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Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition

This document contains the current Clinical and Laboratory Standards Institute-recommended methods for disk susceptibility testing, criteria for quality control testing, and updated tables for interpretive zone diameters.

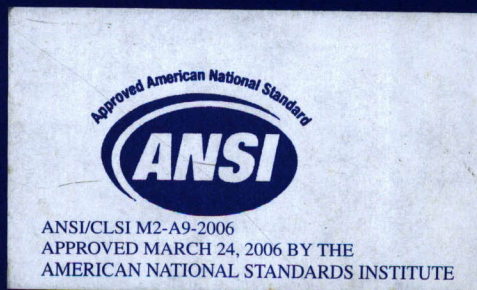
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Abstract

Susceptibility testing is indicated for any organism that contributes to an infectious process warranting antimicrobial chemotherapy, if its susceptibility cannot be reliably predicted from knowledge of the organism's identity. Susceptibility tests are most often indicated when the causative organism is thought to belong to a species capable of exhibiting resistance to commonly used antimicrobial agents.

A variety of laboratory methods can be used to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. In many clinical microbiology laboratories, an agar disk diffusion method is used routinely for testing common, rapidly growing, and certain fastidious bacterial pathogens. Clinical and Laboratory Standards Institute document M2-A9—*Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition* includes a series of procedures to standardize the way disk diffusion tests are performed. The performance, applications, and limitations of the current CLSI-recommended methods are also described.

The supplemental information (M100 tables) presented with this standard represents the most current information for drug selection, interpretation, and quality control using the procedures standardized in M2. These tables, as in previous years, have been updated and should replace tables published in earlier years. Changes in the tables since the most recent edition (M100-S16) appear in boldface type. Additionally, a glossary of antibiotic terms, classes, and abbreviations (for use with antimicrobial susceptibility testing disks and other *in vitro* test devices) has been added to the tables. It is anticipated that this glossary will provide an additional informational resource to users of the M2 standard, as it outlines antimicrobial terminology used in the susceptibility testing documents that may be unfamiliar or confusing to laboratory users. The glossary, as part of the M100 tabular information, will be continuously updated in future (yearly) revisions of M100.

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Contents

Abstract.....	i
Committee Membership.....	iii
Foreword.....	vii
Subcommittee on Antimicrobial Susceptibility Testing Mission Statement	ix
1 Scope.....	1
2 Introduction.....	1
3 Standard Precautions.....	2
4 Definitions	2
5 Indications for Performing Susceptibility Tests.....	2
6 Selection of Antimicrobial Agents for Routine Testing and Reporting.....	3
6.1 Routine Reports	3
6.2 Nonproprietary Names.....	3
6.3 Selection Guidelines	6
6.4 Suggested Guidelines for Routine and Selective Testing and Reporting	6
7 Reagents for the Disk Diffusion Test.....	7
7.1 Mueller-Hinton Agar	7
7.2 Storage of Antimicrobial Disks	9
8 Inoculum Preparation for Disk Diffusion Tests.....	9
8.1 Turbidity Standard for Inoculum Preparation.....	9
8.2 Inoculum Preparation.....	10
9 Procedure for Performing the Disk Diffusion Test.....	10
9.1 Inoculation of Test Plates.....	10
9.2 Application of Disks to Inoculated Agar Plates.....	11
9.3 Reading Plates and Interpreting Results	11
10 Fastidious Organisms.....	12
10.1 <i>Haemophilus influenzae</i> and <i>H. parainfluenzae</i>	12
10.2 <i>Neisseria gonorrhoeae</i>	13
10.3 <i>Neisseria meningitidis</i>	14
10.4 <i>Streptococcus pneumoniae</i> and Other <i>Streptococcus</i> spp.....	14
11 Problem Organisms.....	15
11.1 Staphylococci.....	15
11.2 Enterococci	18
11.3 Extended-Spectrum, β -Lactamase-Producing, Gram-Negative Bacilli	19
12 Inducible Clindamycin Resistance.....	20

Contents (Continued)

13	β -Lactamase Tests	20
	13.1 Purpose	20
	13.2 Selecting a β -Lactamase Test	20
14	Interpretation of Disk Diffusion Test Results	21
	14.1 Zone Diameter Interpretive Standards	21
	14.2 Interpretive Categories	21
	14.3 Equivalent MIC Breakpoints	21
15	Quality Control Procedures	21
	15.1 Purpose	21
	15.2 Reference Strains for Quality Control	22
	15.3 Storing and Testing Quality Control Strains	23
	15.4 Zone Diameter Quality Control Limits	24
	15.5 Frequency of Quality Control Testing	24
	15.6 Corrective Action	25
	15.7 Reporting Patient Results When Out-of-Control Tests Occur	26
	15.8 Verification of Patient Test Results	26
16	Limitations of Disk Diffusion Methods	27
	16.1 Application to Various Organism Groups	27
	16.2 Misleading Results	27
	16.3 Emergence of Resistance	28
17	Screening Tests	28
	References	29
	Appendix A. Quality Control Protocol Flow Charts	30
	Summary of Comments and Subcommittee Responses	32
	The Quality System Approach	34
	Related CLSI/NCCLS Publications	35

Foreword

In this 2006 revision of CLSI document M2—*Performance Standards for Antimicrobial Disk Susceptibility Tests* several sections have been added or revised. In particular, a new section on disk diffusion testing of *Neisseria meningitidis* that relates to a new Table 2J in M100 has been added. The Problem Organisms section now details a procedure for oxacillin salt-agar screening for the detection of MRSA and a procedure for disk approximation testing for detecting inducible clindamycin resistance for *S. aureus*, coagulase-negative *Staphylococcus* spp., and beta-hemolytic streptococci. The latest version of the M100 tables (M100-S16) published as an annual volume is made available with this document to ensure that users are aware of the latest subcommittee guidelines related to both methods and the tabular information that normally is presented in the annual tables. M100-S16 will be updated during subcommittee meetings in 2006 and published again as a separate document in January of 2007.

There are many other editorial and procedural changes in this edition of M2 that have resulted from meetings of the Subcommittee on Antimicrobial Susceptibility Testing since 2003. Specific changes for the M100 tables are summarized at the beginning of the M100-S16 document. The most important changes in the M2 document are summarized below.

It has been an honor to serve as Chairholder of the Subcommittee on Antimicrobial Susceptibility Testing during the last three years. Many members of the subcommittee (which now numbers more than 180 volunteers including members, advisors, and observers) have been indispensable in the preparation of these documents. In addition, I would like to thank the chairholders of the working groups of the Subcommittee on Antimicrobial Susceptibility Testing for their valuable contributions during the last three years. They include Jana Swenson (Text and Table Revision and Acinetobacter Working Groups); Frank Cockerill (Agents of Bioterrorism Working Group); Sharon Cullen and Steve Brown (Quality Control Working Group); Dwight Hardy (*Stenotrophomonas* and *Burkholderia* Working Group); Janet Hindler (M39—Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data Working Group); David Hecht (M11—Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria); Fred Tenover (Fastidious Organisms and Staphylococci Working Groups); Mike Dudley (Enterobacteriaceae Working Group); Jim Jorgensen (M45—Methods for Antimicrobial Dilution and Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria); and Barth Reller (Table 1 Working Group).

Matthew A. Wikler, MD, MBA, FIDSA
Chairholder, Subcommittee on Antimicrobial Susceptibility Testing

Summary of Major Changes in This Document

Additions/Changes

Antimicrobial agents:

- Penem antimicrobial drug class described (Section 6.2.1.4)
- Teicoplanin added to glycopeptide drug class (Section 6.2.2)
- Lipopeptide antimicrobial drug class described (Section 6.2.8)
- Telithromycin and tigecycline added as single-drug class antimicrobial agents (Section 6.2.9)

Reagents:

- Specified problems that can occur if pH of Mueller-Hinton agar is outside acceptable range (Section 7.1.2)
- Outlined the effect that the calcium content of Mueller-Hinton agar can have on daptomycin results (Section 7.1.5)

Summary of Major Changes in This Document (Continued)

Inoculum Preparation:

The absorbance reading for 0.5 McFarland standard modified (Section 8.1)
Recommendation for use of the direct colony suspension method vs. the growth method for inoculum preparation expanded (Section 8.2)

Fastidious Organisms:

Species included in *Haemophilus* spp. organism group modified (Section 10.1)
Recommendations for disk diffusion susceptibility testing of *Neisseria meningitidis* added (Section 10.3)
Separate sections created for *Streptococcus pneumoniae* and *Streptococcus* spp. that describe zone diameter interpretive criteria (Section 10.4)

Problem Organisms:

Testing oxacillin-resistant *Staphylococcus lugdunensis* detailed (Section 11.1.1.1)
Recommendations for testing and reporting of *mecA* resistance for staphylococcal isolates expanded (Sections 11.1.1.1, 11.1.1.2, and 11.1.1.4)
Use of cefoxitin disk as a surrogate for detecting oxacillin resistance in staphylococci described (Section 11.1.1.2)
Procedure for oxacillin salt-agar screening for *S. aureus* added (Section 11.1.2)
Added recommendations for the vancomycin agar BHI screen for *S. aureus* (Section 11.1.4)
Recommendations for the vancomycin agar screen for enterococci modified (Section 11.2.3)

Inducible Clindamycin Resistance:

Recommendations added for detecting inducible clindamycin resistance in *Staphylococcus* spp. and beta-hemolytic streptococci (Section 12)

Quality Control:

Control strains for oxacillin salt-agar screen added (Section 15.2)
Recommendations to test for the presence of plasmid for QC strain *E. coli* ATCC® 35218 added (Section 15.2)
Frequency options for QC clarified (Section 15.4; 15.5)
Incubation temperature range clarified throughout document

It is important for users of M2-A9 and M7-A7 to recognize that commercial susceptibility testing devices are not addressed in these standards. The methods described herein are generic reference procedures that can be used for routine susceptibility testing by clinical laboratories, or that can be used by clinical laboratories to evaluate commercial devices for possible routine use. Results generated by the CLSI reference methods are used by the United States Food and Drug Administration to evaluate the performance of commercial systems before clearance is given for marketing in the United States. Clearance by the FDA indicates that the agency concludes that commercial devices provide susceptibility results that are substantially equivalent to results generated using the CLSI reference methods for the organisms and antimicrobial agents described in the manufacturer's approved package insert. Some laboratories could find that a commercial dilution, antibiotic gradient, colorimetric, turbidimetric, fluorometric, or other method is suitable for selective or routine use.

Subcommittee on Antimicrobial Susceptibility Testing Mission Statement

The Subcommittee on Antimicrobial Susceptibility Testing is composed of representatives from the professions, government, and industry, including microbiology laboratories, government agencies, healthcare providers and educators, and pharmaceutical and diagnostic microbiology industries. Using the CLSI voluntary consensus process, the subcommittee develops standards that promote accurate antimicrobial susceptibility testing and appropriate reporting.

The mission of the Subcommittee on Antimicrobial Susceptibility Testing is to:

- develop standard reference methods for antimicrobial susceptibility tests;
- provide quality control parameters for standard test methods;
- establish interpretive criteria for the results of standard antimicrobial susceptibility tests;
- provide suggestions for testing and reporting strategies that are clinically relevant and cost-effective;
- continually refine standards and optimize the detection of emerging resistance mechanisms through the development of new or revised methods, interpretive criteria, and quality control parameters;
- educate users through multimedia communication of standards and guidelines; and
- foster a dialogue with users of these methods and those who apply them.

The ultimate purpose of the subcommittee's mission is to provide useful information to enable laboratories to assist the clinician in the selection of appropriate antimicrobial therapy for patient care. The standards and guidelines are meant to be comprehensive and to include all antimicrobial agents for which the data meet established CLSI guidelines. The values that guide this mission are quality, accuracy, fairness, timeliness, teamwork, consensus, and trust.

Key Words

Agar diffusion, antibiotic, antimicrobial agents, disk diffusion, Kirby-Bauer method, susceptibility testing

Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition

1 Scope

This document describes the standard agar disk diffusion techniques to be utilized to determine the *in vitro* susceptibility of bacteria that grow aerobically. The document addresses preparation of agar plates, testing conditions (including inoculum preparation and standardization, incubation time, and incubation temperature), interpretation of results, quality control procedures, and limitations of disk diffusion methods. To assist the clinical laboratory, suggestions are provided on the selection of antimicrobial agents for routine testing and reporting. Standards for testing the *in vitro* susceptibility of bacteria that grow aerobically utilizing dilution methods can be found in the most current edition of CLSI document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. Standards for testing the *in vitro* susceptibility of bacteria that grow anaerobically can be found in the most current edition of CLSI/NCCLS document M11—*Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*.

2 Introduction

A variety of laboratory methods can be used to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. In many clinical microbiology laboratories, an agar disk diffusion method is used routinely for testing common, rapidly growing, and certain fastidious bacterial pathogens. This document describes the performance, applications, and limitations of the standardized disk diffusion test method. Recommendations of the International Collaborative Study (ICS)¹ and regulations^{2,3} proposed by the United States Food and Drug Administration (FDA) have been reviewed, and appropriate sections have been incorporated into this standard. Other susceptibility testing methods exist that provide essentially equivalent results to the CLSI methods described herein. The FDA is responsible for the approval of commercial devices used in the United States. CLSI does not approve or endorse commercial products or devices.

Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone are not acceptable for determining antimicrobial susceptibility. Reliable results can only be obtained with disk diffusion tests that use the principle of standardized methodology and zone diameter measurements correlated with minimal inhibitory concentrations (MICs) with strains known to be susceptible or resistant to various antimicrobial agents.

The methods described herein must be followed explicitly to obtain reproducible results. The standardized method currently recommended by the CLSI Subcommittee on Antimicrobial Susceptibility Testing is based on the method originally described by Bauer et al.⁴ This is the most thoroughly described disk diffusion method for which interpretive standards have been developed and supported by laboratory and clinical data.

This document describes methods, quality control, and interpretive criteria recommended presently for disk diffusion susceptibility tests. When new problems are recognized or improvements in these criteria are developed, changes will be incorporated into future editions of this standard and also distributed in annual informational supplements.

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions” and “body substance isolation” practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (Garner JS. Hospital Infection Control Practices Advisory Committee. Guideline for isolation precautions in hospitals. *Infect Control Hosp Epidemiol.* 1996;17(1):53-80). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

4 Definitions

antimicrobial susceptibility test interpretive category – 1) a classification based on an *in vitro* response of an organism to an antimicrobial agent at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent; **2) susceptible antimicrobial susceptibility test interpretive category** – a category that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection; **3) intermediate antimicrobial susceptibility test interpretive category** – the “intermediate” category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g., quinolones and β -lactams in urine) or when a higher than normal dosage of a drug can be used (e.g., β -lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins; **4) resistant antimicrobial susceptibility test interpretive category** – a category that implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate zone diameters that fall in the range where specific microbial resistance mechanisms (e.g., β -lactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

quality control – the operational techniques that are used to ensure accuracy and reproducibility.

5 Indications for Performing Susceptibility Tests

Susceptibility testing is indicated for any organism that contributes to an infectious process warranting antimicrobial chemotherapy, if its susceptibility cannot be reliably predicted from knowledge of the organism’s identity. Susceptibility tests are most often indicated when the causative organism is thought to belong to a species capable of exhibiting resistance to commonly used antimicrobial agents. Mechanisms of resistance include production of drug-inactivating enzymes, alteration of drug targets, and altered drug uptake or efflux. Some organisms have predictable susceptibility to antimicrobial agents, and empiric therapy for these organisms is widely accepted. Susceptibility tests are seldom necessary when the infection is due to a microorganism recognized as susceptible to a highly effective drug (e.g., the continued susceptibility of *Streptococcus pyogenes* to penicillin). For *S. pyogenes* isolates from penicillin-allergic patients, erythromycin or another macrolide may be tested to detect strains resistant to those agents. Susceptibility tests are also important in studies of the epidemiology of resistance and in studies of new antimicrobial agents.

Isolated colonies of each type of organism that may be pathogenic should be selected from primary agar plates and tested for susceptibility. Identification procedures are often performed at the same time. Mixtures of different types of microorganisms should not be tested on the same susceptibility test plate.

The practice of conducting susceptibility tests directly with clinical material (e.g., normally sterile body fluids and urine) should be avoided, except in clinical emergencies when the direct Gram stain suggests a single pathogen. When testing has been carried out directly with the clinical material, results should be reported as preliminary, and the susceptibility test must be repeated using the standardized methodology.

When the nature of the infection is not clear and the specimen contains mixed growth or normal flora (in which the organisms probably bear little relationship to the infectious process being treated), susceptibility tests are often unnecessary and the results may be misleading.

6 Selection of Antimicrobial Agents for Routine Testing and Reporting

Selection of the most appropriate antimicrobial agents to test and report is a decision best made by each clinical laboratory in consultation with the infectious disease practitioners and the pharmacy, as well as the pharmacy and therapeutics and infection control committees of the medical staff. The recommendations in Tables 1 and 1A for each organism group list agents of proven efficacy that show acceptable *in vitro* test performance. Considerations in the assignment of agents to specific test/report groups include clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, FDA clinical indications for usage, and current consensus recommendations for first choice and alternative drugs. Tests of selected agents may be useful for infection control purposes.

6.1 Routine Reports

The agents in Tables 1 and 1A are recommendations that are considered appropriate at present for testing and reporting. To avoid misinterpretation, routine reports to physicians should include only those antimicrobial agents appropriate for therapeutic use, as suggested in Tables 1 and 1A. Agents may be added to or removed from these basic lists as conditions demand. Antimicrobial agents other than those appropriate for use in therapy may also be tested to provide taxonomic data and epidemiologic information, but they should not be included on patient reports. However, such results should be available (in the laboratory) to the infection control practitioner and/or hospital epidemiologist.

6.2 Nonproprietary Names

To minimize confusion, all antimicrobial agents should be reported using official nonproprietary (i.e., generic) names. To emphasize the relatedness of the many currently available antimicrobial agents, they may be grouped together by drug classes as follows:

6.2.1 β -Lactams

β -lactam antimicrobial agents all share the common, central, four-member β -lactam ring and the principal mode of action of inhibition of cell wall synthesis. Additional ring structures or substituent groups added to the β -lactam ring determine whether the agent is classified as a penicillin, cephem, carbapenem, or monobactam.

6.2.1.1 Penicillins

Penicillins are primarily active against non- β -lactamase-producing, aerobic gram-positive, some fastidious, aerobic gram-negative, and some anaerobic bacteria. Aminopenicillins (ampicillin and amoxicillin) are active against additional gram-negative species, including some members of the Enterobacteriaceae. Carboxypenicillins (carbenicillin and ticarcillin) and ureidopenicillins (mezlocillin

and piperacillin) are active against a considerably expanded list of gram-negative bacteria, including many *Pseudomonas* and *Burkholderia* spp. Penicillinase-stable penicillins (cloxacillin, dicloxacillin, methicillin, nafcillin, and oxacillin) are active against predominantly gram-positive bacteria, including penicillinase-producing staphylococci.

6.2.1.2 β -Lactam/ β -Lactamase Inhibitor Combinations

These antimicrobial agents are combinations that include a penicillin class antimicrobial agent and a second agent that has minimal antibacterial activity, but functions as an inhibitor of some β -lactamases. Currently, three β -lactamase inhibitors are in use: clavulanic acid, sulbactam, and tazobactam. The results of tests of only the penicillin portion of the combination against β -lactamase-producing organisms are often not predictive of susceptibility to the two-drug combination.

6.2.1.3 Cephems (Including Cephalosporins)

Different cephem antimicrobial agents exhibit somewhat different spectrums of activity against aerobic and anaerobic gram-positive and gram-negative bacteria. The cephem antimicrobial class includes the classical cephalosporins, as well as the agents in subclasses cephamycin, oxacephem, and carbacephems (see Glossary I). Cephalosporins are often referred to as “first-,” “second-,” “third-,” or “fourth-generation” cephalosporins, based on the extent of their activity against the more antimicrobial agent-resistant, gram-negative aerobic bacteria. Not all representatives of a specific group or generation necessarily have the same spectrum of activity. Because of these differences in activities, representatives of each group may be selected for routine testing.

6.2.1.4 Penems

The penem antimicrobial class which includes two subclasses, the carbapenems and penems, differs slightly in structure from the penicillin class; agents in this class are much more resistant to β -lactamase hydrolysis, which provides them with broad-spectrum activity against many gram-positive and gram-negative bacteria.

6.2.1.5 Monobactams

Monobactam antimicrobial agents are monocyclic β -lactams. At present, aztreonam (which has activity only against aerobic gram-negative bacteria) is the only monobactam antimicrobial agent approved for use by the FDA.

6.2.2 Glycopeptides

Glycopeptide antimicrobial agents, which include vancomycin and teicoplanin, share a complex chemical structure and a principal mode of action of inhibition of cell wall synthesis at a different site than that of the β -lactams. The activity of this group is directed primarily at aerobic gram-positive bacteria. Vancomycin is an accepted agent for treatment of a gram-positive bacterial infection in the penicillin-allergic patient, and it is useful for therapy of infections due to β -lactam-resistant, gram-positive bacterial strains (e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) and some enterococci).

6.2.3 Aminoglycosides

Aminoglycosides are structurally related antimicrobial agents that inhibit bacterial protein synthesis at the ribosomal level. This class includes agents variously affected by aminoglycoside-inactivating enzymes, resulting in some differences in the spectrum of activity among the agents. Aminoglycosides are used primarily to treat aerobic, gram-negative rod infections or in synergistic combinations with cell-wall-

active antimicrobial agents (e.g., penicillin, ampicillin, vancomycin) against some resistant, gram-positive bacteria, such as enterococci.

6.2.4 Macrolides

Macrolides are structurally related antimicrobial agents that inhibit bacterial protein synthesis at the ribosomal level. Several members of this class currently in use may need to be considered for testing against fastidious, gram-negative bacterial isolates. For gram-positive organisms, only erythromycin needs to be tested routinely.

6.2.5 Tetracyclines

Tetracyclines are structurally related antimicrobial agents that inhibit protein synthesis at the ribosomal level of certain gram-positive and gram-negative bacteria. Agents in this group are closely related and, with few exceptions, only tetracycline may need to be tested routinely. Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline or minocycline or both.

6.2.6 Quinolones

Quinolones (quinolones and fluoroquinolones) are structurally related antimicrobial agents that function primarily by inhibiting the DNA-gyrase or topoisomerase activity of many gram-positive and gram-negative bacteria. Some differences in spectrum may require separate testing of the individual agents.

6.2.7 Folate Pathway Inhibitors

Sulfonamides and trimethoprim are chemotherapeutic agents with similar spectra of activity resulting from the inhibition of the bacterial folate pathway. Sulfisoxazole is among the most commonly used sulfonamides in the treatment of urinary tract infections; thus, it may be the appropriate selection for *in vitro* testing. Sulfamethoxazole is usually tested in combination with trimethoprim, because these two antimicrobial agents inhibit sequential steps in the folate pathway of some gram-positive and gram-negative bacteria.

6.2.8 Lipopeptides

Lipopeptides are a structurally related group of antimicrobial agents, whose principal target is the cell membrane. The polymyxin subclass, which includes polymyxin B and colistin, has activity against gram-negative organisms. Daptomycin is a cyclic lipopeptide with activity against gram-positive organisms. Lipopeptide activity is strongly influenced by the presence of divalent cations in the medium used to test them. The presence of excess calcium cations inhibits the activity of the polymyxins, whereas the presence of physiologic levels (50 mg/L) of calcium ions is essential for the proper activity of daptomycin.

6.2.9 Single-Drug Classes

The following antimicrobial agents (antimicrobial class) are currently the only members of their respective classes used in humans that are included in this document, and are appropriate for *in vitro* testing. These include chloramphenicol (phenicols), clindamycin (lincosamides), linezolid (oxazolidinones), quinupristin-dalfopristin (streptogramins), telithromycin (ketolides), and tigecycline (glycylcyclines), all of which inhibit protein synthesis, and rifampin (ansamycins), which is an RNA synthesis inhibitor. Nitrofurantoin (nitrofurans), which is used only in the therapy of urinary tract infections, acts by inhibiting several protein synthesis-and-assembly steps at the ribosomal level.

Fosfomycin (fosfomycins), also approved by the FDA for urinary tract infections, inhibits enzymes involved in cell wall synthesis.

6.3 Selection Guidelines

To make routine susceptibility testing relevant and practical, the number of agents tested should be limited. Tables 1 and 1A list those agents that should fulfill the basic requirements for routine use in most clinical laboratories. The tables are divided into columns based on specific organisms or organism groups, and then the various drugs are indicated in priority for testing to assist laboratories in the selection of their routine testing batteries. The boxes in the table designate clusters of comparable agents that generally need not be duplicated in testing because interpretive results are usually similar and clinical efficacy is usually comparable. In addition, the word “or” designates a related group of agents, which show a nearly identical spectrum of activity and interpretive results, and for which cross-resistance and cross-susceptibility are nearly complete. Therefore, usually only one of the agents within each selection box (cluster or related group) need be selected for testing. With few exceptions, the agent reported must be tested, unless reporting based on testing of another agent provides a more accurate result (e.g., susceptibility of staphylococci to cepheems based on oxacillin testing). When possible, the agents tested should match those included in the hospital formulary, or else the report should include footnotes indicating which other nontested agents may have comparable activity.

6.4 Suggested Guidelines for Routine and Selective Testing and Reporting

As listed in Tables 1 and 1A, agents in Group A are considered appropriate for inclusion in a routine, primary testing panel and for routine reporting of results for the specific organism groups.

Group B comprises agents that may warrant primary testing. However, report the results selectively, such as when the organism is resistant to agents of the same class, as in Group A. Other indications for reporting the result might include a selected specimen source (e.g., a third-generation cephalosporin for enteric bacilli from cerebrospinal fluid [CSF] or trimethoprim-sulfamethoxazole for urinary tract isolates); a polymicrobial infection; infections involving multiple sites; cases of patient allergy, intolerance, or failure to respond to an agent in Group A; or for purposes of infection control.

Group C comprises alternative or supplemental antimicrobial agents that may require testing in those institutions that harbor endemic or epidemic strains resistant to several of the primary drugs (especially in the same class [e.g., β -lactams or aminoglycosides]); for treatment of patients allergic to primary drugs; for treatment of unusual organisms (e.g., chloramphenicol for extraintestinal isolates of *Salmonella* spp. or some vancomycin-resistant enterococci); or for reporting to infection control as an epidemiologic aid.

Group U agents (e.g., nitrofurantoin and certain quinolones) are used only or primarily for treating urinary tract infections; these agents should not be routinely reported against pathogens recovered from other sites of infection. Other agents with broader indications may be included in Group U for specific urinary pathogens (e.g., *Pseudomonas aeruginosa*).

Group O (“other”) agents have a clinical indication for the organism group, but are generally not candidates for routine testing and reporting in the United States.

Group Inv. (“investigational”) agents are undergoing clinical investigation for the organism group and have not yet been approved by FDA for use in the United States.

Each laboratory should decide which agents in Tables 1 and 1A to report routinely (Group A) and which might be reported only selectively (from Group B), in consultation with the infectious diseases practitioners, the pharmacy, as well as the pharmacy and therapeutics and infection control committees of the healthcare institution. Selective reporting should help improve the clinical relevance of test reports

and help minimize the selection of multiresistant, healthcare-associated strains by overuse of broad-spectrum agents. Results for Group B agents not reported routinely should be available on request, or they may be reported for selected specimens. Unexpected resistance, when confirmed, should be reported (e.g., resistance to a secondary agent but susceptibility to a primary agent).

7 Reagents for the Disk Diffusion Test

7.1 Mueller-Hinton Agar

Of the many media available, the subcommittee considers Mueller-Hinton agar the best for routine susceptibility testing of nonfastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing.
- It is low in sulfonamide, trimethoprim, and tetracycline inhibitors.
- It supports satisfactory growth of most nonfastidious pathogens.
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Although Mueller-Hinton agar is generally reliable for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. If a batch of medium does not support adequate growth of a test organism, zones obtained in a disk diffusion test will usually be larger than expected and may exceed the acceptable quality control limits. Only Mueller-Hinton agar formulations that have been tested according to, and that meet the acceptance limits described in, CLSI document M6—*Protocols for Evaluating Dehydrated Mueller-Hinton Agar* should be used.

7.1.1 Preparation of Mueller-Hinton Agar

Mueller-Hinton agar preparation includes the following steps:

- (1) Prepare Mueller-Hinton agar from a commercially available dehydrated base according to the manufacturer's instructions.
- (2) Immediately after autoclaving, allow the agar to cool in a 45 to 50 °C water bath.
- (3) Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 mL of medium for plates with a diameter of 150 mm, and 25 to 30 mL for plates with a diameter of 100 mm.
- (4) Allow the agar plates to cool further to room temperature and, unless the plates are used the same day, store in a refrigerator (2 to 8 °C).
- (5) Use the plates within seven days after preparation unless adequate precautions, such as wrapping in plastic, are taken to minimize drying of the agar.
- (6) A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35 °C for 24 hours or longer.

7.1.2 pH

Check the pH of each batch of Mueller-Hinton agar when the medium is prepared. The exact method used will depend largely on the type of equipment available in the laboratory. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. If the pH is less than 7.2, certain drugs will appear to lose potency (e.g., aminoglycosides, quinolones, macrolides), while other agents may appear to have excessive activity (e.g., tetracyclines). If the pH is greater than 7.4, the opposite effects can be expected. Check the pH by one of the following means:

- Macerate enough agar to submerge the tip of a pH electrode.
- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- Use a surface electrode.

7.1.3 Moisture

If, just before use, excess surface moisture is present on the plates, place them in an incubator (35 °C) or a laminar flow hood at room temperature with lids ajar until excess surface moisture is lost by evaporation (usually ten to 30 minutes). The surface of the plate should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the petri dish covers when the plates are inoculated.

7.1.4 Effects of Thymidine or Thymine

Mueller-Hinton agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim, thus yielding smaller and less distinct zones, or even no zone at all, which may result in false-resistance reports. Use Mueller-Hinton agar that is as low in thymidine content as possible. Should problems with quality control of sulfonamides and trimethoprim occur, it might be necessary to check the Mueller-Hinton agar. To evaluate a new lot of Mueller-Hinton agar, *Enterococcus faecalis* ATCC^{®a} 29212 or, alternatively, *Enterococcus faecalis* ATCC[®] 33186, may be tested with trimethoprim-sulfamethoxazole disks. Satisfactory media will provide essentially clear, distinct zones of inhibition ≥ 20 mm. Unsatisfactory media will produce no zone of inhibition, growth within the zone, or a zone of < 20 mm.

7.1.5 Effects of Variation in Divalent Cations

Variation in divalent cations, principally magnesium and calcium, will affect results of aminoglycoside and tetracycline tests with *P. aeruginosa* strains. Excess cation content will reduce zone sizes, whereas low cation content may result in unacceptably large zones of inhibition. Variation in calcium levels also affects the results of daptomycin tests. For daptomycin, insufficient calcium content reduces zone sizes, whereas high calcium content may increase zone sizes. Excess zinc ions may reduce zone sizes of carbapenems. Performance tests with each lot of Mueller-Hinton agar must conform to the control limits listed in Table 3.

7.1.6 Testing Strains That Fail to Grow Satisfactorily

Only aerobic or facultative bacteria that grow well on unsupplemented Mueller-Hinton agar should be tested on that medium. Certain fastidious species such as *Haemophilus* spp., *Neisseria gonorrhoeae*, *N. meningitidis*, *Streptococcus pneumoniae*, and viridans and beta-hemolytic streptococci do not grow sufficiently on unsupplemented Mueller-Hinton agar. These organisms require supplements or different media to grow, and they should be tested on the media described in Section 10.

^a ATCC[®] is a registered trademark of the American Type Culture Collection.

7.2 Storage of Antimicrobial Disks

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Store disks as follows:

- Refrigerate the cartridges at 8 °C or below, or freeze at -14 °C or below until needed. Do not store the disks in a self-defrosting freezer. Sealed packages of disks that contain drugs from the β -lactam class should be stored frozen, except for a small working supply, which may be refrigerated for at most one week. Some labile agents (e.g., imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
- Remove the sealed packages containing disk cartridges from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This will minimize the amount of condensation that occurs when warm air contacts cold disks.
- Once a cartridge of disks has been removed from its sealed package, place it in a tightly sealed, desiccated container for storage. If a disk-dispensing apparatus is used, it should be fitted with a tight cover and supplied with an adequate desiccant. Allow the dispenser to warm to room temperature before opening. Avoid excessive moisture by replacing the desiccant when the indicator changes color.
- When not in use, refrigerate the dispensing apparatus containing the disks.
- Use only those disks that have not reached the manufacturer's expiration date stated on the label. Discard disks when they reach the expiration date.

8 Inoculum Preparation for Disk Diffusion Tests

8.1 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, a BaSO_4 turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. Prepare a BaSO_4 0.5 McFarland standard as follows:

- (1) Add a 0.5-mL aliquot of 0.048 mol/L BaCl_2 (1.175% w/v $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
- (2) Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
- (3) Transfer the barium sulfate suspension in 4- to 6-mL aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculum.
- (4) Tightly seal the tubes and store in the dark at room temperature.
- (5) Vigorously agitate the barium sulfate turbidity standard on a mechanical vortex mixer before each use, and inspect for a uniformly turbid appearance. Replace the standard if large particles appear. Mix latex particle suspensions by inverting gently, not on a vortex mixer.
- (6) The barium sulfate standards should be replaced or their densities verified monthly.

8.2 Inoculum Preparation

8.2.1 Direct Colony Suspension Method

- (1) The direct colony suspension method is the most convenient method for inoculum preparation. This method can be used with most organisms and is the recommended method for some (see below).
- (2) Prepare the inoculum by making a direct broth or saline suspension of isolated colonies selected from an 18- to 24-hour agar plate (a nonselective medium, such as blood agar, should be used). This results in a suspension containing approximately 1 to 2×10^8 CFU/mL for *E. coli* ATCC® 25922. To perform this step accurately, use either a photometric device or, if performed visually, use adequate light to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.
- (3) Direct colony suspension is the recommended method for testing the fastidious organisms *Haemophilus* spp., *N. gonorrhoeae*, *N. meningitidis*, and streptococci (see Section 10), and for testing staphylococci for potential methicillin or oxacillin resistance.

8.2.2 Growth Method

- (1) The growth method can be used alternatively and is sometimes preferable when colony growth is difficult to suspend directly and a smooth suspension cannot be made. It can also be used for nonfastidious organisms (except staphylococci) when fresh (24-hour) colonies, as required for the direct colony suspension method, are not available.
- (2) Select at least three to five well-isolated colonies of the same morphologic type from an agar plate culture. Touch the top of each colony with a loop or sterile swab and transfer the growth into a tube containing 4 to 5 mL of a suitable broth medium, such as tryptic soy broth.
- (3) Incubate the broth culture at 35 °C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually two to six hours).
- (4) Adjust the turbidity of the actively growing broth culture with sterile saline or broth to achieve a turbidity equivalent to a 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2×10^8 CFU/mL for *E. coli* ATCC® 25922. To perform this step accurately, use either a photometric device or, if performed visually, use adequate light to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

9 Procedure for Performing the Disk Diffusion Test

9.1 Inoculation of Test Plates

- (1) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the adjusted suspension. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
- (2) Inoculate the dried surface of a Mueller-Hinton agar plate by streaking the swab over the entire sterile agar surface. Repeat this procedure by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, swab the rim of the agar.
- (3) The lid may be left ajar for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

NOTE: Avoid extremes in inoculum density. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

9.2 Application of Disks to Inoculated Agar Plates

- (1) Dispense the predetermined battery of antimicrobial disks onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure complete contact with the agar surface. Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 disks should be placed on one 150-mm plate, or more than five disks on a 100-mm plate. In all cases, however, it is best to place disks that give predictably small zones (e.g., gentamicin and vancomycin) next to those that give larger zones (e.g., cephalosporins) in an effort to avoid overlapping zones. It is also important to pay attention to how close the disks are to the edge of the plate, no matter how many disks are dispensed. If disks are placed too close to the edge of the plate, the zones may not be fully round with some drugs. Because some of the drug diffuses almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar. If the D test for inducible clindamycin resistance is being performed, see Section 12 and Tables 2C and 2H for guidance on disk placement.
- (2) Invert the plates and place in an incubator set to 35 ± 2 °C (testing at temperatures above 35 °C may not detect MRS [methicillin-resistant staphylococci]) within 15 minutes after the disks are applied. With the exception of *Haemophilus* spp., *N. gonorrhoeae*, and streptococci (see Section 10), do not incubate the plates in an increased CO₂ atmosphere, because the interpretive standards were developed by using ambient air incubation, and CO₂ will significantly alter the size of the inhibitory zones of some agents.

9.3 Reading Plates and Interpreting Results

- (1) After 16 to 18 hours of incubation, examine each plate. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. Measure the diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk. Measure the zones to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate. Hold the petri plate a few inches above a black, nonreflecting background illuminated with reflected light. If blood was added to the agar base (as with streptococci), measure the zones from the upper surface of the agar illuminated with reflected light, with the cover removed. If oxacillin or vancomycin is being tested against *Staphylococcus* spp. or vancomycin against *Enterococcus* spp., 24 hours of incubation are required before reporting as susceptible; other agents can be read and reported at 16 to 18 hours. Use transmitted light (plate held up to light) to examine the oxacillin and vancomycin zones for light growth of resistant colonies within apparent zones of inhibition. Any discernable growth within the zone of inhibition is indicative of oxacillin or vancomycin resistance.
- (2) The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth. However, when discrete colonies grow within a clear zone of inhibition, the test should be repeated with a pure culture or subculture of a single colony from the primary culture plate. If discrete colonies continue to grow within the zone of inhibition, measure the colony-free inner zone. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., ignore the thin veil of swarming growth in an otherwise obvious zone of inhibition. When blood-supplemented medium for testing streptococci is used, measure the zone of growth inhibition, not the zone of inhibition of hemolysis. With trimethoprim and the sulfonamides, antagonists in the medium may allow some

slight growth; therefore, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter.

- (3) Interpret the sizes of the zones of inhibition by referring to Tables 2A through 2J, and report the organisms as susceptible, intermediate, or resistant to the agents that have been tested (see Section 12). Some agents may be reported only as susceptible, because only susceptible breakpoints are given, since no or very few resistant strains have been identified.

10 Fastidious Organisms

Mueller-Hinton medium described previously for the rapidly growing aerobic pathogens is not adequate for fastidious organisms. If disk diffusion tests are performed with fastidious organisms, the medium, quality control procedures, and interpretive criteria must be modified to fit each organism. Disk diffusion tests for *Haemophilus influenzae*, *N. gonorrhoeae*, *N. meningitidis*, *S. pneumoniae*, and beta-hemolytic and viridans group streptococci have been shown to be accurate for selected agents; they are described here. Other fastidious bacteria may be tested by a dilution method as described in CLSI document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. Anaerobic bacteria should not be tested by the disk diffusion test. See CLSI/NCCLS document M11—*Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* for appropriate anaerobe testing procedures.

10.1 *Haemophilus influenzae* and *H. parainfluenzae*

This method has been validated for *H. influenzae* and *H. parainfluenzae* only. When *Haemophilus* spp. is used below, it applies only to these two species.

10.1.1 Agar Medium

The medium of choice for disk diffusion testing of *Haemophilus* spp. is *Haemophilus* Test Medium (HTM).⁵ Mueller-Hinton chocolate agar is not recommended for routine testing of *Haemophilus* spp.

In its agar form, HTM consists of the following ingredients:

- Mueller-Hinton agar;
- 15 µg/mL β-nicotinamide adenine dinucleotide (NAD);
- 15 µg/mL bovine hematin; and
- 5 g/L yeast extract.

To make HTM, first prepare a fresh hematin stock solution by dissolving 50 mg of bovine hematin powder in 100 mL of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved. Add 30 mL of the hematin stock solution to 1 L of Mueller-Hinton agar with 5 g of yeast extract. After autoclaving and cooling to 45 to 50 °C, aseptically add 3 mL of an NAD stock solution (50 mg of NAD dissolved in 10 mL of distilled water and filter sterilized). The pH should be 7.2 to 7.4.

Haemophilus influenzae (ATCC® 10211) is recommended as a useful additional quality control strain to verify the growth promotion properties of HTM. In particular, manufacturers of HTM are encouraged to use *H. influenzae* ATCC® 10211 as a quality control test strain.

10.1.2 Test Procedure

- (1) Use the direct colony suspension procedure as described in Section 8.2.1 when testing *Haemophilus* spp. Using colonies taken directly from an overnight (preferably 20- to 24-hour) chocolate agar culture plate, prepare a suspension of the test organism in Mueller-Hinton broth or saline. Adjust the

suspension with broth or saline using a photometric device to achieve a turbidity equivalent to a 0.5 McFarland standard. This suspension will contain approximately 1 to 4×10^8 CFU/mL. Exercise care in preparing this suspension, because higher inoculum concentrations may lead to false-resistant results with some β -lactam antimicrobial agents, particularly when β -lactamase-producing strains of *H. influenzae* are tested. Use the suspension for plate inoculation within 15 minutes after adjusting the turbidity.

- (2) Follow the procedure for the disk test as described for nonfastidious bacteria, beginning with Section 9.1, except that, in general, no more than nine disks should be applied to the surface of a 150-mm plate, nor more than four disks on a 100-mm plate.
- (3) Incubate the plates at 35 ± 2 °C in an atmosphere of 5% CO₂ for 16 to 18 hours before measuring the zones of inhibition.
- (4) Consider the zone margin as the area showing no obvious growth visible with the unaided eye. Ignore faint growth of tiny colonies that may appear to fade from the more obvious zone.

10.1.3 Zone Diameter Interpretive Criteria

The antimicrobial agents suggested for routine testing of *Haemophilus* spp. are listed in Table 1A. Specific zone diameter interpretive criteria to be used when testing *Haemophilus* spp. are listed in Table 2E. Disk diffusion testing of *Haemophilus* spp. with other antimicrobial agents is not recommended.

10.2 *Neisseria gonorrhoeae*

10.2.1 Agar Medium

The recommended medium for testing *N. gonorrhoeae* is GC agar to which a 1% defined growth supplement is added after autoclaving.⁶ Cysteine-free growth supplement is not required for disk testing. Enriched chocolate agar is not recommended for susceptibility testing of *N. gonorrhoeae*.

10.2.2 Test Procedure

- (1) Use the direct colony suspension procedure when testing *N. gonorrhoeae* as described in Section 8.2.1. Using colonies taken directly from an overnight chocolate agar culture plate, prepare a suspension in Mueller-Hinton broth or saline to a turbidity equivalent to that of the 0.5 McFarland standard. Inoculate plates within 15 minutes after adjusting the turbidity of the inoculum suspension.
- (2) Follow the disk diffusion test procedure as described in Section 9.1 for nonfastidious bacteria. Place no more than nine antimicrobial disks onto the agar surface of a 150-mm agar plate, or more than four disks onto a 100-mm plate. However, for some agents (e.g., fluoroquinolones or cephalosporins) that produce extremely large zones, only two to three disks may be tested per plate.
- (3) Incubate the plates at 36 ± 1 °C (do not exceed 37 °C) in an atmosphere of 5% CO₂ for 20 to 24 hours before measuring the zones of inhibition.

10.2.3 Zone Diameter Interpretive Criteria

The antimicrobial agents suggested for routine testing of *N. gonorrhoeae* are listed in Table 1A. Specific zone diameter interpretive criteria to be used when testing *N. gonorrhoeae* are listed in Table 2F. Disk diffusion testing of *N. gonorrhoeae* with other agents is not recommended.

NOTE: *N. gonorrhoeae* with 10- μ g penicillin disk zone diameters of ≤ 19 mm generally produce β -lactamase.⁶ However, β -lactamase tests (see Section 13) are faster than disk diffusion tests and are therefore preferred for recognition of this plasmid-mediated penicillin resistance. *N. gonorrhoeae* with plasmid-mediated resistance to tetracycline also have zones of inhibition (30- μ g tetracycline disks) of ≤ 19 mm. Chromosomal mechanisms of resistance to penicillin and tetracycline produce larger zone diameters and can be accurately recognized using the interpretive criteria in Table 2F.

10.3 *Neisseria meningitidis*

Disk diffusion testing of *N. meningitidis* has been validated for detection of possible emerging resistance. To date, resistance has mostly been found in older agents used for therapy (penicillin or ampicillin), or agents used for prophylaxis of case contacts. Because resistance to antimicrobial agents, such as ceftriaxone or cefotaxime that are often employed for therapy of invasive disease, has not been detected, routine testing of isolates by clinical laboratories is not necessary. Meningococci can cause laboratory-acquired infections.

When working with cultures, perform any procedure that potentially generates aerosols in a biological safety cabinet. Consider vaccination of microbiologists that work with invasive meningococcal isolates, although current vaccines are not protective against all serogroups.

10.3.1 Agar Medium

The recommended medium for testing *N. meningitidis* is Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Enriched chocolate agar is not recommended for susceptibility testing of *N. meningitidis*.

10.3.2 Test Procedure

- (1) Use the direct colony suspension procedure when testing *N. meningitidis* as described in Section 8.2.1. Using colonies taken directly from a chocolate agar culture plate incubated for 20 to 22 hours at 35 ± 2 °C in an atmosphere of 5% CO₂, prepare a suspension in Mueller-Hinton broth or saline to a turbidity equivalent to that of the 0.5 McFarland standard. Inoculate plates within 15 minutes after adjusting the turbidity of the inoculum suspension.
- (2) Follow the disk diffusion test procedure as described in Section 9.1 for nonfastidious bacteria. Place no more than five antimicrobial disks onto the agar surface of a 150-mm agar plate, and no more than two on a 100-mm agar plate.
- (3) Incubate the plates at 35 ± 2 °C in an atmosphere of 5% CO₂ for 20 to 24 hours before measuring the zones of inhibition.

10.3.3 Zone Diameter Interpretive Criteria

Specific zone diameter interpretive criteria to be used when testing *N. meningitidis* are listed in Table 2J. Disk diffusion testing of *N. meningitidis* with other agents is not recommended.

10.4 *Streptococcus pneumoniae* and Other *Streptococcus* spp.

10.4.1 Agar Medium

The recommended medium for testing *S. pneumoniae* and other streptococci is Mueller-Hinton agar supplemented with 5% defibrinated sheep blood.

10.4.2 Test Procedure

- (1) Use the direct colony suspension procedure as described in Section 8.2.1 when testing streptococci. Using colonies taken from an overnight (18- to 20-hour) sheep blood agar plate, prepare a suspension in Mueller-Hinton broth or saline to achieve a turbidity equivalent to a 0.5 McFarland standard. Use the inoculum suspension for plate inoculation within 15 minutes after adjusting the turbidity.
- (2) Follow the disk diffusion procedure steps described, beginning with Section 9.1 for nonfastidious bacteria, except that not more than nine disks should be placed on a 150-mm agar plate, or more than four disks on a 100-mm plate.
- (3) Incubate the plates at 35 ± 2 °C in an atmosphere of 5% CO₂ for 20 to 24 hours before measuring the zones of inhibition.

10.4.3 *Streptococcus pneumoniae* Zone Diameter Interpretive Criteria

The antimicrobial agents suggested for routine testing of pneumococci are indicated in Table 1A. Specific zone diameter interpretive criteria to be used when testing *S. pneumoniae* are listed in Table 2G.

NOTE: Isolates of *S. pneumoniae* with oxacillin zone sizes of ≥ 20 mm are susceptible (MIC ≤ 0.06 $\mu\text{g/mL}$) to penicillin. Because zones of ≤ 19 mm with the oxacillin disk screening test occur with penicillin-resistant, intermediate, and certain susceptible strains, a penicillin, and cefotaxime, ceftriaxone, or meropenem MIC should be determined on isolates of *S. pneumoniae* for which the oxacillin zones are ≤ 19 mm.⁷ Isolates should not be reported as penicillin resistant or intermediate based solely on an oxacillin zone of ≤ 19 mm.

10.4.4 Other *Streptococcus* spp. Zone Diameter Interpretive Criteria

The antimicrobial agents suggested for routine testing of other streptococci are indicated in Table 1A. Specific zone diameter interpretive criteria to be used when testing other streptococci are listed in Table 2H.

Oxacillin disk testing to determine penicillin susceptibility of streptococci other than *S. pneumoniae* is not recommended. A penicillin or ampicillin disk may be used to predict susceptibility for beta-hemolytic streptococci only. A penicillin MIC should be determined on isolates of viridans group streptococci from normally sterile body sites (e.g., cerebrospinal fluid, blood, bone). Penicillin and oxacillin disk diffusion tests are not reliable with viridans group streptococci.

Inducible clindamycin resistance can be identified in beta-hemolytic streptococci using the method described in Section 12.

11 Problem Organisms

11.1 Staphylococci

11.1.1 Methicillin/Oxacillin Resistance

11.1.1.1 Classification

Historically, resistance to the antistaphylococcal, penicillinase-stable penicillins has been referred to as “methicillin resistance,” and the acronyms MRSA (methicillin-resistant *S. aureus*) and MRS (methicillin-resistant staphylococci) are still commonly used, even though methicillin is no longer the agent of choice

for testing or treatment. In this document, resistance to these agents may be referred to using several terms (e.g., “MRS,” “methicillin resistance,” or “oxacillin resistance”).

Most resistance to oxacillin in staphylococci is mediated by the *mecA* gene, which codes for the production of a supplemental penicillin-binding protein, PBP 2a, and is expressed either homogeneously or heterogeneously. Homogeneous resistance is easily detected with standard testing methods, whereas heterogeneous expression may be more difficult to detect with some methods, since only a fraction of the population (e.g., 1 in 100 000 cells) expresses the resistance phenotype. In the past, the presence of resistance to other classes of agents was an indication of oxacillin resistance. However, some MRSA, such as those found in community-associated infections, are not multiply-resistant.

Oxacillin-resistant strains of *Staphylococcus lugdunensis* are not accurately detected by disk diffusion using oxacillin. The presence of *mecA*-mediated resistance in *S. lugdunensis* is detected more accurately using the cefoxitin disk and *S. aureus* interpretive criteria.

11.1.1.2 Methods

- Of the penicillinase-stable penicillins, oxacillin is preferred for *in vitro* testing. Oxacillin susceptibility test results can be applied to the other penicillinase-stable penicillins (i.e., cloxacillin, dicloxacillin, flucloxacillin, methicillin, nafcillin). Oxacillin is more resistant to degradation and is more likely to detect heteroresistant staphylococci strains than is methicillin or nafcillin. Cloxacillin disks should not be used because they may not detect oxacillin-resistant *S. aureus*. Cefoxitin may be used instead of oxacillin (see the following points).
- The cefoxitin disk test is the preferred method for testing *S. aureus* and *S. lugdunensis* for resistance to the penicillinase-stable penicillins. For *S. aureus* and *S. lugdunensis*, the cefoxitin disk test is comparable to the oxacillin disk test for prediction of *mecA*-mediated resistance to oxacillin; however, the cefoxitin disk test is easier to read and thus is the preferred method. In this case, cefoxitin is used as a surrogate to report oxacillin.
- The cefoxitin disk test is also the preferred method for testing coagulase-negative staphylococci. Although oxacillin interpretive criteria for coagulase-negative staphylococci correlate with the presence or absence of the gene encoding methicillin/oxacillin resistance (*mecA*) in *S. epidermidis*, these criteria may overestimate resistance for other coagulase-negative staphylococci (e.g., *S. saprophyticus*). The cefoxitin disk test has higher specificity than and equal sensitivity to the oxacillin disk test for coagulase-negative staphylococci; however, the cefoxitin disk test is easier to read and thus is the preferred method. In this case, cefoxitin is used as a surrogate to report oxacillin.
- Tests for *mecA* or the protein encoded by *mecA*, the penicillin-binding protein 2a (PBP 2a, also called PBP 2'), are the most accurate methods for prediction of resistance to oxacillin, and could be used to confirm results for isolates of staphylococci from serious infections.

11.1.1.3 Testing Issues

- Prepare the inoculum using the direct colony suspension method (see Section 8.2.1) rather than the growth method (see Section 8.2.2).
- Incubate tests to detect MRS for a full 24 hours (rather than 16 to 18 hours) at 35 ± 2 °C (testing at temperatures above 35 °C may not detect MRS) before reporting as susceptible. Resistance may be reported any time growth is observed after a minimum of 16 hours incubation.
- For all staphylococci, read the zone of inhibition around the cefoxitin disk using reflected light. If an oxacillin disk is used, examine the zone of inhibition around the oxacillin disk for light growth using

transmitted light (plate held up to light); any discernable growth within the zone of inhibition of oxacillin is indicative of oxacillin resistance.

11.1.1.4 Reporting

When using cefoxitin as a surrogate for detecting oxacillin resistance, report oxacillin as susceptible or resistant based on the cefoxitin result.

Report isolates of staphylococci that carry *mecA*, or that produce PBP 2a, the *mecA* gene product, as oxacillin resistant. Report isolates that do not carry *mecA* or do not produce PBP 2a as oxacillin susceptible. Because of the rare occurrence of resistance mechanisms other than *mecA*, if MIC tests are performed in addition to disk diffusion, report isolates for which oxacillin MICs are ≥ 4 $\mu\text{g}/\text{mL}$ and that are *mecA* negative as oxacillin resistant.

MRSA and methicillin-resistant, coagulase-negative staphylococci should be reported as resistant to all other penicillins, carbapenems, cepheems, and β -lactam/ β -lactamase inhibitor combinations, regardless of *in vitro* test results with those agents. This is because most cases of documented methicillin-resistant infections have responded poorly to β -lactam therapy, and convincing clinical data have yet to be presented that document clinical efficacy for β -lactams versus MRS.

For oxacillin-susceptible strains, results for parenteral and oral cepheems, β -lactam/ β -lactamase inhibitor combinations, and carbapenems, if tested, should be reported according to the results generated using routine interpretive criteria.

11.1.2 Oxacillin Agar Screen

The oxacillin salt-agar screening-plate procedure can be used for the detection of MRSA. Perform the test by inoculating an *S. aureus* isolate onto Mueller-Hinton agar that has been supplemented with NaCl (4% w/v; 0.68 mol/L) and that contains 6 μg oxacillin/mL. Inoculate the agar from a direct colony suspension equivalent to a 0.5 McFarland standard using either a 1- μL loop or a swab. Using a 1- μL loop, spread the inoculum in an area 10 to 15 mm in diameter. Alternatively, use a swab, expressing excess fluid as for the disk diffusion test and then spotting an area at least 10 to 15 mm in diameter or streaking an entire quadrant. Incubate the plate at temperatures no higher than 35 °C for 24 hours and examine carefully with transmitted light for evidence of small colonies (>1 colony) or a light film of growth, indicating oxacillin resistance (see Table 2C).⁸ Do not reuse plates after incubation.

11.1.3 Vancomycin Resistance and Reduced Susceptibility to Vancomycin

11.1.3.1 Vancomycin Resistance

From 2002 to 2005, a total of four *S. aureus* strains for which the vancomycin MICs ranged from 32 to 1024 $\mu\text{g}/\text{mL}$ were reported from the United States. All of these strains contained a *vanA* gene similar to that found in enterococci.^{9,10} These strains can be detected using the disk diffusion procedure when zones are examined using transmitted light following a full 24 hours of incubation at 35 ± 2 °C.

11.1.3.2 Reduced Susceptibility to Vancomycin

Strains of *S. aureus* and coagulase-negative staphylococci for which the vancomycin and teicoplanin MICs are in the intermediate range have been described.^{11,12} The first occurrence of a strain of *S. aureus* with reduced susceptibility to vancomycin (MICs 4 to 16 $\mu\text{g}/\text{mL}$) was reported from Japan in 1997,¹³ followed by reports from the United States and France.¹⁴ The exact mechanisms of resistance that result in elevated MICs are unknown, although they likely involve alterations in the cell wall and changes in

several metabolic pathways. To date, most vancomycin-intermediate *S. aureus* strains appear to have developed from MRSA.

Strains of staphylococci with reduced susceptibility to vancomycin (MICs 4 to 16 µg/mL) are not detected by disk diffusion. MIC testing must be performed. The detection of such strains is improved when the vancomycin agar screen test is used (see below); however, the performance of the vancomycin agar screen test has not been rigorously studied with strains for which the vancomycin MICs are 4 µg/mL.¹⁵

11.1.4 Vancomycin Agar Screen

The vancomycin agar screen can be used for the detection of *S. aureus* with resistance or reduced susceptibility to vancomycin. Perform the test by inoculating an isolate of *S. aureus* onto Brain Heart Infusion (BHI) agar that has been supplemented with 6 µg/mL of vancomycin. Preferably, inoculate the agar from a direct colony suspension equivalent to a 0.5 McFarland standard, using a micropipette to deliver a 10 µL drop to the agar surface. Alternatively, use a swab from which the liquid has been expressed as for the disk diffusion test. Spot an area at least 10 to 15 mm in diameter. Growth may be more difficult to detect using the swab method. Incubate the plate at 35 ± 2 °C in ambient air for a full 24 hours and examine carefully for evidence of small colonies (>1 colony) or a film of growth, suggesting reduced susceptibility to vancomycin (see Table 2C). Organisms present in quantities >1 colony should be reidentified and the MICs confirmed by a CLSI reference dilution method or equivalent. Use of a susceptible quality control strain, such as *Enterococcus faecalis* ATCC® 29212, is critical to ensure specificity. *E. faecalis* ATCC 51299® may be used as a positive control. Until further data on the prevalence or clinical significance of these isolates are known, laboratories may choose to examine MRSA strains carefully for elevated MICs to vancomycin. Currently, there are insufficient data to recommend using this agar screen test for coagulase-negative staphylococci.

11.1.5 Clindamycin Resistance

Inducible clindamycin resistance can be identified in staphylococci using the procedure described in Section 12.

11.2 Enterococci

11.2.1 Penicillin/Ampicillin Resistance

Enterococci may be resistant to penicillin and ampicillin because of production of low-affinity, penicillin-binding proteins (PBPs) or, rarely, because of the production of β-lactamase. The disk diffusion test accurately detects isolates with altered PBPs, but it will not reliably detect isolates that produce β-lactamase. The rare β-lactamase-producing strains of enterococci are detected best by using a direct, nitrocefin-based β-lactamase test (see Section 13). A positive β-lactamase test predicts resistance to penicillin, and amino-, carboxy-, and ureidopenicillins. Certain enterococci may possess high-level resistance to penicillin (MICs ≥128 µg/mL) or ampicillin (MICs ≥64 µg/mL). The disk test will not differentiate those with normal resistance from those with high-level resistance. Enterococci with lower levels of resistance to penicillin (MICs ≤64 µg/mL) or ampicillin (MICs ≤32 µg/mL) may be susceptible to synergistic killing by these penicillins in combination with gentamicin or streptomycin (in the absence of high-level resistance to gentamicin or streptomycin) if high doses of the penicillin are used, whereas strains with higher levels of penicillin (MICs ≥128 µg/mL) or ampicillin (MICs ≥64 µg/mL) resistance may not be susceptible to the synergistic effect.^{16,17} Physicians' requests to determine the actual MICs of penicillin or ampicillin for blood and CSF isolates of enterococci should be considered.

11.2.2 Vancomycin Resistance

Accurate detection of vancomycin-resistant enterococci by the disk diffusion test requires the incubation of plates for a full 24 hours (rather than 16 to 18 hours) and careful examination of any zone surrounding the vancomycin disk with transmitted light for evidence of small colonies or a light film growing within the zone. Verify an intermediate category result by the disk diffusion test by determining the vancomycin MIC, as described in CLSI document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. A vancomycin agar screen test may also be used, as described below.

11.2.3 Vancomycin Agar Screen

The vancomycin agar screening-plate procedure can be used for the detection of vancomycin-resistant enterococci. Perform the test by inoculating an enterococcal isolate onto BHI agar that has been supplemented with 6 µg vancomycin/mL.¹⁸ Inoculate the agar from a direct colony suspension equivalent to a 0.5 McFarland standard, using either a 1- or 10-µL loop or a swab.¹⁹ Using a loop, spread the inoculum in an area 10 to 15 mm in diameter. Alternatively, use a swab, expressing as for the disk diffusion test and then spotting an area at least 10 to 15 mm in diameter. Incubate the plate at 35 ± 2 °C for 24 hours and examine carefully for evidence of small colonies (>1 colony) or a film of growth, indicating vancomycin resistance (also see Table 2D).

11.2.4 High-Level Aminoglycoside Resistance

High-level resistance to gentamicin and/or streptomycin indicates that an enterococcal isolate will not be killed by the synergistic action of a penicillin or glycopeptide combined with that aminoglycoside.¹⁶ To screen for this type of resistance, use special, high-content gentamicin (120 µg) and streptomycin (300 µg) disks. No zone of inhibition indicates high-level resistance, and zones of ≥10 mm indicate a lack of high-level resistance. Strains that yield zones of 7 to 9 mm should be examined using a dilution screen test, as outlined in CLSI document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. See Table 3 for quality control of the special high-content disk tests. Other aminoglycosides need not be tested, because their activities against enterococci are not superior to those of gentamicin or streptomycin.

11.3 Extended-Spectrum, β-Lactamase-Producing, Gram-Negative Bacilli

Extended-spectrum β-lactamases (ESBLs) are enzymes that arise by mutations in genes for common plasmid-encoded β-lactamases, such as TEM-1, SHV-1, and OXA-10, or may be only distantly related to a native enzyme, as in the case of the CTX-M β-lactamases. ESBLs may confer resistance to penicillins, cephalosporins, and aztreonam in clinical isolates of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Proteus mirabilis*,²⁰ and other genera of the family Enterobacteriaceae.²¹ Some of these strains will show zones of inhibition smaller than the normal susceptible population, but still will be interpreted as susceptible by standard breakpoints for certain extended-spectrum cephalosporins or aztreonam; such strains may be screened for potential ESBL production by using the screening breakpoints listed in the table at the end of Table 2A. Other strains may test intermediate or resistant by standard breakpoints to one or more of these agents. In all strains with ESBLs, the zone diameters for one or more of the extended-spectrum cephalosporins or aztreonam should increase in the presence of clavulanic acid (see the ESBL table at the end of Table 2A). Report all ESBL-producing strains of *K. pneumoniae*, *K. oxytoca*, *E. coli*, and *P. mirabilis* as resistant to all penicillins, cephalosporins, and aztreonam. Susceptible or intermediate results for β-lactam/β-lactamase inhibitor combinations, cephamycins, oxacephems, and carbapenems should not be changed. For current recommendations regarding testing and reporting, see Table 1 and the table located at the end of Table 2A for ESBL screening and confirmatory tests.

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12 Inducible Clindamycin Resistance

Macrolide-resistant isolates of *S. aureus*, coagulase-negative *Staphylococcus* spp., and beta-hemolytic streptococci may have constitutive or inducible resistance to clindamycin (methylation of the 23S rRNA encoded by the *erm* gene, also referred to as MLS_B [macrolide, lincosamide, and type-B streptogramin] resistance) or may be resistant only to macrolides (efflux mechanism encoded by the *msrA* gene in staphylococci or a *mef* gene in streptococci).

Inducible clindamycin resistance can be detected using a disk approximation test, which may be done as part of the disk diffusion procedure.^{22,23} For current recommendations regarding procedures for disk placement, see Table 2C (for *Staphylococcus* spp.) or Table 2H (for beta-hemolytic streptococci). Following incubation, report organisms that do not show flattening of the clindamycin zone adjacent to the erythromycin disk as “clindamycin susceptible.” Organisms that show flattening of the clindamycin zone adjacent to the erythromycin disk (referred to as a “D” zone) have inducible clindamycin resistance. Report such isolates as “clindamycin resistant.” A comment that, “This isolate is presumed to be resistant based on detection of inducible clindamycin resistance. Clindamycin may still be effective in some patients.” may be included. Recommendations for quality control/quality assessment are given in Table 3.

13 β-Lactamase Tests

13.1 Purpose

A rapid β-lactamase test may yield clinically relevant results earlier than a disk diffusion test with *Haemophilus* spp., *N. gonorrhoeae*, and *Moraxella catarrhalis*; a β-lactamase test is the only reliable test for detecting β-lactamase-producing *Enterococcus* spp.

A positive β-lactamase test result predicts the following:

- resistance to penicillin, ampicillin, and amoxicillin among *Haemophilus* spp., *N. gonorrhoeae*, and *M. catarrhalis*; and
- resistance to penicillin, and amino-, carboxy-, and ureidopenicillins among staphylococci and enterococci.

A negative β-lactamase test result does not rule out β-lactam resistance due to other mechanisms. Do not use β-lactamase tests for members of the Enterobacteriaceae, *Pseudomonas* spp., and other aerobic, gram-negative bacilli, because the results may not be predictive of susceptibility to the β-lactams most often used for therapy.

13.2 Selecting a β-Lactamase Test

Nitrocefin-based tests are the preferred method for testing *Haemophilus* spp., *N. gonorrhoeae*, *M. catarrhalis*, staphylococci, and enterococci.²⁴ Acidimetric β-lactamase tests have generally produced acceptable results with *Haemophilus* spp., *N. gonorrhoeae*, and staphylococci. Iodometric tests may be used for testing *N. gonorrhoeae*, but only nitrocefin-based tests should be used to test *M. catarrhalis*.²⁵ Accurate detection of β-lactamase in staphylococci may require induction of the enzyme and incubation of a nitrocefin-based test for up to one hour. Induction can be easily accomplished by testing the growth from the zone margin surrounding an oxacillin or cefoxitin disk test. Care must be exercised when using these assays to ensure accurate results, including testing of known positive and negative control strains at the time clinical isolates are examined.

14 Interpretation of Disk Diffusion Test Results

14.1 Zone Diameter Interpretive Standards

Zone diameter interpretive criteria to categorize the levels of susceptibility of organisms to various antimicrobial agents are given in Tables 2A through 2J. For most agents, these criteria are developed by first comparing zone diameters to MICs of a large number of isolates, including those with known mechanisms of resistance relevant to the particular class of drug. Second, the MICs and correlated zone sizes are analyzed in relation to the pharmacokinetics of the drug from normal dosing regimens. Finally, when feasible, the tentative *in vitro* interpretive criteria are analyzed in relation to studies of clinical efficacy and microbiologic eradication efficacy in the treatment of specific pathogens, as outlined in CLSI/NCCLS document M23—*Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters*.

14.2 Interpretive Categories

14.2.1 Susceptible

The “susceptible” category implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent tested when the recommended dosage is used for the site of infection.

14.2.2 Intermediate

The “intermediate” category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g., quinolones and β -lactams in urine) or when a higher than normal dosage of a drug can be used (e.g., β -lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

14.2.3 Resistant

The “resistant” category implies that isolates are not inhibited by the usually achievable systemic concentrations of the agent when normal dosage schedules are used and/or they may have zone diameters that fall within the range where specific microbial resistance mechanisms are likely (e.g., β -lactamases) and clinical efficacy has not been reliable in treatment studies.

14.3 Equivalent MIC Breakpoints

Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests. Tables 2A through 2J list the MIC breakpoint correlates corresponding to the zone diameter interpretive criteria. These MIC correlates are based on zone diameter versus MIC comparisons and are generally identical to the MIC interpretive criteria defined in CLSI document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. A few minor discrepancies may exist, due to technical differences in the original databases.

15 Quality Control Procedures

15.1 Purpose

The goals of a quality control program are to monitor the following:

- the precision (repeatability) and accuracy of susceptibility test procedures;
- the performance of reagents used in the tests; and
- the performance of persons who carry out the tests and read the results.

These goals are achieved by, but not limited to, the testing of quality control strains with known susceptibility to the antimicrobial agents to be tested.

15.2 Reference Strains for Quality Control

To control the precision (repeatability) and accuracy of the disk diffusion test procedures, obtain the following recommended quality control strains from a reliable source:

- *Enterococcus faecalis* ATCC[®] 29212;
- *Escherichia coli* ATCC[®] 25922;
- *Escherichia coli* ATCC[®] 35218;
- *Haemophilus influenzae* ATCC[®] 49247;
- *Haemophilus influenzae* ATCC[®] 49766;
- *Klebsiella pneumoniae* ATCC[®] 700603;
- *Neisseria gonorrhoeae* ATCC[®] 49226;
- *Pseudomonas aeruginosa* ATCC[®] 27853;
- *Staphylococcus aureus* ATCC[®] 25923; and
- *Streptococcus pneumoniae* ATCC[®] 49619.

Enterococcus faecalis ATCC[®] 29212 (or alternatively *Enterococcus faecalis* ATCC[®] 33186) is used to monitor Mueller-Hinton agar for unacceptable levels of thymidine when trimethoprim or sulfonamides are tested.

Enterococcus faecalis ATCC[®] 29212 is also used for control of high-content aminoglycoside disks (see Table 3).

Escherichia coli ATCC[®] 35218 is recommended only as a control organism for β -lactamase inhibitor combinations, such as those containing clavulanic acid, sulbactam, or tazobactam.

The *E. coli* ATCC[®] 35218 control strain contains a plasmid-encoded β -lactamase (non-ESBL); therefore, the organism is resistant to many penicillinase-labile drugs, but susceptible to β -lactam/ β -lactamase inhibitor combinations. The plasmid must be present in the control strain for the quality control test to be valid; however, the plasmid may be lost during storage at refrigerator or freezer temperatures. To ensure that the plasmid is present, the strain must be tested with a β -lactam agent alone (ampicillin, amoxicillin, piperacillin, or ticarcillin) in addition to a β -lactam/ β -lactamase inhibitor agent (e.g., amoxicillin-clavulanate). If the strain loses the plasmid, it will be susceptible to the β -lactam agent when tested alone,

indicating that the quality control test is invalid and a new culture of *E. coli* ATCC® 35218 must be used. Proper maintenance is important for this strain (see Section 15.3).

Haemophilus influenzae ATCC® 49247 is an ampicillin-resistant, β -lactamase-negative organism (BLNAR).

Haemophilus influenzae ATCC® 49766 is an ampicillin-susceptible organism that is more reproducible for controlling selected β -lactams than is *H. influenzae* ATCC® 49247.

Klebsiella pneumoniae ATCC® 700603 is used only as a control for ESBL tests (see Table 2A). Proper maintenance is important for this strain (see Section 15.3).

Staphylococcus aureus ATCC® 29213 and *Staphylococcus aureus* ATCC® 43300 are used as control organisms for oxacillin salt agar screening tests (see Table 2C).

15.3 Storing and Testing Quality Control Strains

- Test the quality control strains by the standard disk diffusion test procedure described herein, using the same materials and methods that are used to test clinical isolates.
- For prolonged storage, maintain stock cultures at -20 °C or below (preferably at -60 °C or below or in liquid nitrogen) in a suitable stabilizer (e.g., 50% fetal calf serum in broth, 10 to 15% glycerol in tryptic soy broth, defibrinated sheep blood, or skim milk) or in a freeze-dried state without significant risk of altering their antimicrobial susceptibility.
- Store working control cultures on tryptic soy agar (nonfastidious strains) or on enriched chocolate agar slants (fastidious strains) at 2 to 8 °C, and subculture each week for no more than three successive weeks. Prepare new working cultures at least monthly from frozen, freeze-dried, or commercial cultures.
- Before testing, subculture the strains onto agar plates to obtain isolated colonies. Subculture frozen or freeze-dried cultures twice prior to testing.
- Grow organisms and prepare direct colony or log phase suspensions for testing according to the recommended inoculum preparation procedures.
- A quality control culture can be used to monitor precision (repeatability) and accuracy of the disk test as long as no significant change in the mean zone diameter not attributable to faulty methodology occurs. If an unexplained result suggests a change in the organism's inherent susceptibility, a fresh culture of the control strain should be obtained.
- Careful attention to organism maintenance (e.g., minimal subcultures) and storage (e.g., -60 °C or below) is especially important for quality control strains *E. coli* ATCC® 35218 and *K. pneumoniae* ATCC® 700603, because spontaneous loss of the plasmid encoding the β -lactamase has been documented. Plasmid loss leads to quality control results outside the acceptable limit, such as increased zone diameters for *E. coli* ATCC® 35218 with enzyme-labile penicillins (e.g., ampicillin, piperacillin, ticarcillin) and increased zone diameters for *K. pneumoniae* ATCC® 700603 with cephalosporins and aztreonam.

15.4 Zone Diameter Quality Control Limits

Acceptable zone diameter quality control limits for a single quality control test (single-drug/single-organism combination) are listed in Tables 3 and 3A. Monitor the overall performance of the test system using these ranges by testing the appropriate control strains each day the test is performed or, if satisfactory performance is documented (see Section 15.5.2.1), test the control strains weekly (see below).

15.5 Frequency of Quality Control Testing (Also refer to Appendix A and Table 3B)

The weekly quality control testing option outlined below is applicable to routine testing only. Quality control testing should be performed each test day for disk diffusion tests performed less than once a week.

15.5.1 Daily Testing

Performance is satisfactory for daily QC testing when no more than 3 out of 30 consecutive results for each antimicrobial agent/organism combination are outside the acceptable limit stated in Tables 3 and 3A. Corrective action by the laboratory is required when this frequency is exceeded.

15.5.2 Weekly Testing

15.5.2.1 Demonstrating Satisfactory Performance for Conversion From Daily to Weekly Quality Control Testing

- Test all applicable control strains for 20 or 30 consecutive test days and document results.
- To convert from daily to weekly quality control testing, no more than 1 out of 20 or 3 out of 30 zone diameters for each antimicrobial agent/organism combination may be outside the acceptable zone diameter limits stated in Tables 3 and 3A.

15.5.2.2 Implementing Weekly Quality Control Testing

- Weekly quality control testing may be performed once satisfactory performance has been documented (see Section 15.5.2.1).
- Perform quality control testing once per week and whenever any reagent component of the test (e.g., a new lot of agar or a new lot of disks from the same or a different manufacturer) is changed.
- If any of the weekly quality control results are out of the acceptable range, corrective action is required (see Section 15.6).
- If a new antimicrobial agent is added or agar from a different manufacturer is used, it must be tested for 20 or 30 consecutive days and satisfactory performance documented before it can be tested on a weekly schedule. In addition, 20 or 30 days of testing is required if a major change in the method of reading test results is implemented, such as conversion from manual zone measurements to an automated zone reader.

15.6 Corrective Action

15.6.1 Out-of-Control Result Due to an Obvious Error

If out-of-control results occur for obvious reasons, document the reason and retest the strain on the day that the error is observed. If the repeated result is within range, no further corrective action is required. Obvious reasons for out-of-control results include:

- use of the wrong disk;
- use of the wrong control strain;
- obvious contamination of the strain; or
- inadvertent use of the wrong incubation temperature or conditions.

15.6.2 Out-of-Control Result Not Due to an Obvious Error

15.6.2.1 Immediate Corrective Action

If no obvious reason for the out-of-control result is apparent, immediate corrective action is required.

- Test the out-of-control antimicrobial agent/organism combination on the day the error is observed and monitor for a total of five consecutive test days. Document all results.
- If all five zone diameter measurements for the antimicrobial agent/organism combination are within the acceptable ranges, as defined in Tables 3 and 3A, no additional corrective action is necessary.
- If any of the five zone diameter measurements are outside the acceptable range, additional corrective action is required (see Section 15.6.2.2).
- Daily control tests must be continued until final resolution of the problem is achieved.

15.6.2.2 Additional Corrective Action

When immediate corrective action does not resolve the problem, the problem is likely due to a system rather than a random error. The following common sources of error should be investigated to verify that:

- zone diameters were measured and transcribed correctly;
- the turbidity standard has not expired, is stored properly, meets performance requirements (see Section 8.1), and was adequately mixed prior to use;
- all materials used are within their expiration dates and stored at the proper temperature;
- the incubator was at proper temperature and atmosphere;
- other equipment used (e.g., pipettors) was functioning properly;
- disks were stored desiccated and at proper temperature;
- the control strain has not changed and was not contaminated;

- inoculum suspensions were prepared and adjusted correctly; and
- inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

If necessary, obtain a new quality control strain (either from freezer storage or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. If the problem appears to be related to a manufacturer, contact and provide the manufacturer with the test results. It is also helpful to exchange quality control strains and materials with another laboratory using the same method. Until the problem is resolved, it may be necessary to use an alternate test method.

Once the problem is corrected, documentation of satisfactory performance for another 20 or 30 consecutive days is required in order to return to weekly quality control testing (see Section 15.5.2.1).

15.7 Reporting Patient Results When Out-of-Control Tests Occur

Whenever an out-of-control result occurs or corrective action is necessary, careful assessment of whether to report patient test results should be made on an individual patient basis, taking into account if the source of the error, when known, is likely to have affected relevant patient test results. Options to consider include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternate test method or a reference laboratory until the problem is resolved.

15.8 Verification of Patient Test Results

Multiple test parameters are monitored by following the quality control recommendations described in this standard. However, acceptable results derived from testing quality control strains do not guarantee accurate results when testing patient isolates. It is important to review all of the results obtained from all drugs tested on a patient's isolate prior to reporting the results. This should include but not be limited to ensuring that:

- the antimicrobial susceptibility results are consistent with the identification of the isolate;
- the results from individual agents within a specific drug class follow the established hierarchy of activity rules (e.g., third-generation cephalosporins are more active than first- or second-generation cephalosporins against Enterobacteriaceae); and
- the isolate is susceptible to those agents for which resistance has not been documented (e.g., vancomycin and *Streptococcus* spp.) and for which only "susceptible" interpretive criteria exist in M100.

Unusual or inconsistent results should be verified by checking for the following:

- accurate zone measurements;
- transcription errors;
- contamination of the test;
- use of a defective agar plate (e.g., too thick or too thin);
- improper disk placement (e.g., inadequate contact with the agar); and

- previous results on the patient (e.g., Did the patient have the same isolate with an unusual antibiogram previously?).

If a reason for the unusual or inconsistent result cannot be ascertained, a repeat of the susceptibility test or the identification or both is in order. Sometimes it is helpful to use an alternative test method for the repeat test. A suggested list of results that may require verification is included in Table 4 of M100. Each laboratory must develop its own policy for verification of unusual or inconsistent antimicrobial susceptibility test results. This policy should emphasize those results that may significantly impact patient care.

16 Limitations of Disk Diffusion Methods

16.1 Application to Various Organism Groups

The disk diffusion methods described in this document are standardized for testing rapidly growing pathogens, which include *Staphylococcus* spp., *Enterococcus* spp., the Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter* spp., *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, and *Vibrio cholerae*, and they have been modified for testing fastidious organisms such as *Haemophilus* spp., *N. gonorrhoeae*, *N. meningitidis*, and streptococci. For organisms excluded from Tables 2A through 2J (e.g., *Campylobacter*, *Corynebacterium* spp., *Bacillus* spp.) and organisms not covered in other CLSI guidelines or standards such as CLSI document M45—*Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria*, studies are not yet adequate to develop reproducible, definitive standards to interpret results. These organisms may require different media, require different atmospheres of incubation, or show marked strain-to-strain variation in growth rate. For these microorganisms, consultation with an infectious disease specialist is recommended for guidance in determining the need for susceptibility testing and in the interpretation of results. Published reports in the medical literature and current consensus recommendations for therapy of uncommon microorganisms may obviate the need for testing of such organisms. If testing is necessary, a dilution method usually will be the most appropriate testing method, and this may require submitting the organism to a reference laboratory.

16.2 Misleading Results

Dangerously misleading results can occur when certain antimicrobial agents are tested and reported as susceptible against specific organisms. These combinations include, but may not be limited to, the following:

- first- and second-generation cephalosporins and aminoglycosides against *Salmonella* and *Shigella* spp.;
- all β -lactam antimicrobial agents (except oxacillin, methicillin, and nafcillin) against methicillin-resistant staphylococci;
- cephalosporins, aminoglycosides (except testing for high-level resistance), clindamycin, and trimethoprim-sulfamethoxazole against enterococci;
- cephalosporins against *Listeria* spp.; and
- cephalosporins and aztreonam against ESBL-producing *K. pneumoniae*, *K. oxytoca*, *E. coli*, and *P. mirabilis*.

16.3 Emergence of Resistance

Some antimicrobial agents are associated with the emergence of resistance during prolonged therapy. Therefore, isolates that are initially susceptible may become resistant after initiation of therapy. This occurs within three to four days, most frequently in *Enterobacter*, *Citrobacter*, and *Serratia* spp. with third-generation cephalosporins; in *P. aeruginosa* with all antimicrobial agents; and in staphylococci with quinolones and with vancomycin (VISAs).

In certain circumstances, repeat testing to detect emerging resistance might be warranted earlier than within three to four days, and the decision to do so requires knowledge of the specific situation and the severity of the patient's condition. Laboratory guidelines on when to perform repeat susceptibility testing should be determined after consultation with the medical staff.

17 Screening Tests

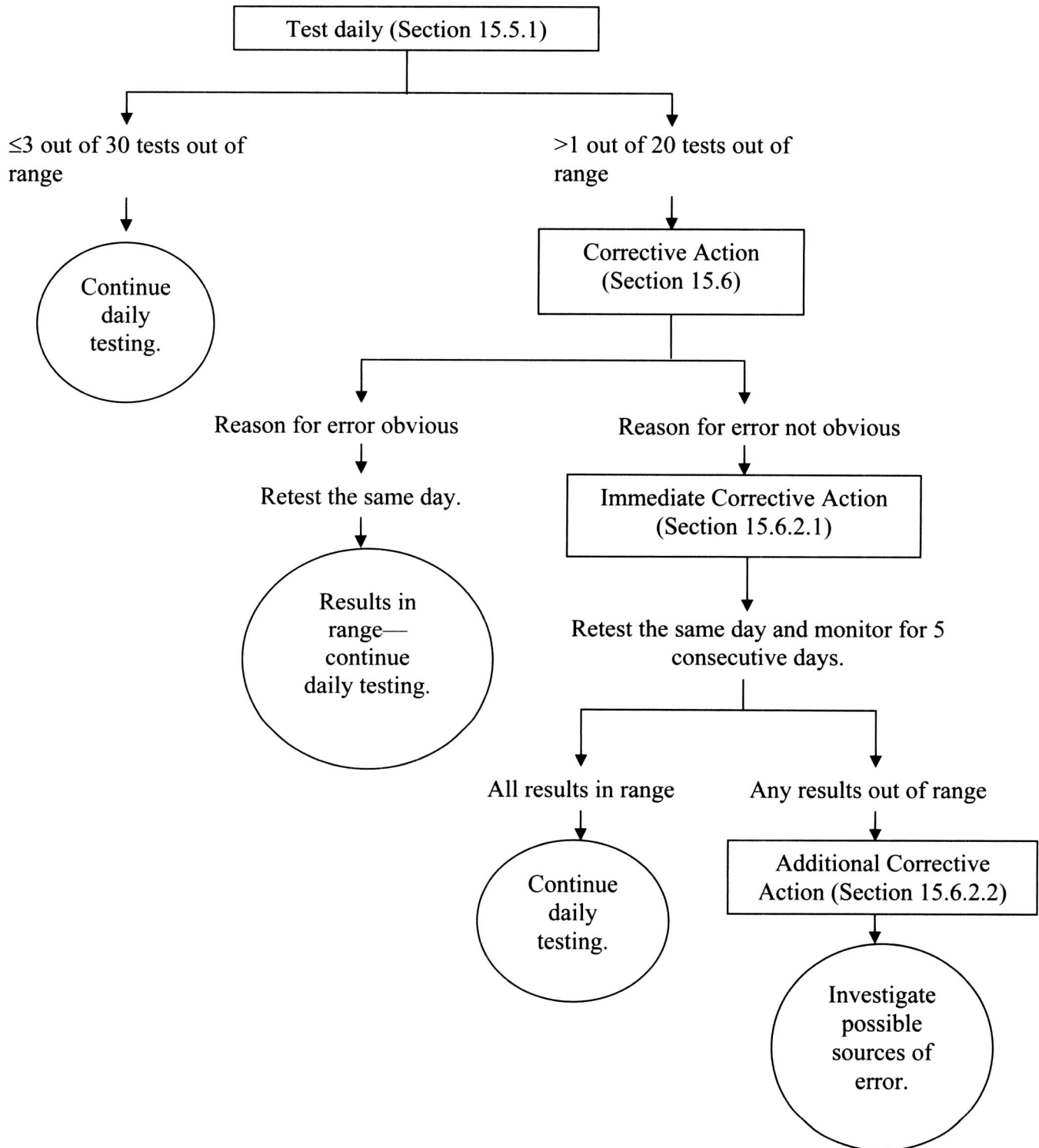
Screening tests for oxacillin-resistant *S. aureus* and high-level aminoglycoside-resistant enterococci have been shown to be comparable in reliability to standard methods for detecting clinically significant resistance; additional confirmatory tests are unnecessary. Limitations of other screening tests [e.g., for vancomycin resistance in enterococci (Table 2D) and *S. aureus* (Table 2C) and ESBLs in certain Enterobacteriaceae (Table 2A)] and the necessity for further confirmation are given in the individual tables.

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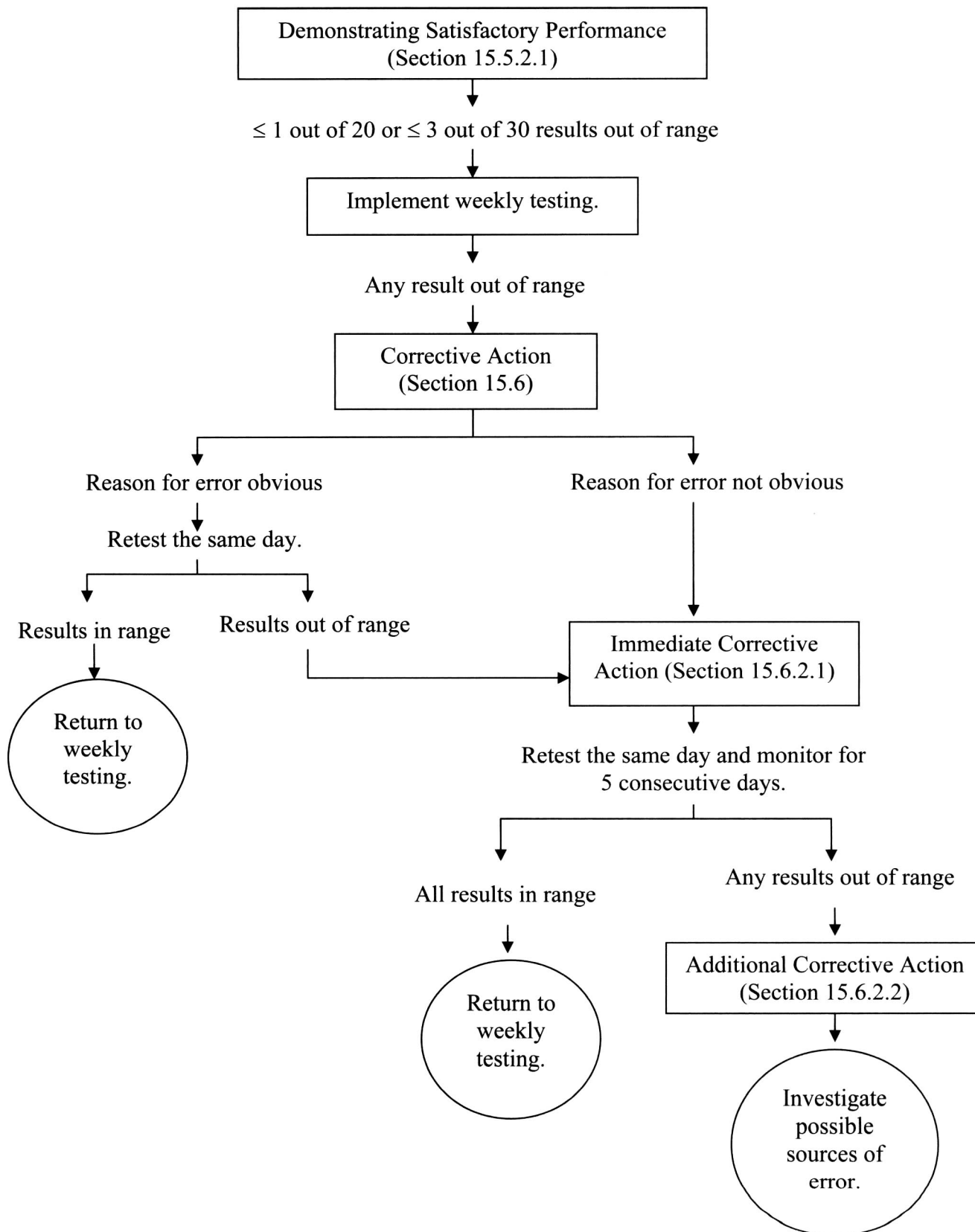
Appendix A. Quality Control Protocol Flow Charts

Disk Diffusion Daily Quality Control Testing Protocol



Appendix A. (Continued)

Disk Diffusion Weekly Quality Control Testing Protocol



Clinical and Laboratory Standards Institute consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact CLSI or visit our website at www.clsi.org.

Summary of Comments and Subcommittee Responses

M2-A8: *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Eighth Edition*

General

1. Could you please clarify a point for me about cefuroxime testing? Your standard gives different zone size criteria for the parenteral and oral forms of the drug and specifies that different discs should be used. Is the implication that you could get an isolate testing sensitive with the one disk and resistant with the other? If this is true, could you use the parenteral (cefuroxime sodium) disk to predict sensitivity for both forms, accepting that you may miscall some strains resistant that would respond to oral, or is it the other way around? The standard gives no details about these particular recommendations.
- **Although there are two formulations for cefuroxime, one for parenteral and one for oral administration, there is only one disk for laboratory testing. Different interpretive criteria were developed based on the different pharmacodynamic/pharmacokinetic data and clinical indications for the two formulations. Tables 2A through 2J should be used to guide interpretation for individual organisms.**
2. Regarding the intention of the “Warning” on page 23 of M100-S14 regarding not routinely reporting clindamycin, macrolides, etc. for bacteria isolated from the “CSF”: Should this be interpreted in its narrowest sense or does CLSI consider all central nervous system (CNS) sites similarly to CSF? Specifically, would it be incorrect to report clindamycin, etc. for a bacterial brain abscess aspirate? Infectious disease textbooks consider clindamycin as an acceptable alternative to treat brain abscesses.
- **The WARNING box in M100 refers only to bacterial meningitis. Clindamycin is mentioned among alternative therapies useful for brain abscess (*Principles and Practice of Infectious Diseases*, Mandell GL, Bennett JE, Dolin R, eds, 5th edition, Churchill Livingstone, Inc., Philadelphia, 2000), so it would not be incorrect to report clindamycin susceptibility test results on an aspirate from a brain abscess. There are insufficient data to provide comprehensive guidance on which test results to report routinely for pathogens isolated from brain abscesses.**

Table 2A

3. Could you clarify the comment in M100 regarding the “Warning” for *Salmonella* and *Shigella* in Table 2A, comment (4) on page 35? The comment states that first- and second-generation cephalosporins should not be reported as susceptible. Does that comment include the cephamycins also?
- **Yes, the statement has been clarified.**

Table 2C

4. There appears to be a discrepancy between the disk diffusion and MIC sections of the current M100 document as related to oxacillin:

For disk diffusion testing of non-*S. epidermidis* coagulase negative staphylococci, disk diffusion “R” isolates that are *mecA*/PBP2a negative should be reported as “S” (pg. 42).

However, for MIC testing of these same non-*S. epidermidis* coagulase negative staphylococci, isolates that are *mecA*/PBP 2a negative should be reported as “S” if oxacillin MICs are between 0.5 to 2 mcg/ml, but as “R” if MICs are > 4 mcg/ml (pg. 105).

If moderately to highly oxacillin-resistant *mecA*/PBP2a negative isolates should be reported as “R” when performing the MIC test, shouldn’t this also be the case when doing disk diffusion? Put another way, shouldn’t the disk diffusion criterion also have a zone size below which a report of “R” be made, regardless of *mecA*/PBP 2a findings?

- **The reason for reporting strains exhibiting MICs ≥ 4 $\mu\text{g/mL}$ as oxacillin resistant despite *mecA*/PBP2a status is based on pharmacokinetic/pharmacodynamic data and the possibility that other resistance mechanisms not yet discovered may be responsible for the increased MICs. Unfortunately, there is no zone diameter or range of zone diameters that correlates exactly with MICs ≥ 4 $\mu\text{g/mL}$. The data from the CLSI study used to establish the revised interpretive criteria (Tenover, et al. *J Clin Microbiol* 37: 4051-4058) show that 108 of 110 (98.2%) isolates with zone diameters of 6 mm (i.e., no zone) were *mecA* positive. Of 42 strains with zone diameters in the 7 to 17 mm range, 15 (35.7%) were *mecA* positive and 26 (64.3%) were *mecA* negative. Therefore, based on these data, it would be possible to do the following: if there is no zone to oxacillin, report as resistant; if there is any zone ≥ 7 mm, then perform a *mecA* test or a cefoxitin disk test for a definitive answer. However, recent studies show that using the cefoxitin disk in place of the oxacillin disk gives better correlation with *mecA* status for coagulase-negative staphylococci, and the cefoxitin zone is much easier to read.**

Table 2E

5. I am aware that the antibiotic tested is the one to be reported; however, I need clarification on how to address the reporting of doxycycline with the fastidious organisms when the tetracycline interpretation is resistant or intermediate. In Tables 2A, 2B, 2C, and 2D of M100-S15, all tetracycline comments end by stating, “...However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline...” But in Tables 2E (*Haemophilus*) and 2G (*S. pneumo*), this statement is not part of the comment. Am I to assume this statement does not hold true for the fastidious organisms, or can I deduce that it does? Literature leads me to believe that it does. Should tetracycline R or I be equated with doxycycline resistance? Will CLSI be developing zone sizes for doxycycline in the future?

Some physicians are hesitant to use doxycycline because of presumed inactivity. Clinicians assume isolates reported resistant to tetracycline are also doxycycline resistant. Our pharmacologist notes this assumption does not agree with the literature and that extrapolating our tetracycline susceptibility data to doxycycline has no direct application. It doesn’t relate to therapy or help in clinical studies. Citing literature, she says doxycycline is consistently active against all common typical and atypical bacterial causes of pneumonia. She is concerned this assumption of tetracycline resistance (which is common for *S. pneumo*) implying doxycycline resistance as well is sending those not familiar with infectious disease in the wrong direction (i.e., towards the use of more expensive alternatives with more harmful side effects).

- **Tetracycline susceptible isolates of various species are susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline or minocycline. Currently, there are no interpretive criteria for doxycycline and minocycline against pneumococci and *H. influenzae*. Until additional studies are undertaken or reviewed by CLSI, it is premature to use tetracycline to predict doxycycline or minocycline resistance.**

Table 2G

6. Reading the CLSI documents of 2005, I wonder why the D-test that looks for inducible clindamycin resistance is not described for *Streptococcus pneumoniae*. Can you give me the reason behind this?
- **Isolates of *Streptococcus pneumoniae* can have *erm*-mediated resistance to erythromycin. However, the vast majority of these isolates are also resistant to clindamycin (i.e., the constitutive resistance phenotype). Rare isolates of pneumococci may have inducible resistance; however the clinical significance of this has not been established. Therefore, routine testing for inducible clindamycin resistance is not recommended for this species.**

The Quality System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*. The quality management system approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any healthcare service’s path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The quality system essentials (QSEs) are:

Documents & Records Organization Personnel	Equipment Purchasing & Inventory Process Control	Information Management Occurrence Management Assessment	Process Improvement Service & Satisfaction Facilities & Safety
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M2-A9 addresses the quality system essentials (QSEs) indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
GP2					X M6 M7 M11 M23 M29						M29

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

M2-A9 addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Preexamination				Examination			Postexamination	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
				X M7 M11	X M7 M11	X M7 M11	X M7 M11	

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

Related CLSI/NCCLS Publications*

- GP2-A4** **Clinical Laboratory Technical Procedure Manuals; Approved Guideline—Fourth Edition (2002).** This guideline addresses the design, preparation, maintenance, and use of technical procedure manuals (whether they are in paper or electronic formats) for use by the patient-testing community.
- M6-A2** **Protocols for Evaluating Dehydrated Mueller-Hinton Agar; Approved Standard—Second Edition (2005).** This document describes procedures for evaluating production lots of Mueller-Hinton agar, and for the development and application of reference media.
- M7-A7** **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition (2006).** M7-A7 discusses reference methods for the determination of minimal inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.
- M11-A6** **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth Edition (2004).** This standard provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by broth macrodilution, broth dilution, and agar dilution.
- M23-A2** **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Second Edition (2001).** This document addresses the required and recommended data needed for the selection of appropriate interpretive standards and quality control guidelines for antimicrobial agents.
- M29-A3** **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on U.S. regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.
- M39-A2** **Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data; Approved Guideline—Second Edition (2005).** This document describes methods for the recording and analysis of antimicrobial susceptibility test data, consisting of cumulative and ongoing summaries of susceptibility patterns of epidemiologically significant microorganisms.
- M45-P** **Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Proposed Guideline (2005).** This document provides guidance to clinical microbiology laboratories for standardized susceptibility testing of infrequently isolated or fastidious bacteria that are not presently included in CLSI documents M2, M7, or M11. The tabular information in this document presents the most current information for drug selection, interpretation, and quality control for the infrequently isolated or fastidious bacterial pathogens included in this guideline.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most recent editions.

NOTES

NOTES

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