-old man presented to the emergency room with a fever of 102°F, myalgias, and shaking chills. One week earlier, he noted burning on urination and discoloration of his urine, but did not see his physician. On examination, he was

noted to have a petechial rash on his trunk, splinter hemorrhages under his fingernails, and a new heart murmur. Urine and blood cultures were obtained, and the patient was started on ampicillin and gentamicin. The following day, all cultures were positive for gram-positive cocci in pairs and short chains. The organism was identified as *Enterococcus faecium* and a laboratory report was issued (see below). The patient was diagnosed with bacterial endocarditis. The surgical resident overseeing the patient noted that the organism was reported as ampicillin resistant but gentamicin synergy screen susceptible. She inquired if this meant that gentamicin alone would be adequate therapy.

Lab Report

Specimen

Source: blood

Results: *Enterococcus faecium*

Antimicrobial	MIC (mcg/mL)	Susceptibility
Ampicillin	64	R
Vancomycin	≥0.5	S
Gentamicin synergy		S
Streptomycin synergy		S

How would you respond to the physician? After working through this chapter you will be able to answer this question.

Resistance in Enterococci

Types of Resistance in Enterococci	
Intrinsic	Acquired
<p><i>Enterococcus</i> spp. have naturally occurring, or intrinsic, resistance to:</p> <ul style="list-style-type: none"> • Cephalosporins • Clindamycin • Penicillinase-stable penicillins (e.g., oxacillin) • Trimethoprim-sulfamethoxazole • Low or therapeutic concentrations of aminoglycosides 	<p>Enterococci may acquire resistance to many antimicrobial agents. Among the most important are:</p> <ul style="list-style-type: none"> • Ampicillin, penicillin, and vancomycin • High-level gentamicin and streptomycin

Resistance to Ampicillin and Penicillin

Resistance to ampicillin and penicillin in enterococci is primarily due to changes in the penicillin-binding proteins (PBPs) which decrease the affinity of the PBP target proteins for beta-lactam drugs. Since the drugs do not bind to their cellular targets, they no longer initiate destruction of the cell wall. *Enterococcus faecalis* strains typically are susceptible to ampicillin and penicillin, while *Enterococcus faecium* often are resistant. Resistance due to beta-lactamase production is rare.

Resistance to High-level Aminoglycosides

Some enterococci produce aminoglycoside-modifying enzymes that confer high-level aminoglycoside resistance (HLAR). These include aminoglycoside adenyltransferases (AAD), aminoglycoside phosphotransferases (APH), and aminoglycoside acetyltransferases (AAC). When the enzyme modifies the aminoglycoside, the aminoglycoside cannot be transported into the cell to exert an antibacterial effect. Thus there is **no synergy with cell wall-active agents**. Aminoglycoside-modifying enzymes may modify one or more aminoglycosides, depending on the activity spectrum of the enzyme. Some enterococci may produce multiple enzymes. Review the table below to see the common aminoglycoside-modifying enzymes and the aminoglycosides that they modify.

Aminoglycoside-modifying Enzymes in *Enterococcus* spp.

Enzyme	Aminoglycoside ^a				
	Str	Gm	Tob	Net	Amk/Kan
AAD (6')	Y ^b	N ^c	N	N	N
APH (3')	N	N	N	N	Y
AAC (6') + APH (2')	N	Y	Y	Y	Y
AAC (6') ^d	N	N	Y	Y	Y

^a Streptomycin (str), gentamicin (gm), tobramycin (tob), netilmicin (net), amikacin/kanamycin (amk/kan)

^b Y, does modify the respective aminoglycoside

^c N, does not affect the activity of the respective aminoglycoside

^d Found in virtually all *E. faecium*

HLAR to streptomycin may be due to either aminoglycoside-modifying enzymes or to alteration of the ribosomes, which decreases the ability of the ribosome to bind the aminoglycoside

Working together, beta-lactams and gentamicin can exert a **bactericidal synergy** on enterococci.

Vancomycin Genotype and Phenotype Designations

Vancomycin resistance can be classified by genotype or phenotype.

Genotype. The *vanA*, *vanB*, *vanC*, *vanE* and *vanG* genes together with several supplementary genes mediate vancomycin resistance.

Phenotype. The phenotype is the measurable effect of gene expression. For example, if the organism carries the *vanA* gene cluster, it will have high vancomycin (and teichoplanin) MICs. The genotype is *vanA*. (All genes are designated in lower case and italics). The phenotype is the high level MICs for these drugs and is designated VanA.

Technical tip: Teichoplanin is not therapeutically used in the USA, but can be helpful to determine the vancomycin phenotype of an enterococcus.

Acquired Resistance to Vancomycin

Isolates of some enterococcal species can become resistant to vancomycin by acquisition of *vanA* or *vanB* or less frequently *vanD*, *vanE* or *vanG* genes. These strains with **acquired vancomycin resistance** usually are referred to as “VRE.” Their containment deserves special attention from infection control personnel. *Enterococcus faecium* and *E. faecalis* are the most common VRE. *E. faecium* is more likely to be VRE than *E. faecalis*.

Intrinsic Resistance to Vancomycin

Intrinsic low-level resistance in enterococci usually is due to the presence of *vanC* genes. These genes inhibit the organism from binding vancomycin. Intrinsic resistance is unlikely to be spread from patient to patient and usually is not a concern for infection control

Resistance—Typical Patterns of *Enterococcus* species

Typical susceptibility patterns by species include:

- *E. faecalis*
Usually susceptible to ampicillin and penicillin.
Can acquire resistance to vancomycin, usually due to *vanA* or *vanB*.
Occasionally produce beta-lactamase.
- *E. faecium*
Often resistant to ampicillin and penicillin.
Can acquire resistance to vancomycin, usually due to *vanA* or *vanB*.
- *E. gallinarum* and *E. casseliflavus*
Have **intrinsic** low level vancomycin resistance due to the *vanC* gene.
- *E. raffinosus*, *E. avium* and *E. durans*
Can acquire vancomycin resistance due to the *vanA* or *vanB* genes or, less frequently, the *vanD*, *vanE*, or *vanG* genes.

Testing Strategy

NCCLS suggests the following strategy for enterococci (NCCLS M100, Table 1, Group A).

- Ampicillin or penicillin should be tested and reported routinely.
- Vancomycin and linezolid should be tested routinely and reported selectively (NCCLS Group B).
- Only *E. faecium* isolates should be tested for quinupristin-dalfopristin.
- Susceptibility tests generally are performed on enterococci isolated from sterile body sites. Isolates from other sites may be tested depending on the institution's practices and the patient population.

Methods

NCCLS has specific standards for testing *Enterococcus* spp. For vancomycin the routine disk diffusion and MIC methods are used with the modifications listed below:

Modifications for testing *Enterococcus* spp. with vancomycin

Method	Medium	Incubation time (h)
Disk diffusion ^a	MHA ^b	24
Broth or agar dilution	CAMHB ^c	24

^a Any zone of inhibition must be examined with transmitted light and ANY growth is considered significant. Isolates that produce zones of inhibition in the intermediate range should be tested by an MIC method.

^b Mueller-Hinton agar.

^c Cation-adjusted Mueller-Hinton broth.

Interpreting Results

In NCCLS M100, Table 2D (in both the disk diffusion and MIC sections) there are specific interpretive criteria for enterococci tested with ampicillin and penicillin. The MIC interpretive criteria for both ampicillin and penicillin are:

<8 mcg/mL = Susceptible

>16 mcg/mL = Resistant

NOTE: There is no intermediate breakpoint for either antimicrobial agent.

In *E. faecium*, low-level resistance to ampicillin or penicillin is associated with MICs of 16–32 mcg/ml or 32–64 mcg/ml, respectively. However, *E. faecium* can acquire high-level beta-lactam resistance (ampicillin MICs > 64 mcg/ml and penicillin MICs > 128 mcg/ml). *E. faecium* with low level ampicillin or penicillin resistance may continue to show synergy with a susceptible aminoglycoside, whereas those with high level resistance generally do not show synergy.

Beta-lactamase Testing

Beta-lactamase testing in enterococci is best performed by using the chromogenic cephalosporin test (nitrocefin). Because the amount of beta-lactamase produced by enterococci is low, standard disk diffusion and MIC tests often do not correctly classify these strains as resistant to penicillin and ampicillin. Isolates that produce beta-lactamase should be reported as resistant to ampicillin and penicillin despite susceptible disk diffusion or MIC results.

Vancomycin Agar Screen Test

The vancomycin agar screen test is performed on enterococcus isolates available in pure culture, or when isolated colonies are available. It is a simple and inexpensive method to screen for VRE. The screen plate contains 6 mcg/mL of vancomycin in brain heart infusion agar (BHIA).

To perform the test:

1. Use the growth or direct colony suspension method to prepare an inoculum suspension standardized to the turbidity of a 0.5 McFarland standard.
2. Using a device that delivers 1 to 10 μL of the standardized suspension, inoculate the test medium in a “spot” on the agar surface (if care is taken to avoid cross contamination, up to eight isolates can be tested on one 100-mm plate,).
3. Incubate the plate for a full 24 h before reporting susceptible results.
4. Consider >1 colony as “presumptive” resistance. Presumptive resistance means that the isolate may have acquired vancomycin resistance; however, it may be a species with intrinsic vancomycin resistance.

Technical tip: Identification tests together with disk diffusion or MIC tests are needed to differentiate isolates with intrinsic versus acquired vancomycin resistance.

Positive and negative **controls** must be used each time the test is performed.

- Negative control: *E. faecalis* ATCC 29212 (vancomycin susceptible)
- Positive control: *E. faecalis* ATCC 51299 (vancomycin resistant).

Synergy Screen tests for High-level Aminoglycoside Resistance

High level aminoglycoside resistance-synergy screen tests are performed with a special disk or a single concentration of gentamicin or streptomycin to determine if the aminoglycoside will act synergistically in combination with a cell wall-active agent. Testing of gentamicin and streptomycin predicts the activities of other aminoglycosides.

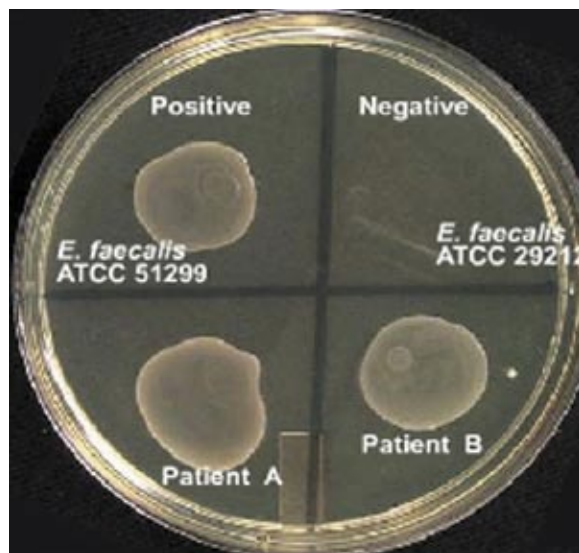


Figure 9.1—A vancomycin screen plate with positive and negative controls

The disk diffusion synergy screen test

- Uses the standard disk diffusion procedure
- Uses high content disks: gentamicin 120 mcg and streptomycin 300 mcg.
- Is incubated 16–18 h at 35°C in ambient air.

Results—Interpreting zone diameters

- <6 mm = resistant
- >10 mm = susceptible.
- 7–9 mm = inconclusive and indicate that an MIC test must be performed.

MIC Screening Tests for High-level Aminoglycoside Resistance

Procedure

For both the broth and agar dilution methods, follow standard NCCLS procedures with the following modifications:

1. For broth and agar dilution tests, test the following single concentrations:
 - 500 mcg/mL gentamicin in CAMHB or MHA
 - 1,000 mcg/mL streptomycin in CAMHB
 - 2,000 mcg/mL streptomycin in MHA
2. For the agar screen, spot 10 µl of a 0.5 McFarland standardized suspension of the organism being tested onto the agar surface.
3. Incubate gentamicin tests for 24 h at 35°C in ambient air
4. Incubate streptomycin tests for 24–48 h (if susceptible at 24 h, reincubate the test for an additional 24 h) at 35°C in ambient air.

Result Interpretation

- No growth is a susceptible result
- Agar: growth of >1 colony indicates resistance
- Broth: any turbidity or growth indicates resistance

Quality Control

To ensure that the results of the high-level aminoglycoside screening tests are accurate, it is essential to include the following controls with each test.

- The negative control, *E. faecalis* ATCC 29212, is susceptible to gentamicin at 500 mcg/mL and streptomycin at 1,000 mcg/mL (broth) or 2,000 mcg/mL (agar).
- The positive control, *E. faecalis* ATCC 51299, is resistant to the above concentrations of antibiotic.



Figure 9.2—Yellow pigment of *Enterococcus casseliflavus* on a cotton swab

These recommendations for screen tests for high-level aminoglycoside resistance and vancomycin resistance are found in the MIC portion of NCCLS document M100 Table 2D. This table also includes suggestions for distinguishing isolates with intrinsic versus acquired vancomycin resistance.

Differentiation of *Enterococcus* spp.

Tests for pigment and motility usually are sufficient to differentiate enterococci with acquired vancomycin resistance (usually *E. faecalis* or *E. faecium*) from those with intrinsic vancomycin resistance (*E. gallinarum* or *E. casseliflavus*). As seen in the table below, the methyl-alpha-D-glucopyranoside (MGP) test is useful when results from pigment and motility are equivocal.

Tests to differentiate *Enterococcus* species

Species	Motility	Yellow Pigment ^a	MGP ^b
<i>E. faecalis</i>	—	—	—
<i>E. faecium</i>	—	—	—
<i>E. casseliflavus</i>	+ ^c	+	+
<i>E. gallinarum</i>	+ ^c	—	+

^a Pigment can be seen on a cotton swab.

^b Acidification of methyl-alpha-D-glucopyranoside.

^c Exceptions may occur.

Commercial identification test systems and conventional biochemical tests can be used to obtain a definitive species identification of enterococci. Species identification is recommended for enterococci that are vancomycin resistant.

REPORTING RESULTS—STERILE SITES

Report results of testing cell wall-active agents and high-level aminoglycoside on isolates from normally sterile body sites (e.g. blood, cerebrospinal fluid) for which combination therapy may be needed. Review the table below that indicates how to predict synergy by examining the test results of cell wall-active agents and high-level aminoglycosides.

Aminoglycoside ^a	Cell wall-active drug	
	Susceptible	Resistant
Susceptible	Synergy	No synergy
Resistant	No synergy	No synergy

^a Requires special tests for high-level resistance to gentamicin and streptomycin.

The above table demonstrates that both the cell wall-active agent (ampicillin, penicillin, or vancomycin) and the aminoglycoside must show susceptible results for synergy to occur. If there is resistance to either drug there will be no synergy.

Technical tip: When reporting results, a note should be appended to the report (see example below) to emphasize the need for combination therapy for serious enterococcal infections such as endocarditis.

Lab Report

Specimen
Source: blood
Results: *Enterococcus faecalis**

Antimicrobial	MIC (mcg/mL)	Susceptibility
Ampicillin	2	S
Vancomycin	0.5	S
Gentamicin synergy		S
Streptomycin synergy		S

* Comment: Enterococcal endocarditis requires combined therapy with high-dose penicillin (or high-dose ampicillin, or vancomycin or teicoplanin) plus gentamicin or streptomycin for bactericidal action.

REPORTING RESULTS—URINE

Report the results of additional antimicrobial agents (e.g. fluoroquinolones and nitrofurantoin) that are useful for treating enterococcal urinary tract infections.

It is unnecessary to report the results of aminoglycoside synergy tests since combination therapy is not warranted for uncomplicated enterococcal urinary tract infections (see the following report).

Lab Report

Specimen
Source: Urine
Results: *Enterococcus* spp.

Antimicrobial Agent	Susceptibility
Ampicillin	S
Ciprofloxacin	S
Nitrofurantoin	S
Tetracycline	S
Vancomycin	S

REPORTING RESULTS—WARNING

Results for cephalosporins, clindamycin, trimethoprim-sulfamethoxazole and aminoglycosides (except for high-level resistance or synergy screening), should NOT be reported even though they may demonstrate susceptible results in vitro. These drugs are not clinically useful against enterococci.

Technical tip: Reporting susceptible results on these drugs might encourage a physician to prescribe one of these agents which would be *dangerously misleading*. It is imperative to take precautions to avoid such erroneous reporting.

REPORTING RESULTS—SUPPLEMENTAL AGENTS FOR VRE

The following report for VRE isolated from the blood of a liver transplanted patient, although accurate, does not provide guidance on antimicrobial agents to consider for treating the patient.

Lab Report

Specimen
Source: blood
Results: *Enterococcus faecium*

Antimicrobial Agent	MIC (mcg/mL)	Susceptibility
Ampicillin	>64	R
Vancomycin	>32	R
Gentamicin synergy		R
Streptomycin synergy		R

Supplemental agents to consider reporting for VRE (after consultation with infectious disease physicians, pharmacists, and clinical staff) may include chloramphenicol, erythromycin, linezolid, nitrofurantoin (urine isolates only), quinupristin-dalfopristin, rifampin and tetracycline (or doxycycline).

See the expanded supplemental report below.

Lab Report

Specimen
Source: blood
Results: *Enterococcus faecium**

Antimicrobial Agent	MIC (mcg/mL)	Susceptibility
Ampicillin	>64	R
Chloramphenicol	8	S
Doxycycline	2	S
Erythromycin	>8	R
Linezolid	1	S
Quinopristin-dalfopristin	0.5	S
Rifampin	8	R
Vancomycin	>32	R*
Gentamicin synergy		R
Streptomycin synergy		R

* Comment: VRE isolated. Please notify infection control staff.

Because the most appropriate therapy for VRE infections depends on a variety of clinical factors in addition to the susceptibility test report, physicians should be encouraged to consult infectious disease practitioners for patient management.

Quality Control

Review the QA/QC chapter for specific instructions for QC of tests for *Enterococcus* spp.

NCCLS-recommended QC strains for the vancomycin agar screen test and the high-level aminoglycoside screen tests are:

- *E. faecalis* ATCC 51299, positive (resistant) control strain
- *E. faecalis* ATCC 29212, negative (susceptible) control strain

The QC strain recommended for MIC testing of *Enterococcus* spp. is *E. faecalis* ATCC 29212

Note: There are no published acceptable disk diffusion or MIC QC ranges for *E. faecalis* ATCC 51299

Commentary on Case Study

Now you should be able to respond to the physician's inquiry: "Can gentamicin alone be used for therapy? My patient is receiving ampicillin and gentamicin and the laboratory report (see below) shows that the isolate is resistant to ampicillin."

Lab Report

Specimen

Source: blood

Results: *Enterococcus faecium*

Antimicrobial Agent	MIC (mcg/mL)	Susceptibility
Ampicillin	64	R
Vancomycin	≤0.5	S
Gentamicin synergy		S
Streptomycin synergy		S

Questions on Case Study

1. Based on the laboratory report, can gentamicin alone be used for therapy in this patient with bacterial endocarditis?
 - A. Yes
 - B. No

Answers and comment:

- A. Incorrect. Combination therapy with a cell wall-active agent and an aminoglycoside is necessary for bactericidal synergy. Since this isolate is ampicillin resistant, vancomycin would be the alternative cell wall-active agent. Gentamicin alone does not penetrate enterococcal cells and cannot be used as a single agent for treating enterococcal infections.
 - B. Correct.
2. Which therapy might be considered for the patient in the case study?
 - A. Ampicillin and gentamicin
 - B. Vancomycin and gentamicin
 - C. Vancomycin alone
 - D. Ampicillin alone

Answer and comment:

- B. is the correct answer. The physician switched therapy from ampicillin to vancomycin and continued treatment for 4 weeks at which time the patient no longer had symptoms of endocarditis.

VRE Surveillance

To improve detection of VRE-colonized patients, your infection control practitioner may request that surveillance cultures be taken from selected patients to identify those colonized with VRE. The surveillance cultures most frequently collected are rectal swabs and stool specimens.

VRE rectal surveillance specimens generally are inoculated onto a medium that suppresses normal stool flora, while enhancing the growth of VRE. One example of a selective medium is bile esculin azide agar (BEA) containing 6–10 mcg/mL of vancomycin.

Potential VRE must be confirmed biochemically, and the resistance pattern confirmed, to ensure that the enterococcal isolates represent acquired vancomycin resistance.

The laboratory report for VRE surveillance should be limited to:

- VRE isolated
- No VRE isolated.

The isolation of VRE from a stool or rectal swab specimen indicates that the patient is colonized with VRE and does not mandate the use of antimicrobial therapy.

VRE and other surveillance cultures should be performed only when specifically requested by infection control personnel. Do not look for VRE in stool specimens sent to the microbiology laboratory for identification of enteric pathogens, unless specifically requested by infection control personnel.

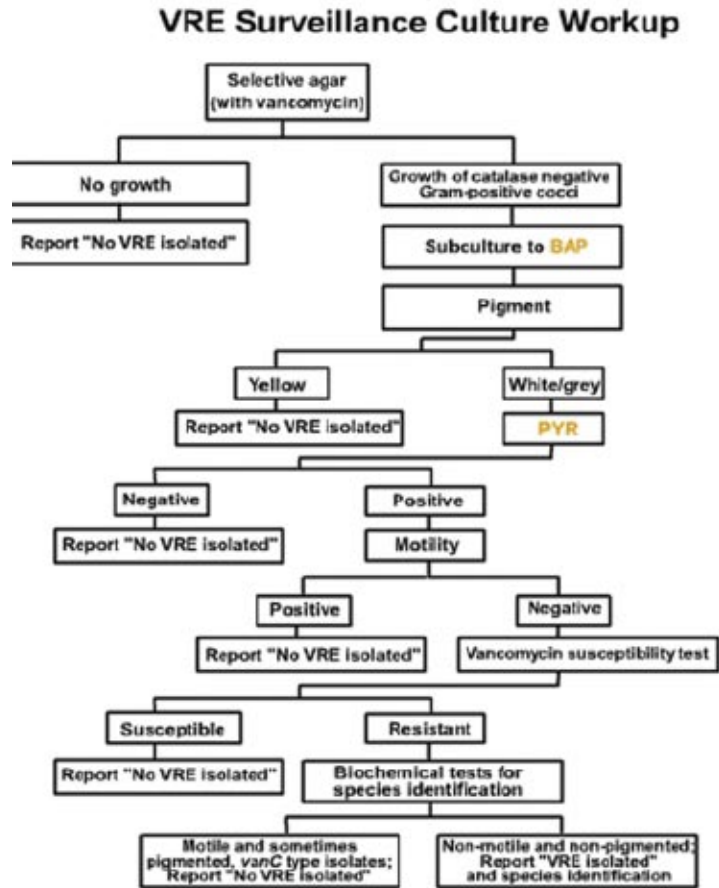


Figure 9.3—Flow chart for workup of VRE surveillance culture

REVIEW

The reader should now be knowledgeable about recommendations for routine antimicrobial susceptibility testing and reporting for *Enterococcus* spp.

Remember to:

- Use the most current NCCLS standards (M2, M7 and M100) for instructions for testing *Enterococcus* spp.
- Perform high-level aminoglycoside synergy screen tests on isolates for which combination therapy might be warranted (e.g. isolates associated with enterococcal endocarditis).
- Perform identification tests to differentiate isolates with intrinsic vancomycin resistance (e.g. *E. gallinarum* and *E. casseliflavus*) from isolates with acquired vancomycin resistance (e.g. *E. faecium* and *E. faecalis*).
- Develop VRE testing and reporting policies in conjunction with infection control personnel.

Summary of Methods for Susceptibility Testing of Enterococci

1. Disk diffusion
 - A. Mueller-Hinton agar
 - B. Incubation 16–18 h in ambient air at 35–37°C. For vancomycin incubate 24 h.
2. Broth MIC
 - A. Cation-adjusted Mueller-Hinton broth
 - B. Incubation 16–18 h in ambient air at 35–37°C. For vancomycin incubate 24 h.
3. Inoculum preparation
 - A. Use direct standardization method
 - B. Select fresh colonies from a 20–24 hour blood plate
 - C. Suspend colonies in broth or saline
 - D. Standardize suspension immediately to 0.5 McFarland and immediately inoculate susceptibility test medium
4. MIC testing
 - A. Ensure adequate growth in growth control well
 - B. Read MIC as lowest concentration of agent that inhibits visible growth
5. Beta-lactamase testing
 - A. Chromogenic cephalosporinase
 - B. If positive the ampicillin and penicillin report must be revised to “Resistant”
6. Vancomycin agar screen
 - A. Brain heart infusion agar containing 6 mcg/ml of vancomycin
 - B. Use growth in broth or direct colony method to standardize the inoculum to 0.5 McFarland
 - C. Deliver 1–10 mL in a spot on the agar surface.
 - D. Incubate for a full 24 h in ambient air 35–37°C
 - E. Greater than 1 colony indicates possible resistance.
 - F. Identification and disk diffusion or MIC tests must be performed to further classify the isolate.
7. High-Level aminoglycoside resistance-synergy screen
 - A. Standard disk diffusion procedure
 1. Special high content disks
 2. Incubate 16–18 h at 35°C in ambient air.
 - B. MIC tests
 1. Agar screen method
 2. Broth dilution tests
 3. Incubate gentamicin for 24 h at 35°C in ambient air.
 4. Incubate streptomycin for 24–48 hr (susceptible strains-incubate the additional 24 h) at 35°C in ambient air.
8. Interpretation
 - A. Use NCCLS M100 document Table 2D
 - B. Breakpoints for ampicillin or penicillin <8 = susceptible and >16 = resistant.
 - C. No intermediate breakpoint is available for ampicillin or penicillin
 - D. *E. faecium* with low-level ampicillin (16–32 mcg/mL) or penicillin (32–64 mcg/mL) resistance may have synergy with aminoglycosides, while high-level resistance will not.
9. Perform quality control
 - A. MIC control strain *E. faecalis* ATCC 29212
 - B. Vancomycin and high-level aminoglycoside agar screen QC.
 1. *E. faecalis* ATCC 51299, positive (resistant) control strain
 2. *E. faecalis* ATCC 29212, negative (susceptible) control strain.

SELF-ASSESSMENT QUESTIONS

1. What is the most common species of VRE?
 - A. *E. faecalis*
 - B. *E. faecium*
 - C. *E. casseliflavus*
 - D. *E. gallinarum*
2. Why are enterococci with acquired vancomycin resistance (e.g. *vanA* or *vanB* type) more important from an infection control perspective than those with intrinsic vancomycin resistance (e.g. *vanC* type)?
 - A. The genetic elements responsible for acquired vancomycin resistance can spread to other bacteria.
 - B. Intrinsically resistant isolates never cause infections.
 - C. Isolates with acquired vancomycin resistance are often multiply resistant and difficult to treat, therefore their spread among patient must be controlled.
3. What should you do if your disk diffusion test yields an intermediate result for vancomycin with an enterococcus?
 - A. Report the result as resistant.
 - B. Perform a vancomycin MIC test.
4. If an enterococcus has high-level resistance to gentamicin and streptomycin would it be appropriate to perform synergy tests with tobramycin or amikacin?
 - A. Yes
 - B. No
5. Which of the following tests are performed in a clinical laboratory to determine if synergy will occur between a specific cell wall-active drug (e.g. ampicillin, penicillin, or vancomycin) and an aminoglycoside (e.g. gentamicin or streptomycin) when testing enterococci?
 - A. Synergy tests with both the cell wall-active drug and the aminoglycoside in the same tube.
 - B. Individual tests for the cell wall-active agent and for the high concentrations of the aminoglycoside.
 - C. Individual tests for the cell wall active agent and for the aminoglycoside at standard (e.g. therapeutic) concentrations.
6. Why can ampicillin be used alone to treat acute uncomplicated urinary tract infections caused by enterococci?
 - A. Nearly all isolates that cause enterococcal urinary tract infections are susceptible to ampicillin.
 - B. Ampicillin is concentrated in the urine, and levels typically exceed the levels required to inhibit the growth of *E. faecalis* and *E. faecium*.
7. If a patient's isolate from urine grew on the vancomycin agar screen test what should you do next?
 - A. Report the isolate as vancomycin-resistant
 - B. Perform pigment and motility tests.

10

Streptococcus pneumoniae

OBJECTIVES

When readers have completed this chapter, they should be able to:

- Describe a practical strategy of antimicrobial susceptibility testing (AST) of *Streptococcus pneumoniae* isolated from cerebrospinal fluid (CSF), blood and other body sites
- List the testing conditions, including inoculum preparation, test medium, duration of incubation, and atmosphere of incubation, recommended for disk diffusion and MIC testing of *S. pneumoniae*.
- Describe the proper use of the oxacillin disk test to screen for susceptibility to beta-lactam agents.
- Describe the rationale for establishing separate breakpoints for cefepime, cefotaxime, and ceftriaxone for organisms from meningeal and non-meningeal sites.
- Discuss an effective strategy for reporting AST results for *S. pneumoniae*.

BACKGROUND

S. pneumoniae is a leading cause of bacterial meningitis in the world. The mortality rate is approximately 25% with higher rates in untreated patients. If treatment of children is delayed neurological sequelae often occur. *S. pneumoniae* also is the leading bacterial cause of community-acquired pneumonia and accounts for up to 50% of otitis media in children in the United States.

When patients present to their physician with pneumococcal pneumonia, it is not always possible to isolate the organism from respiratory secretions or blood. The ability of this organism to lyse spontaneously may account for this. Blood cultures are positive in about 25% of cases of pneumococcal pneumonia. Carriage of *S. pneumoniae* in the upper respiratory tract of 5–20% of healthy adults complicates the interpretation of expectorated sputum cultures.

Antibiotic resistance in *S. pneumoniae* has been increasing in recent years.

Decreased susceptibility to penicillin was first identified in 1967 and is now encountered worldwide. Many penicillin-resistant strains are also resistant to macrolides, tetracyclines, and trimethoprim/sulfamethoxazole. Resistance to fluoroquinolones is uncommon but increasing.

A polyvalent polysaccharide vaccine is available for the serotypes of pneumococci that most commonly cause invasive disease. Vaccination is recommended for those >65 years of age and those with impaired immune function. A new conjugant vaccine is available for use in children <2 years of age.

CASE STUDY A

A 52-year-old woman sought medical care following worsening flu-like illness that had persisted for several days. Along with a temperature of 103°F, she experienced shaking chills, significant congestion, and difficulty in breathing. Blood were cultures obtained in the emergency room and an expectorated sputum specimen was sent to the laboratory for Gram stain, culture, and susceptibility testing. A chest x-ray revealed a left lower lobe infiltrate. The physician admitted the patient to the hospital and prescribed ceftriaxone 1 gm every 24 hours. The patient responded to antimicrobial therapy and was discharged after 48 hours. The sputum culture grew many *S. pneumoniae* and blood cultures also were positive for *S. pneumoniae*.

The laboratory report for the isolate from the patient's blood culture is below:

Lab Report

Specimen source: Blood

Results: *Streptococcus pneumoniae*

	MIC mcg/ml	Interpretation
Ceftriaxone (meningitis)	1	I*
Ceftriaxone (nonmeningitis)	1	S
Erythromycin	2	R
Levofloxacin	0.5	S
Penicillin	2	R
Trimethoprim-Sulfamethoxazole	>4/76	R
Vancomycin	0.5	S

* Patients with meningitis require therapy with maximum doses of ceftriaxone.

Why were there two different interpretations for ceftriaxone in the final blood culture report? After working through this chapter the reader will be able to answer this question.

Beta-lactam Resistance

Penicillins

S. pneumoniae becomes resistant to penicillin through alterations in the penicillin binding proteins (PBPs) of the cell wall. These altered PBPs have decreased affinity for beta-lactam drugs. Since beta-lactams do not bind to their cellular targets (primarily PBP 2b), they do not initiate cell lysis.

Extended-spectrum Cephalosporins

Alterations in PBP 1a and PBP 2x can result in resistance to extended spectrum cephalosporins such as cefepime, cefotaxime, and ceftriaxone. In several regions in the US the incidence of resistance to these drugs exceeds 20%.

As a result of increasing beta-lactam resistance in *S. pneumoniae*, the recommended initial therapy for pneumococcal meningitis includes an extended-spectrum cephalosporin plus vancomycin. If cephalosporin resistances rates in the region are high, rifampin may be added. If the isolate is susceptible to extended-spectrum cephalosporins, vancomycin can be discontinued.

Resistance to Macrolides, Lincosamides, and Streptogramins

Resistance of *S. pneumoniae* to macrolides, lincosamides and streptogramins may be through several mechanisms.

- Production of ribosomal methylase
Synthesis of this enzyme is mediated by the *ermB* gene that confers resistance to macrolides, lincosamides (e.g. clindamycin) and streptogramin B agents. This is termed MLSB resistance
- Efflux of macrolides
This is mediated by the *mefA* gene and confers resistance to macrolides. This is referred to as the M (macrolide) phenotype. These organisms remain susceptible to clindamycin.
- Mutations in ribosomal RNA genes
This resistance mechanism is rare but often results in macrolide and streptogramin resistance. It is referred to as the MS phenotype

Resistance to Fluoroquinolones

Resistance to the newer fluoroquinolones, such as gatifloxacin, levofloxacin and moxifloxacin, which have good activity against *S. pneumoniae*, is still uncommon (<3%) in US but is observed in >15% of isolates in some areas of the world. Mutations in *parC* and *gyrA* genes, which code for enzymes that are involved in DNA unwinding and partitioning, result in decreased susceptibility to fluoroquinolones. High-level resistance occurs with multiple mutations in *parC*, *parE*, *gyrA*, and *gyrB* genes. Resistance in pneumococci can also be mediated by efflux mechanisms.

Changes in fluoroquinolone MICs ($\mu\text{g/mL}$) due to introduction of *parC* or *parC* and *gyrA* mutations in *S. pneumoniae*.

	Mutation		
	None	<i>parC</i>	<i>parC</i> & <i>gyrA</i>
Ciprofloxacin*	1	8	64
Gatifloxacin	0.25 S	0.5 S	4 R
Levofloxacin	1 S	2 S	16 R
Moxifloxacin	0.12 S	0.5 S	4 R

* No NCCLS interpretive criteria defined

Resistance to Other Classes of Drugs

Resistance to chloramphenicol, tetracyclines and trimethoprim/sulfamethoxazole also can occur and is more common in penicillin-resistant strains. Resistance to these drugs is particularly common in pediatric isolates of serotypes 6, 14, 19 and 23F. Some penicillin-resistant strains that are susceptible in vitro to chloramphenicol may not be killed in vivo thus precluding its use in meningitis caused by penicillin-resistant strains.

Testing Strategy

Isolates associated with **meningitis:**

For *S. pneumoniae* isolated from patients with meningitis, MIC tests for penicillin and cefotaxime or ceftriaxone should be performed. The oxacillin disk screen should not be performed since this will take an additional day and may unnecessarily delay the institution of appropriate antimicrobial therapy.

Isolates associated with **nonmeningeal infections:**

If the zone of inhibition surrounding an oxacillin 1 µg disk is ≤19 mm, MIC tests for penicillin and cefotaxime or ceftriaxone should be performed. In general, MIC tests should be performed for drugs that cannot be reliably tested by disk diffusion, e.g. cefotaxime, ceftriaxone, and other beta-lactams. If the zone of inhibition is ≥20 mm with the 1 µg oxacillin disk the strain is considered susceptible to beta-lactam agents.

For other agents, e.g. erythromycin and trimethoprim/sulfamethoxazole, disk diffusion or MIC methods can be performed. Note that these agents are not considered appropriate therapy for meningitis and should not be reported for meningeal infections regardless of the susceptibility results.

Methods

The disk diffusion method for *S. pneumoniae* is performed using Mueller-Hinton agar with 5% sheep blood. Incubation is for 20–24 hours at 35–37°C in 5% CO₂. The broth MIC method is performed using cation-adjusted Mueller-Hinton broth containing 2–5% lysed horse blood. Incubation is for 20–24 hours at 35–37°C in ambient air.

Inoculum preparation notes:

S. pneumoniae typically produce autolysins (i.e. enzymes that initiate lysis of the cell wall) that lead to a rapid reduction in the number of viable cells in late log and early stationary phases of growth. To avoid false susceptible results from underinoculation of the medium due to autolysis, the inoculum should be prepared using the direct standardization method. Fresh colonies from a 20–24 hour blood plate are selected and suspended in broth or saline. The suspension is then immediately standardized to match the turbidity of a 0.5 McFarland standard. For disk diffusion tests, the plates are immediately inoculated from this standardized suspension. For microdilution tests, the standardized suspension is diluted to a concentration that yields a final concentration in each well of 5 × 10⁵ CFU/mL.

Technical Tip: Prepare the inoculum suspension just prior to inoculating the MIC broth or disk diffusion plate. Do not allow pneumococci to remain suspended in liquid medium for more than a few minutes.

An alternative disk diffusion test is needed for beta lactams. The standard disk diffusion test is not reliable for pneumococci when testing with beta-lactams such as penicillin and cephalosporins because the results do not correlate with MIC results. However, an alternate disk test using a 1-µg oxacillin disk to screen for penicillin and beta-lactam resistance may be performed. The presence of adequate growth is demonstrated by a lawn of bacteria. Individual colonies should not be apparent. Zones of inhibition should be read from the agar side using reflected light. Zones of hemolysis should not be read. If a double zone is encountered the inner zone should be read. An oxacillin zone of ≥20 mm indicates susceptibility to most beta-lactam agents. In contrast, zone diameters that are ≤19 mm indicate possible resistance. In this event, MIC testing should be performed.

MIC testing of pneumococci should be done in Mueller-Hinton broth supplemented with lysed horse blood. It is important that adequate growth be observed in the growth control well. The MIC is read as lowest concentration of agent that inhibits visible growth. *S. pneumoniae* may produce a slight greening of the media in the microdilution wells as it grows. The presence or absence of this greenish colorization should not be used alone to determine MIC endpoints.

Penicillin-susceptible *S. pneumoniae* also are susceptible to the following:

Amoxicillin	Cefepime	Ceftibuten
Amoxicillin-clavulanic acid	Cefetamet	Ceftizoxime
Ampicillin	Cefixime	Ceftriaxone
Ampicillin-sulbactam	Cefotaxime	Cefuroxime
Cefaclor	Cefpodoxime	Imipenem
Cefdinir	Cefprozil	Loracarbef

Interpretation of Results

NCCLS M100 document Table 2G should be used to interpret the results of disk diffusion and MIC tests on *S. pneumoniae*.

Vancomycin and Linezolid

Both methods only have a susceptible breakpoint for vancomycin and linezolid. If non-susceptible results are obtained with either of these drugs the identification of the isolate and susceptibility results should be confirmed. If the results are confirmed, the isolate should be saved and sent to a reference lab that performs the NCCLS reference broth microdilution method.

Beta-lactams

As shown in the table below there are now two sets of interpretative criteria for MIC testing of cefotaxime, ceftriaxone and cefepime. The original breakpoints for penicillin and extended-spectrum cephalosporins were established with the treatment of meningitis in mind and were based on the achievable levels in cerebrospinal fluid. Later, clinical and pharmacokinetic data suggested that some nonmeningeal pneumococcal infections, such as community-acquired pneumonia, could be treated with extended-spectrum cephalosporins even when the MICs were as high as 1 µg/mL. Thus, a second set of breakpoints were established for nonmeningeal pneumococcal infections as shown in the following table.

For isolates from patients with meningitis, the original breakpoints (≤ 0.5 µg/mL susceptible, 1 µg/mL intermediate, 2 µg/mL resistant) should be used. For isolates from non-meningitis infections, the new breakpoints (≤ 1 µg/mL susceptible, 2 µg/mL intermediate, 4 µg/mL resistant) should be used. In addition, for CSF isolates, report only results for agents appropriate to treat meningitis, such as cefotaxime or ceftriaxone (meningitis breakpoints), meropenem, penicillin, and vancomycin.

Even if the following antimicrobial agents yield susceptible test results do not report them because they are not clinically effective for treating meningitis: first and second generation cephalosporins, clindamycin, most fluoroquinolones, macrolides and tetracyclines.

MIC ($\mu\text{g/mL}$) interpretive criteria for <i>S. pneumoniae</i>				
Antimicrobial Agent	MIC ($\mu\text{g/mL}$) Interpretive Standard			Comments
	S	I	R	
Penicillin	≤ 0.06	0.12–1	≥ 2	Penicillin should not be used for therapy in meningitis for isolates in the I or R category
Cefepime (meningitis)	≤ 0.5	1	≥ 2	Report only interpretations for nonmeningitis and include the nonmeningitis notation on the report. Cefepime does not have a U.S. FDA-approved indication for meningitis.
Cefepime (non-meningitis)	≤ 1	2	≥ 4	
Cefotaxime (meningitis)	≤ 0.5	1	≥ 2	For CSF isolates, report only meningitis interpretations because these patients require maximum doses. For all other isolates report both interpretations.
Cefotaxime (non-meningitis)	≤ 1	2	≥ 4	
Ceftriaxone (meningitis)	≤ 0.5	1	≥ 2	Report as described above for cefotaxime.
Ceftriaxone (non-meningitis)	≤ 1	2	≥ 4	

For non-CSF isolates (e.g. respiratory specimens) other drugs, such as clindamycin, macrolides, fluoroquinolones, tetracyclines, and trimethoprim/sulfamethoxazole may be reported as well. For non-CSF isolates, both meningitis and non-meningitis breakpoint interpretations for cefotaxime and ceftriaxone should be reported since some patients with pneumococcal meningitis may have *S. pneumoniae* isolated from sources other than CSF.

Quality Control

See Chapter 6 QA/QC of this manual for specific instructions for QC of tests for *S. pneumoniae*. The NCCLS-recommended QC strain is *S. pneumoniae* ATCC 49619 for which the penicillin MIC is in the intermediate category.

REVIEW

There are many important points to consider when performing routine antimicrobial susceptibility tests on *S. pneumoniae*.

Remember to:

- Use the most current NCCLS standards (M2 and M7) for instructions on testing *S. pneumoniae*. Use the current NCCLS M100 document for tables for interpretative criteria and QC ranges
- Handle *S. pneumoniae* carefully as they tend to autolyze if allowed to remain in liquid suspension for more than a few minutes prior to inoculation of disk diffusion plates or MIC panels.
- Use MIC methods for testing beta-lactams such as penicillin, cefotaxime and ceftriaxone. There are no disk diffusion interpretative criteria for these agents.

- Report both the meningitis and nonmeningitis interpretations for isolates from sources other than CSF. For CSF isolates, report meningitis interpretations only.
- Read the package insert carefully before using any commercial product. Some commercial systems are not reliable for testing *S. pneumoniae*.

SELF-ASSESSMENT QUESTIONS

1. Which of the following pairs of antimicrobial agents and resistance mechanisms are correct? Select all that apply.
 - A. Levofloxacin/mutation in *parC*
 - B. Erythromycin/altered PBP
 - C. Cefotaxime/efflux
 - D. Erythromycin and clindamycin/ribosomal methylase
2. Which of the following susceptibility profiles are based on known resistance mechanisms in *S. pneumoniae*? Select all that apply.

Drug	A	B	C	D
Ceftriaxone	S	R	R	S
Clindamycin	S	R	S	R
Erythromycin	S	R	S	S
Levofloxacin	R	S	S	S
Penicillin	S	R	S	S
Trimethoprim/sulfamethoxazole	S	R	R	S
Vancomycin	S	R	S	S

3. For case study A: why were there two different results for ceftriaxone in the final blood culture report?
 - A. A lower dose of ceftriaxone can be used for treatment of meningeal infections and separate interpretative criteria can help physicians determine if a lower dose would be appropriate.
 - B. Nonmeningeal *S. pneumoniae* infections (such as pneumonia) caused by strains for which the ceftriaxone MIC is 1 µg/mL can be effectively treated with routine doses of ceftriaxone and are categorized as susceptible.
 - C. It is more important to detect emerging resistance in *S. pneumoniae* isolates causing meningitis than in those associated with other types of infections. Thus, more conservative interpretative criteria should be applied to isolates causing meningitis.
4. Which of the following may result in an inadequate lawn of growth when performing a disk diffusion test with *S. pneumoniae*? Select all that apply.
 - A. Using fresh colonies for inoculum preparation but allowing the suspension to remain at room temperature for two hours before inoculating the Mueller-Hinton agar plate.

- B. Using log phase suspension of organisms grown in Mueller-Hinton broth for two hours at 35°C.
 - C. Incubating the inoculated test plate in ambient air for 20 hours.
5. Which of the following methods are acceptable for determining penicillin susceptibility in *S. pneumoniae*? Select all that apply.
- A. Ampicillin MIC test
 - B. Oxacillin disk test
 - C. Penicillin MIC test
 - D. Beta-lactamase test
6. A *S. pneumoniae* isolate tested with an oxacillin 1 µg disk produces a zone of inhibition of 16 mm. How will you proceed?
- A. Report as oxacillin resistant
 - B. Report as penicillin resistant
 - C. Perform penicillin and cefotaxime or ceftriaxone MIC tests
 - D. Perform an oxacillin MIC test
7. You are working in a small community hospital that routinely performs disk diffusion testing and sends isolates that require MIC testing to a reference laboratory. A physician asks you to test an isolate of *S. pneumoniae* for cefotaxime resistance, since the oxacillin screen result was 14 mm. There are no disk diffusion interpretative criteria for cefotaxime and *S. pneumoniae*. What should you do?
- A. Inform the physician that the test cannot be performed.
 - B. Send the isolate to a reference laboratory for a cefotaxime MIC test
 - C. Perform a disk diffusion test and use the cefotaxime interpretative criteria that appear in the *Enterobacteriaceae* table.

Indicate if the following questions are True or False

- 8. In the United States, cefepime results should be reported for CSF isolates.
- 9. Erythromycin is not reported for CSF isolates of *S. pneumoniae* because it can only be administered orally
- 10. The oxacillin disk diffusion test for predicting penicillin susceptibility in *S. pneumoniae* isolates is performed in most clinical laboratories in the United States before other susceptibility tests are performed.
- 11. A fluoroquinolone should be routinely tested against clinical isolates of *S. pneumoniae*.
- 12. Meningitis interpretations for cefotaxime and ceftriaxone should be reported for *S. pneumoniae* isolated from sputum, blood, and other sites.

OBJECTIVES

After completion of this chapter the reader should be able to:

- Describe a practical strategy for antimicrobial susceptibility testing (AST) of *Streptococcus* sp. including media used, inoculum preparation, incubation atmosphere and duration of incubation.
- Describe documents outlining testing methods for disk diffusion and MIC testing for streptococci.
- Discuss the appropriate methods for testing and reporting results including interpretative criteria for the viridans group streptococci and beta-hemolytic streptococci from various infections.
- Discuss the rationale for AST testing of streptococci including the reasons routine testing is unnecessary for beta-hemolytic streptococci.

BACKGROUND

Nonpneumococcal streptococci are classified into two groups according to their ability to hemolyze sheep red blood cells. Those isolates that completely lyse or hemolyze red blood cells are called beta-hemolytic streptococci. Based upon antigenic characteristics of the C carbohydrate located in their cell wall the beta-hemolytic streptococci are further classified into groups A, B, C, D, F and G. Those species that only partially hemolyze red blood cells are called viridans group streptococci. There are at least 20 species of viridans streptococci. The viridans streptococci are members of the normal flora of the gastrointestinal and respiratory tracts of humans. Common species include *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus salivarius* and *Streptococcus sanguis*.

Group A streptococci (*Streptococcus pyogenes*) are responsible for “strep throat” and also are associated with scarlet fever and streptococcal toxic shock syndrome. Sequelae of *S. pyogenes* infections include rheumatic fever and glomerulonephritis. Early treatment can minimize these serious complications from Group A infections. Group B streptococci (*Streptococcus agalactiae*) often colonize the vagina and are a major cause of neonatal disease in the United States. Other beta hemolytic streptococci cause a variety of infections.

Beta-hemolytic streptococci have not demonstrated resistance to penicillin which is the drug of choice for these infections. Unlike the beta-hemolytic streptococci, some viridans group streptococci are not susceptible to penicillin because

they contain altered penicillin-binding proteins. Resistance to other antimicrobial agents, such as erythromycin and tetracycline, varies from strain to strain.

Viridans group streptococci are among the leading causes of bacterial endocarditis. Therapy generally includes penicillin and an aminoglycoside and often is guided by the degree of penicillin susceptibility (as seen in the table below). Therefore, availability of an accurate penicillin MIC is important for patient care.

Suggested therapy for endocarditis caused by viridans group streptococci

Penicillin MIC (mcg/mL)	Suggested therapy
≤0.1	Penicillin +/- Gentamicin, or Ceftriaxone
>0.1–<0.5	Penicillin + Gentamicin
≥0.5	Penicillin or Ampicillin + Gentamicin
≥4.0	Vancomycin +/- Gentamicin

CASE STUDY

A 72-year-old woman sought medical care following several days of malaise and a fever. She had undergone extensive dental work ten days prior to onset of fever. Because she had a prosthetic aortic valve in place she had been given a single oral dose of amoxicillin one hour prior to her dental work.

Examination of the patient and review of the patient's history suggested the diagnosis of subacute bacterial endocarditis. Two sets of blood cultures were obtained. A trans-esophageal echocardiogram revealed a 4-mm vegetation on her mitral valve. The patient was started on a regimen of penicillin and gentamicin. Two days later the laboratory reported growth of viridans group streptococci from both blood cultures. The physician requested that penicillin susceptibility testing be performed on the isolate.

What would be the best way to proceed?

The reader will be able to answer this question after working through this chapter.

Resistance—Penicillin

Beta-hemolytic Streptococci

Resistance to penicillin has not yet been observed among beta-hemolytic streptococci. Typical penicillin MICs for groups A, C and G are < 0.03 mcg/mL. Penicillin MICs for Group B streptococci are often higher but are in the susceptible range between 0.06–0.12 mcg/mL.

Viridans Group Streptococci

Resistance to penicillin (i.e., MIC >4mcg/mL) among viridans group streptococci has been increasing throughout the world. Resistance is due to alterations in the penicillin binding proteins and is most common among strains of *S. mitis* and *S. sanguis*.

Resistance—Extended-Spectrum Cephalosporins

Beta-hemolytic Streptococci:

Resistance to extended spectrum cephalosporins has not been reported among beta-hemolytic streptococci.

Viridans Group Streptococci:

Viridans group streptococci that are resistant to penicillin may exhibit reduced susceptibility or resistance (i.e., >4mcg/mL) to extended-spectrum cephalosporins. Such resistant strains are rare.

Resistance—Macrolides, Lincosamides & Streptogramin B

Beta-hemolytic Streptococci

Group A—the incidence of resistance to erythromycin varies considerably in different geographic regions and has exceeded 50% in some regions. This seems to reflect erythromycin use. Susceptibility to erythromycin can be used to predict susceptibility to both azithromycin and clarithromycin.

Group B—resistance to erythromycin has been reported to be as high as 25% in the United States; the incidence of clindamycin resistance is about half of this.

Mechanisms of macrolide resistance in beta-hemolytic streptococci include:

- Production of a ribosomal erythromycin resistance methylase that is mediated by the *ermA* group and the *ermB* genes. The *erm* genes confer resistance to macrolides, lincosamides (e.g., clindamycin) and streptogramin B agents. This resistance can be inducible or constitutive and is termed MLSB resistance.
- Efflux of macrolides is mediated by the *mefA* gene. The resistance often is called the “M phenotype” because only macrolides are affected. These strains remain susceptible to clindamycin.
- Mutations in ribosomal RNA genes are relatively rare causes of resistance that usually confer macrolide and streptogramin resistance (MS phenotype).

Resistance—Other Agents

Resistance to other agents such as quinupristin-dalfopristin and fluoroquinolones is low among *Streptococcus* spp. Group B streptococci usually are resistant to tetracyclines. Streptococci have not shown resistance to vancomycin or linezolid.

Testing Strategy

Beta-hemolytic Streptococci

Since all beta hemolytic streptococci are susceptible to penicillin, there is no need to routinely test these isolates. Because some patients cannot tolerate penicillin, in the case of serious infections, it is useful to test Group A streptococci for erythro-

mycin resistance and Group B streptococci for both clindamycin and erythromycin resistance.

When testing beta-hemolytic streptococci for susceptibility to erythromycin and clindamycin, a normal disk diffusion procedure should be incorporated using a 2µg clindamycin disk placed 12 mm (measured edge to edge) from a 15µg erythromycin disk. This is known as the disk approximation test, “D test.” Following incubation, zones that show a positive “D test” have flattening of the clindamycin zone next to the erythromycin disk as seen in Figure 8.7. These strains demonstrate inducible resistance to clindamycin and therefore should be reported as resistant to clindamycin. The strains with a clindamycin zone diameter \geq 19 mm and no flattening of the zone may be reported as susceptible.

Reports of *S. agalactiae* that are “not susceptible” to penicillin have appeared in the literature, usually from laboratories using the disk diffusion test method. Strains that show reproducible MICs above the susceptible breakpoint of 0.12 mcg/mL, or zone diameters $<$ 24 mm, should be sent to a reference laboratory for confirmation

Viridans Group Streptococci

Viridans group streptococci are not uniformly susceptible to penicillin or ampicillin. Testing should be performed when these organisms are found in serious infections (e.g. bacteremia, endocarditis or pyogenic abscesses). Penicillin or ampicillin should be routinely tested and reported for such infections. Other drugs that may be tested include cefepime, cefotaxime, or ceftriaxone. Vancomycin resistance has not been reported in the viridans group streptococci. Some species that are intrinsically resistant to vancomycin may show colonial morphologies similar to those of the viridans group streptococci. These include *Pediococcus*, *Leuconostoc*, *Lactobacillus* and *Erysipelothrix rhusiopathiae*. Therefore, prior to susceptibility testing, the Gram stain and appropriate biochemical tests should confirm the identification of viridans group streptococci

Technical Tips:

- The disk diffusion method is not acceptable for testing the viridans group streptococci against penicillin because the results do not correlate with results of MIC tests. However, disk diffusion is acceptable for testing cefepime, cefotaxime and ceftriaxone. Interpretative criteria are in NCCLS M100 Table 2H
- The oxacillin disk screening test for detecting penicillin susceptibility among *Streptococcus pneumoniae* is NOT appropriate for other *Streptococcus* spp.

The procedures for *S. pneumoniae* are generally applicable to other *Streptococcus* spp. Additional guidance in measuring zones of inhibition and MIC endpoints is available in Chapter 10 on *S. pneumoniae*.

Methods

NCCLS has specific standards for testing nonpneumococcal *Streptococcus* spp. Routine disk diffusion and MIC methods are used with the following modifications.

Method	Medium	Incubation	
		Time (h)	Atmosphere
Disk diffusion	SB-MHA ^a	20–24	CO ₂ (5%)
Broth MIC	MH-LHB ^b	20–24	Ambient air
Agar MIC	SB-MHA	20–24	Ambient air ^c

^a Mueller-Hinton agar containing 5% sheep blood.

^b Cation-adjusted Mueller-Hinton broth containing 2–5% (v/v) laked horse blood.

^c CO₂, if needed.

Interpretation of Results

NCCLS M100 Tables 2H (in both the disk diffusion chapter and the MIC chapter) contain interpretive criteria for “*Streptococcus* spp. other than *S. pneumoniae*.” Since there are significant differences in susceptibility to beta-lactams between beta-hemolytic streptococci and viridans group streptococci there are separate sets of interpretive criteria for both groups with disk diffusion and MIC methods. The tables below present examples of the information in Table 2H.

Interpretative criteria for beta-hemolytic group and viridans group with disk diffusion test

Drug	Disk content	Interpretation of Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Penicillin (beta group)	10 units	– ^a	–	≥24
Penicillin (viridans group)	10 units	NA ^b	NA	NA
Ceftriaxone (beta group)	30 (mcg)	–	–	≥24
Ceftriaxone (viridans group)	30 (mcg)	≤24	25–26	≥27

^a Because all strains have been susceptible other interpretations have not been developed.

^b NA, not applicable. Disk diffusion results with penicillin are not reliable for viridans streptococci.

Interpretive criteria for MIC tests of beta-hemolytic group and viridans group

Drug	Interpretation of MIC (mcg/mL)		
	Susceptible	Intermediate	Resistant
Penicillin (beta group)	≤0.12	–	–
Penicillin (viridans group)	≤0.12	0.25–2	≥4
Ceftriaxone (beta group)	≤0.5	–	–
Ceftriaxone (viridans group)	≤1	2	≥4

Disk diffusion and MIC interpretive criteria for all *Streptococcus* spp.

Method/Drug	Disk content (mcg)	Interpretation		
		Resistant	Intermediate	Susceptible
Disk Diffusion				
Linezolid	30	– ^a	–	≥21
Vancomycin	30	–	–	≥17
MIC (mcg/mL)				
Linezolid		≤2	–	–
Vancomycin		≤1	–	–

^a Since strains resistant to linezolid and vancomycin have not been recognized, only susceptible break-points are listed for linezolid and vancomycin)

For **large colony beta hemolytic streptococci** (containing group A, B, C or G antigens) use interpretive criteria for beta hemolytic streptococci.

For **viridans streptococci and small colony beta hemolytic strains** with group A, C, F or G antigens, use the interpretive criteria for viridans group streptococci.

When laboratories encounter an isolate that does not test susceptible to a drug classified with “susceptible only” interpretive criteria they should:

- Confirm the identification.
- Confirm the susceptibility result.
- Save any isolate that is not susceptible and submit it to a reference laboratory that performs an NCCLS reference dilution method.

When measuring zone diameters of a streptococcus on a blood agar medium, the light source should be reflected, not transmitted. It is important to measure the zone of growth inhibition and not the zone of hemolysis around the disk.

Reporting Results

A streptococcal isolate that is susceptible to penicillin can be considered susceptible to other beta-lactam agents. (See list below.) Laboratories may want to add a comment on the report that mentions the beta-lactam agents that are on their institution’s formulary.

Penicillin-susceptible streptococci can be considered susceptible to the following agents:

Ampicillin	Cefepime	Cefuroxime
Ampicillin-sulbactam	Cefotaxime	Cephalothin
Amoxicillin	Cefpodoxime	Cephapirin
Amoxicillin-clavulanic acid	Cefprozil	Cephradine
Cefaclor	Ceftibutin	Imipenem
Cefazolin	Ceftizoxime	Loracarbef
Cefdinir	Ceftriaxone	Meropenem

Since some physicians may not be aware of the universal susceptibility of beta-hemolytic streptococci to penicillin the laboratory report could mention this.

NCCLS suggests adding a comment to the laboratory report to emphasize the need for combination therapy when treating serious viridans group infections caused by strains with penicillin MICs in the intermediate range. See the following example.

Specimen Source: Blood
Results: Viridans group streptococcus

Antimicrobial Agents	MIC mcg/mL	Interpretation
Ceftriaxone	0.5	S
Penicillin	1	I
Vancomycin	≤0.5	S

Comment: streptococci that are “Intermediate” to penicillin may require combined therapy with an aminoglycoside to achieve bactericidal action.

Quality Control

Refer to Chapter 6 QA/QC for specific instructions for QC of tests for *Streptococcus* spp.

The NCCLS-recommended QC strain is *S. pneumoniae* ATCC 49619. The penicillin MIC for this strain is in the intermediate range.

REVIEW

The reader should now understand routine antimicrobial susceptibility testing and reporting recommendations for non-pneumococcal *Streptococcus* spp.

Remember to:

- Use the most current NCCLS standards (M2 and M7) for testing *Streptococcus* sp. The M100 standards are updated annually and contain the most recent tables and reporting suggestions.
- Perform a penicillin MIC test for viridans group streptococci isolated from normally sterile sites (e.g., isolates associated with endocarditis).
- Investigate all “nonsusceptible” penicillin results on beta-hemolytic streptococci, since penicillin resistance in this group has not been observed.

SELF-ASSESSMENT QUESTIONS

1. From the three profiles below, select which is the best match for *S. agalactiae*, *S. pyogenes*, and *S. mitis*.

	A	B	C
Clindamycin	S	S	S
Erythromycin	S	R	S
Penicillin	R	S	S
Tetracycline	R	R	S
Vancomycin	S	S	S

2. Select the conditions to be used for broth microdilution MIC testing of viridans group streptococci.

Inoculum preparation:

- A. Log phase suspension (grown until turbid)
- B. Direct colony suspension

Incubation length:

- A. 16–20 h
- B. 20–4 h

Incubation atmosphere:

- A. Ambient air
- B. CO₂

3. Your laboratory isolated *S. agalactiae* from a vaginal/rectal specimen from a pregnant woman. The physician requested that susceptibility testing be performed on this isolate because the patient is allergic to ampicillin.

What additional drugs will you test and report?

- A. Clindamycin, erythromycin
 - B. Ciprofloxacin, erythromycin
 - C. Erythromycin, tetracycline
 - D. Erythromycin, vancomycin
 - E. Vancomycin
4. A physician asks you to perform a penicillin susceptibility test for an isolate of *S. pyogenes*. What would be the most appropriate response?
- A. Test by disk diffusion.
 - B. Test by MIC.
 - C. Explain to the physician that all *S. pyogenes* are susceptible to penicillin and therefore that laboratory does not routinely test this species.
5. What would be the most appropriate response if you encountered a viridans group streptococcus for which the vancomycin MIC was > 32 mcg/mL?
- A. Repeat the vancomycin susceptibility test using the disk diffusion method.
 - B. Repeat the vancomycin susceptibility test using the same MIC method as for the initial test.
 - C. Confirm the identification of the isolate.
 - D. Repeat the vancomycin susceptibility test using the same MIC method and confirm the identification of the isolate.
6. The physician in the case study requested a penicillin susceptibility test for the viridans group streptococcus isolated from the patient's blood cultures. What would be the most appropriate response to this request?
- A. Test by disk diffusion.
 - B. Test by MIC.
 - C. Explain to the physician that all viridans group streptococci are susceptible to penicillin. Therefore, the laboratory does not routinely test this species.

IV

Gram-Negative Organisms

12

Enterobacteriaceae

OBJECTIVES

After completing this chapter readers should be able to:

- Discuss a practical strategy for antimicrobial susceptibility testing of *Enterobacteriaceae* in their laboratory.
- Describe where to find recommendations for disk diffusion and MIC testing of *Enterobacteriaceae*, including inoculum preparation, test medium, duration of incubation, and atmosphere of incubation.
- Modify routine testing methods to screen for and confirm production of extended-spectrum beta-lactamases (ESBLs) in *Enterobacteriaceae*.
- Compare and contrast inducible beta-lactamases and ESBLs.
- Describe a strategy for testing *Salmonella* and *Shigella* spp. including the appropriate antimicrobial agents to test and report for these organisms.

BACKGROUND

The Family *Enterobacteriaceae* contains many species of aerobic or facultatively anaerobic, gram-negative, nonspore-forming rods. Approximately 50 per cent of clinically significant bacteria isolated in a clinical bacteriology laboratory are *Enterobacteriaceae* and 20 species account for most *Enterobacteriaceae* isolated from clinical specimens. *Enterobacteriaceae* are part of the normal gastrointestinal flora; however, several species can cause gastrointestinal disease.

One of the most common types of infections caused by *Enterobacteriaceae* in the outpatient setting is acute, uncomplicated cystitis in which *Escherichia coli* is the predominant pathogen. Among hospitalized patients, *Enterobacteriaceae* often cause infections and it is not uncommon for outbreaks to result from spread of multidrug resistant strains of *Enterobacteriaceae*.

CASE STUDY

A 68-year-old woman was placed in the surgical intensive care unit (SISC) following repair of an aortic aneurism. On the sixth day post surgery, she experienced a fever of 101°F. The attending physician ordered two sets of blood cultures and started the patient on cefotaxime and vancomycin. Within six hours, the patient showed limited improvement, but remained febrile.

On day two of the fever, both sets of blood cultures were positive and a Gram stain revealed a gram-negative bacillus. This report and the fact that the patient remained febrile prompted the physician to order two additional blood cultures. The vancomycin was discontinued because gram-negative bacilli typically are resistant to vancomycin. Gentamicin was added to the therapeutic regimen. The laboratory report subsequently indicated that the gram-negative bacillus was *Enterobacter cloacae*.

Following addition of gentamicin, the patient became afebrile and showed marked improvement.

Blood cultures obtained on day 2 also were positive for *E. cloacae*.

Lab Report

Specimen source: blood (obtained on day 1)

Results: *Enterobacter cloacae*

Ampicillin	R
Cefazolin	R
Cefotaxime	S
Ciprofloxacin	S
Gentamicin	S
Imipenem	S
Piperacillin	R
Trimethoprim-sulfamethoxazole	R

Do you see any differences in the antimicrobial susceptibility test results on the *E. cloacae* isolated from the second set of blood cultures compared to the results from the initial blood cultures?

Lab Report

Specimen source: blood (obtained on day 2)

Results: *Enterobacter cloacae*

Ampicillin	R
Cefazolin	R
Cefotaxime	R
Ciprofloxacin	S
Gentamicin	S
Imipenem	S
Piperacillin	R
Trimethoprim-sulfamethoxazole	R

After working through this chapter the reader should be able to explain what happened.

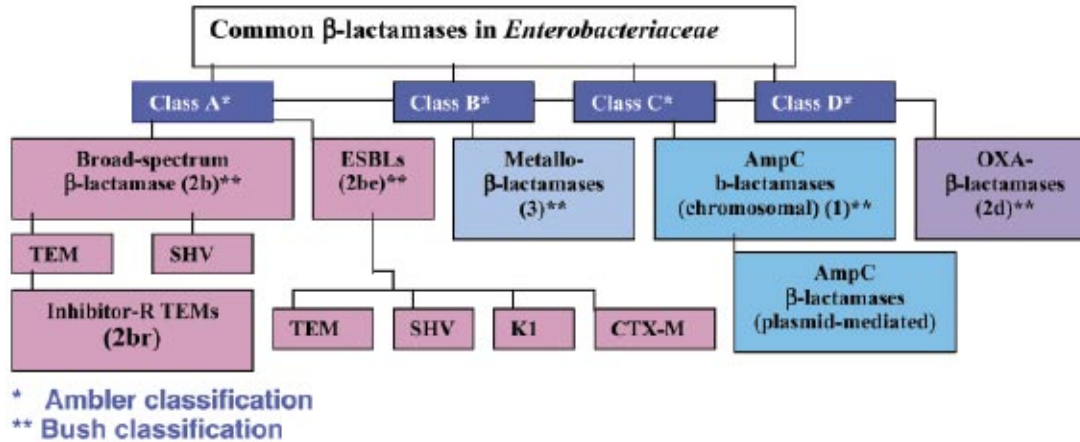


Figure 12.1—A positive disk diffusion confirmation test for ESBL production

Beta-lactamases in *Enterobacteriaceae*

As shown in this diagram members of the *Enterobacteriaceae* produce many different types of beta-lactamases. Each type of beta-lactamase in this chart is described below. For more information on overall classification of beta-lactamases, including Classes A-D, see Chapter 2 Beta-lactamases in this manual.

Broad-spectrum Beta-lactamase (Class 2b):

Many familiar beta-lactamases are clustered in Bush Group 2b, including the basic plasmid-mediated TEM-1, TEM-2 and SHV-1 enzymes. These enzymes are inhibited by clavulanic acid. As the level of expression of broad spectrum beta lactamases increases, resistance to some other beta lactams such as cephalothin and cefazolin occurs.

- TEM: TEM-1 is responsible for ampicillin resistance in *E. coli* and in some other *Enterobacteriaceae*.
 - Inhibitor-R TEMs (2br): Some of the genes encoding the TEM β -lactamase undergo mutations that change the amino acid sequences of the enzymes. These novel enzymes are inhibited by clavulanic acid, but to a significantly lesser degree than the original TEM-1 beta lactamase.
- SHV: SHV-1 and similar enzymes produced by *K. pneumoniae* confer resistance to ampicillin and related penicillins.

ESBLs (Class 2be): Mutations of the genes encoding the TEM-1, TEM-2, and SHV-1 beta lactamases are becoming widespread in isolates of *E. coli*, *Klebsiella pneumoniae*, and in some other gram-negative bacilli including *Burkholderia cepacia*, *Capnocytophaga ochracea*, *Citrobacter* spp., *Enterobacter* spp., *Morganella morganii*, *Proteus* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Serratia marcescens*, and *Shigella dysenteriae*. These mutations result in production of beta-lactamases known as extended-spectrum beta lactamases, or ESBLs.

Properties of ESBLs: ESBLs hydrolyze all penicillins, aztreonam, and all cephalosporins (but not cephamycins e.g. cefoxitin and cefotetan). ESBL genes commonly are located on transmissible plasmids that often encode other resistance determinants (e.g., aminoglycoside or trimethoprim/sulfamethoxazole). ESBLs are inhibited by beta lactamase inhibitors (e.g. clavulanic acid).

Types of ESBLs recognized as of 2004:

- TEM: There are approximately 150 types of TEM ESBLs.
- SHV: There are approximately 50 types of SHV ESBLs.
- K1: *Klebsiella oxytoca* strains produce the K1 beta lactamase that confers resistance to ampicillin. Hyper-producers of the K1 enzyme also are resistant to other penicillins, cefuroxime, aztreonam, and ceftriaxone, but are susceptible to ceftazidime. Cefotaxime MICs are moderately elevated.
- CTX-M: The plasmid-mediated CTX-M beta lactamases resemble K1 enzymes and have been found in *E. coli*, *K. pneumoniae*, and a variety of other *Enterobacteriaceae*. Isolates that produce CTX-M typically are resistant to cefotaxime and some may have reduced susceptibility to inhibitors of beta lactamases.

Metallo-beta-lactamases (Class 3)

Most metallo-beta-lactamases require zinc or other cations for activity. These enzymes are capable of hydrolyzing carbapenems (e.g. imipenem, meropenem) and other beta lactams (except monobactams).

Metallo beta-lactamases are not inhibited by clavulanic acid and infrequently occur in *Enterobacteriaceae*.

AmpC Beta-lactamases (chromosomal) (Class 1)

One of the most widespread groups of beta-lactamases is the AmpC enzymes. These are encoded by *ampC* genes that are typically located on the chromosomes of most *Enterobacteriaceae*. Nearly all members of the family *Enterobacteriaceae* (with the exception of *Salmonella* spp. and *Klebsiella* spp.) produce low levels of AmpC enzymes. However, in some species such as *Enterobacter cloacae* and *Citrobacter freundii* the presence of beta-lactams induces production of much higher levels of AmpC beta-lactamases. Species with higher levels of AmpC production are resistant to ampicillin and first-generation cephalosporins. Not all gram-negative species carry an inducible *ampC* gene. Expression of *ampC* in *E. coli* and *Shigella* spp. is constitutively low, not inducible, and only small amounts of the enzyme are produced under any circumstances. Beta-lactam antibiotics vary from strong inducers to non-inducers of AmpC beta-lactamases.

Additional information:

- Induction of AmpC beta-lactamases: cefoxitin and the carbapenems (e.g. imipenem and meropenem) can induce production of very high levels of AmpC beta-lactamases.

Chromosomal beta-lactamases and their expression in *Enterobacteriaceae*

Organism	Name	Modes of Expression			
		Inducible	Constitutive		
			Minimal	Moderate	High
<i>E. coli</i>	AmpC	–	N		R
<i>Shigellae</i>	AmpC	–	N		R
<i>Enterobacter</i> spp.	AmpC	N	R		F
<i>C. freundii</i>	AmpC	N	R		F
<i>M. organii</i>	AmpC	N	–		F
<i>Providencia</i> spp.	AmpC	N	R	–	R
<i>Serratia</i> spp.	AmpC	N		–	F
<i>K. pneumoniae</i>	SHV-1			N	R*
<i>K. oxytoca</i>	K-1		–	N	F
<i>Proteus mirabilis</i>			N		–
<i>Proteus vulgaris</i>	Group 2e	N	–	–	R

N Normal mode of production, typical of the species.

F Frequently encountered, variable among countries, hospitals, and units, but seen in 10 to 50% of isolates in most recent surveys.

R Rare, seen in fewer than 10% of isolates.

– Unknown or isolated reports only.

Minimal production indicates that enzyme is detectable but causes no significant resistance; **moderate** indicates that the enzyme contributes to resistance to good substrates; **high** indicates huge levels of enzyme—up to 3% of total cell protein in some *Enterobacter* – able to confer resistance even to weak substrates.

* Frequent when SHV-1 enzyme is plasmid-mediated.

Adapted from Livermore, D. 1995. Clin. Microbiol Rev. 8:564.

List of Enterobacteriaceae that produce inducible beta-lactamases:

<i>C. freundii</i> .	<i>S. marcescens</i>
<i>Enterobacter</i> spp.	<i>Hafnia alvei</i>
<i>M. organii</i>	<i>P. vulgaris</i>
<i>Providencia</i> spp	<i>Proteus penneri</i>

The *ampC* gene mutation:

The *ampC* gene in *Enterobacteriaceae* can undergo mutation that results in enhanced beta-lactamase production. This is independent of exposure to an inducing agent and generally occurs in one in 10⁶ to 10⁸ cells. The new resistant cell often does not survive among a large population of susceptible cells unless selective pressure, such as prolonged antimicrobial therapy, enhances the proliferation of resistant mutants.

To review induction and selection processes see Chapter 1 Antimicrobial Modes of Action in this manual.

AmpC Beta-lactamases (plasmid-mediated)

The *ampC* genes have been discovered on plasmids in several species of *Enterobacteriaceae*. These *ampC* genes were likely derived from chromosomal *ampC* genes in *C. freundii*, *E. cloacae*, and *M. organii*.

These isolates produce large amounts of AmpC beta-lactamase, conferring resistance to extended-spectrum cephalosporins, cephamycins, penicillins, and beta-lactamase inhibitor combinations. Expression usually is high level and constitutive.

OXA-beta-lactamases (2d)

The OXA beta-lactamases, originally detected in *P. aeruginosa*, hydrolyze oxacillin and cloxacillin. They also mediate low-level resistance to penicillins and many are not blocked by beta-lactamase inhibitors. Other OXA beta-lactamases are ES-BLs and carbapenemases.

Resistance—Aminoglycosides

Aminoglycosides (e.g., amikacin, gentamicin, tobramycin) are generally active against *Enterobacteriaceae*

Aminoglycoside resistance in *Enterobacteriaceae*:

- Usually is mediated by production of enzymes that add an acetyl, adenylyl, or phosphate group to the aminoglycoside so that it no longer can bind to a ribosome.
- Rarely is due to modification of ribosomes so that there is a decreased affinity for binding the aminoglycoside (this only affects spectinomycin).

Many aminoglycoside resistance genes are on plasmids and transposable elements.

Likelihood of various aminoglycoside profiles in *Enterobacteriaceae* in the United States^a

Likelihood	Gentamicin	Tobramycin	Amikacin
Common	R	S	S
Common	R	R	S
Uncommon	S	R	R
Uncommon	R	S	R
Rare	R	R	R
Unlikely ^b	S	S	R

^a The frequency of resistant phenotypes in each country can be very different. For example, in Argentina the most common resistant phenotype is the one labeled "Rare" in this table (personal communication, Dr. Marcelo Galas).

^b Probably an error.

Resistance—Fluoroquinolones

The incidence of fluoroquinolone resistance in *Enterobacteriaceae* varies greatly from one country to another. At this time it is uncommon in the United States; but its frequency is increasing, especially in *E. coli*.

Resistance to fluoroquinolones is independent of resistance to other classes of antimicrobial agents. Occasionally you may find an *E. coli* that is susceptible to all agents on your panel except fluoroquinolones (e.g., ciprofloxacin, levofloxacin, etc.).

Testing Strategy

NCCLS M100 Table 1 suggests drugs for testing and reporting against *Enterobacteriaceae*.

Enterobacteriaceae can display a variety of antimicrobial susceptibility profiles. Thus, susceptibility testing must be performed on all clinically significant isolates within this group of organisms.

Testing Strategy—ESBLs

Treatment of bacteremia caused by ESBL-producing organisms with an extended-spectrum cephalosporin (e.g., ceftazidime) may result in clinical failure. Thus, it is imperative to screen and confirm the presence of ESBLs in all *E. coli* and *Klebsiella* spp.

The NCCLS has not addressed the subject of screening for ESBLs in species other than *E. coli* and *Klebsiella* spp. A large study of non-*E. coli* and non-*Klebsiella* *Enterobacteriaceae* from patients in the United States found that ESBL-producers are relatively rare in these organisms. In preliminary ESBL screening of 690 isolates 355 were positive but confirmatory tests were positive in only 15 (2.2%) isolates. The authors concluded that ESBL screening of non-*E. coli* and non-*Klebsiella* *Enterobacteriaceae* is not warranted in the United States. (Schwaber, M.J. et al., J. Clin. Microbiol. 2004. Vol. 42 (1) p. 294–298.) However, the situation could be very different in other regions. A similar study needs to be done with isolates from across Latin America.

Uncomplicated urinary tract infections caused by ESBL-producing *Enterobacteriaceae* may be treated with extended-spectrum beta-lactams because of the high concentration of drug attainable in the urine. However, urosepsis caused by ESBL-producing strains should not be treated with extended-spectrum beta-lactam agents.

Currently, NCCLS suggests (in M100) that the decision to perform ESBL screening tests on all urine isolates should be made on an institutional basis.

Methods for Testing

NCCLS has specific standards for testing *Enterobacteriaceae*. Routine disk diffusion and MIC methods are used, including standard inoculum preparation, inoculation, and incubation procedures. There are some modifications to the standard procedure when testing for ESBLs. These will be covered later in this chapter.

When using a commercial system, check to see if there are any limitations for reporting certain antimicrobial agent/organism combinations.

Methods—Interpreting Results

NCCLS M100 Table 2A in both the disk diffusion and MIC sections contains interpretive criteria for *Enterobacteriaceae*.

NCCLS emphasizes the potential for resistance development (and suggestions for retesting) among *Enterobacteriaceae* with the comment:

“*Enterobacter*, *Citrobacter*, and *Serratia* spp. may develop resistance during prolonged therapy with third-generation cephalosporins. Therefore, isolates that are initially susceptible may become resistant within

three to four days after initiation of therapy. Testing of repeat isolates may be warranted.” A comment to reflect this concern may be added to the patient report.

In addition to beta-lactamase production, porin changes may contribute to resistance to third- and fourth-generation (extended-spectrum) cephalosporins and the cephamycins.

Methods—ESBL Tests

NCCLS standards describe both disk diffusion and MIC tests for detecting ESBLs in *E. coli* and *Klebsiella* species.

ESBL Screening Tests

To identify potential ESBL producers use novel disk diffusion (see Table 1 below) and MIC (see Table 2 below) breakpoints for aztreonam, cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone. An ESBL-producing strain may hydrolyze one or more of these agents. Testing of several of these agents will increase the sensitivity of detection of the variety of ESBLs that may be encountered.

ESBL Screening Test by Disk Diffusion

Perform the standard disk diffusion test according to NCCLS recommendations using the testing conditions and disk content specified for *Enterobacteriaceae*.

Table 1. interpretive criteria to detect potential ESBL producers

Antimicrobial	Disk diffusion Breakpoint (mm)
Aztreonam	≤27
Cefotaxime	≤27
Cefpodoxime	≤17
Ceftazidime	≤22
Ceftriaxone	≤25

Interpretation: If an isolate produces a zone of inhibition less than or equal to the zone diameter specified above for one or more of the agents, it is considered to be a potential ESBL producer.

ESBL Screening Test by MIC

Perform MIC tests according to NCCLS recommendations for *Enterobacteriaceae*.

Table 2. MIC interpretive criteria (mcg/mL) to detect potential ESBL producers:

Antimicrobial	MIC Breakpoint (mcg/mL)
Aztreonam	≤2
Cefotaxime	≤2
Cefpodoxime	≤8
Ceftazidime	≤2
Ceftriaxone	≤2

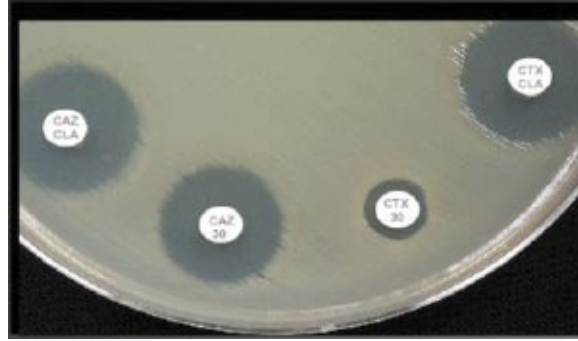


Figure 12.2—A positive disk diffusion confirmation test for ESBL production.

Interpretation: If the MIC is ≥ 8 mcg/mL for cefpodoxime and/or if the MIC is ≥ 2 mcg/mL for one or more of the other four agents, it is considered a potential ESBL producer.

ESBL Confirmatory Tests

Potential ESBL producers are tested with both cefotaxime and ceftazidime alone and in combination with clavulanic acid. If the isolate produces an ESBL, the clavulanic acid will inhibit the enzyme's activity and restore the activity of the cefotaxime or ceftazidime. Either a disk diffusion (see Table 3 below) or MIC method (see Table 4 below) can be used.

Table 3. Confirmatory disk test to detect potential ESBL producers

Antimicrobial	Disk content
Cefotaxime	30 mcg
Cefotaxime	30 mcg + clavulanic acid 10 mcg
Ceftazidime	30 mcg
Ceftazidime	30 mcg + clavulanic acid 10 mcg

Interpretation: A ≥ 5 mm increase in the zone diameter for cefotaxime *or* ceftazidime when tested in combination with clavulanic acid, compared to the zone diameter when tested without clavulanic acid, confirms ESBL production.

Perform confirmatory MIC tests for ESBL producers according to NCCLS recommendations for *Enterobacteriaceae* and include a range of concentrations (two-fold dilutions) specified below.

Table 4. Confirmatory MIC tests for ESBL producers

Antimicrobial	Range of concs. mcg/mL
Cefotaxime	0.25–64
Cefotaxime/clavulanic acid	0.25/4–64/4
Ceftazidime	0.25–128
Ceftazidime/clavulanic acid	0.25/4–128/4

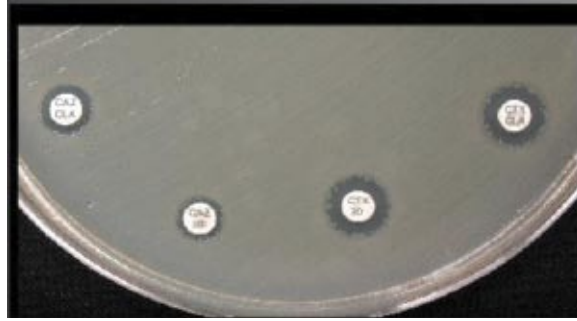


Figure 12.3—A negative disk diffusion confirmation test for ESBL production.

Interpretation: ESBL production is confirmed when there is a diminution ≥ 3 twofold dilutions in the MIC of either cefotaxime or ceftazidime when tested in combination with clavulanic acid, compared to the MIC when tested without clavulanic acid.

Methods—ESBL Test Results

View Fig. 12.3 the photo of a negative disk diffusion confirmatory test for ESBL production that was performed on an *E. coli*. The disks with clavulanic-acid do not show enhanced activity compared to the disks with only cefotaxime or ceftazidime.

This negative result may be due to one or more of the following:

- Production of plasmid-mediated AmpC beta-lactamase
- Over-expression of chromosomal AmpC beta-lactamase
- Presence of a combination of resistance mechanisms:
 - ESBL + inhibitor resistant TEM or SHV
 - ESBL + plasmid mediated AmpC beta-lactamase
 - ESBL + porin mutation

Reporting Results—ESBL Tests

ESBL-producing bacteria sometimes appear susceptible to some cephalosporins, penicillins, and aztreonam when traditional breakpoints are used for interpretation of results. However, clinical data suggest that infections caused by ESBL-producing isolates do not respond to these agents.

“NCCLS states that for ESBL-producers “the test interpretation should be reported as resistant for all penicillins, cephalosporins (but not cephamycins), and aztreonam.”

Technical tip: Remember that the cephamycins include ceftoxitin and cefotetan.

Reporting Results—ESBL Tests

A preliminary report should be issued for isolates of *E. coli* or *Klebsiella* spp. that have a positive ESBL screening test, while the confirmatory results are pending.

Results for cephalosporins (but not cephamycins), penicillins, and aztreonam must not be reported as susceptible on a potential ESBL producer. To facilitate reporting, a note could be added to the preliminary report as seen below.

Lab Report

Source: blood

Results: *Klebsiella pneumoniae*

	MIC (mcg/mL)	Interpretation
Amikacin	8	S
Ampicillin	>32	R
Cefazolin	>32	R
Cefoxitin	2	S
Ceftazidime	>32	R
Ciprofloxacin	0.5	S
Gentamicin	>16	R
Imipenem	≤0.25	S
Piperacillin-tazobactam	16	S
Tobramycin	>16	R
Trimethoprim-sulfamethoxazole	>4/76	R

Comment: This *K. pneumoniae* is a potential extended-spectrum beta-lactamase (ESBL) producer. Results for additional extended-spectrum cephalosporins and penicillins are pending.

Lab Report

Source: blood

Results: *Klebsiella pneumoniae*

	MIC (mcg/mL)	Interpretation
Amikacin	8	S
Ampicillin	>32	R
Cefazolin	>32	R
Cefotaxime		R*
Cefoxitin	2	S
Ceftazidime		R*
Ciprofloxacin	0.5	S
Gentamicin	>16	R
Imipenem	≤0.25	S
Piperacillin-tazobactam	16	S
Tobramycin	>16	R
Trimethoprim-Sulfamethoxazole	>4/76	R

* Comment: Confirmatory tests demonstrate that this *K. pneumoniae* is an extended-spectrum beta-lactamase (ESBL) producer. Interpretations have been modified.

If confirmatory test results are positive, the report should clearly state that the isolate is an ESBL producer. ESBL producers must be reported as resistant to all penicillins, cephalosporins (but not cephamycins) and aztreonam regardless of the in vitro result.

If the ESBL confirmatory test is negative, results of all tests are reported without modification and an additional comment should be added to the final report:

Comment: This *Klebsiella pneumoniae* is NOT an extended-spectrum beta-lactamase (ESBL) producer. Results are definitive. Testing completed.

ESBL Testing—Points to Remember

- ESBL production is most commonly associated with *E. coli* and *Klebsiella* spp. As of January 2005 *P. mirabilis* will be added to the NCCLS algorithm for *E. coli* and *Klebsiella* spp.. Tests for ESBL production in other species have not been standardized.
- The activity of extended-spectrum beta-lactams, including cefotaxime and ceftazidime, against ESBL producers is restored in the presence of clavulanic acid.
- ESBL producers are often resistant to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole.
- ESBL producers must be reported as resistant to all penicillins, cephalosporins (but not ceftioxin or cefotetan), and aztreonam regardless of the in vitro result.

Comparing AmpC, inducible, and Extended-Spectrum Beta-lactamases:

Summary of Characteristics of Beta-lactamases

Characteristic	AmpC ^a (plasmid mediated)	Inducible	ESBL
Location of genes for resistance	Plasmid	Chromosome (<i>ampC</i>)	Plasmid or chromosome
Most commonly found in	<i>E. coli</i> , <i>Klebsiella</i> spp.	<i>Enterobacter</i> spp., <i>C. freundii</i> , <i>S. marcescens</i> , <i>M. organii</i> , <i>Providencia</i> , spp., <i>Proteus rettgeri</i>	<i>E. coli</i> , <i>Klebsiella</i> spp.
Inhibited by clavulanic acid	No	No	Yes
Host organism generally susceptible to which beta-lactam drugs?	Carbapenems	Carbapenems	Cephamycins (cefotetan and ceftioxin) and carbapenems
Host organism generally resistant to which beta-lactam drugs?	All beta-lactams including cephamycins but excluding carbapenems	All beta-lactams including cephamycins but excluding carbapenems	All cephalosporins, penicillins, and aztreonam
Reporting notes	Report results as obtained	Report results as obtained	Report as resistant to all cephalosporins, penicillins, and aztreonam

^a *ampC* generally is located on the chromosome of a variety of *Enterobacteriaceae*. However, it can be transferred to a plasmid and result in hyperproduction in *E. coli* and *Klebsiella* spp.

Other Enterobacteriaceae—*Salmonella* and *Shigella* spp.

There are three important comments in NCCLS *Enterobacteriaceae* tables that pertain to reporting results for *Salmonella* spp. and/or *Shigella* spp.

“For fecal isolates of *Salmonella* and *Shigella* spp., only ampicillin, a fluoroquinolone, and trimethoprim-sulfamethoxazole should be tested and reported routinely. In addition, chloramphenicol and a third-generation cephalosporin should be tested and reported for extraintestinal isolates of *Salmonella* spp.”

“Extraintestinal isolates of *Salmonella* should also be tested for resistance to nalidixic acid. For isolates that test susceptible to fluoroquinolones and resistant to nalidixic acid, the physician should be informed that the isolate may not be eradicated by fluoroquinolone treatment. A consultation with an infectious disease practitioner is recommended.”

“Warning: For *Salmonella* spp. and *Shigella* spp., aminoglycosides and 1st and 2nd generation cephalosporins may appear active in vitro but they are not effective clinically and should not be reported as susceptible.”

Quality control

Refer to Chapter 6 QA/QC for specific instructions regarding QC of tests for *Enterobacteriaceae*.

NCCLS-recommended QC strains for routine disk diffusion and MIC tests are:

- *P. aeruginosa* ATCC 27853
- *E. coli* ATCC 25922
- *E. coli* ATCC 35218 (for beta-lactam/beta-lactamase inhibitor combinations)

NCCLS-recommended QC strains for ESBL screening and confirmatory tests are:

- *E. coli* ATCC 25922
- *K. pneumoniae* ATCC 700603 (ESBL-producing strain)

CASE STUDY COMMENTARY

Now the reader should be able to explain why the patient's initial isolate of *E. cloacae* was susceptible to cefotaxime and why the *E. cloacae* isolate from day 2 was resistant to cefotaxime.

What is the most likely explanation?

- A. Patient became infected with a new strain of *E. cloacae*.
- B. Isolate developed resistance during therapy.
- C. The cefotaxime susceptibility result was in error.

Correct answer:

- B. The *E. cloacae*, initially susceptible to cefotaxime, is now resistant. The patient remained febrile while receiving cefotaxime and vancomycin which suggests that the isolate developed resistance to cefotaxime.

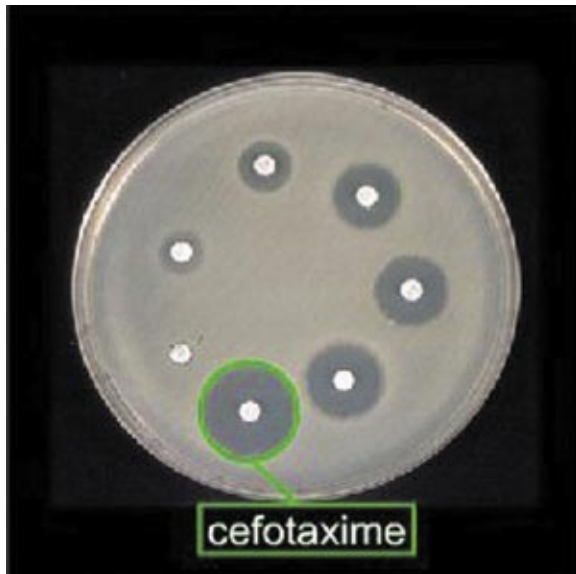


Figure 12.4—A test of isolates from the initial blood culture shows a large zone around the cefotaxime disk.

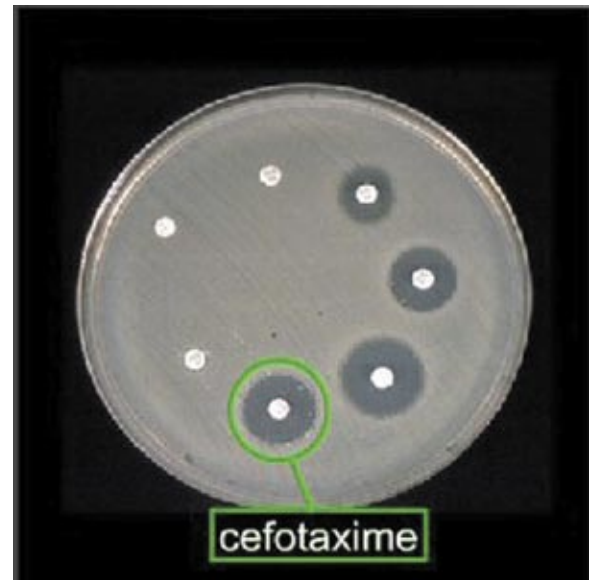


Figure 12.5—A test of isolates from the blood culture taken on day 2 shows a smaller zone around the cefotaxime disk.



Figure 12.6—A close-up of the cefotaxime zone seen in Figure 12.5; note colonies within the zone. Do not ignore these when measuring zones.



Figure 12.7—Test of colonies from within the zone in Figure 12.6 after they were subcultured and retested. There is no zone around the cefotaxime disk.

View the photos (Figures 12.4 to 12.7) of the disk diffusion tests on the *E. cloacae* isolates. (Note: the green criteria circles remain constant on each photograph.)

Colonies within a zone often represent resistant subpopulations. Review Chapter 4 Disk Diffusion in this manual for suggestions on dealing with these inner colonies.

Sometimes with broth microdilution MIC testing of extended-spectrum beta-lactams against organisms with resistant subpopulations the growth will skip wells.

REVIEW

Now the reader should be knowledgeable about recommendations for routine antimicrobial susceptibility testing and reporting for *Enterobacteriaceae*.

Remember to:

- Use the most current NCCLS M2, M7 and M100 standards for instructions for testing *Enterobacteriaceae*.
- For confirmed ESBL-producing *E. coli* and *Klebsiella* spp. report all penicillins, cephalosporins (but not cephamycins), and aztreonam as resistant despite in vitro results
- Refrain from reporting aminoglycosides and first- and second-generation cephalosporin results on *Salmonella* spp. and *Shigella* spp.
- Test extraintestinal *Salmonella* spp. with nalidixic acid to detect reduced susceptibility to fluoroquinolones.

SELF-ASSESSMENT QUESTIONS

1. Which of the following species do not produce inducible beta-lactamases?
 - A. *C. freundii*
 - B. *E. coli*
 - C. *E. cloacae*
 - D. *M. organii*
 - E. *S. marcescens*
2. Select the organism/resistance mechanisms listed below that best match with the susceptibility profiles A, B, C, and D.
 1. Plasmid-mediated AmpC producing *E. coli*
 2. K1 hyper producing *K. oxytoca*
 3. ESBL producing *K. pneumoniae*
 4. TEM-producing *E. coli*
3. Answer the following as True or False.
 - A. TEM-1 is an ESBL enzyme.
 - B. ESBL-producing isolates are usually cefoxitin susceptible.
 - C. Metallo beta-lactamases are abundant in *Enterobacteriaceae*.
 - D. ESBLs are only found in *E. coli* and *Klebsiella* spp.
 - E. AmpC beta-lactamases are inhibited by clavulanic acid.
4. Which of the following testing conditions are correct for testing *Enterobacteriaceae* by disk diffusion?
5. You have five sets of ESBL disk diffusion screening test results from testing five *E. coli* isolates with both cefpodoxime and ceftazidime. Which of these are potential ESBL producers? Select all that apply.

<i>E. coli</i> isolates	Cefpodoxime	Ceftazidime
A	20	19
B	28	24
C	24	9
D	8	10
E	28	28

6. Which of the following sets of zone diameters (mm) from testing three *E. coli* isolates confirms the production of an ESBL? Select all that apply.

<i>E. coli</i> isolates	Ceftazidime – CA*	Ceftazidime	Cefotaxime – CA	Cefotaxime
A	24	24	27	26
B	28	8	23	18
C	18	6	14	8

* CA, clavulanic acid

7. Which of the following sets of MIC (mcg/mL) results from testing three *K. pneumoniae* isolates confirms the production of an ESBL?

<i>K. pneumoniae</i> isolates	Ceftazidime – CA*	Ceftazidime	Cefotaxime – CA	Cefotaxime
A	32	8	16	1
B	8	8	1	1
C	16	1	1	1

* CA, clavulanic acid

8. Should clinical laboratories perform the disk diffusion test for inducible beta-lactamases?

- A. Yes
B. No

Enterobacteriaceae that produce inducible beta-lactamases

Citrobacter freundii
Enterobacter spp.
Morganella morganii
Providencia spp.
S. marcescens
H. alvei

9. A *Shigella* spp. was isolated from the stool of a 28-year-old man. Which antimicrobial agents would you report? Select all that apply.

- A. Ampicillin
B. Cefazolin
C. Cefotaxime
D. Ciprofloxacin
E. Gentamicin
F. Imipenem
G. Piperacillin
H. Trimethoprim-sulfamethoxazole

13

Non-*Enterobacteriaceae*

OBJECTIVES

After completing this chapter the reader should be able to:

- Discuss a practical strategy for antimicrobial susceptibility testing and reporting of *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Stenotrophomonas maltophilia* and other non-*Enterobacteriaceae*.
- Describe primary resistance concerns in *P. aeruginosa*, *Acinetobacter* spp., and *S. maltophilia*.
- List the recommended disk diffusion testing conditions for *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia* and *Burkholderia cepacia*.
- List the recommended conditions for broth microdilution MIC testing for *P. aeruginosa*, *Acinetobacter* spp., and *S. maltophilia*.
- Discuss limitations of disk diffusion testing for some non-*Enterobacteriaceae* and explain why MIC testing is required.

PSEUDOMONAS AERUGINOSA

BACKGROUND

P. aeruginosa is an opportunistic pathogen and a frequent cause of healthcare-associated infections. It also is a major cause of infections in cystic fibrosis patients. It tends to inhabit sites where moisture accumulates, including ventilator tubing. The organism has a characteristic grape-like odor and contains the pigment pyocyanin, which gives it a bluish-green color on culture media.

P. aeruginosa is intrinsically resistant to narrow-spectrum penicillins, first- and second-generation cephalosporins, trimethoprim, and sulfonamides. The anti-pseudomonal agents include extended-spectrum penicillins, such as ticarcillin and piperacillin; extended-spectrum cephalosporins, such as ceftazidime and cefepime; carbapenems; aminoglycosides; and fluoroquinolones. However, *P. aeruginosa* isolates that are resistant to one or more of these agents are becoming common.

CASE STUDY A

An 8-year-old girl with cystic fibrosis was admitted to the hospital with respiratory distress and a temperature of 102°F. Two distinct colony types of *P. aeruginosa* were isolated from a sputum culture; one of the colonies was very mucoid. How will you proceed with susceptibility testing of these isolates?

Resistance—Beta-Lactams

Beta-lactam resistance in *P. aeruginosa* is due to a combination of:

- Beta-lactamases
- Efflux systems
- Changes in outer membrane proteins (permeability barriers)
- Changes in penicillin-binding proteins

The anti-pseudomonal penicillins include:

- Carboxypenicillins—carbenicillin, ticarcillin
- Ureidopenicillins—mezlocillin, piperacillin

Ureidopenicillins are more active than the carboxypenicillins. Piperacillin-tazobactam does not offer any significant advantage over piperacillin against *P. aeruginosa* because the beta-lactamase inhibitor, tazobactam, does not inhibit most beta-lactamases produced by *P. aeruginosa*.

Resistance to cefotaxime, ceftriaxone, and other extended-spectrum cephalosporins usually is due to the chromosomally mediated type 1 (AmpC) beta-lactamase. Wild-type strains of *P. aeruginosa* can produce AmpC at such high levels that the *in vivo* MICs approach the therapeutic limits for these antibiotics. Therefore, use of these antibiotics is not recommended for treatment of *P. aeruginosa* infections, particularly when other alternatives, such as ceftazidime, that have better activity are available. *Cefepime* retains some activity against organisms producing these beta-lactamases unless the enzymes are hyper-produced.

In the U.S. cefepime activity is comparable to that of ceftazidime for *P. aeruginosa*. This may not be the case in your country. Aztreonam is slightly less active than ceftazidime against *P. aeruginosa*.

Carbapenems usually are not inactivated by the AmpC beta-lactamases produced by *P. aeruginosa*, but inactivation can occur with unique carbapenem-hydrolyzing enzymes. Resistance also can be due to efflux. Of all the beta-lactams the carbapenems have the broadest spectrum of activity against *P. aeruginosa*.

Resistance—Aminoglycosides

P. aeruginosa can become resistant to gentamicin, tobramycin, and amikacin in several ways:

- Low-level aminoglycoside resistance is due to lack of permeability of the outer membrane to these drugs.
- High-level aminoglycoside resistance is due to aminoglycoside-modifying enzymes.
- Some isolates are resistant as a result of both impermeability and aminoglycoside-modifying enzymes.

Resistance to only amikacin (but not gentamicin and tobramycin) is highly unusual.

Resistance—Fluoroquinolones

Ciprofloxacin remains the most active of the fluoroquinolones against *P. aeruginosa*. Resistance to fluoroquinolones is due to impermeability, efflux or mutations affecting the DNA gyrase and topoisomerase IV enzymes.

Technical tip: Fluoroquinolones are contraindicated for children therefore most laboratories do not routinely report them on patients less than 12 years old.

Testing Strategy

Table 1 in NCCLS M100 suggests agents for routine testing and reporting against *P. aeruginosa*. In the disk diffusion tables, this Table 1 listing is applicable to *P. aeruginosa* and *Acinetobacter* spp. only. Note that M100-S14 (2004) includes disk diffusion interpretive criteria for minocycline, levofloxacin and trimethoprim-sulfamethoxazole vs. *S. maltophilia* and ceftazidime, meropenem and minocycline for *B. cepacia*. No other non-*Enterobacteriaceae* can be reliably tested by disk diffusion.

In the MIC tables, the Table 1 listing includes *P. aeruginosa* and other non-*Enterobacteriaceae*. These non-*Enterobacteriaceae* are defined in a footnote to include *Acinetobacter* spp., *S. maltophilia*, *Pseudomonas* spp. and other non-fastidious, glucose non-fermenting, gram-negative bacilli.

Methods

NCCLS has specific standards for disk diffusion and MIC testing of *P. aeruginosa*. Standard inoculum preparation, inoculation and incubation procedures are used. NCCLS Table 1 lists drugs suggested for testing and reporting.

NCCLS addresses testing of *P. aeruginosa* from patients with cystic fibrosis with the comment:

“The susceptibility of *Pseudomonas aeruginosa* from patients with cystic fibrosis can be reliably determined by the disk diffusion, reference agar dilution, or frozen reference broth microdilution methods, but may require extended incubation up to 24 hours.”

Methods—Interpreting Results

In NCCLS M100 Table 2B both the disk diffusion and MIC sections contains interpretive criteria for non-*Enterobacteriaceae*, including *P. aeruginosa*.

Some agents listed, such as ampicillin-sulbactam, are inappropriate for *P. aeruginosa* but are listed for other species in the non-*Enterobacteriaceae* group.

Several beta-lactams, such as piperacillin-tazobactam, have separate sets of interpretive criteria for *P. aeruginosa* and other non-*Enterobacteriaceae*.

Methods—Reporting Results

NCCLS Table 2B contains an “Rx” comment to emphasize the need for combination therapy in treating serious *P. aeruginosa* infections. Remember that you should consider adding this comment or a variation of the comment to the laboratory report. The decision to do this must be based on your institution’s policies.

Quality Control

Check Chapter 6 QA/QC in this manual for specific instructions for QC of tests for *P. aeruginosa*.

NCCLS-recommended QC strains are:

- *P. aeruginosa* ATCC 27853
- *E. coli* ATCC 25922
- *E. coli* ATCC 35218 (for beta-lactam/beta-lactamase inhibitor combinations)

ACINETOBACTER SPP.

BACKGROUND

Acinetobacter spp. are opportunistic pathogens frequently associated with outbreaks in healthcare settings, particularly among immunocompromised patients.

Differentiation among the 21 DNA homology groups (known as genomospecies) of *Acinetobacter* spp. is difficult. However, members of the *A. baumannii* complex typically are the most common isolates from hospital outbreaks and tend to be more resistant to antimicrobial agents than other *Acinetobacter* spp.

CASE STUDY B

A 34-year-old woman suffered extensive head injuries after slipping from a cliff while hiking. After extensive head and neck surgery, she was placed on a ventilator in the neurosurgical intensive care unit. Five days later she became febrile and developed a pulmonary infiltrate. Cultures of blood and respiratory secretions yielded the same organism identified as *Acinetobacter baumannii* which was resistant to ceftazidime, ciprofloxacin, gentamicin, piperacillin, tobramycin, and trimethoprim-sulfamethoxazole.

Should further testing be done on this isolate to verify the results obtained? What, if any, additional antimicrobial agents should be tested? The reader will be able to answer these questions after working through this chapter.

Resistance—Beta-lactams

Beta-lactam resistance in *Acinetobacter* spp. can be due to beta-lactamases (carbapenemases), alterations in penicillin binding proteins, over-expression of multidrug efflux systems and decreased outer membrane permeability caused by the loss or reduced expression of porins. Imipenem/meropenem resistance can occur in healthcare-associated strains that carry a combination of several mechanisms in the same organism.

Resistance—Aminoglycosides

Acinetobacter spp. possesses a wide variety of aminoglycoside-modifying enzymes, and susceptibility profiles to aminoglycosides can vary considerably among clinical isolates. Strains can be resistant to amikacin, gentamicin, and tobramycin.

Resistance—Fluoroquinolones

Although fluoroquinolones show activity against many isolates of *Acinetobacter* spp., resistance is becoming common. Resistance to fluoroquinolones is due to efflux pumps and mutations affecting the DNA gyrase and topoisomerase IV enzymes.

Resistance—Other Agents

Acinetobacter spp. can develop resistance to a wide variety of antimicrobial agents during relatively short periods of time. Multiply resistant strains, susceptible only to polymyxin B or colistin, have been noted in hospitals around the world. Note that there are no NCCLS guidelines for interpretation of zone sizes or MICs for either polymyxin B or colistin. Multiresistance in *Acinetobacter* spp. is a consequence of multiple efflux pumps, altered porin changes that reduce uptake of antimicrobial agents, altered penicillin binding proteins, and a multitude of beta-lactamases and aminoglycoside-modifying enzymes.

Testing Strategy

NCCLS M100 Table 1 suggests agents for routine testing and reporting against *Acinetobacter* spp. In the disk diffusion section, the listing in Table 1 is applicable to only *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia* and *B. cepacia*.

In MIC Table 1, the listing includes *P. aeruginosa* and other non-*Enterobacteriaceae*. These non-*Enterobacteriaceae* are defined in a footnote to include *Acinetobacter* spp., *S. maltophilia*, *Pseudomonas* spp. and other non-fastidious, glucose non-fermenting gram-negative bacilli.

Methods

NCCLS has specific standards for disk diffusion and MIC testing of *Acinetobacter* spp. However, the results from these two tests may not always agree, particularly for beta-lactam drugs like piperacillin. When standardized procedures are used for inoculum preparation, inoculation, and incubation, the results for carbapenems, fluoroquinolones, and amikacin are consistent for both methods. Table 1 in NCCLS 100 lists drugs suggested for testing and reporting.

Methods—Interpreting and Reporting Results

NCCLS Table 2B in both the disk diffusion and MIC sections of M100 contains interpretive criteria for non-*Enterobacteriaceae* including *Acinetobacter* spp.

In the disk diffusion Table 2B, several beta-lactams, such as piperacillin, have separate interpretive criteria listed for *P. aeruginosa* and *Acinetobacter* spp. Some agents listed, such as ampicillin-sulbactam, have specific recommendations for *Acinetobacter* spp.

In the MIC Table 2B, interpretive criteria for non-*Enterobacteriaceae* apply to *Acinetobacter* spp.

Quality Control

The NCCLS-recommended QC strains are as listed for *P. aeruginosa*:

- *P. aeruginosa* ATCC 27853
- *E. coli* ATCC 25922
- *E. coli* ATCC 35218 (for QC beta-lactam/beta-lactamase inhibitor combinations)

Commentary on Case Study B

As noted previously, a very resistant *A. baumannii* isolate has been obtained from blood and sputum cultures of the woman in the neurosurgical ICU. This isolate is resistant to all antimicrobial agents routinely reported. If the prevalence of multi-resistant strains is low in your patient population, several issues should be considered in determining what to do with the isolate. Although *A. baumannii* with the resistant profile demonstrated in Case Study B has been reported previously, you should consider verifying these results by:

- Confirming the identification
- Confirming the susceptibility

If there is suspicion that the methods used may have led to erroneous results, an alternative method should be used to confirm results.

Technical Tip: Make certain the tests were not contaminated. Testing mixed populations of bacteria can lead to what may appear to be highly resistant bacteria.

STENOTROPHOMONAS MALTOPHILIA

BACKGROUND

S. maltophilia, formerly known as *Pseudomonas maltophilia* and *Xanthomonas maltophilia*, is an opportunistic pathogen that has become increasingly prevalent as a cause of healthcare-associated infections, primarily among immunosuppressed patients. Although *S. maltophilia* can cause many different types of infections, it often colonizes the respiratory tract, which is the source of most clinical isolates of *S. maltophilia*.

S. maltophilia is inherently resistant to many broad-spectrum antimicrobial agents. The agent of choice for *S. maltophilia* infections is trimethoprim-sulfamethoxazole, which may be prescribed in combination with ticarcillin-clavulanic acid or rifampin. Other agents sometimes considered for therapy include chloramphenicol or minocycline.

CASE STUDY C

A 19-year-old woman with carcinoma of the liver developed fever, abdominal pain, and vomiting. She was admitted to the local medical center where blood cultures were drawn. She was started on cefuroxime and gentamicin.

The following day, *S. maltophilia* was isolated from her blood cultures and she remained febrile. The patient was allergic to sulfa drugs so trimethoprim-sulfa-

methoxazole therapy was not possible. The physician asked the laboratory for additional susceptibility results. What agents should be tested?

Resistance—Beta-lactams

Beta-lactam resistance in *S. maltophilia* often is due to two distinct beta-lactamases, designated L1 and L2,

- L1 is a metalloenzyme (contains Zn⁺⁺ at the active site of the enzyme) found in virtually all *S. maltophilia*. L1 confers resistance to imipenem and meropenem.
- L2 is a cephalosporinase and is inhibited by beta-lactamase inhibitors such as clavulanic acid.

Other beta-lactamases have been identified in *S. maltophilia*, and sometimes beta-lactam resistance may also be due to porin changes. Mutants resistant to meropenem can be isolated with high frequency in vitro; therefore the use of this antibiotic in patients should be avoided.

Resistance—Aminoglycosides

Resistant to aminoglycosides among *S. maltophilia* is believed to be due to lack of permeability of the outer membrane to these drugs. Resistance due to aminoglycoside-modifying enzymes is uncommon in this species. *S. maltophilia* typically is resistant to all aminoglycosides.

Resistance—Fluoroquinolones

Although *S. maltophilia* may appear susceptible to fluoroquinolones, resistance can develop rapidly as a result of mutations in genes that encode the outer-membrane proteins. Consequently, fluoroquinolones are not used as single agents in treating infections caused by *S. maltophilia*.

Resistance—Other Agents

Resistance to trimethoprim-sulfamethoxazole occurs in approximately 2–5% of *S. maltophilia* isolates. *S. maltophilia* strains frequently are resistant to minocycline and chloramphenicol, probably due to the presence of efflux pumps or changes in outer-membrane proteins.

Testing Strategy

S. maltophilia was recently added to NCCLS M100 disk diffusion Table 1 and MIC Table 1.

NCCLS M100 Table 1 lists drugs for testing and reporting for non-*Enterobacteriaceae*. Several are listed specifically for non-*Enterobacteriaceae* other than *P. aeruginosa*, which include: chloramphenicol, tetracycline, ticarcillin-clavulanic acid, and trimethoprim-sulfamethoxazole. In addition, Table 2B containing MIC interpretive criteria lists moxalactam as an option for *S. maltophilia*. However, this agent is not available for routine use in the United States.

Methods

NCCLS MIC methods have been standardized for testing *S. maltophilia*. Disk diffusion testing has been standardized only for the following antimicrobial agents: minocycline, levofloxacin and trimethoprim-sulfamethoxazole. Standardized procedures for inoculum preparation, inoculation, and incubation are used for disk diffusion and MIC testing.

There are ongoing concerns about the lack of correlation of in vitro results with in vivo response for *S. maltophilia*. The ability to perform prospective studies and provide additional guidance is limited by:

- Difficulty in identifying an acceptable test methodology.
- Difficulty distinguishing infection from colonization in patients.
- The need to treat *S. maltophilia* infections with combination therapy.

Methods—Interpreting and Reporting Results

Table 2B in the disk diffusion section of NCCLS M100 contains a recent general comment that only results for minocycline, levofloxacin, and trimethoprim-sulfamethoxazole should be reported for *S. maltophilia*. Disk diffusion should not be used for testing other antimicrobial agents.

Table 2B in the MIC section of NCCLS M100 contains interpretive criteria for non-*Enterobacteriaceae* including *S. maltophilia*. Several beta-lactams have separate interpretive criteria listed for *P. aeruginosa* and other non-*Enterobacteriaceae*. The interpretive criteria for “other non-*Enterobacteriaceae*” apply to *S. maltophilia*. (See ticarcillin-clavulanic acid showing two sets of interpretive criteria and a comment for *S. maltophilia*.)

Quality Control

NCCLS-recommended QC strains are as listed for *P. aeruginosa*:

- *P. aeruginosa* ATCC 27853
- *E. coli* ATCC 25922
- *E. coli* ATCC 35218 (for beta-lactam/beta-lactamase inhibitor combinations)

OTHER NON-ENTEROBACTERIACEAE

Non-*Enterobacteriaceae*, other than *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, and *B. cepacia* should only be tested by MIC methods because there are no standardized disk diffusion methods for testing these bacteria.

Testing recommendations are the same as those for *Acinetobacter* spp., *S. maltophilia* and *B. cepacia*. There are no unique comments for the other non-*Enterobacteriaceae*.

REVIEW

You should now be knowledgeable about routine antimicrobial susceptibility testing recommendations for *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, *B. cepacia*, and other non-*Enterobacteriaceae*.

Remember to:

- Use the most current NCCLS M2 and M7 standards for instructions for testing non-*Enterobacteriaceae*.
- Use MIC methods for members of the non-*Enterobacteriaceae* other than *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia* or *B. cepacia*.
- Thoroughly read the package insert for any commercial product before using it for testing non-*Enterobacteriaceae*. Some commercial systems are not satisfactory for testing these organisms.

SELF-ASSESSMENT QUESTIONS

1. From the list below, select five beta-lactams that are often active against *P. aeruginosa*.
 - A. Ampicillin
 - B. Ampicillin-sulbactam
 - C. Cefazolin
 - D. Cefepime
 - E. Ceftazidime
 - F. Imipenem
 - G. Piperacillin
 - H. Ticarcillin
2. A broth microdilution MIC test on a *P. aeruginosa* isolate has yielded good growth in the wells with ceftazidime concentrations <1.0 mcg/mL. However, wells with 2.0–32 mcg/mL contain light growth described as a “haze” A carefully standardized repeat test on the same isolate did not contain any growth at >2.0 mcg/mL. Could the initial result have been due to an inoculation problem?
 - A. Yes
 - B. No
3. You have performed the disk diffusion test on a mucoid strain of *P. aeruginosa* from the sputum of an elderly patient. On initial and repeat testing, growth was light and insufficient to interpret the results. What will you do?
 - A. Contact the physician and explain that this isolate grows poorly using the routine testing procedure. Determine whether MIC results are needed for the care of the patient.
 - B. Measure zones, interpret, and report results.
 - C. Repeat the disk diffusion test a third time.
4. You obtained a piperacillin MIC of 64 mcg/mL on a *P. aeruginosa* isolate. The interpretive criteria for piperacillin are found in Table 2B. How would you interpret the result?
 - A. Susceptible
 - B. Intermediate
 - C. Resistant
5. How will you proceed with antimicrobial susceptibility testing of the mucoid and nonmucoid colonies isolated from a culture of the same specimen?
 - A. Combine the mucoid and nonmucoid colony strains and perform a single susceptibility test.
 - B. Test the mucoid colony type only.

- C. Perform separate tests on the mucoid and nonmucoid strains.
 D. Refrain from performing any susceptibility tests.
6. Assuming the following are available to you, which can you use to obtain reliable results on the isolates in Question 5? Select all that apply.
- A. Disk diffusion only
 B. Broth microdilution only
 C. Disk diffusion or broth microdilution MIC
 D. An automated commercial MIC method
7. Below is the final report for the two colony types in Question 5.

Specimen Source:Sputum

Results: Heavy growth mucoid *Pseudomonas aeruginosa* (1) Heavy growth non-mucoid *Pseudomonas aeruginosa*(2)Normal Respiratory flora.

	(1)	(2)
Ceftazidime	S	R
Gentamicin	S	R
Imipenem	–	S
Piperacillin	S	R
Tobramycin	–	S

Comment: Patients with serious *P. aeruginosa* infections may require maximum doses of a beta-lactam in combination with an aminoglycoside.

- Why were three drugs reported on the mucoid isolate and five on the nonmucoid isolate?
- A. Imipenem should never be reported on mucoid *P. aeruginosa*.
 B. The mucoid strain is susceptible to the primary agents and, according to the laboratory's selective reporting policies, secondary agents are not to be reported.
 C. Tobramycin should never be reported on a gentamicin-susceptible *P. aeruginosa*.
8. Review the organisms listed below. Select the susceptibility profile that best matches each organism.
- A. A hospital-acquired strain of *A. baumannii*
 B. A community-acquired strain of *A. baumannii* associated with colonization of the patient
 C. *A. lwoffii*

	(1)	(2)	(3)
Cefotaxime	R	S	S
Gentamicin	R	S	S
Imipenem	R	S	S
Piperacillin	R	S	R
Tobramycin	R	S	S
Trimethoprim-sulfamethoxazole	R	S	S

9. What, if any, additional antimicrobial agents should be tested against isolate (1) in Question 8? Select all that apply.
- A. Ampicillin-sulbactam
 - B. Polymyxin B
 - C. Vancomycin

10. **Lab Report**

Specimen Source: Expecterated sputum

Results: Few *Stenotrophomonas maltophilia*.

Normal respiratory flora.

Would you perform antimicrobial susceptibility tests on this isolate?

- A. Yes
- B. No

11. **Lab Report**

Specimen Source: tracheal aspirate

Results: *Stenotrophomonas maltophilia*

Comments: Susceptibility testing is not routinely performed on *S. maltophilia*. Trimethoprim-sulfamethoxazole is the drug of choice for treating infections caused by *S. maltophilia*.

Why is this reporting approach reasonable? Select all that apply.

- A. *S. maltophilia* is difficult to test and correlation of in vitro results with in vivo outcome is limited.
 - B. *S. maltophilia* does not grow well in routine MIC test systems.
 - C. Trimethoprim-sulfamethoxazole is the drug of choice for *S. maltophilia* and there is little resistance to this agent.
12. A physician asked for results on imipenem since it was not included in the lab report seen in Question 11. Imipenem is tested routinely on gram-negative bacteria in your laboratory. How should you respond?
- A. Check results for imipenem and release them to the physician.
 - B. Inform the physician that imipenem is not active against *S. maltophilia* and this is the reason the laboratory did not report it.
13. What antimicrobial agents will you report on a *S. maltophilia* isolated from blood? Select all that apply
- A. Ticarcillin
 - B. Ticarcillin-clavulanic acid
 - C. Trimethoprim-sulfamethoxazole
 - D. Chloramphenicol
 - E. Tetracycline
14. If this patient is known to be allergic to sulfa what additional antimicrobial agents might be reported? Select all that apply.
- A. Ceftazidime
 - B. Ciprofloxacin
 - C. Meropenem

The antimicrobial agents reported may vary depending on your laboratory's policies and should reflect agents that might be considered in combination therapy for *S. maltophilia*.

OBJECTIVES

When readers have completed this chapter, they should be able to:

- Discuss a practical strategy for antimicrobial susceptibility testing of *Haemophilus* spp. in their laboratory.
- Describe where to find recommendations for testing conditions, including inoculum preparation, test medium, duration of incubation, and atmosphere of incubation for disk diffusion and MIC testing of *Haemophilus* spp.
- Describe methods that can be used to detect beta-lactamase producing *H. influenzae* and strains that are beta-lactamase negative, ampicillin resistant (BLNAR).
- Explain the rationale for using two quality control strains when performing antimicrobial susceptibility tests with *Haemophilus* spp.

HAEMOPHILUS INFLUENZAE VS. OTHER HAEMOPHILUS SPP.

The methods described in this chapter are standardized for *Haemophilus influenzae* and other *Haemophilus* spp. Because *Haemophilus* spp. other than *H. influenzae* are infrequently tested in the routine clinical laboratory, the emphasis of the chapter will be on antimicrobial susceptibility testing and reporting for *H. influenzae*.

BACKGROUND

Before *H. influenzae* type B vaccine became available, *H. influenzae* was one of the primary causes of meningitis in children in the United States. Meningitis and other systemic diseases due to this serotype are now uncommon in the United States.

CHILDREN VS. ADULTS

H. influenzae, particularly the nontypeable strains, continue to cause otitis media and respiratory tract infections in children. In adults, *H. influenzae* is also associated with respiratory tract infections. *H. influenzae* may also cause bacteremia in AIDS patients.

The antimicrobial agents commonly used to treat infections caused by *H. influenzae* include amoxicillin, amoxicillin-clavulanic acid, cephalosporins, macrolides, and tetracyclines. In addition, for children, trimethoprim-sulfamethoxazole is sometimes prescribed. For adults, a fluoroquinolone may be used.

CASE STUDY

A 28-year-old patient with AIDS was admitted to the hospital with a productive cough, extreme lethargy, and a fever of 103°C. Two sets of blood cultures were drawn and the patient was started on ceftriaxone and gentamicin. The following day, *H. influenzae* was isolated from both sets of blood cultures. The patient's physician noted that the final laboratory report (see report below) indicated that the organism was beta-lactamase positive. No additional susceptibility test results were reported.

Lab Report

Specimen

Source: blood

Results: *Haemophilus influenzae*

Comments: beta-lactamase positive; amoxicillin and ampicillin resistant.

Given the beta-lactamase result the physician was concerned that the isolate may not be susceptible to ceftriaxone.

Does the beta-lactamase test provide sufficient information for therapy of this patient? How would you respond to the physician?

After working through this chapter the reader should be able to answer these questions and to comment on the laboratory's testing strategy for blood isolates of *H. influenzae*.

Resistance—Amoxicillin and Ampicillin

- In the United States, approximately 40% of *H. influenzae* isolates produce a plasmid-mediated beta-lactamase that confers resistance to amoxicillin and ampicillin. TEM-1 and ROB-1 beta-lactamases are found in *H. influenzae*.
- Because amoxicillin is frequently used for treatment of respiratory tract infections, testing *H. influenzae* for beta-lactamase production is useful, since amoxicillin is hydrolyzed or inactivated by beta-lactamase.
- Occasional strains of *H. influenzae* do not produce beta-lactamase but nonetheless are resistant to amoxicillin and ampicillin due to changes in penicillin binding proteins (PBPs). These strains are designated as "beta-lactamase negative, ampicillin resistant" or BLNAR strains.

Resistance—Other Agents

- Resistance to cephalosporins and newer macrolides, which are commonly used to treat respiratory tract infections, is uncommon in *H. influenzae*.
- Resistance to extended-spectrum cephalosporins has been reported in rare cases but has not been confirmed.

- Fluoroquinolone resistance also is rare.
- Approximately 10–20% of isolates of *H. influenzae* are resistant to trimethoprim-sulfamethoxazole.

Testing Methods

NCCLS Table 1A provides useful suggestions for developing a testing strategy for *H. influenzae*.

**Group A
Primary Test and Report**

Haemophilus spp.

Ampicillin

Trimethoprim-sulfamethoxazole

Group A of the Table contains two agents, ampicillin and trimethoprim-sulfamethoxazole.

Although beta-lactamase testing will identify most ampicillin-resistant strains, it is still important to test ampicillin by an MIC or disk-diffusion method for serious infections caused by beta-lactamase-negative strains.

Testing trimethoprim-sulfamethoxazole may be warranted depending on usage policies and incidence of resistance in a particular geographic area.

Agents in Group B are used primarily to treat serious infections, such as meningitis, bacteremia, and epiglottitis. These agents are administered parenterally. Results from testing one of the third-generation cephalosporins and meropenem should be reported on isolates from cerebral spinal fluid (CSF).

**Group B Primary Test
Report Selectively**

Cefotaxime or ceftazidime or ceftizoxime or
ceftriaxone

Cefuroxime sodium (parenteral)

Chloramphenicol

Meropenem

Group C includes many oral agents. However, these agents are rarely tested in the laboratory. See footnote below from Table 1A.

“[These agents] may be used as empiric therapy for respiratory tract infections due to *Haemophilus* spp. The results of susceptibility tests with these agents are often not useful for management of individual patients. However, susceptibility testing of *Haemophilus* spp. with these compounds may be appropriate for surveillance or epidemiologic studies.”

**Group C Supplemental
Report Selectively**

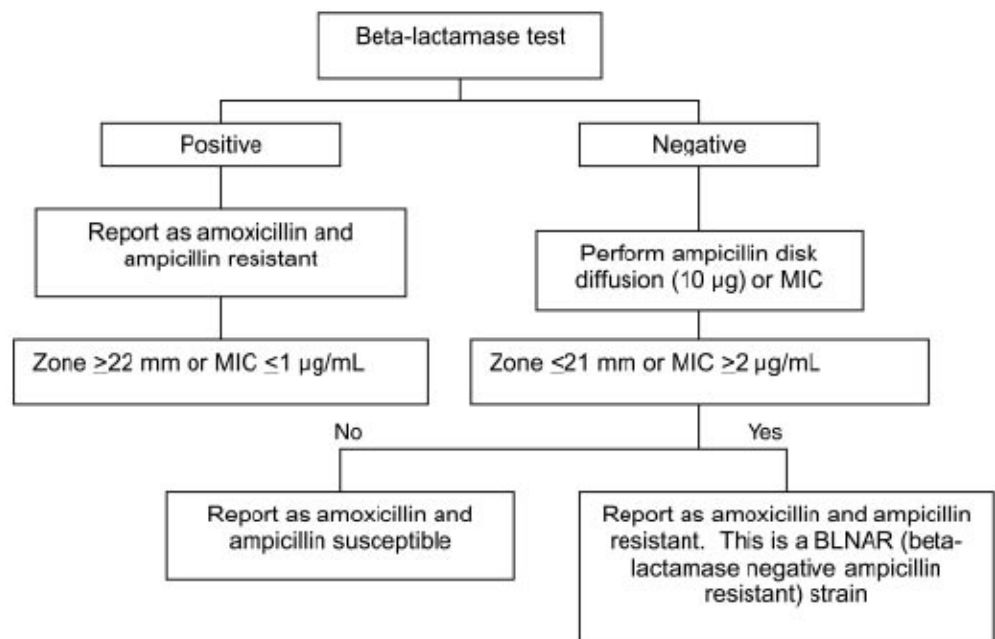
Azithromycin or clarithromycin
 Aztreonam
 Cefaclor or cefprozil or loracarbef
 Cefdinir or cefixime or cefpodoxime
 Cefonicid
 Cefuroxime axetil (oral)
 Ciprofloxacin or gatifloxacin or levofloxacin or lomefloxacin or
 moxifloxacin or ofloxacin or sparfloxacin
 Gemifloxacin
 Ertapemen or imipenem
 Rifampin
 Tetracycline

Methods—Beta-lactamase Test

Most standard rapid beta-lactamase tests are satisfactory for *H. influenzae*. The algorithm (shown below) shows how the beta-lactamase test is used for deducing results for ampicillin and amoxicillin.

See Chapter 2 Beta-Lactamases in this manual for instructions on performing a beta-lactamase test.

Beta-lactamase Testing for Amoxicillin and Ampicillin Resistance



Methods—Incubation Conditions

NCCLS has specific standards for testing *Haemophilus* spp.

Routine disk diffusion and MIC methods are done with these modifications

Method	Medium	Incubation	
		Time (h)	Atmosphere
Disk diffusion	HTM* agar	16–18	CO ₂ (5%)
Broth MIC	HTM* broth	20–24	Ambient air

* HTM. *Haemophilus* test medium

Methods—Inoculum Preparation Notes

Results of routine disk diffusion and MIC tests for *Haemophilus* spp. and beta-lactam agents are significantly affected by the number of bacteria in the test inoculum, so inoculum standardization is very important.

A heavy inoculum may lead to false resistance, particularly with beta-lactam agents.

Technical tip:

- Use the direct standardization method for inoculum preparation.
- Select colonies from a plate no older than 24 h (preferably 20–24 h).
- Ideally, use a photometric device to standardize the inoculum suspension.

Methods—Measuring Zones

HTM agar is translucent and slightly more yellow than Mueller-Hinton agar (MHA). If you encounter double zones, measure and report the results of the inner zone.

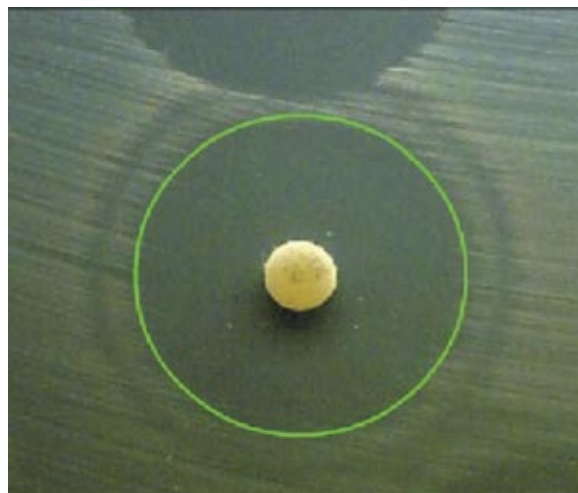


Figure 14.1 A photo of double zones of inhibition with a circle indicating the true zone diameter

Methods—Interpretation of Results

- NCCLS document M100 Tables 2E, in both the disk diffusion and MIC sections, contain interpretive criteria for *Haemophilus* spp.
- Table 2E applies to *H. influenzae* and other *Haemophilus* spp. that grow comparably to *H. influenzae*.
- For several antimicrobial agents, including cefotaxime and ceftriaxone (see table below), there are “susceptible only” interpretive criteria. Resistance to these agents among *Haemophilus* spp. has not been reported to date.

Haemophilus spp. Interpretive Criteria—Cefotaxime & Ceftriaxone

	Disk Content (μg)	Disk Diffusion (mm)		
		Susceptible	Intermediate	Resistant
Cefotaxime	30	≥ 26	–	–
Ceftriaxone	30	≥ 26	–	–

	MIC ($\mu\text{g}/\text{mL}$)		
	Susceptible	Intermediate	Resistant
Cefotaxime	≤ 2	–	–
Ceftriaxone	≤ 2	–	–

Technical tip: If you encounter a *Haemophilus* spp. that is not susceptible to cefotaxime or any other drug for which there are only susceptible interpretive criteria:

- Confirm the identification.
- Confirm the susceptibility results.
- Send isolate to a reference laboratory for an MIC test by the NCCLS broth microdilution reference method.
- Save the isolate.

Reporting Results—BLNAR

Isolates that are resistant to ampicillin and amoxicillin due to changes in penicillin binding proteins frequently test as susceptible to agents in vitro even though patients fail to respond to these drugs clinically.

Thus, the following antimicrobial agents should be reported as resistant regardless of their in vitro test results, presuming that they are normally reported by the laboratory:

Amoxicillin-clavulanic acid	Cefonicid
Ampicillin-sulbactam	Cefprozil
Cefaclor	Cefuroxime
Cefamandole	Loracarbef
Cefetamet	Piperacillin-tazobactam

Quality Control

Check Chapter 6 QA/QC in this manual for specific instructions on QC of tests for *Haemophilus* spp.

Two quality control strains are recommended for disk diffusion and MIC testing of *Haemophilus* spp.

- *H. influenzae* ATCC 49247 (ampicillin-R beta-lactamase negative) is used for QC of most agents but does not perform well with carbapenems and certain cepheims. Consequently, a second QC strain is necessary when these agents are tested
- *H. influenzae* ATCC 49766 is used for QC of cefaclor, cefamandole, cefdinir, cefonicid, cefprozil, cefuroxime, imipenem, loracarbef, and meropenem.

Based on NCCLS suggestions, and to meet physicians' needs, a practical strategy for testing *H. influenzae* isolates associated with life-threatening infections would be to:

- Perform a beta-lactamase test.
- Perform disk diffusion or MIC tests with the specific antimicrobial agent(s) the physician is considering for therapy.

REVIEW

Now the reader should be familiar with antimicrobial susceptibility testing methods for *H. influenzae* and other *Haemophilus* spp.

Remember to:

- Use the most current NCCLS standards (M2 and M7) for instructions for testing *Haemophilus* spp. The M100 standards are updated annually and contain the recent tables, including reporting suggestions.
- Perform a beta-lactamase test as a rapid way to determine if the isolate is resistant to amoxicillin or ampicillin.
- Consider testing ampicillin (for beta-lactamase-negative strains), a third-generation cephalosporin, and chloramphenicol on isolates causing life-threatening infections. Test meropenem if it is on your institution's formulary.

SELF-ASSESSMENT QUESTIONS

1. Are the results of a beta-lactamase test sufficient for isolates from blood?
 - A. Yes
 - B. No
2. What agents does NCCLS suggest for testing against *H. influenzae* isolates from patients with life-threatening infections, such as meningitis? Select all that apply.
 - A. Ampicillin
 - B. A third-generation cephalosporin
 - C. Chloramphenicol
 - D. Meropenem

3. What approach does NCCLS suggest for testing *H. influenzae* isolates from other body sites (e.g., respiratory isolates)? Table 1A in NCCLS document M100 will help you with this.
 - A. Test for beta-lactamase only
 - B. Test ampicillin and trimethoprim-sulfamethoxazole only.
 - C. Test ampicillin, amoxicillin-clavulanic acid, and trimethoprim-sulfamethoxazole only.
4. Why do some laboratories perform only a beta-lactamase test rather than strictly following the NCCLS suggests when testing *H. influenzae*? Check all that apply.
 - A. Ampicillin/amoxicillin resistance is the major concern for respiratory tract isolates of *H. influenzae* and those results are easily predicted by beta-lactamase.
 - B. Disk diffusion and MIC testing for *H. influenzae* require HTM agar or broth, which is expensive.
 - C. BLNAR are uncommon.
 - D. To date, no resistance to third-generation cephalosporins has been reported.
5. Why would some ampicillin-resistant *H. influenzae* not be detected by beta-lactamase?
 - A. The test lacks sensitivity and specificity for *H. influenzae*.
 - B. Rare isolates are ampicillin resistant by an alternative resistance mechanism.
 - C. Some *H. influenzae* isolates are pigmented and cannot be reliably tested with a colorimetric test.

OBJECTIVES

When readers have completed this chapter they should be able to:

- Describe primary resistance concerns in *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Moraxella catarrhalis*.
- List the recommended conditions for disk diffusion and agar dilution MIC testing of *N. gonorrhoeae*, such as inoculum preparation, agar medium, incubation atmosphere, and incubation length.
- Discuss limitations of antimicrobial susceptibility testing of *N. meningitidis*.
- Describe a practical strategy for testing and reporting the antimicrobial susceptibility of *N. gonorrhoeae*, *N. meningitidis* and *M. catarrhalis*.
- Discuss the pros and cons of the beta-lactamase test for *M. catarrhalis*.

NEISSERIA GONORRHOEAE**BACKGROUND**

N. gonorrhoeae continues to be a common cause of sexually transmitted disease throughout the world. Penicillin was the drug of choice for treating uncomplicated urethral, cervical, and rectal infections caused by *N. gonorrhoeae*. However, due to emerging resistance to penicillin, current recommendations for empiric therapy include ceftriaxone, cefixime, or a fluoroquinolone such as ciprofloxacin or ofloxacin. Spectinomycin, an aminocyclitol, is no longer recommended for *N. gonorrhoeae* in the United States because of its high cost and the availability of more effective agents.

It is uncommon for *N. gonorrhoeae* to cause disease outside the genitourinary tract, but it occasionally causes disseminated infection, including septic arthritis. During delivery a woman can transmit *N. gonorrhoeae* to her child, causing ophthalmia neonatorum, an infection of the eyes. To prevent this condition, clinicians administer silver nitrate, erythromycin, or tetracycline eye drops to all newborns in the United States and in some other countries. This treatment also will prevent ophthalmia neonatorum caused by *Chlamydia trachomatis*.

CASE STUDY—N. GONORRHOEAE

A 21-year-old soldier, upon returning to the United States from Southeast Asia, presented to the local emergency room complaining of painful urination and purulent discharge from his penis. Urethral specimens were collected for detection of

N. gonorrhoeae and *C. trachomatis* using DNA-based methods. The patient was given a single 500-mg oral dose of ciprofloxacin. The tests were positive for *N. gonorrhoeae* but negative for *C. trachomatis*. When the patient's symptoms did not improve after two days, he returned to the emergency room.

What test(s) would be most appropriate to perform?

Since the patient had just completed a tour of duty in Southeast Asia where fluoroquinolone-resistant *N. gonorrhoeae* is prevalent, and had not responded to initial therapy, the physician was concerned that the patient had a fluoroquinolone-resistant isolate. A repeat culture was performed and the isolate was sent to the state health department for agar dilution MIC testing. This time the patient was given a single dose of cefixime; the patient's symptoms subsided within 48 hours of therapy. The agar dilution ciprofloxacin MIC reported (see Table below) for the *N. gonorrhoeae* isolate by the state health department was 2 mcg/mL.

Lab Report

Specimen Source: Urethral discharge

Results: *N. gonorrhoeae*

Drug	MIC ($\mu\text{g/mL}$)	Interpretation
Ceftriaxone	≤ 0.12	S
Ciprofloxacin	2	R
Tetracycline	≤ 0.25	S

After working through this chapter the reader will be able to answer questions on this case.

Resistance—Penicillin

Penicillin resistance in *N. gonorrhoeae* is due to either:

- Plasmid-mediated beta-lactamase production (referred to as penicillinase-producing *N. gonorrhoeae* or PPNG).
- Altered penicillin binding proteins encoded by genes on the chromosome (referred to as chromosomally-mediated resistant *N. gonorrhoeae* or CMRNG).

Incidence of each resistance mechanism in the United States (2001):

- PPNG—2.0%
- CMRNG—6.4%

Neither of the above mechanisms mediates resistance to extended-spectrum cephalosporins, such as ceftriaxone or cefixime. Resistance to these cephalosporins has yet to be documented in *N. gonorrhoeae*.

Resistance—Other Agents

- Tetracycline resistance in *N. gonorrhoeae* usually is plasmid mediated, but it also can be chromosomally mediated.
- Fluoroquinolone resistance is uncommon in the United States; however, high percentages (up to 80%) of fluoroquinolone-resistant organisms have been noted in other geographic areas, particularly Southeast Asia.
- Spectinomycin resistance is low in the United States and abroad.

Testing Strategy—*N. gonorrhoeae*

NCCLS M100 Table 1A provides useful suggestions for developing a testing strategy. No drugs are listed under Groups A and B since routine testing of *N. gonorrhoeae* is not recommended by NCCLS. Drugs that would be appropriate for testing in selected situations are listed in Group C.

Note: Using a network of laboratories located throughout the United States, the CDC performs ongoing surveillance for the development and spread of antimicrobial resistance in *N. gonorrhoeae*. These monitoring efforts help assess the need for periodic changes in CDC recommendations for empiric therapy of gonococci infections.

Testing Standards—*N. gonorrhoeae*

Agar dilution is the MIC reference method for *N. gonorrhoeae*; broth methods are not reliable because *N. gonorrhoeae* may lyse in broth media.

NCCLS has specific standards for testing *N. gonorrhoeae*.

Method ^a	Medium	Incubation	
		Time (h)	Atmosphere
Disk diffusion	Agar ^b + 1% defined supplement	20–24	CO ₂ (5%)
Agar dilution MIC ^c	Agar + 1% defined supplement	20–24	CO ₂ (5%)

^a For these procedures see the Disk Diffusion and MIC chapters.

^b GC agar base with a 1% defined supplement. It is similar in appearance to Mueller-Hinton agar.

^c When the agar dilution method is used for carbapenems or clavulanate, the GC agar base with 1% defined supplement must be free of cysteine, because cysteine interferes with the test for these agents. No problem occurs with cysteine in the disk diffusion test.

Interpretation of Results—*N. gonorrhoeae*

Penicillin zone interpretation:

Smaller penicillin zone diameters, i.e., ≤ 19 mm, suggest beta-lactamase mediated resistance. Larger zones, i.e., ≥ 20 mm, are more likely to be a result of chromosomally-mediated resistance. Chromosomally-mediated penicillin resistance typically produces lower MICs than resistance mediated by beta-lactamase.

Interpretive criteria:

- NCCLS M100 Tables 2F contain interpretive criteria for *N. gonorrhoeae* in both the disk diffusion and MIC chapters
- For several extended-spectrum cephalosporins there are only “susceptible” interpretive criteria. Resistance to these agents among *N. gonorrhoeae* either has not been reported to date or is extremely rare.

Technical Tip: If you encounter a strain of *N. gonorrhoeae* that is not susceptible to cefotaxime or any other drug with only susceptible interpretive criteria:

- Confirm the identification and the susceptibility test results.
- Send isolate to a reference laboratory for confirmation using the NCCLS agar dilution reference method.
- Save the isolate.

Quality Control—*N. gonorrhoeae*

Consult Chapter 6 of this manual for specific instructions on QC of tests for *N. gonorrhoeae*.

The NCCLS-recommended QC strain is:

N. gonorrhoeae ATCC 49226

This is a chromosomally mediated penicillin-resistant strain that also gives intermediate results for tetracycline.

NEISSERIA MENINGITIDIS**BACKGROUND**

N. meningitidis continue to cause both epidemic and sporadic diseases around the world. *N. meningitidis* can cause pneumonia or systemic diseases such as meningitis and bacteremia that have relatively high mortality rates.

Disseminated disease requires prompt treatment with an extended-spectrum cephalosporin or chloramphenicol.

Because *N. meningitidis* can be highly contagious, close contacts of the infected patient are treated prophylactically with rifampin, a fluoroquinolone, or ceftriaxone.

A meningococcal vaccine, primarily active against serotypes A and C, is available for high-risk populations.

CASE STUDY—*N. MENINGITIDIS*

A 19-year-old football player became ill and left practice early. He went to his fraternity house, slept for several hours, but woke feeling feverish, confused, and disoriented. He was taken to an emergency room, where physicians noted a petechial rash over much of his body.

The patient's symptoms suggested a diagnosis of meningococcal meningitis. A cerebrospinal fluid (CSF) specimen was obtained for Gram stain and culture and the patient was started on ceftriaxone. The final report (see table below) was issued without susceptibility results.

Lab Report

Specimen Source: CSF

Direct Gram stain: Rare gram-negative diplococci

Many WBCs

Culture Report: *Neisseria meningitidis*

Why did the laboratory not perform susceptibility tests on this isolate?

The reader should be able to answer this question after working through this chapter.

Penicillin resistance vs. decreased susceptibility

Although MIC interpretive criteria have not been standardized for *N. meningitidis*, the generally accepted interpretive criteria for penicillin are:

- Susceptible ≤ 0.06 mcg/mL
- Intermediate 0.1–1 mcg/mL
- Resistant ≥ 2.0 mcg/mL

In the early 1980's, four isolates of beta-lactamase-producing *N. meningitidis* were reported: two from South Africa and one each from Canada and Spain. The penicillin MICs for all four organisms were ≥ 128 mcg/mL. Two additional beta-lactamase-producing isolates from Spain have been described recently.

Isolates with “decreased susceptibility” to penicillin (sometimes referred to as “relatively resistant” or “insensitive”) have been reported in the literature.

These strains:

- Show penicillin MICs ranging from 0.1 to 1.2 mcg/mL.
- Appear to have an altered penicillin-binding protein 2 (PBP 2) that accounts for the decreased susceptibility.
- Remain susceptible to extended-spectrum cephalosporins.
- Are relatively uncommon.

Resistance—Other Agents

Ceftriaxone resistance has not been reported in *N. meningitidis*. By late 2004 reduced susceptibility to fluoroquinolones has been noted in only four isolates. Chloramphenicol resistance has been documented but is uncommon. Rifampin resistance in *N. meningitidis* also is relatively uncommon; it may be due to either of the following:

Mutations in the RNA polymerase gene (*rpoB*)
Decreases in permeability of the drug into the cell

Resistance to sulfonamides occurs frequently. With the availability of more desirable alternatives, sulfonamides are no longer recommended for prophylaxis.

Testing Strategy—*N. meningitidis*

Although NCCLS suggests a method for antimicrobial susceptibility testing (in M100, Table 7), there are no QC organism recommendations and no interpretive criteria for *N. meningitidis* results.

Because meningococcal disease is treated empirically with drugs that continue to be effective, routine testing is not warranted at this time. However, for cases in which treatment failures are suspected, isolates should be tested by one of the methods suggested by NCCLS.

Testing Standards—*N. meningitidis*

NCCLS has defined standard methods for testing *N. meningitidis*.

Method	Medium	Incubation	
		Time (h)	Atmosphere
Broth dilution	MH-LHB*	24	35°C, CO ₂ (5%)
Agar dilution	SB-MHA**	24	35°C, CO ₂ (5%)

* Cation-adjusted Mueller-Hinton broth containing 2–5% (v/v) lysed horse blood.

** SB-MHA. Mueller-Hinton agar containing 5% sheep blood

Testing and Reporting Beta-lactam MIC Results—*N. meningitidis*

When testing *N. meningitidis* isolates from CSF, some laboratories use pneumococcal interpretive criteria as a tentative guide for penicillin and cephalosporins. This is because the *S. pneumoniae* criteria are based on CSF levels, and NCCLS has no interpretive criteria for *N. meningitidis*. However, a comment that no standard criteria exist is important.

Physicians should obtain assistance from infectious disease specialists when using MIC results for which there are no interpretive criteria. To understand the results, the physicians should refer to the literature or experienced colleagues.

Interpretation of Results—*N. meningitidis*

If your laboratory does susceptibility testing on *N. meningitidis*, the results you report should be qualified (see report example below).

Lab report

Specimen

Source: CSF

Direct Gram stain: Rare gram-negative diplococci

Many WBCs

Culture report: *Neisseria meningitidis*

Drug	MIC (mcg/mL)
Ceftriaxone	≤ 0.03
Penicillin	0.25

Comments: Susceptibility results are presumptive; no interpretive criteria are available for *N. meningitidis*.

Quality Control—*N. meningitidis*

No standard quality control strain of *N. meningitidis* has been described by NCCLS. QC of the testing system (agar dilution or broth microdilution) should be performed using *Streptococcus pneumoniae* ATCC 49619.

MORAXELLA CATARRHALIS

BACKGROUND

M. catarrhalis often is associated with community-acquired respiratory tract infections; however *S. pneumoniae* and *H. influenzae* are more frequent causes of these infections.

M. catarrhalis may cause sinusitis, otitis media, bronchitis, and pneumonia. Systemic disease due to *M. catarrhalis* is rare. The agents typically used to treat *M. catarrhalis* infections include amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole, oral cephalosporins, macrolides, tetracyclines, and fluoroquinolones.

Susceptibility testing usually is not warranted.

Case study—*M. catarrhalis*

A 32-year-old teacher sought medical care following two weeks of progressively worsening sinus-related headaches and purulent nasal discharge. The physician requested a culture and sent the patient home with amoxicillin-clavulanic acid treatment. The laboratory report (see below) indicated *M. catarrhalis*. The patient's symptoms improved within 24 hours and she had a full recovery within six days.

Lab report

Specimen
Source: sinus drainage
Results: *Moraxella catarrhalis*

Is susceptibility testing useful for *M. catarrhalis* in this case? You will be able to answer this question after working through this chapter.

Resistance – beta-lactamases – *M. catarrhalis*

Between 85 and 95% of human clinical isolates of *M. catarrhalis* produce a beta-lactamase. The beta-lactamases of *M. catarrhalis* usually are either BRO-1 or BRO-2. These differ from the beta-lactamase TEM-1 that is a very common type found in many bacteria.

Not all beta-lactamase tests are satisfactory for detecting beta-lactamases produced by *M. catarrhalis*. The chromogenic cephalosporin method has been most reliable.

Resistance in *M. catarrhalis* to other agents that would be considered for treating respiratory tract infections is uncommon.

Testing Strategy—*M. catarrhalis*

There is no NCCLS standard method for antimicrobial susceptibility testing and reporting of *M. catarrhalis*.

Because *M. catarrhalis* generally is associated with respiratory tract infections and typically is susceptible to agents that would be prescribed, routine susceptibility testing is not warranted. Although most *M. catarrhalis* are beta-lactamase positive, some laboratories perform a beta-lactamase test and report results to further educate physicians that beta-lactamase-labile penicillins (e.g., ampicillin, amoxicillin, and penicillin) are inappropriate for therapy.

REVIEW

Remember to:

- Use the most current NCCLS M2 and M7 standards for testing *N. gonorrhoeae* and *N. meningitidis*. The M100 standards are updated annually and contain the most recent tables and reporting suggestions.
- Maintain an awareness of resistance issues for *N. gonorrhoeae* in your community and suggest culture and susceptibility testing for patients that appear to be failing therapy.
- When specifically requested to do so by a physician, perform broth dilution or agar dilution MIC tests on *N. meningitidis* isolated from normally sterile body sites or send isolate to a reference laboratory for testing.
- Refrain from performing antimicrobial susceptibility tests on *M. catarrhalis* because there is no NCCLS guideline for doing this and susceptibility test results generally are not necessary for patient care.

SELF-ASSESSMENT QUESTIONS

1. Which of the following statements are true regarding the absence of drugs in Group A and B for *N. gonorrhoeae* in NCCLS M100 Table 1A. Select all that apply.
 - A. It is not necessary to routinely test any drugs against *N. gonorrhoeae*.
 - B. Beta-lactamase is the only test needed.
 - C. The methods for testing *N. gonorrhoeae* have not been standardized.
2. Which inoculum preparation method should be used for either the agar dilution MIC test or disk diffusion test?
 - A. Direct standardization
 - B. Log phase growth
 - C. Stationary phase growth

3. Should beta-lactamase testing of *N. gonorrhoeae* be performed routinely by all clinical laboratories?
 - A. Yes
 - B. No
4. Referring to the case study in which the patient did not respond to ciprofloxacin, what tests are now warranted?
 - A. Repeat direct test for *N. gonorrhoeae* only.
 - B. *N. gonorrhoeae* culture only.
 - C. *N. gonorrhoeae* culture and susceptibility tests.
5. Which rationale might the laboratory give for NOT performing susceptibility tests routinely on *N. gonorrhoeae*? Select all that apply.
 - A. All *N. gonorrhoeae* are penicillin susceptible, so testing is unnecessary.
 - B. Uncomplicated genital infections with *N. gonorrhoeae* are usually treated empirically and typically respond.
 - C. Routine analysis using DNA-based methods eliminates the availability of an isolate for susceptibility testing.
6. Given the resistance and susceptibility patterns described in this chapter, what agent might be used for treatment for systemic infections caused by *N. meningitidis*?
 - A. Extended-spectrum cephalosporin
 - B. Chloramphenicol
 - C. Penicillin
 - D. Sulfonamides
7. Based on the laboratory report for the case study (above) on *N. meningitidis* what potential reasons might the laboratory cite for not performing susceptibility tests on this isolate? Select all that apply.
 - A. *N. meningitidis* remain universally susceptible to ceftriaxone.
 - B. The laboratory did not have access to beta-lactamase test reagents, the best way to test for penicillin resistance in *N. meningitidis*.
 - C. *N. meningitidis* are very fastidious and cannot be grown easily in the laboratory.
 - D. There is no standard NCCLS interpretive criterion for *N. meningitidis*.
8. Reviewing the case study on *M. catarrhalis*, why is it unnecessary to perform routine susceptibility tests on this organism?
 - A. *M. catarrhalis* grow poorly on all media used for susceptibility testing.
 - B. *M. catarrhalis* infections do not require antimicrobial therapy.
 - C. Infections caused by *M. catarrhalis* generally respond to empiric therapy.

16

Anaerobes

OBJECTIVES

After completing this chapter the reader should be able to:

- Discuss a strategy for obtaining antimicrobial susceptibility test results on anaerobes isolated in your laboratory.
- Describe where to find recommendations for MIC testing of anaerobic bacteria, including inoculum preparation, test medium, duration of incubation, and atmosphere of incubation.
- List those situations for which antimicrobial susceptibility testing should be performed on anaerobic isolates.

BACKGROUND

Anaerobic bacteria are part of the endogenous flora of the skin, gastrointestinal tract, and genitourinary tract. Approximately 100 different species of anaerobes are encountered in clinical specimens. In the colon, anaerobes outnumber aerobes 1000 to 1, and in the saliva the ratio is 10 to 1.

Strict anaerobes, such as *Fusobacterium*, *Prevotella* and *Porphyromonas* spp., may not survive exposure to air for longer than 10–30 minutes. Isolates of these genera are found throughout the body and may be involved in a variety of infections ranging from brain abscesses to diabetic foot ulcers. *Actinomyces* and *Propionibacterium* spp. are relatively aerotolerant and are typically involved in infections of the skin and oral cavity. Some isolates of *Bacteroides* and *Clostridium* spp., also may be aerotolerant and often are found in infections of normally sterile tissue adjacent to the gastrointestinal tract and soft tissue infections at body sites below the waist.

One third of the anaerobes isolated from specimens submitted to the routine clinical microbiology laboratory are members of the *Bacteroides fragilis* group (including *B. fragilis*, *B. thetaiotaomicron*, *B. distasonis*, and seven other species), another third are peptostreptococci, and the remaining third include *Prevotella*, *Fusobacterium*, *Clostridium* spp., and the nonspore-forming gram-positive rods.

The classic anaerobic infections include gas gangrene caused by *Clostridium perfringens* and other *Clostridium* spp., tetanus caused by *Clostridium tetani*, and botulism caused by *Clostridium botulinum*. Most anaerobic infections are polymicrobial. Management of anaerobic infections includes surgical drainage of pus, removal of necrotic tissue, and administration of antimicrobial agents active against both aerobes and anaerobes. Because some anaerobes are slow growing and results from anaerobic cultures and antimicrobial susceptibility tests are often not available for several days, therapy is generally empiric.

Anaerobic infections usually are treated with broad-spectrum antimicrobial agents that show activity against a variety of anaerobic species. The most common-

ly used agents include ampicillin-sulbactam, cefoxitin, imipenem, metronidazole, and piperacillin-tazobactam.

Chloramphenicol remains highly active against most anaerobes, but is infrequently prescribed in the United States because of its potential side effects. Clindamycin is also used for treating anaerobic infections, but emerging resistance within the *B. fragilis* group and the availability of agents for which there is little resistance has limited its use in recent years.

CASE STUDY

An 85-year-old-woman was admitted to the hospital with severe abdominal pain and a temperature of 102°F. Two sets of blood cultures were drawn and the patient was started on cefoxitin and gentamicin. She was taken to surgery where a ruptured diverticulum was discovered.

On the second day of the patient's hospitalization, the blood cultures showed growth of gram-negative bacilli. The patient remained febrile and on the third day her therapy was switched to piperacillin-tazobactam. Later that day, the anaerobic subculture of the blood cultures revealed colonies consistent with *B. fragilis* group while the aerobic plates were sterile. The anaerobic colonies were negative by the spot indole test, but catalase positive. Later that day the organism was identified as *B. fragilis* using a commercial kit.

The laboratory does not perform antimicrobial susceptibility tests on anaerobic bacteria, but has a policy of sending isolates to a reference laboratory if testing is requested by an infectious disease clinician and approved by the microbiology laboratory director. The infectious disease clinician requested that this *B. fragilis* be sent to the reference laboratory for antimicrobial susceptibility testing.

Is it likely that the laboratory director will approve this request?

Resistance—Penicillins

Over 99% of *B. fragilis* group isolates are resistant to penicillin. Most produce beta-lactamase; however, some are resistant due to changes in the organism's penicillin-binding proteins. Approximately 50% of *Prevotella* spp. produce beta-lactamase. Beta-lactamase production in other gram-negative anaerobic species is uncommon.

Penicillin resistance among gram-positive anaerobic bacteria occurs infrequently. Occasionally strains of some *Clostridium* spp., produce beta-lactamase.

Resistance—Cephems & Other Beta-lactams

First- and second-generation cephalosporins have limited activity against the anaerobic bacteria encountered in clinical specimens. However, cephamycins, including cefoxitin and cefotetan, are active against anaerobes. Cefoxitin is generally more active than cefotetan against *B. fragilis* group isolates.

In vitro, ceftizoxime, ceftriaxone, and cefotaxime have limited activity against anaerobes; however, ceftizoxime appears to be effective in treating intra-abdominal infections. Ceftazidime and cefepime have poor activity against most anaerobes. Beta-lactamase inhibitor combination agents, including ampicillin-sulbactam, piperacillin-tazobactam, and ticarcillin-clavulanic acid, are active against most anaerobic bacteria, including the *B. fragilis* group, which are beta-lactamase producers. Imipenem remains highly active against anaerobes.

Resistance—Quinolones

The older fluoroquinolones, including ciprofloxacin and ofloxacin, have poor activity against anaerobic bacteria.

Some of the newer fluoroquinolones, such as gatifloxacin, levofloxacin, and moxifloxacin, are considered active against a variety of anaerobic species, although data in the literature are scanty. Resistance occurs in some isolates of *B. fragilis* group and *Clostridium* spp., other than *C. perfringens*.

Resistance—Other Agents

Because aminoglycosides require oxygen for their transport into bacterial cells, none of these agents are active against anaerobes.

Metronidazole is very active against anaerobes, but does not have any activity against aerobic bacteria or aerotolerant anaerobes, such as *Actinomyces* and *Propionibacterium* spp.

Historically, clindamycin was one of the primary choices for treating anaerobic infections. However, because of increasing resistance, particularly among *B. fragilis* group isolates and some clostridia, it is no longer recommended as a first-line drug for treating anaerobic infections. Approximately 15–20% of *B. fragilis* isolates and 50% of *B. fragilis* group isolates are clindamycin resistant. Many clostridia and some peptostreptococci and *Prevotella* spp. are also clindamycin resistant.

Chloramphenicol remains highly active against anaerobes.

Many anaerobes are resistant to tetracycline; however, doxycycline and minocycline may show activity against a variety of species.

Testing Strategies

NCCLS provides standards for testing anaerobic bacteria in document M11 entitled “Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria.”

In this document NCCLS recommends antimicrobial susceptibility testing when therapy decisions are critical. These include:

- Organisms with known antimicrobial resistance.
- Patients whose infections persist despite adequate treatment with appropriate antimicrobial agents.
- Situations in which empiric therapy for serious anaerobic infections is unclear due to multiple sites of infection or lack of clinical experience with these types of infections.

Additional reasons for testing clinical isolates are to:

- Confirm appropriate therapy of severe infections or for those that may require long-term therapy.
- Periodically monitor local and regional resistance patterns.
- Determine patterns of susceptibility of anaerobes to new antimicrobial agents.

Testing Strategy—Routine Testing

NCCLS provides examples of specific situations when antimicrobial susceptibility testing is warranted.

These include situations from which anaerobes have been isolated:

- Brain abscesses
- Endocarditis
- Osteomyelitis
- Joint infections
- Infections involving prosthetic devices or grafts
- Bacteremia

Isolates from normally sterile body sites should be tested unless they are thought to be contaminants.

Anaerobes that are known to cause human infections and that have unpredictable susceptibility profiles include *Bacteroides*, *Prevotella*, *Fusobacterium*, *Clostridium*, *Bilophila*, and *Sutterella* spp. When these organisms are isolated from significant clinical specimens, antimicrobial susceptibility tests should be performed.

Methods

NCCLS document M11 describes specific standards for testing anaerobes using either an agar dilution MIC or broth microdilution MIC procedure. Agar dilution can be used for all anaerobes that grow satisfactorily in this test system. Broth microdilution is recommended for *B. fragilis* only.

Table 1 in M11 includes suggestions for selecting antimicrobial agents to test against anaerobic bacteria. These are divided into primary choices and supplemental choices.

The broth microdilution and agar dilution MIC methods that are described for anaerobes are similar to those for aerobes. Specific recommendations for anaerobic MIC tests include:

Inoculum preparation:

- Use the direct colony suspension method and a 0.5 McFarland standard.
- The final inoculum concentration for broth microdilution is $1-2 \times 10^6$ CFU/mL.
- The final inoculum for agar dilution is 10^5 CFU/spot.

NOTE: The inoculum for anaerobic MIC tests is higher than that for aerobic MIC tests.

Medium:

- Broth microdilution: Brucella broth + vitamin K1 + hemin + laked sheep blood
- Agar dilution: Brucella agar + vitamin K1 + hemin + laked sheep blood

Incubation

- 48 hours incubation at 35°C in an anaerobic environment (chamber, jar or pouch)

Methods—Additional Notes

Currently, NCCLS recommendations do not include a pre-reduction step for agar plates or broth microdilution MIC trays prior to testing. This may change, particularly if future recommendations for broth microdilution testing address additional species beyond *B. fragilis* group.

Brucella broth and Brucella agar support the growth of most anaerobes encountered in clinical specimens that require susceptibility testing. Because not all manufacturers use the same ingredients for Brucella base, the formula is provided in the M11 document.

As with MIC testing of aerobes, it is essential to perform a purity check plate of the inoculum immediately after inoculation of the broth microdilution MIC test. A sample of the inoculum should be subcultured to two plates, one incubated anaerobically and the other aerobically. For agar dilution tests, drug-free plates should be inoculated for aerobic and anaerobic incubation after every drug-containing set of plates.

Since essentially all gram-negative anaerobes are metronidazole susceptible, a resistant result should always be confirmed.

Methods—Beta-Lactamase Testing

Members of the *B. fragilis* group produce beta-lactamase and are resistant to the penicillinase-labile penicillins (e.g., ampicillin, penicillin). However, beta-lactamase testing can be performed on other gram-negative and gram-positive anaerobes if penicillin therapy is being considered. The chromogenic cephalosporin method should be used for beta-lactamase testing of anaerobes. See Chapter 2 for details of this procedure

Beta-lactamase positive isolates should be considered resistant to ampicillin and penicillin. Some anaerobes (including strains of *B. fragilis* and *B. distasonis*) may be resistant to ampicillin and penicillin by a mechanism other than beta-lactamase production. Thus, a negative beta-lactamase result does not guarantee susceptibility to beta-lactams. If therapy with ampicillin or penicillin is considered, an MIC test should be performed.

Methods—Interpretation of Results

MIC results are interpreted as susceptible, intermediate or resistant using the specific interpretive criteria for anaerobe tests in NCCLS document M11. The interpretations are valid only if testing is performed according to NCCLS recommendations or by a method that produces results comparable to the NCCLS method.

Many MIC results for the *B. fragilis* group cluster around the breakpoint for susceptibility. This is especially true for the cephamycins and clindamycin. Also, some MIC endpoints are difficult to read. The intermediate category provides a

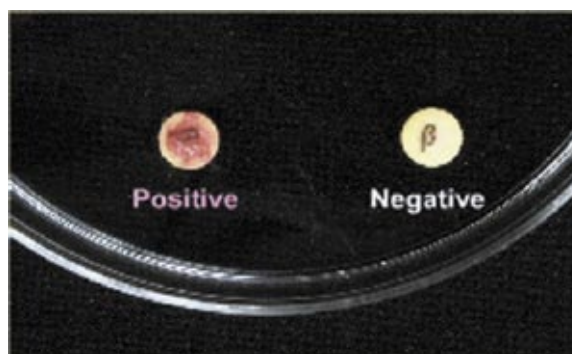


Figure 16.1—Positive and Negative beta-lactamase tests.

buffer zone that helps prevent false-susceptible or false-resistant interpretive errors from being reported.

Quality Control

Check Chapter 6 QA/QC in this manual for specific instructions regarding QC of tests for anaerobes.

NCCLS-recommended QC strains for anaerobic broth microdilution and agar dilution MIC tests are:

Bacteroides fragilis ATCC 25285

Bacteroides thetaiotaomicron ATCC 29741

Eggerthella (Eubacterium) lentum ATCC 43055

For broth microdilution testing, at least one QC strain should be included in each testing run. The isolate selected should have on-scale endpoints for the drugs tested.

For agar dilution testing, at least two QC strains should be included in each run. Long-term storage of anaerobe QC strains is similar to that for aerobes.

CASE STUDY COMMENTARY

Now you should be able to predict the response of the microbiology laboratory director to the request by the infectious disease clinician for antimicrobial susceptibility testing on the *B. fragilis* isolate from the elderly patient's blood.

Is it likely that the laboratory director will approve the request? (Correct answer: A)

- A. Yes
- B. No

The laboratory director approved the request and the isolate was sent to a reference laboratory that performs susceptibility testing using the NCCCLS broth microdilution MIC method. The following report was received:

Lab Report

Specimen Source: blood

Results: *Bacteroides fragilis*

	MIC ($\mu\text{g}/\text{mL}$)	Interpretation
Ampicillin-sulbactam	8	S
Cefotetan	>64	R
Cefoxitin	>64	R
Chloramphenicol	8	S
Imipenem	0.5	S
Metronidazole	1	S
Penicillin	16	R
Piperacillin-tazobactam	2/4	S

Now the reason for the patient's failure to respond to initial therapy with cefoxitin and gentamicin is clearer. The patient's *B. fragilis* isolate is resistant to cefoxitin. Since aminoglycosides are not active against anaerobes, the gentamicin offered virtually no benefit. However, the *B. fragilis* is susceptible to piperacillin-tazobactam.

REVIEW

The reader should now be familiar with routine antimicrobial susceptibility testing and reporting recommendations for anaerobic bacteria.

Remember to:

- Use the most current NCCLS standard (M11) for instructions on antimicrobial susceptibility testing of anaerobes and to help you develop a strategy for deciding which isolates to test in your laboratory.
- When using a commercial system read the package insert thoroughly and follow the manufacturer's instructions precisely.
- Identify a resource for cumulative antimicrobial susceptibility test data on anaerobes, particularly if limited data are available from your laboratory.
- If penicillin therapy is being considered note that beta-lactamase testing can be useful for anaerobes other than *B. fragilis* group isolates. Virtually all *B. fragilis* group isolates are beta-lactamase positive.

SELF-ASSESSMENT QUESTIONS

1. Why are anaerobic infections generally treated empirically? Select all that apply.
 - A. There is very little resistance among anaerobes and therefore most antimicrobial agents are effective in treating anaerobic infections.
 - B. Anaerobes grow slowly and the antimicrobial susceptibility test results that would assist clinicians in determining the best therapy are often delayed.
 - C. Many anaerobic infections are polymicrobial, which necessitates the use of broad-spectrum agents targeted against many aerobic and anaerobic species.
 - D. All anaerobes are susceptible to penicillins.
2. For which of the following isolates should antimicrobial susceptibility tests be performed? Select all that apply.
 - A. *Fusobacterium nucleatum*, *B. fragilis* and *C. perfringens* isolated from a peritoneal fluid that also grew *E. coli* and *Enterobacter* spp.
 - B. *Peptostreptococci* isolated from cerebrospinal fluid that also grew coagulase-negative staphylococci and *Corynebacterium* spp.
 - C. *Clostridium septicum* from a blood culture.
 - D. *Bacteroides thetaiotaomicron* from pleural fluid.
3. Do the results on the laboratory report shown below look reasonable for a *B. fragilis*?

Lab Report

Specimen Source: blood

Results *Bacteroides fragilis*

	MIC ($\mu\text{g/mL}$)	Interpretation
Ampicillin-sulbactam	8	S
Cefotetan	8	S
Cefoxitin	8	S
Chloramphenicol	8	S
Imipenem	0.5	S
Metronidazole	>32	R
Penicillin	16	R
Piperacillin-tazobactam	2/4	S

4. Why should antimicrobial susceptibility tests be performed on a *B. fragilis* isolated from the blood of an elderly woman? Select all that apply:
 - A. *B. fragilis* group isolates have variable susceptibility patterns to antimicrobial agents commonly prescribed for treating anaerobic infections.
 - B. The patient did not respond as expected to initial antimicrobial therapy and surgical intervention. This suggests the patient's isolate may have been resistant to the agents prescribed.
 - C. Anaerobic bacteremia is a very serious condition.
5. What would be a reasonable number of anaerobic susceptibility tests per month to justify performing the testing in house?
 - A. Any number
 - B. 5
 - C. 20
6. If your laboratory obtains susceptibility results on anaerobe isolates infrequently, how can you determine if resistance emerges in isolates infecting patients in your community? Select all that apply.
 - A. Obtain information from other institutions
 - B. Save isolates that are likely to be contributing to patients' infections and perform antimicrobial susceptibility testing once a year.
 - C. Review the literature.
7. You have isolated *B. fragilis* and a *Peptostreptococcus* spp. from a liver abscess aspirate from a 48-year-old man. Which of the isolates, if any, should you test for susceptibility?
 - A. Neither
 - B. Both
 - C. *B. fragilis*

Answers For All Chapters

CHAPTER 1—ANTIMICROBIAL MODES OF ACTION

1. True.
2. B.
3. B, C, D and E.
4. A, C, E and F are true.
5. A and B.
6. A. (2); B. (3); C. (4); D. (1).
7. A. (2); B. (1); C. (2); D. (1).
8. A. (4); B. (3); C. (1); D. (2).

CHAPTER 2—BETA-LACTAMASES

1. A, C and D are true.
2. A, B, and F.

CHAPTER 3—NCCLS

1. C. Correct. NCCLS is a private organization that convenes groups of experts from industry, academia, and government agencies on different topics and develops guidelines for clinical laboratory testing.
2. B. Correct.
3. A. Incorrect.
B. Correct. M2 and M7 provide instructions for reference methods, not for commercial systems. If you are using a commercial system for MIC testing, you should follow the manufacturer's directions.
4. C. Correct.
5. C. Correct.
6. A. Incorrect.
B. Correct. Results for ampicillin are outside of the acceptable limits. The ampicillin test is not working properly and must be investigated before releasing ampicillin results in patient isolates. QC results for the other two drugs are acceptable, and, therefore, tests with these agents could be reported on patient isolates.
7. A. Incorrect.
B. Correct. Penicillin is not listed in the *Enterobacteriaceae* column because it is not effective against members of the *Enterobacteriaceae* which includes *E. coli*.
8. C. Correct.
9. True.

10. False.
11. True.

CHAPTER 4—DISK DIFFUSION TESTING

1. True.
2. True.
3. True.
4. False.
5. Direct colony method: 18–24; 0.5.
Log phase method: 2–8; 0.5.
6. *Enterobacteriaceae*: C.
Staphylococci: B.
Fastidious bacteria: B
7. B. Correct.
8. B. Correct.
9. A. Correct. The colonies within the zone likely represent a mixed culture.
B. Correct. This is a less likely explanation.
C. Incorrect. The lawn of growth appears acceptable.
10. C. Correct. The test should be repeated from colonies on the primary plates or a subculture of these.

CHAPTER 5—MIC TESTING

1. NCCLS document M7.
2. NCCLS document M100 S14 (higher numbers in the S series are later editions).
3. A. The incubation time must be extended to 24 hours for staphylococci with oxacillin and vancomycin and for enterococci with vancomycin and high level gentamicin and streptomycin. If results are negative for high level streptomycin resistance incubate for an additional 24 hours.
B. The end point for trimethoprim, sulfonamides, and trimethoprim-sulfamethoxazole is read at the concentration where there is a >80% reduction in growth.
C. Bacteriostatic agents may exhibit “trailing” so a very light haze or buttons of growth <2mm are ignored. Exceptions are staphylococci with oxacillin or vancomycin and enterococci with vancomycin.
4. A. Incorrect
B. Correct. In most situations a susceptible, intermediate or resistant result is sufficient. However, some clinicians may prefer MIC results to help guide therapy for specific illnesses such as endocarditis, osteomyelitis and septic arthritis.

CHAPTER 6 QA/QC

There is no separate section with answers and comments for Chapter 6. All answers and comments are integrated into the text. Because of this chapter’s design we feel that students and readers will benefit most from having the answers and comments immediately available.

CHAPTER 7—COMMERCIAL SYSTEMS

1.
 - A. Incorrect. M2 describes the reference disk diffusion method.
 - B. Incorrect. M7 describes the reference broth and agar dilution methods.
 - C. Incorrect. M100 contains tables with interpretive criteria and quality control ranges for both disk diffusion and MIC methods.
 - D. Correct. NCCLS' role is to develop generic reference-testing procedures, i.e., NOT to evaluate, endorse, or recommend commercial antimicrobial susceptibility test systems.
2.
 - A. Incorrect
 - B. Incorrect
 - C. Incorrect
 - D. Correct. E-test is a commercial antibiotic gradient test system.
3.
 - A. Incorrect. The limitation means that the manufacturer has not demonstrated that the system can produce accurate results for *B. cepacia*. Therefore, you cannot use the method even with a qualifying statement.
 - B. Incorrect. Even if results look typical for the species, this does not guarantee that they are accurate. The limitation means that the manufacturer has not demonstrated that the system can produce accurate results for *B. cepacia*.
 - C. Correct.
4.
 - A. Correct. It is very important that the system can detect important types of resistance, such as that in ORSA, VRE, and ESBLs, that are likely to have a clinical impact.
 - B. Correct. If, for example, your laboratory tests large numbers of *P. aeruginosa* because you are affiliated with a burn center, it is important to test a significant number and variety of *P. aeruginosa* isolates during your study.
 - C. Incorrect. Although it is important to test a variety of species, it would be difficult and impractical to include equal numbers of each species and still test a good sampling of isolates with select types of resistance.
 - D. Correct. Information derived from testing isolates with MICs above the highest concentration or below the lowest concentration of drug tested for a specific agent is less useful than information derived from testing isolates with MICs that fall "on-scale" or within the range of concentrations tested.
5.
 - A. Correct. When reviewing journal articles, note the version of the system (materials and software) that was evaluated. If the study was not performed recently, it is possible that a newer version of the system is currently in use and its performance may differ from that reported in the article.
 - B. Correct. It is important to learn the source of the data published in the manufacturer's literature. Such data may represent unpublished studies that were compiled as part of the FDA clearance request.
 - C. Correct. Many find it extremely valuable to discuss the product with current users to obtain information related to performance and use in a clinical laboratory setting.
 - D. Incorrect. NCCLS does not provide any information on commercial antimicrobial susceptibility test systems.
6.
 - A. Incorrect. Testing QC strains alone will not guarantee satisfactory performance with clinical isolates.
 - B. Incorrect. It is also necessary to evaluate performance of the system with QC strains. Use the QC strains recommended by the manufacturer.

- C. Incorrect. It would be impossible to appropriately assess performance of the system in your laboratory by testing only 20–30 clinical isolates.
- D. Correct. It is important to make certain that the system performs satisfactorily with the QC strains and produces accurate results with clinical isolates. It is essential to ensure that the system can detect isolates with specific types of resistance.
7. A. Correct. Although the very major error rate of 10% is excessive, this represents an error with only one isolate. All data available should be reviewed to better assess the magnitude of the perceived problem.
- B. Correct. It is important to determine if the error is reproducible.
- C. Correct. Additional data would be useful. It is best to select ORSA from various locations in your institution to minimize the possibility of testing isolates from the same clone.
- D. Incorrect. It is important to analyze all data from the study, unless the reason for the aberrant results is known (e.g., the wrong organism was tested, or sporadic contamination occurred.)
8. The FDA
9. Data from their system are compared with those from a dilution reference method such as an agar or broth MIC method. Hundreds of strains from across the country including those with unusual or high levels of resistance to related drugs must be included in the evaluations.
10. Laboratories should conduct in-house parallel testing of the new system with an NCCLS reference method, such as disk diffusion or a broth micro-dilution MIC. For nonfastidious bacteria, they should test a minimum of 100–200 randomly selected fresh clinical isolates representing a variety of gram-positive and gram-negative species. Strains with known resistance also should be run in both systems. QC strains should be tested by both methods for 30 consecutive runs.
11. The package insert provides the laboratorian with extensive background information as well every detail for using the system. If the directions in the package insert are followed exactly, the user is very likely to receive results that agree with a reference method.
12. This informs the user about organisms as well as organism/drug combinations for which the system should not be used. Pre-FDA evaluation tests revealed that results from these organisms are not reliable.
13. Any method other than disk diffusion, agar dilution and macro-broth or micro-broth dilution. Examples of non-reference methods include any automated system or the E-test.
14. A thin strip containing an antibiotic gradient is labeled according to the levels that correspond to MICs. After the strip is placed on a freshly inoculated agar plate (and incubated overnight) the antibiotic diffuses from the strip and inhibits growth above the level of the strip that correlates with the MIC.

CHAPTER 8 *STAPHYLOCOCCUS* SPP.

1. A. ORSA
B. ORSA
C. BORSA
D. ORSA
E. BORSA
2. A. Correct

- B. Correct.
 - C. Incorrect. Results from oxacillin and penicillin can be used to deduce results for other beta-lactams, including cephalosporins.
- 3.
- A. Incorrect. Aminoglycosides are not considered first-line agents against staphylococci.
 - B. Correct. Clindamycin may be used instead of macrolides for skin infections, especially if anaerobes are present.
 - C. Incorrect. Not often tested routinely; however, testing would be warranted if a fluoroquinolone is being considered. An example would be a staphylococcus isolated from patients with osteomyelitis.
 - D. Correct. Glycopeptides include vancomycin, an important anti-staphylococcal agent used when first-line agents are resistant.
 - E. Correct. Erythromycin is used for skin infections in patients who are allergic to penicillin.
 - F. Incorrect. Tetracyclines are not first-line agents.
 - G. Correct. Trimethoprim-sulfamethoxazole occasionally is used for less serious staphylococcal infections.
- 4.
- A. Incorrect.
 - B. Correct. The length of incubation in this scenario is only 16 hours. Oxacillin resistance in staphylococci may be subtle in some isolates and 24 hours of incubation is often needed to detect resistance. Of course, if resistance is evident earlier, you can report it.
- 5.
- A. Correct. The direct colony suspension method is optimal for detecting oxacillin resistance in heteroresistant strains.
 - B. Incorrect. If heteroresistant ORSA are grown in broth, the susceptible population may overgrow the resistant population. This may compromise detection of the isolate as oxacillin resistant.
 - C. Correct. Sodium chloride acts as an osmotic stabilizer and enables heteroresistant populations to express oxacillin resistance more readily. Without this enhancement, some resistant strains may not be detected.
 - D. Incorrect. Oxacillin tests must be incubated a full 24-h before a susceptible result is reported.
 - E. Correct. 24-h incubation provides additional time for slower growing oxacillin-resistant cells to be detected as resistant.
 - F. Correct. For broth microdilution this is true. However, because disk diffusion is inadequate in detecting vancomycin resistance, there is no recommendation for examining vancomycin disk diffusion tests following 24-h incubation.
- 6.
- A. Correct. ORSA with *mecA* typically are resistant to multiple classes of anti-staphylococcal drugs, including macrolides and lincosamides. They also often are resistant to tetracyclines, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole.
 - B. Correct. However, this profile occurs less frequently than that showing resistance to several other classes of drugs. Strains causing community-acquired infections often have this profile.
 - C. Incorrect. Oxacillin-resistant staphylococci always are resistant to penicillin.
- 7.
- A. Incorrect.
 - B. Incorrect.
 - C. Correct. The clindamycin result should be reported based on the results of the induction test.

- D. Correct. This an alternate approach to reporting that is favored by many infectious disease specialists.
8. A. False. VISA appear vancomycin susceptible in disk diffusion tests.
B. True.
C. True. Isolates with very unusual resistance profiles such as VISA always should be saved for further studies.
D. True. Borderline-susceptible results are rare and should be confirmed.
E. True. Previous studies have demonstrated that all VISA isolates have grown on this medium.
F. False. At the start of 2000 less than 50 isolates of VISA had been confirmed.
9. A. Correct.
B. Incorrect.
C. Correct.
D. Incorrect.
10. A. Incorrect.
B. Incorrect.
C. Incorrect.
D. Correct. (If this protocol has been established in your institution.)
E. Incorrect. The *mecA* or PBC2a tests are reliable.
11. A. False. Only the direct colony suspension method should be used for preparing inocula for susceptibility testing of staphylococci because this will improve detection of oxacillin resistance in heteroresistant strains.
B. False. The oxacillin-salt agar screen plate is only reliable for detection of oxacillin resistance in *S. aureus*.
C. True. Oxacillin-susceptible staphylococci are considered susceptible to cepheids; oxacillin-resistant staphylococci are considered resistant to cepheids.
D. True. To date, the standard vancomycin disk diffusion test demonstrated zones in the susceptible range for VISA.

CHAPTER 9—ENTEROCOCCI

1. A. Incorrect.
B. Correct. *E. faecium* is the most common species of VRE. Isolates may show high-level MIC >256 mcg/mL (vanA phenotype) or moderate level MIC 16–128 mcg/mL (vanB phenotype) resistance.
C. Incorrect.
D. Incorrect.
2. A. Correct.
B. Incorrect. Isolates with intrinsic vanC type resistance, such as *E. gallinarum* and *E. casseliflavus* can cause infections; however, this occurs infrequently.
C. Correct. Enterococci with acquired resistance have been associated with outbreaks in many institutions.
3. A. Incorrect.
B. Correct. Vancomycin MIC tests should be performed on isolates with intermediate disk diffusion results and the MIC results should be reported. Many isolates with intermediate vancomycin disk diffusion results will have susceptible MICs, according to NCCLS document M2

4. A. Incorrect.
B. Correct. Enterococci that show high-level gentamicin resistance typically produce a bifunctional enzyme, AAC (6')/APH (2''), which modifies the activity of all aminoglycosides, except streptomycin. Therefore, tobramycin and amikacin need not be tested, since the lack of synergy can be deduced from the gentamicin results because this isolate also has high-level streptomycin resistance, no aminoglycoside would show synergism with penicillin or ampicillin. In such cases, the best therapy choices must be determined on an individual patient basis.
5. A. Incorrect.
B. Correct.
C. Incorrect. Special high concentrations of the aminoglycoside are tested to detect the presence of aminoglycoside-modifying enzymes.
6. A. Incorrect. Some urinary tract infections are caused by *E. faecium*, which typically is resistant to ampicillin.
B. Correct.
7. A. Incorrect. Intrinsically vancomycin-resistant enterococci can grow on the vancomycin agar screen plate. The isolates should not be reported as VRE. In addition, other genera and species that morphologically resemble enterococci may grow on this medium. These include: *Erysipelothrix rhusiopathiae*, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp.
B. Correct. These tests will distinguish the intrinsically vancomycin-resistant species from those with acquired vancomycin resistance.

CHAPTER 10—S. PNEUMONIAE

1. A. Correct.
B. Incorrect.
C. Incorrect.
D. Correct.
2. A. Correct. *S. pneumoniae* resistant to only fluoroquinolones have been reported.
B. Incorrect. To date there has not been any report of *S. pneumoniae* that is not susceptible to vancomycin.
C. Incorrect. *S. pneumoniae* resistant to ceftriaxone are not susceptible to penicillin.
D. Incorrect. *S. pneumoniae* resistant to clindamycin also are resistant to erythromycin. However, *S. pneumoniae* may be resistant to erythromycin but susceptible to clindamycin.
3. A. Correct. The number of viable cells in a broth or saline suspension of *S. pneumoniae* decreases rapidly at room temperature.
B. Correct. The growth method of inoculum preparation should NOT be used for *S. pneumoniae*. Additionally *S. pneumoniae* is unlikely to grow satisfactorily in Mueller-Hinton broth without additional nutrients.
C. Correct. Since many strains of *S. pneumoniae* do not grow adequately on an agar medium when incubated in ambient air, CO₂ incubation is required for disk diffusion testing.
4. A. Incorrect. There are no interpretive criteria for ampicillin.
B. Correct. A zone of inhibition of ≥20 mm around an oxacillin disk indicates that the isolate is penicillin susceptible. However, if the oxacillin zone measures ≤19 mm, a penicillin MIC test must be performed to determine if the isolate is susceptible, intermediate, or resistant to penicillin.

- C. Correct. This is the optimal method of testing penicillin.
 - D. Incorrect. *S. pneumoniae* do not produce beta-lactamase. The mechanism of resistance for strains that are not penicillin-susceptible is altered penicillin-binding proteins.
5. A. Incorrect.
B. Incorrect.
C. Correct.
D. Incorrect.
6. A. Incorrect. Cefotaxime is an important drug for many types of pneumococcal infections; therefore susceptibility results should be obtained by an MIC test.
B. Correct.
C. Incorrect. Disk diffusion interpretive criteria for cefotaxime for *S. pneumoniae* have not been established. Using the interpretive criteria developed for gram-negative bacilli will likely produce erroneous results.
7. A. Incorrect. Meningitis should be treated with maximum doses of ceftriaxone.
B. Correct.
C. Incorrect. Detecting emerging resistance is important for any isolate.
8. False. In the United States, cefepime is not approved by the FDA for treating pneumococcal meningitis. NCCLS M100 lists interpretive criteria for cefepime and meningitis because NCCLS documents are used outside of the United States where cefepime may be approved for therapy of meningitis.
9. False. Erythromycin can be administered both orally and parenterally; however, its penetration into CSF is poor. Thus erythromycin should not be used to treat meningitis.
10. False. Because the incidence of combined penicillin-intermediate and resistant *S. pneumoniae* in the United States is between 15–30%, some laboratories have eliminated the oxacillin screen test and now proceed directly with MIC testing of penicillin and ceftriaxone to avoid delays in reporting results. For CSF isolates of *S. pneumoniae*, MICs of cefotaxime, ceftriaxone, meropenem, or penicillin should be performed as soon as sufficient colonies are available for testing.
11. True. The overall incidence of *S. pneumoniae* resistance to gatifloxacin, levofloxacin, and moxifloxacin in the United States is less than 3%; however, emerging resistance of *S. pneumoniae* to fluoroquinolones is a concern. Gatifloxacin, levofloxacin, and moxifloxacin are listed in NCCLS Test/Report Group B (these are tested routinely and reported selectively).
12. True. *S. pneumoniae* may be isolated from body sites other than CSF in patients with pneumococcal meningitis. By receiving sets of interpretive criteria, clinicians can apply the appropriate criteria for the site of infection.

CHAPTER 11 *STREPTOCOCCUS* SPP.

1. A. *S. mitis*.
B. *S. agalactiae*
C. *S. pyogenes*
2. Inoculum preparation:
A. Incorrect. Viridans group streptococci often grow unpredictably in broth.
B. Correct

- Incubation length:
- A. Incorrect
 - B. Correct
- Incubation atmosphere:
- A. Correct
 - B. Incorrect
3. A. Correct. These are the alternative agents that are used in pregnant women to eliminate colonization with *S. agalactiae* from the vagina prior to delivery. B., C., D., and E. are Incorrect
 4. A. Incorrect
B. Incorrect
C. Correct
 5. A., B., and C. are Incorrect
D. Correct. Some organisms, such as *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Erysipelothrix rhusiopathiae*, are intrinsically resistant to vancomycin and may demonstrate colonial morphology similar to that of viridans group streptococci. In contrast to these genera, the viridans group streptococci have not been reported as vancomycin resistant.
 6. A. Incorrect. The penicillin disk diffusion test is not valid for the viridans group streptococci. There are no interpretive criteria available for penicillin disk diffusion results for these organisms.
B. Correct.
C. Incorrect. Many viridans group streptococci are no longer susceptible to penicillin.

CHAPTER 12—ENTEROBACTERIACEAE

1. B. Correct
2. A. 4
B. 1
C. 3
D. 2
3. A. False. TEM-1 is a beta-lactamase that does not mediate resistance to extended spectrum cephalosporins.
B. True. Cefoxitin, a cephamycin, is not an extended spectrum cephalosporin.
C. False. Metallo beta-lactamases that hydrolyze imipenem and meropenem are uncommon in *Enterobacteriaceae*.
D. False. ESBLs have been detected in other gram negative species such as *Citrobacter*, *Enterobacter*, and *Salmonella*; however there currently is no NCCLS-approved method for detecting and reporting the presence of ESBLs in these other genera.
E. False. In fact, clavulanic acid can induce production of some AmpC beta-lactamases.
4. Inoculum preparation: B. Correct
Incubation length: A. Correct
5. A. C. and D. Correct
6. B. and C. Correct
7. A. and C. Correct
8. B. Correct answer is No. Since nearly all isolates of the species capable of producing inducible beta-lactamase can produce this enzyme, it is unnecessary to do the test. To avoid the potential for resistance development,

- physicians should be educated to cautiously use beta-lactams that have a high potential for induction or selection of resistant mutations.
9. A.D. and H. Correct. Remember, fluoroquinolones should not be reported on patients <12 years old.

CHAPTER 13—NON-ENTEROBACTERIACEAE

1. Correct answers:
 - D.
 - E.
 - F.
 - G.
 - H.
2. A. Correct. Growth like this may be due to over inoculation and makes the end point difficult to read.
 - B. Incorrect.
3. A. Correct. Antimicrobial susceptibility test results are not always essential for patient care. Prior to using significant resources when problematic organisms are encountered, the situation should be discussed with the patient's physician.
 - B. Incorrect. This organism has not demonstrated satisfactory growth and the results may not be accurate.
 - C. Incorrect. An organism that grows poorly twice is unlikely to produce satisfactory results when tested a third time. If the physician informs you that results are essential, another method such as broth microdilution should be done.
4. A. Correct.
 - B. Incorrect.
 - C. Incorrect.
5. A. Incorrect. Although some laboratories have adopted this practice, there are no standard guidelines documenting its accuracy.
 - B. Incorrect. Because there is heavy growth of both colony types of *P. aeruginosa*, susceptibility testing is necessary for each of them. Frequently, the antimicrobial susceptibility profiles are different, with the mucoid variety often being more susceptible.
 - C. Correct.
 - D. Incorrect. *P. aeruginosa* often contributes to an infectious process and presents with a variety of susceptibility profiles. Therefore, susceptibility test results are important to guide therapy.
6. A. Incorrect.
 - B. Incorrect.
 - C. Correct.
 - D. Correct. Providing the method is FDA cleared and the package insert clearly states that the method is acceptable for testing mucoid and nonmucoid *P. aeruginosa*. Be sure and note any restrictions that might apply.
7. A. Incorrect. Imipenem is an acceptable agent to test and report against all varieties of *P. aeruginosa*, providing the strain grows satisfactorily and there are no restrictions for testing imipenem with the test system.
 - B. Correct.
 - C. Incorrect. Both tobramycin and imipenem are acceptable for reporting on *P. aeruginosa*.

8. A. 1. Highly resistant *A. baumannii* have been associated with nosocomial transmission and multiple outbreaks in healthcare settings.
B. 3. *Acinetobacter lwoffii* generally is more susceptible to antimicrobial agents than is *A. baumannii*.
C. 2. Highly resistant *A. baumannii* is ubiquitous but wild type strains resistant to only a few narrow spectrum agents are sometimes encountered among patients' specimens.
9. A. Correct. *A. baumannii* is unique and some strains are inhibited by the sulbactam component. Sulbactam typically acts as a beta-lactamase inhibitor and has little, if any, antibacterial activity against other species.
B. Correct. Polymyxin B has been used to treat infections caused by *A. baumannii* resistant to virtually all other classes of antimicrobial agents. Polymyxins are not often prescribed because of toxicity. Because disk diffusion testing is unreliable for this drug, testing should be done by an MIC method. There are no NCCLS interpretive criteria for Polymyxin B; however, one study suggests an MIC >4 mcg/mL indicates resistance. (Gales et al. 2001. J. Clin. Microbiol. 39:183.)
C. Incorrect. Vancomycin is active only against gram-positive bacteria and has no activity against *Acinetobacter* spp.
(*The laboratory tested ampicillin-sulbactam and the organism was susceptible. Ampicillin-sulbactam was prescribed and the patient's condition cleared. The patient was placed under barrier precautions in an effort to contain this highly resistant organism.*)
10. A. Incorrect. Since the specimen is expectorated sputum and only a few colonies are present along with normal flora, the *S. maltophilia* likely represents colonization. Consequently, testing should not be routinely performed.
B. Correct.
11. A. Correct.
B. Incorrect. Most strains will grow well in routine broth microdilution MIC panels containing cation-adjusted Mueller-Hinton broth.
C. Correct. Trimethoprim-sulfamethoxazole is the drug of choice for *S. maltophilia* and there is little resistance to this agent.
12. A. Incorrect. Despite imipenem's activity against most gram-negative bacteria it is not active against *S. maltophilia*. If a susceptible result were obtained for *S. maltophilia*, it is likely due to a technical problem since virtually all *S. maltophilia* are imipenem resistant.
B. Correct.
13. A. Incorrect. Ticarcillin alone has not been an effective drug against *S. maltophilia* and NCCLS does not recommend testing and reporting this agent.
B. Correct.
C. Correct. If a patient has sulfa allergies this result will not help the physician. Nevertheless, it would be important for infection control purposes to note if the isolate has a typical susceptibility profile (e.g., trimethoprim-sulfamethoxazole susceptible).
D. Correct.
E. Correct. However, studies have shown that strains may be resistant to tetracycline, but susceptible to minocycline. Your physician may want results for minocycline. Since you cannot perform the disk diffusion test on *S. maltophilia* and since minocycline probably is not on your routine panels, you may have to send the isolate to a reference laboratory to obtain minocycline results.

14. A. Correct.
- B. Correct. However, *S. maltophilia* has demonstrated rapid development of resistance to fluoroquinolones. Some newer fluoroquinolones (e.g., levofloxacin) may be more active than ciprofloxacin against *S. maltophilia*.
- C. Incorrect. *S. maltophilia* typically is resistant to meropenem as well as imipenem.

CHAPTER 14—HAEMOPHILUS SPP.

1. A. Incorrect
- B. Correct. When *H. influenzae* is isolated from patients with life-threatening infections (e.g. meningitis, bacteremia, and epiglottitis) disk diffusion or MIC susceptibility tests should be performed.
2. A. Correct if the isolate is beta-lactamase negative. By definition, beta-lactamase positive isolates are ampicillin resistant.
- B. Correct. An extended-spectrum cephalosporin (cefotaxime or ceftriaxone) should be tested by either disk diffusion or an MIC method. Reports of extended-spectrum cephalosporin resistance in *H. influenzae* are currently unsubstantiated.
- C. Correct
- D. Correct if meropenem is on your hospital's formulary.
3. A. Incorrect. In NCCLS M100, Table 1A, both ampicillin and trimethoprim-sulfamethoxazole are listed in Group A (Primary Test and Report).
- B. Correct.
- C. Incorrect
4. A. Correct. Although rare isolates may be BLNAR, which would only be detected with conventional disk diffusion or MIC tests.
- B. Correct. HTM is used exclusively for *Haemophilus* spp. and most labs have a low volume of *Haemophilus* spp. isolates that would warrant testing. Costs typically are high for low volume tests.
- C. Correct
- D. Correct
5. A. Incorrect
- B. Correct. Some strains are ampicillin resistant due to altered penicillin-binding proteins.
- C. Incorrect

CHAPTER 15—NEISSERIA AND MORAXELLA

1. A. Correct. Uncomplicated genital infections caused by *N. gonorrhoeae* are treated empirically. Resistance to the drugs of choice, such as ceftriaxone, cefixime, or a fluoroquinolone, is generally low in the United States.
- B. Incorrect. Beta-lactamase tests are primarily used to test penicillin resistance. Penicillin is no longer recommended for treating infections caused by *N. gonorrhoeae* in the United States because this organism has a high incidence of resistance.
- C. Incorrect. NCCLS published standard methods for both disk diffusion and agar dilution MIC testing of *N. gonorrhoeae*.
2. A. Correct.
- B. Incorrect. As for other organisms that grow unpredictably in broth, a growth method should not be used.

- C. Incorrect. As for other organisms that grow unpredictably in broth, a growth method should not be used.
3. A. Incorrect. Since beta-lactamase results generally are not used for patient care, many health departments have informed clinical laboratories that routine beta-lactamase testing is not necessary. However, some health departments may require this information for epidemiologic purposes.
B. Correct.
4. A. Incorrect.
B. Incorrect.
C. Correct.
5. A. Incorrect. First, many isolates of *N. gonorrhoeae* are penicillin resistant. Second, penicillin is not the drug of choice for treating gonorrhea in most countries.
B. Correct.
C. Correct.
6. A, B and C are correct.
7. A. Correct.
B. Incorrect.
C. Incorrect. *N. meningitidis* grows well in CAMBH with 5% lysed horse blood with CO₂ incubation.
D. Correct.
8. A. Incorrect.
B. Incorrect. Respiratory tract and other infections caused by *M. catarrhalis* may require antimicrobial therapy.
C. Correct.

CHAPTER 16—ANAEROBES

1. A. Incorrect. There is significant resistance among many commonly encountered anaerobic species and resistance rates are increasing.
B. Correct.
C. Correct.
D. Incorrect. Members of the *B. fragilis* group (the most common anaerobes encountered in clinical specimens) and other anaerobic species are frequently penicillin resistant. Penicillin usually is not the primary drug used to treat anaerobic infections unless the etiologic pathogen is known to be susceptible to the agent.
2. A. Incorrect. Antimicrobial susceptibility tests should not be performed on isolates from a peritoneal fluid specimen containing mixed anaerobic and aerobic flora. However, there may be an occasion (e.g., brain abscess) when isolates from mixed cultures may warrant testing.
B. Incorrect. They probably represent skin contamination.
C. Correct.
D. Correct.
3. A. Incorrect.
B. Correct. Anaerobic gram-negative bacilli are typically susceptible to metronidazole. The resistant result suggests the test may be contaminated with an aerotolerant anaerobe (*Propionibacterium* spp.) or an aerobe.
4. A. Correct.
B. Correct.
C. Correct. If anaerobic bacteremia is not treated appropriately, the mortality rate is nearly 60%. The mortality rate decreases to approximately 15% when bacteremia is treated with appropriate antimicrobial agents.

5.
 - A. Incorrect.
 - B. Incorrect. Generally this would not be cost-effective since the number of QC tests required per run would be large. In addition, it is difficult to maintain expertise in a particular test if it is performed infrequently.
 - C. Correct.
6.
 - A. Correct. Although this is not ideal because the patient populations may differ and different types of anaerobes may be encountered, it can provide useful information for empiric therapy.
 - B. Correct. NCCLS suggests this as a strategy.
 - C. Correct. There are publications that describe surveillance for emerging resistance in anaerobes, particularly for *B. fragilis*. These data are useful but not ideal because susceptibility profiles may vary in different geographic areas and for individual patients.
7.
 - A. Incorrect.
 - B. Incorrect.
 - C. Correct. Even though *B. fragilis* is present with another organism it is likely to be significant in this situation. Because of its varied susceptibility profiles, testing is warranted. *Peptostreptococcus* spp. have predictable patterns of susceptibility and are susceptible to drugs that would be prescribed for *B. fragilis*. Frequently, when antimicrobial therapy is chosen to cover the *B. fragilis*, other anaerobes that might be contributing to the infection also are inhibited.

Appendix

BASIC BIOSAFETY RULES

Take the following safety precautions when performing antimicrobial susceptibility tests:

1. Follow Bio-Safety Level (BSL) 2 practices.* Gloves or face shields are not required when working with BSL-2 bacteria in pure culture.
2. Wear a buttoned laboratory coat.
3. Disinfect your workbench before work, after any spill, and at the end of the workday.
4. Avoid the creation of aerosols. For disk diffusion testing, express the excess inoculum from the swab prior to inoculating the plate. If vortexing is required, use tightly sealed screw-capped tubes.
5. Use a sharps container for disposal of pipettes, plastic loops, sticks, other sharp items and cotton swabs.
6. Dispose of all contaminated materials in waste containers for biohazardous materials.

*An excellent manual with essential information on biosafety BSL-2 practices is available free on the web in Spanish as well as English:

“*Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition.*” <http://www.cdc.gov/od/ohs/biosfty/biosfty.htm>

It also can be purchased from the address below:

U.S. Government Printing Office

Washington, D.C. 20402

Stock number: 017-040-00547-4

PREPARATION OF A 0.5 MCFARLAND STANDARD

Reagents

1% (wt/vol) anhydrous Barium Chloride (BaCl_2)

1% (vol/vol) cold pure sulfuric acid (H_2SO_4)

To prepare a 0.5 McFarland Standard:

Add 0.5 mL of 1.0% BaCl_2 to 99.5 mL of 1% H_2SO_4 solution.

Stir to maintain a suspension.

Thoroughly mix immediately before the next step:

Distribute about 5 mL of the 0.5 McFarland Standard into screw-top tubes.

The diameter of these tubes should be the same as those used for adjusting the density of culture suspensions prior to inoculation.

When these standards are thoroughly shaken the turbidity equals that of a culture containing about 1.5×10^8 cells.

Store the tubes containing the 0.5 McFarland Standard in a dark at room temperature.

See NCCLS document M2 for more details on this recipe and for quality control of the turbidity.

...from NCCLS M100-S14 (M7)

The following table reflects the drugs listed for testing against the respective organisms in Tables 2A-2J in M100 and gives some examples to consider for verification protocols at a given institution. The list includes phenotypes that 1) have never been documented, 2) are uncommon, and/or 3) represent results that could easily occur from technical errors and which may have significant clinical consequences.

Table 8. Suggestions for Verification of Antimicrobial Susceptibility Test Results and Confirmation of Organism Identification

Organism or Group	Category I ^a Phenotypes that have not been reported, are uncommon, and/or result from technical errors	Category II ^b Phenotypes that may be uncommon at a given institution and/or result from technical errors
Gram-negative organisms		
Enterobacteriaceae (any)	carbapenem-I or R	amikacin-R fluoroquinolone-R
<i>Citrobacter freundii</i> <i>Enterobacter</i> spp. <i>Serratia marcescens</i>	ampicillin, cefazolin, or cephalothin-S	
<i>Escherichia coli</i>		ESBL confirmed positive
<i>Klebsiella</i> spp.	ampicillin-S	ESBL confirmed positive
<i>Proteus vulgaris</i> <i>Providencia</i> spp.	ampicillin-S	
<i>Pseudomonas aeruginosa</i>		concurrent gentamicin and tobramycin and amikacin-R
<i>Stenotrophomonas maltophilia</i>	carbapenem-S	trimethoprim-sulfamethoxazole-R
<i>Haemophilus influenzae</i>	aztreonam-NS carbapenem-NS 3rd-generation cephalosporin ^c -NS fluoroquinolone-NS	ampicillin-R and β -lactamase-negative, amoxicillin-clavulanic acid-R
<i>Neisseria gonorrhoeae</i>	3rd-generation cephalosporin-R	fluoroquinolone-R
Any organism	Resistant to all agents routinely tested	

^a Category I

When results listed in this category are observed on individual patient isolates, they should be verified by one or more of the following:

1. Ensuring the unusual results are not due to transcription errors, contamination, or use of a defective panel, plate, or card.
2. Checking previous reports on the patient to determine if the isolate was encountered and verified earlier.
3. Confirming the identification of the isolate.
4. Repeating the susceptibility test to confirm results. Sometimes it is helpful to use an alternative test method for the repeat test.
5. For isolates that show results other than susceptible for those antimicrobial agents for which only susceptible interpretive criteria are provided in Tables 2A-2J (listed with an "NS" above) and for staphylococci with vancomycin intermediate or resistant results: 1) confirm the organism identification; 2) confirm the antimicrobial susceptibility test results; 3) save the isolate; and 4) submit the isolate to a reference laboratory that will test it by an NCCLS reference dilution method.

^b Category II

When results listed in this category are observed on individual patient isolates, the verification steps as outlined for Category I should be considered if the resistance is uncommon in a given institution.

^c For these antimicrobial agent/organism combinations, resistance has not been documented to date.

Table 8. (continued)

Organism or Group	Category I ^a Phenotypes that have not been reported, are uncommon, and/or result from technical errors	Category II ^b Phenotypes that may be uncommon at a given institution and/or result from technical errors
Gram-positive organisms		
<i>Enterococcus</i> spp.		vancomycin-R
<i>Enterococcus faecalis</i>	ampicillin or penicillin-R quinupristin-dalfopristin-S linezolid-R	high-level aminoglycoside-R (particularly if isolate from sterile body site)
<i>Enterococcus faecium</i>	linezolid-R	high-level aminoglycoside-R (particularly if isolate from sterile body site) quinupristin-dalfopristin-R
<i>Staphylococcus aureus</i>	linezolid-NS quinupristin-dalfopristin-I or R vancomycin-I or R	oxacillin-R
<i>Staphylococcus</i> , coagulase-negative	linezolid-NS vancomycin-I or R	
<i>Streptococcus pneumoniae</i>	fluoroquinolone-R linezolid ^c -NS vancomycin ^c -NS	penicillin-R 3rd-generation cephalosporin-R
<i>Streptococcus</i> , beta group	ampicillin or penicillin ^c -NS 3rd-generation cephalosporin-NS linezolid-NS vancomycin ^c -NS	
<i>Streptococcus</i> , viridans group	linezolid-NS vancomycin-NS	penicillin-I or R
Any organism	Resistant to all agents routinely tested	

^a Category I

When results listed in this category are observed on individual patient isolates, they should be verified by one or more of the following:

1. Ensuring the unusual results are not due to transcription errors, contamination, or use of a defective panel, plate, or card.
2. Checking previous reports on the patient to determine if the isolate was encountered and verified earlier.
3. Confirming the identification of the isolate.
4. Repeating the susceptibility test to confirm results. Sometimes it is helpful to use an alternative test method for the repeat test.
5. For isolates that show results other than susceptible for those antimicrobial agents for which only susceptible interpretive criteria are provided in Tables 2A–2J (listed with an “NS” above) and for staphylococci with vancomycin intermediate or resistant results: 1) confirm the organism identification; 2) confirm the antimicrobial susceptibility test results; 3) save the isolate; and 4) and submit the isolate to a reference laboratory that will test it by an NCCLS reference dilution method.

^b Category II

When results listed in this category are observed on individual patient isolates, the verification steps as outlined for Category I should be considered if the resistance is uncommon in a given institution.

^c For these antimicrobial agent/organism combinations, resistance has not been documented to date.

Disk Diffusion Susceptibility Test Troubleshooting Guide

ABERRANT RESULT	PROBABLE CAUSES	CORRECTIVE ACTION (record and note all errors)	EFFECT ON CURRENT DAY'S REPORT
Tetracycline zone too large and clindamycin zone too small with <i>Escherichia coli</i> or <i>Staphylococcus aureus</i> control strains	pH of medium too low	Adjust pH to 7.2–7.4 before pouring media Use a new lot	DO NOT report test results until corrective action has been taken and a new batch of medium demonstrates acceptable results with control strains.
	pH of medium too high	Commercial media should not have pH problems	
Aminoglycoside zone too small with <i>Pseudomonas aeruginosa</i> control strain	Ca ⁺⁺ and/or Mg ⁺⁺ too high in medium	(Incubation in CO ₂ may alter agar surface pH)	DO NOT report aminoglycoside results on <i>Ps. aeruginosa</i> or <i>Acinetobacter</i> sp. until zone sizes meet QC standard
Aminoglycoside zone too large with <i>Pseudomonas aeruginosa</i> control strain	Ca ⁺⁺ and/or Mg ⁺⁺ too low in medium	Acquire a new lot of agar medium that will meet QC criteria.	
Zones universally too large on control plates	Inoculum too light	Adjust inoculum to McFarland 0.5 turbidity standard	Hold results until repeat QC is within limits
	Nutritionally poor medium	Use only Mueller-Hinton agar medium	DO NOT report until Mueller-Hinton agar is used
	Slow-growing organisms (not seen with controls)	Use minimum inhibitory concentration (MIC) procedure only	DO NOT report disk results from any slow-grower
	Improper medium depth (too thin)	Use 4 to 5 mm depth	DO NOT use this batch of medium for testing
Zones universally too small on control plates	Inoculum too heavy	Adjust inoculum to a 0.5 McFarland standard	Hold results until repeat QC is within limits
	Agar depth too thick	Use 4 to 5 mm depth	DO NOT use this batch of medium for testing
Single disk result above or below the limits for control organisms	Error in reading fuzzy zone edge. Transcription error. Bad disk. (Bad disks usually exhibit a gradual trend toward deterioration) Disk may not be pressed firmly onto the agar surface	Note error. Recheck reading and ask for a second opinion. Statistically, one may expect an occasional out-of-range result. Values usually fall within range on retesting.	Report results with other disks following standard protocol. Repeat the test for the out-of-control drug with the control strain and patients' isolates before reporting actual test results.

ABERRANT RESULT	PROBABLE CAUSES	CORRECTIVE ACTION (record and note all errors)	EFFECT ON CURRENT DAY'S REPORT
Colonies within zone of inhibition	Mixed culture	Isolate, identify and retest pure culture only	DO NOT report results of this plate
	Resistant mutants within zone	Gram stain or do other test to rule out contaminant. Re-test.	Measure colony-free zone and interpret
Very large zones with anaerobes		DO NOT use a disk agar diffusion procedure to test anaerobes	
<i>S. aureus</i> from patient was resistant to oxacillin one day and sensitive the next	May be two different organisms. Temperature shift from 37°C to 35°C can dramatically alter the zone size in this case.	Check testing temperature. Test must be performed at 35°C and incubated for a full 24 hours. Perform ceftioxin disk test if not already set up (refer to NCCLS document).	Report result obtained after incubation for a full 24 hours at 35°C
Zones overlap	Disks too close together	Use no more than 12 disks on a 150 mm plate and 4 to 5 disks on a 100 mm plate. Place disks no closer than 15 mm from edge of plate.	Repeat test
Zones indistinct with single colonies noted on plate.	Poorly streaked plate. Inadequate inoculum.	Use properly adjusted inoculum and repeat test	Repeat test before reporting.
"Zone within a zone" phenomenon	A swarming <i>Proteus</i>	Read the wide, distinct zone and disregard the swarming within it	Report results from outer distinct zone Ignore the swarming.
	Feather edges of zones around penicillin or ampicillin disks usually occur with beta-lactamase negative strains of <i>S. aureus</i> .	Measure the point at which you can see an obvious demarcation between growth and no growth. Avoid straining to see the tiniest colonies.	Report zone as described
	Sulfonamides	Ignore the light growth in the zone. Measure the zone where there is an 80 % reduction in growth.	Report zone as described
	Beta-lactamase positive <i>Haemophilus influenzae</i> with penicillin or ampicillin	Use inside zone	Call physician if the diagnosis is meningitis
Indistinct zones with sulfa-methoxazole with or without trimethoprim or with trimethoprim alone	Thymidine in medium allows organisms to by-pass the metabolic targets of these antibiotics	Use commercial thymidine-free plates. Measure the zone at the point where there is an 80% reduction in growth.	Report as usual if confident of results

NOTE: For more information, refer to Table 8, "Suggestions for Verification of Antimicrobial Susceptibility Test Results and Confirmation of Organism Identification," of the NCCLS document M100.

Glossary

A

antibiogram

The overall susceptibility profile of a bacterium to a battery of antimicrobial agents.

antibiotic

Any antimicrobial agent produced by a living organism. It inhibits the metabolism and/or growth of a microorganism and can kill it—e.g. penicillin from *Penicillium notatum*. Vast numbers of antibiotics exist in nature but only a small number are safe for human use.

antimicrobial

Any substance of natural, semi-synthetic, or synthetic origin which inhibits the metabolism and/or growth of a microorganism and can kill it.

B

bactericidal

Kills bacteria.

bacteriostatic

Inhibits the growth of bacteria without killing them.

beta-lactamases

Enzymes produced by microorganisms that destroy the activity of beta-lactam agents by hydrolyzing the beta-lactam ring portion of the molecules. There are many types of beta-lactamases each with activity against specific beta-lactam agents.

beta-lactamase test

Only has use in the clinical laboratory to detect beta-lactamases encountered in *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Enterococcus* spp., *Staphylococcus* spp, and some anaerobic bacteria.

positive beta-lactamase test

Indicates that the bacterium is resistant to penicillins that are beta-lactamase labile including ampicillin, amoxicillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin.

negative beta-lactamase test

When found with *H. influenzae*, *M. catarrhalis* and *Staphylococcus* spp. a negative test suggests that the organism is susceptible to the beta-lactamase labile penicillins listed above. For *N. gonorrhoeae*, *Enterococcus* spp., and anaerobic bacteria, non-beta-lactamase-mediated resistance mechanisms are often encountered, which also confer resistance to beta-

lactamase labile penicillins. This resistance is detected with conventional disk diffusion and MIC tests.

breakpoints

Synonymous with “interpretive criteria” in NCCLS tables. MIC and disk diffusion values that distinguish susceptible, intermediate and resistant test results.

C**cascade reporting**

(see selective reporting.)

C&S

Culture and sensitivity (susceptibility).

colonizers

Microorganisms present in a host that are not contributing to an infectious process.

colony count

Number of colonies noted on culture medium following plating of a specimen. Each colony arises from a single bacterium unless they develop as clumps or chains (e.g. staphylococci or streptococci). Colony counts often are used to determine the significance of the quantity of organisms in certain specimen types such as urines.

CFU (colony forming units)

One colony forming unit is assumed to arise from one bacterium. See colony count above.

cytocentrifuge

A type of centrifuge that deposits a film of specimen on a microscopic slide.

D**disk diffusion (Kirby-Bauer) test**

A type of antimicrobial susceptibility test in which disks of filter paper are impregnated with various antimicrobial agents and placed on the surface of an agar plate that has been inoculated with the patient’s bacteria. Following overnight incubation, zones of inhibited growth around each disk are measured and interpreted as susceptible, intermediate or resistant (based on pre-established criteria).

E**E test**

A type of commercial antimicrobial susceptibility test in which plastic strips impregnated with a gradient of antimicrobial concentrations are placed on the surface of an agar plate that has been inoculated with the patient’s bacteria. Following overnight incubation, antimicrobial activity is seen as an eclipse of inhibited growth around the strip. The upper surface of the E test strip has a scale so that an MIC value for the isolate is obtained where the eclipse intersects the scale.

ESBL

Extended-spectrum beta-lactamases are enzymes produced by some *E. coli* and *Klebsiella* spp. (and sometimes other Enterobacteriaceae) that inactivate penicillins, expanded-spectrum cephalosporins and aztreonam.

F

fastidious

Refers to microorganisms that require special nutrients or environmental conditions for their growth.

G

GC

Gonococcus (*Neisseria gonorrhoeae*).

GISA

Glycopeptide intermediate *S. aureus* (also see VISA).

H

hierarchical reporting (see selective reporting).

Intermediate (indeterminate) interpretation of susceptibility results that implies clinical utility in body sites in which the drugs are physiologically concentrated (e.g. quinolones and beta-lactams in urine) or when high dosage of drug can be used (e.g. beta-lactams).

I

in vitro

in a laboratory.

in vivo

in a patient's body.

K

Kirby Bauer test (see disk diffusion test).

M

MIC

See minimal inhibitory concentration.

microdilution MIC

MIC test performed in a small plastic microdilution tray that usually includes 96 wells (12 columns of 8 wells). Each well contains a standard volume (e.g. 0.1 mL) of antimicrobial solutions prepared in two-fold dilutions. A battery of antimicrobial agents (e.g. 8–12) is included in each tray which is inoculated with a patient's isolate.

MicroScan

A commercially available instrument used in many routine clinical microbiology laboratories for identification and antimicrobial susceptibility testing of rapidly growing bacteria.

minimal inhibitory concentration

The lowest concentration of an antimicrobial agent required to inhibit the growth of a microorganism.

MRSA

Methicillin-resistant *S. aureus*. It is synonymous with oxacillin-resistant *S. aureus* (oxacillin is used for in vitro testing because it is the superior agent to detect resistance to the penicillinase-stable penicillins).

N

NCCLS

The Clinical and Laboratory Standards Institute, formerly known as “The National Committee for Clinical Laboratory Standards (NCCLS),” is a nonprofit organization with members representing multiple disciplines. It is an educational organization that provides a communication forum for the development, promotion and use of national and international standards that describe antimicrobial susceptibility tests, their appropriate use and interpretation of test results.

NCCLS Antimicrobial Susceptibility Testing Documents.

The NCCLS produces documents addressing various topics in clinical laboratory science, such as analysis of glucose in serum samples and protection of laboratory workers from blood-borne pathogens. Documents for routine antimicrobial susceptibility testing and reporting are developed by a subcommittee that includes experts in infectious diseases, pharmaceuticals, and clinical laboratory practices.

normal flora

Microorganisms commonly present in specific body sites (eg., throat) that generally are beneficial to the host.

O

oxacillin agar screen test

A test performed on an agar plate containing 6 mcg/mL oxacillin and 4% sodium chloride in Mueller Hinton agar. This medium is used to screen for oxacillin resistance in *S. aureus*.

P

PBPs

Penicillin binding proteins are a group of membrane-bound enzymes that are responsible for cross-linking of peptidoglycans in the cell wall. Their active sites are available in the periplasmic space.

PCR

Polymerase chain reaction. This reaction permits direct detection and identification of organisms based on amplification of DNA sequences unique to particular microorganisms.

R

resistant

In therapeutic terms, resistance means that a microorganism is not inhibited by the concentration of an antimicrobial agent that can be attained in body fluids following standard therapeutic doses.

S

selective reporting

Reporting of antimicrobial susceptibility results based on organism identification, its overall susceptibility profile and the patient’s body site. Secondary (broader spectrum, more costly, more toxic) agents are reported only if they

offer significant clinical advantages or if the organism is resistant to the primary agents.

susceptible (“sensitive”)

In therapeutic terms, susceptible means that a microorganism is inhibited by a concentration of antimicrobial agent that can be attained in body fluids following standard therapeutic doses.

synergy screen

Tests performed to detect high level aminoglycoside resistance (to gentamicin and/or streptomycin) in enterococci to determine if the aminoglycoside will act synergistically in combination with a cell wall-active agent.

V

vancomycin agar screen test

Test performed on an agar plate containing 6 mcg/mL vancomycin in brain heart infusion agar. This is used to screen for vancomycin resistance in enterococci.

VISA

Vancomycin-intermediate *S. aureus* (also see GISA).

Vitek

A commercially available instrument that is used in many routine microbiology laboratories for identification and antimicrobial susceptibility testing of rapidly growing bacteria.

VRE

Vancomycin resistant enterococcus.

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