CLINICAL AND LABORATORY STANDARDS INSTITUTE®

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# M02-A12

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

This standard 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.

A standard for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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

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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 M02-A12—*Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Twelfth 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[1] tables) presented with this standard represents the most current information for drug selection, interpretation, and QC using the procedures standardized in M02. These tables, as in previous years, have been updated and should replace tables published in earlier years. Changes in the tables since the previous edition (M100-S24) appear in boldface type and are also summarized in the front of the document.

Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Twelfth Edition.* CLSI document M02-A12 (ISBN 1-56238-985-8 [Print]; ISBN 1-56238-986-6 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2015.



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## Foreword

In this revision of M02, several sections were added or revised as outlined below in the Summary of Changes. One of the main updates is the reformatting of the document to follow a laboratory's path of workflow—defined as the sequential processes of preexamination, examination, and postexamination. An overview of the disk diffusion susceptibility testing process is provided in the beginning of the document in the new Figure 1 (see Chapter 3) with various testing methods shown in easy-to-follow step-action tables throughout the document.

The most current edition of CLSI document M100,[1] published as an annual volume of tables, 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 presented in the annual tables.

Many other editorial and procedural changes in this edition of M02 resulted from meetings of the Subcommittee on Antimicrobial Susceptibility Testing since 2012. Specific changes to the M100[1] tables are summarized at the beginning of CLSI document M100.[1] The most important changes in M02 are summarized below.

### Summary of Changes

**Formatting Changes Throughout the Document:**

- Main sections are now referred to as "Chapters." Sections within the chapters are referred to as "Subchapters."

- Easy-to-follow step-action tables are introduced, consistent with CLSI's goal to make standards and guidelines more user friendly. Most of these tables strictly reflect reformatting of text that previously appeared in M02. Any changes to the testing recommendations are highlighted here in the Summary of Changes. The new step-action tables within the document include:
  - Subchapter 3.3.2, Storage of Antimicrobial Disks
  - Subchapter 3.4.2, Direct Colony Suspension Method for Inoculum Preparation
  - Subchapter 3.4.3, Growth Method for Inoculum Preparation
  - Subchapter 3.5, Inoculation of Test Plates
  - Subchapter 3.6, Application of Disks to Inoculated Agar Plates
  - Subchapter 3.9.1.7.2, Vancomycin Agar Screen (*Staphylococcus aureus*)
  - Subchapter 3.9.2.3, Vancomycin Agar Screen (*Enterococcus* spp.)

**Subchapter 1.4.1, Definitions**
Added definitions for susceptible-dose dependent, test method, and test system.

Expanded the definition of quality control.

**Subchapter 2.3, Suggested Guidelines for Routine and Selective Testing and Reporting**
Provided additional information on the location of Test and Report Group designations in M100.[1]

Noted cefazolin is a surrogate agent in Test and Report Group U and is not reported exclusively on urine isolates.

**Chapter 3, Susceptibility Testing Process**
Added a flow chart that provides an overview of the disk diffusion susceptibility testing process.

**Subchapter 3.6, Application of Disks to Inoculated Agar Plates**
Modified recommendation from "5" to "6 or fewer" as the number of disks that can be placed on a 100-mm plate.

**Subchapter 3.7, Special Considerations for Fastidious Organisms**
Added table that summarizes special testing requirements (eg, media, incubation time, and temperature) for fastidious organisms.

**Subchapter 3.8, Reading Plates and Interpreting Results**
Clarified time of incubation for testing of cefoxitin against *Staphylococcus* spp.: 24 hours for coagulase-negative *Staphylococcus* spp.; 16 to 18 hours for *S. aureus*.

Noted that the penicillin zone edge test can be useful for determining β-lactamase production in *S. aureus* strains with penicillin zones ≥29 mm.

Added susceptible-dose dependent to the list of disk diffusion and minimal inhibitory concentration (MIC) interpretive categories.

**Subchapter 3.9.1.2, Methicillin/Oxacillin Resistance**
Expanded explanation of mechanisms and generic determinants of oxacillin resistance in staphylococci, which includes *mecC* in *S. aureus*.

**Subchapter 3.9.1.4, Methods for Detection of Oxacillin Resistance**
Expanded the discussion of oxacillin resistance and added a table that summarizes the tests available to detect oxacillin resistance in staphylococci.

**Subchapter 3.9.1.6, Reporting**
Clarified several reporting recommendations to include: application of oxacillin results to other penicillinase-stable penicillins and reporting results for *mecA*- and/or penicillin-binding protein 2a–negative *S. aureus* with oxacillin MICs ≥4 µg/mL.

**Subchapter 3.9.1.7.4, Reporting**
Further emphasized the need to confirm and communicate results to appropriate authorities when *S. aureus* and coagulase-negative staphylococci with vancomycin MICs of ≥ 8 µg/mL and ≥ 32 µg/mL, respectively, are encountered.

**Subchapter 3.9.1.10, Mupirocin Resistance**
Noted that use of mupirocin is known to increase rates of high-level mupirocin resistance in *S. aureus*.

**Subchapter 3.9.2.4, High-Level Aminoglycoside Resistance**
Noted that high-level resistance to both gentamicin and streptomycin implies resistance to all aminoglycosides.

**Subchapter 3.9.3.1, Extended-Spectrum β-Lactamases**
Updated discussion of extended-spectrum β-lactamases.

**Subchapter 3.9.3.3, Carbapenemases (Carbapenem-Resistant Gram-Negative Bacilli)**
Added reference to the Carba NP colorimetric microtube assay to detect carbapenemase activity.

**Subchapter 3.10.1, Inducible Clindamycin Resistance**
Noted that infections due to streptococci with inducible clindamycin resistance may fail to respond to clindamycin therapy.

**Subchapter 4.3, Selection of Strains for Quality Control**
Expanded description of routine and supplemental QC strains.

**Subchapter 4.4, Maintenance and Testing of Quality Control Strains**
Introduced terms "F1," "F2," and "F3" to relate to "frozen" or "freeze-dried" subcultures of QC strains and provided enhanced recommendations for handling QC strains.

**Subchapter 4.7.2, Performance Criteria for Reducing Quality Control Frequency to Weekly**
Introduced for the first time in M02 the 15-replicate (3 × 5 day) QC plan as an alternative to the 20- or 30-day QC plan.

**Appendix A, Quality Control Protocol Flow Charts**
Revised and expanded flow charts to better convey the QC testing process and added flow charts that depict the new 15-replicate (3 × 5 day) QC option to convert from daily to weekly QC testing.

**Appendix E, Quality Control Strain Maintenance**
Revised schematic that depicts stages of subculture and testing of QC strains that originate from "frozen" or "freeze-dried" stock cultures.

## Summary of CLSI Processes for Establishing Interpretive Criteria and Quality Control Ranges

The Clinical and Laboratory Standards Institute (CLSI) is an international, voluntary, not-for-profit, interdisciplinary, standards-developing, and educational organization accredited by the American National Standards Institute that develops and promotes the use of consensus-developed standards and guidelines within the health care community. These consensus standards and guidelines are developed to address critical areas of diagnostic testing and patient health care, and are developed in an open and consensus-seeking forum. CLSI is open to anyone or any organization that has an interest in diagnostic testing and patient care. Information about CLSI is found at www.clsi.org.

The CLSI Subcommittee on Antimicrobial Susceptibility Testing reviews data from a variety of sources and studies (eg, *in vitro,* pharmacokinetics/pharmacodynamics, and clinical studies) to establish antimicrobial susceptibility test methods, interpretive criteria, and QC parameters. The details of the data required to establish interpretive criteria, QC parameters, and how the data are presented for evaluation are described in CLSI document M23.[2]

Over time, a microorganism's susceptibility to an antimicrobial agent may decrease, resulting in a lack of clinical efficacy and/or safety. In addition, microbiological methods and QC parameters may be refined to ensure more accurate and better performance of susceptibility test methods. Because of this, CLSI continually monitors and updates information in its documents. Although CLSI standards and guidelines are developed using the most current information and thinking available at the time, the field of science and medicine is ever changing; therefore, standards and guidelines should be used in conjunction with clinical judgment, current knowledge, and clinically relevant laboratory test results to guide patient treatment.

Additional information, updates, and changes in this document are found in the meeting summary minutes of the Subcommittee on Antimicrobial Susceptibility Testing at www.clsi.org.

## CLSI Reference Methods vs Commercial Methods and CLSI vs US Food and Drug Administration Interpretive Criteria (Breakpoints)

It is important for users of M02-A12, M07-A10,[3] and the M100 Informational Supplement to recognize that the standard methods described in CLSI documents are reference methods. These methods may be used for routine antimicrobial susceptibility testing of clinical isolates, for evaluation of commercial devices that will be used in clinical laboratories, or by drug or device manufacturers for testing of new agents or systems. Results generated by reference methods, such as those contained in CLSI documents, may be used by regulatory authorities to evaluate the performance of commercial susceptibility testing devices as part of the approval process. Clearance by a regulatory authority indicates that the commercial susceptibility testing device provides susceptibility results that are substantially equivalent to results generated using reference methods for the organisms and antimicrobial agents described in the device manufacturer's approved package insert.

CLSI breakpoints may differ from those approved by various regulatory authorities for many reasons, including the following: different databases, differences in interpretation of data, differences in doses used in different parts of the world, and public health policies. Differences also exist because CLSI proactively evaluates the need for changing breakpoints. The reasons why breakpoints may change and the manner in which CLSI evaluates data and determines breakpoints are outlined in CLSI document M23.[2]

Following a decision by CLSI to change an existing breakpoint, regulatory authorities may also review data in order to determine how changing breakpoints may affect the safety and effectiveness of the antimicrobial agent for the approved indications. If the regulatory authority changes breakpoints, commercial device manufacturers may have to conduct a clinical laboratory trial, submit the data to the regulatory authority, and await review and approval. For these reasons, a delay of one or more years may be required if an interpretive breakpoint change is to be implemented by a device manufacturer. In the United States, it is acceptable for laboratories that use US Food and Drug Administration (FDA)–cleared susceptibility testing devices to use existing FDA interpretive breakpoints. Either FDA or CLSI susceptibility interpretive breakpoints are acceptable to clinical laboratory accrediting bodies in the United States. Policies in other countries may vary. Each laboratory should check with the manufacturer of its antimicrobial susceptibility test system for additional information on the interpretive criteria used in its system's software.

Following discussions with appropriate stakeholders, such as infectious diseases practitioners and the pharmacy department, as well as the pharmacy and therapeutics and infection control committees of the medical staff, newly approved or revised breakpoints may be implemented by clinical laboratories. Following verification, CLSI disk diffusion test breakpoints may be implemented as soon as they are published in M100.[1] If a device includes antimicrobial test concentrations sufficient to allow interpretation of susceptibility and resistance to an agent using the CLSI breakpoints, a laboratory could choose to, after appropriate verification, interpret and report results using CLSI breakpoints.

## 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, health care 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 detection of emerging resistance mechanisms through development of new or revised methods, interpretive criteria, and quality control parameters.
- Educate users through multimedia communication of standards and guidelines.
- 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, susceptibility testing

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

## Chapter 1: Introduction

This chapter includes:

- Document scope and applicable exclusions
- Background information pertinent to the document content
- Standard precautions information
- Terms and definitions used in the document
- Abbreviations and acronyms used in the document

### 1.1 Scope

This document describes the standard agar disk diffusion techniques used to determine the *in vitro* susceptibility of bacteria that grow aerobically. It addresses preparation of agar plates, testing conditions (including inoculum preparation and standardization, incubation time, and incubation temperature), interpretation of results, QC 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 using dilution methods are found in CLSI document M07[3]; standards for testing the *in vitro* susceptibility of bacteria that grow anaerobically are found in CLSI document M11.[4] Guidelines for standardized susceptibility testing of infrequently isolated or fastidious bacteria that are not included in CLSI documents M02, M07,[3] or M11[4] are available in CLSI document M45.[5]

The susceptibility testing methods provided in this standard can be used in laboratories around the world including, but not limited to:

- Medical laboratories
- Public health laboratories
- Research laboratories
- Food laboratories
- Environmental laboratories

### 1.2 Background

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[6] and regulations[7,8] proposed by the US Food and Drug Administration (FDA) have been reviewed, and appropriate sections were 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 clearance of antimicrobial agent disks and for the approval of commercial devices used in the United States, including specific devices for disk testing such as zone readers. 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.[9] This method is the most thoroughly described disk diffusion method for which interpretive standards have been developed and supported by laboratory and clinical data.

This document, along with M100,[1] describes methods, QC, and interpretive criteria currently recommended for disk diffusion susceptibility tests. 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, *in vitro* interpretive criteria are analyzed in relation to studies of clinical efficacy and microbiological eradication efficacy in the treatment of specific pathogens, as outlined in CLSI document M23.[2]

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 (M100[1]).

## 1.3 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 known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of bloodborne pathogens. The Centers for Disease Control and Prevention (CDC) address this topic in published guidelines that address the daily operations of diagnostic medicine in human and animal medicine while encouraging a culture of safety in the laboratory.[10] For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.[11]

## 1.4 Terminology

### 1.4.1 Definitions

**antimicrobial susceptibility test interpretive category** – 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.

1) **susceptible (S)** – a category that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

2) **susceptible-dose dependent (SDD)** – a category that implies that susceptibility of an isolate is dependent on the dosing regimen that is used in the patient. In order to achieve levels that are likely to be clinically effective against isolates for which the susceptibility testing results (either minimal inhibitory concentrations [MICs] or disk diffusion) are in the SDD category, it is necessary to use a dosing regimen (ie, higher doses, more frequent doses, or both) that results in higher drug exposure than the dose that was used to establish the susceptible breakpoint. Consideration should be given to the maximum approved dosage regimen, because higher exposure gives the highest probability of adequate coverage of an SDD isolate. The dosing regimens used to set the SDD interpretive criterion are provided in Appendix E in M100.[1] The drug label should be consulted for recommended doses and adjustment for organ function.

   **NOTE:** The SDD interpretation is a new category for antibacterial susceptibility testing, although it has been previously applied for interpretation of antifungal susceptibility test results (see CLSI document M27-S4,[12] the supplement to CLSI document M27[13]). The concept of SDD has been included within the intermediate category definition for antimicrobial agents. However, this is often overlooked or not understood by clinicians and microbiologists when an intermediate result is reported. The SDD category may be assigned when doses well above those used to calculate the susceptible breakpoint are approved and used clinically, and where sufficient data to justify the designation exist and have been reviewed. When the intermediate category is used, its definition remains unchanged.

3) **intermediate (I)** – a category that 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; **NOTE:** The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (eg, quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (eg, 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 (R)** – 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 in which specific microbial resistance mechanisms (eg, β-lactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

5) **nonsusceptible (NS)** – a category used for isolates for which only a susceptible interpretive criterion has been designated because of the absence or rare occurrence of resistant strains. Isolates for which the antimicrobial agent MICs are above or zone diameters below the value indicated for the susceptible breakpoint should be reported as nonsusceptible; **NOTE 1:** An isolate that is interpreted as nonsusceptible does not necessarily mean that the isolate has a resistance mechanism. It is possible that isolates with MICs above the susceptible breakpoint that lack resistance mechanisms may be encountered within the wild-type distribution subsequent to the time the susceptible-only breakpoint is set; **NOTE 2:** For strains yielding results in the "nonsusceptible" category, organism identification and antimicrobial susceptibility test results should be confirmed. (See M100[1] Appendix A.)

**breakpoint//interpretive criteria** – minimal inhibitory concentration (MIC) or zone diameter value used to indicate susceptible, intermediate, and resistant, as defined above.

For example, for antimicrobial agent X with interpretive criteria of:

| | MIC (μg/mL) | Zone Diameter (mm) |
|---|---|---|
| Susceptible | ≤4 | ≥20 |
| Intermediate | 8–16 | 15–19 |
| Resistant | ≥32 | ≤14 |

"Susceptible breakpoint" is 4 μg/mL or 20 mm.
"Resistant breakpoint" is 32 μg/mL or 14 mm.

**D-zone test** – a disk diffusion test using clindamycin and erythromycin disks placed in close proximity to detect the presence of inducible clindamycin resistance in staphylococci and streptococci.[14,15]

**quality assurance (QA)** – part of quality management focused on providing confidence that quality requirements will be fulfilled (ISO 9000[16]); **NOTE:** The practice that encompasses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. In the testing environment, this includes monitoring all the raw materials, supplies, instruments, procedures, sample collection/transport/storage/processing, recordkeeping, calibrating and maintaining of equipment, quality control, proficiency testing, training of personnel, and all else involved in the production of the data reported.

**quality control (QC)** – the operational techniques and activities that are used to fulfill requirements for quality (modified from ISO 9000[16]); **NOTE 1:** In health care testing, the set of procedures designed to monitor the test method and the results to ensure test system performance; **NOTE 2:** QC includes testing control materials, charting the results and analyzing them to identify sources of error, and evaluating and documenting any remedial action taken as a result of this analysis.

**saline** – a solution of 0.85% to 0.9% NaCl (w/v).

**test method** – the method (ie, either the routine laboratory method or automated method) that is compared with the reference method.

**test system** – system that includes instructions and all of the instrumentation, equipment, reagents, and/or supplies needed to perform an assay or examination and generate test results.

### 1.4.2 Abbreviations and Acronyms

| | |
|---|---|
| AST | antimicrobial susceptibility testing |
| ATCC®* | American Type Culture Collection |
| BHI | Brain Heart Infusion |
| BLNAR | β-lactamase negative, ampicillin resistant |
| BSC | biological safety cabinet |
| BSL-2 | Biosafety Level 2 (USA) |
| BSL-3 | Biosafety Level 3 (USA) |
| CDC | Centers for Disease Control and Prevention |
| CFU | colony-forming unit(s) |
| CMRNG | chromosomally mediated penicillin-resistant *Neisseria gonorrhoeae* |
| CoNS | coagulase-negative staphylococci |

* ATCC® is a registered trademark of the American Type Culture Collection.

| | |
|---|---|
| CSF | cerebrospinal fluid |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| ESBL | extended-spectrum β-lactamase |
| FDA | US Food and Drug Administration |
| HLAR | high-level aminoglycoside resistance |
| HTM | *Haemophilus* Test Medium |
| hVISA | heteroresistant vancomycin-intermediate *Staphylococcus aureus* |
| KPC | *Klebsiella pneumoniae* carbapenemase |
| MHA | Mueller-Hinton agar |
| MHB | Mueller-Hinton broth |
| MHT | modified Hodge test |
| MIC | minimal inhibitory concentration |
| MRS | methicillin-resistant staphylococci |
| MRSA | methicillin-resistant *Staphylococcus aureus* |
| NAD | nicotinamide adenine dinucleotide |
| NDM | New Delhi metallo-β-lactamase |
| PBP | penicillin-binding protein |
| PBP 2a | penicillin-binding protein 2a |
| QA | quality assurance |
| QC | quality control |
| RNA | ribonucleic acid |
| SDD | susceptible-dose dependent |
| TEM | Temoneira (first patient from whom a TEM β-lactamase–producing strain was reported) |
| VRE | vancomycin-resistant enterococci |

# Chapter 2: Indications for Performing Susceptibility Tests

This chapter includes:

- Indications for when susceptibility testing is necessary
- Guidelines for selecting appropriate antimicrobial agents for testing and reporting
- Descriptions of the various antimicrobial agent classes

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 microporganism recognized as susceptible to a highly effective drug (eg, 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 antimicrobial 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 individually 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. Conducting susceptibility tests directly with clinical material (eg, normally sterile body fluids and urine) is not standardized and should not be done.

## 2.1 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 diseases practitioners and the pharmacy, as well as the pharmacy and therapeutics and infection control committees of the medical staff. The recommendations in M100[1] Tables 1A and 1B for each organism group list antimicrobial agents of proven efficacy that show acceptable *in vitro* test performance. Considerations in the assignment of antimicrobial 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 antimicrobial agents may be useful for infection control purposes. Refer to Appendix B in M100,[1] which lists intrinsic resistance properties of the more commonly encountered bacteria to assist in the selection process.

### 2.1.1 Routine Reports

The antimicrobial agents in M100[1] Tables 1A and 1B are recommendations that are considered appropriate for testing and reporting (note that some antimicrobial agents listed in M100[1] Tables 1A and 1B are recommended for MIC testing only). To avoid misinterpretation, routine reports to physicians should include those antimicrobial agents appropriate for therapeutic use, as suggested in M100[1] Tables 1A and 1B. Antimicrobial 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 epidemiological 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.

### 2.1.2 Antimicrobial Agent Classes

To minimize confusion, all antimicrobial agents should be reported using official nonproprietary (ie, generic) names. To emphasize the relatedness of the many currently available antimicrobial agents, they may be grouped together by drug classes. Brief descriptions of antimicrobial agent classes are given below along with examples of agents within each class (see M100[1] Glossary I, Parts 1 and 2 for the complete list).

#### 2.1.2.1 β-Lactams (see M100[1] Glossary I, Part 1)

β-lactam antimicrobial agents all share the common, central, four-member β-lactam ring and inhibition of cell wall synthesis as the primary mode of action. 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.

- **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* such as *Escherichia coli* and *Proteus mirabilis*.

  - Carboxypenicillins and ureidopenicillins are active against a considerably expanded list of gram-negative bacteria, including many *Pseudomonas* and *Burkholderia* spp.

  - Penicillinase-stable penicillins are active against predominantly gram-positive bacteria, including penicillinase-producing staphylococci.

- **β-lactam/β-lactamase inhibitor combinations**

  These antimicrobial agents are combinations that include a β-lactam class antimicrobial agent and a second agent that has minimal antibacterial activity, but functions as an inhibitor of some β-lactamases.

  β-lactamase inhibitors generally do not have antimicrobial activity on their own, but will potentiate the activity of the β-lactam antimicrobial agent combined with it. Currently, three β-lactamase inhibitors are in use:

  - Clavulanate
  - Sulbactam
  - Tazobactam

  The results of tests of only the β-lactam portion of the combination against β-lactamase–producing organisms are often not predictive of susceptibility to the two-drug combination. Several other β-lactamase inhibitors and combinations are currently in development.

- **Cephems (including cephalosporins)**

  Different cephem antimicrobial agents exhibit somewhat different spectrums of activity against aerobic and anaerobic, and gram-positive and gram-negative bacteria. The cephem antimicrobial class includes:

  - Classical cephalosporins
  - Cephamycin
  - Oxacephem
  - Carbacephems
  - Cephalosporins with anti–methicillin-resistant *Staphylococcus aureus* (MRSA) activity

  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.

- **Penems**

  The penem antimicrobial class includes two subclasses that differ slightly in structure from the penicillin class:

  - Carbapenems
  - Penems

  Antimicrobial 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.

- **Monobactams**

  Monobactam antimicrobial agents are monocyclic β-lactams. Aztreonam, which has activity only against aerobic, gram-negative bacteria, is the only monobactam antimicrobial agent approved for use in the United States by the FDA.

2.1.2.2 Non-β-Lactams (see M100[1] Glossary I, Part 2)

- **Aminoglycosides**

  Aminoglycosides are structurally related antimicrobial agents that inhibit bacterial protein synthesis at the ribosomal level. This class includes antimicrobial 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 (eg, penicillin, ampicillin, vancomycin) against some resistant gram-positive bacteria, such as enterococci.

- **Folate pathway inhibitors**

  Sulfonamides and trimethoprim are chemotherapeutic antimicrobial agents with similar spectra of activity resulting from the inhibition of the bacterial folate pathway. 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.

- **Glycopeptides**

  Glycopeptide antimicrobial agents, which include vancomycin (in the glycopeptide subclass) and teicoplanin (in the lipoglycopeptide subclass), 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 (eg, MRSA and some enterococci).

- **Lipopeptides**

  Lipopeptides are a structurally related group of antimicrobial agents for which the 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 physiological levels (50 mg/L) of calcium ions is essential for the proper activity of daptomycin.

- **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 be appropriate for testing against fastidious, gram-negative bacterial isolates. For gram-positive organisms, only erythromycin is generally tested routinely. The macrolide group of antimicrobial agents consists of several subgroups, including ketolide and fluoroketolide subgroups.

- **Nitroimidazoles**

  Nitroimidazoles, which include metronidazole and tinidazole, are bactericidal agents that are converted intracellularly in susceptible organisms to metabolites that disrupt the host DNA; they are active only against strictly anaerobic bacteria.

- **Oxazolidinones**

  Members of the oxazolidinone class have a unique mechanism of action that inhibits protein synthesis. The first agent approved in this class was linezolid, which has activity against gram-positive organisms.

- **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 antimicrobial agents.

- **Streptogramins**

  Streptogramins, which include quinupristin-dalfopristin and linopristin-flopristin, are combinations of two cyclic peptides produced by *Streptomyces* spp. They work synergistically to inhibit protein

synthesis, mainly in gram-positive organisms, although they do have limited activity against some gram-negative and anaerobic organisms.

- **Tetracyclines**

Tetracyclines are structurally related antimicrobial agents that inhibit protein synthesis at the ribosomal level of certain gram-positive and gram-negative bacteria. Antimicrobial 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, minocycline, or both. Tigecycline, a glycylcycline, is a derivative of minocycline that has activity against organisms that may be resistant to other tetracyclines.

- **Single-drug classes**

Several antimicrobial agents are currently the only members of their respective classes included in this document that are available for human use and appropriate for *in vitro* testing. These antimicrobial agents are listed below by mechanism of action with class designation in parentheses.

Inhibit protein synthesis:

- Chloramphenicol (phenicols)
- Clindamycin (lincosamides)
- Fusidic acid (steroidal)
- Mupirocin (pseudomonic acid)
- Spectinomycin (aminocyclitols)

Inhibit RNA synthesis:

- Fidaxomicin (macrocyclics)
- Rifampin (ansamycins)

Inhibits protein synthesis-and-assembly steps at the ribosomal level:

- Nitrofurantoin (nitrofurans): used only in the therapy of urinary tract infections

Inhibits enzymes involved in cell wall synthesis:

- Fosfomycin (fosfomycins): approved by the FDA for urinary tract infections only

## 2.2 Selection Guidelines

To make routine susceptibility testing relevant and practical, the number of antimicrobial agents tested should be limited. M100[1] Tables 1A and 1B list those antimicrobial agents that 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 groups for testing (see Subchapter 2.3) to assist laboratories in the selection of their routine testing batteries.

The listing of drugs together in a single box designates clusters of antimicrobial agents for which interpretive results (susceptible, intermediate, or resistant) and clinical efficacy are similar. Within each box, an "or" between agents designates those agents for which cross-resistance and cross-susceptibility are nearly complete. This means combined major and very major errors are fewer than 3% and minor

errors are fewer than 10%, based on a large collection of random clinical isolates tested (see CLSI document M23[2]). In addition, to qualify for an "or," at least 100 strains with resistance to the agents in question must be tested, and a result of "resistant" must be obtained with all agents for at least 95% of the strains. "Or" is also used for comparable antimicrobial agents when tested against organisms for which "susceptible-only" interpretive criteria are provided (eg, cefotaxime or ceftriaxone with *Haemophilus influenzae*). Thus, results from one agent connected by an "or" could be used to predict results for the other agent. For example, *Enterobacteriaceae* susceptible to cefotaxime can be considered susceptible to ceftriaxone. The results obtained from testing cefotaxime would be reported and a comment could be included on the report that the isolate is also susceptible to ceftriaxone. When no "or" connects agents within a box, testing of one agent cannot be used to predict results for another, either owing to discrepancies or insufficient data.

## 2.3 Suggested Guidelines for Routine and Selective Testing and Reporting

Test and Report Groups A, B, C, and U are noted in M100[1] Table 1A, and Groups A, B, and C are noted in M100[1] Table 1B. These group designations are restated in the M100[1] Table 2 series that lists the interpretive criteria for each organism group. The M100[1] Table 2 series contains two additional test and report group designations, "O" and "Inv."

**Group A** includes antimicrobial agents that are considered appropriate for inclusion in a routine, primary testing panel as well as for routine reporting of results for the specified organism groups.

**Group B** includes antimicrobial agents that may warrant primary testing, but they may be reported only selectively, such as when the organism is resistant to antimicrobial agents of the same class, as in Group A. Other indications for reporting the result might include a selected specimen source (eg, a third-generation cephalosporin for enteric bacilli from 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 antimicrobial agent in Group A; or for purposes of infection control.

**Group C** includes 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, eg, β-lactams); for treatment of patients allergic to primary drugs; for treatment of unusual organisms (eg, chloramphenicol for extraintestinal isolates of *Salmonella* spp.); or for reporting to infection control as an epidemiological aid.

**Group U** includes certain antimicrobial agents (eg, nitrofurantoin and certain quinolones) that are used only or primarily for treating urinary tract infections. These antimicrobial agents should not be routinely reported against pathogens recovered from other sites of infection. An exception to this rule is for *Enterobacteriaceae* in M100[1] Table 1A, where cefazolin is listed as a surrogate agent for oral cephalosporins. Other antimicrobial agents with broader indications may be included in Group U for specific urinary pathogens (eg, *Pseudomonas aeruginosa* and ofloxacin).

**Group O ("other")** includes antimicrobial agents that 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")** includes antimicrobial agents that are investigational for the organism group and have not yet been approved by the FDA for use in the United States.

Each laboratory should decide which antimicrobial agents in M100[1] Tables 1A and 1B to report routinely (Group A) and which might be reported only selectively (Group B) in consultation with the infectious diseases practitioners, the pharmacy, and the pharmacy and therapeutics and infection control committees of the health care institution. Selective reporting should improve the clinical relevance of test

reports and help minimize the selection of multiresistant, health care–associated strains by overuse of broad-spectrum agents. Results for Group B antimicrobial agents tested but not reported routinely should be available on request, or they may be reported for selected specimen types. Unexpected resistance, when confirmed, should be reported (eg, resistance to a secondary agent but susceptibility to a primary agent, such as a *P. aeruginosa* isolate resistant to amikacin but susceptible to tobramycin; as such, both drugs should be reported). In addition, each laboratory should develop a protocol to address isolates that are confirmed as resistant to all antimicrobial agents on its routine test panel. This protocol should include options for testing additional agents in-house or sending the isolate to a reference laboratory.

## Chapter 3: Susceptibility Testing Process

This chapter includes:

- An overview of the disk diffusion susceptibility testing process
- Suggested media for disk diffusion testing including supplements for fastidious organisms
- Instructions for acquiring disks and proper disk storage
- Description of the methods for inoculum preparation and standardization, plate inoculation, and application of disks
- Testing considerations for fastidious organisms including recommended medium and incubation conditions
- Instructions for measuring zones and interpreting the results
- Discussion of organism-specific resistance mechanisms and special testing methods that may be appropriate
- Description of the limitations of disk diffusion methods

Figure 1 provides an overview of the disk diffusion susceptibility testing process. Detailed information for each step is provided in each designated chapter/subchapter.

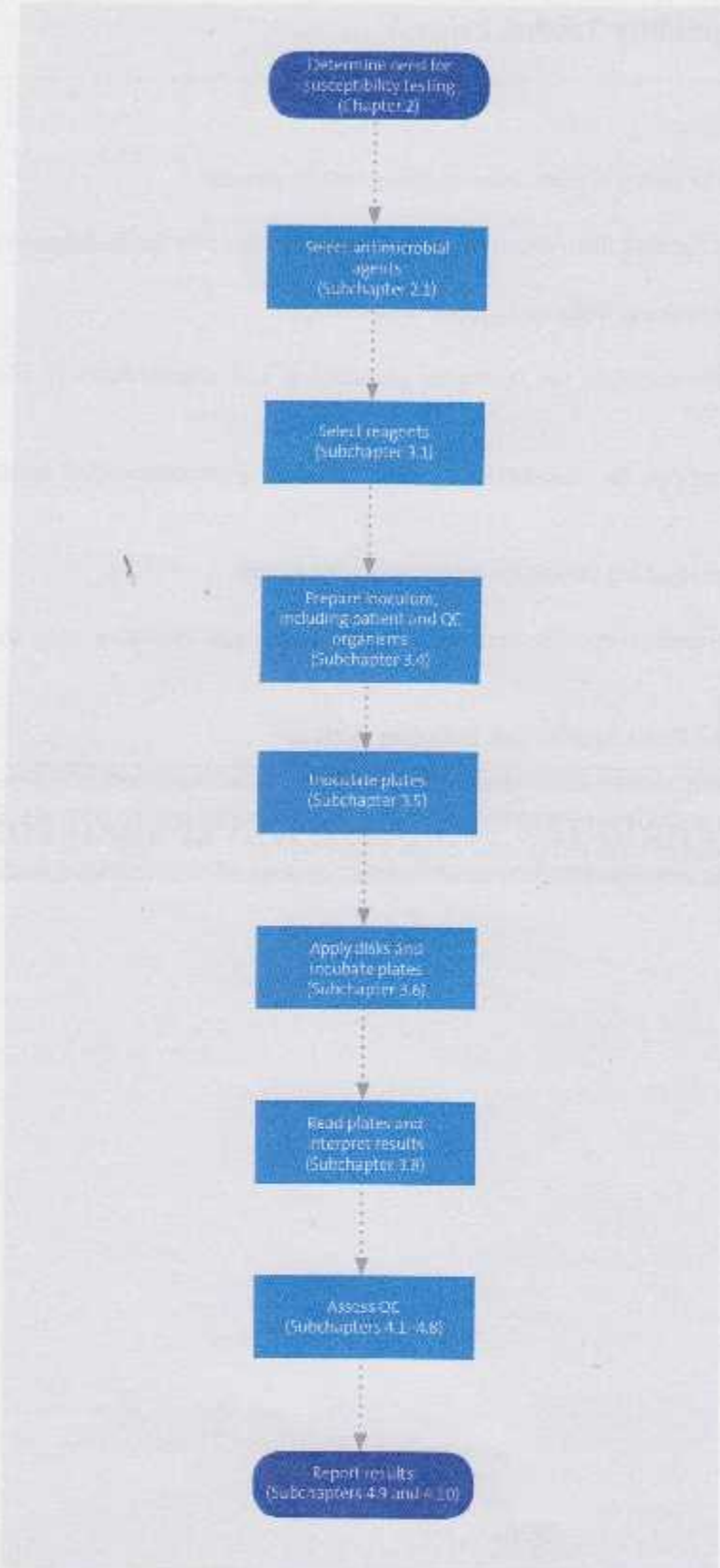



**Figure 1. Disk Diffusion Susceptibility Testing Process**
Abbreviation: QC, quality control.

## 3.1 Reagents for the Disk Diffusion Test

### 3.1.1 Mueller-Hinton Agar

Of the many media available, the subcommittee considers Mueller-Hinton agar (MHA) the best for routine susceptibility testing of nonfastidious bacteria because:

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

Although MHA 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 will likely be erroneously large and results with QC strains may exceed the acceptable QC limits. Only MHA formulations that have been tested according to, and that meet the acceptance limits described in, CLSI document M06[17] should be used. Commercially prepared plates may be used or they may be prepared as described in Appendix B.

### 3.1.2 pH

The agar medium should have a pH between 7.2 and 7.4 at room temperature. The method to assess pH is provided in Appendix B (see B1.1).

### 3.1.3 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 removed by evaporation (usually 10 to 30 minutes). The surfaces of the plates should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the Petri plate covers when the plates are inoculated.

### 3.1.4 Effects of Thymidine or Thymine

MHA 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 MHA that is as low in thymidine content as possible. If problems with QC of sulfonamides and trimethoprim occur, it might be necessary to check the MHA. To evaluate a lot of MHA, *Enterococcus faecalis* ATCC® 29212 or, alternatively, *E. faecalis* ATCC® 33186, may be tested with trimethoprim-sulfamethoxazole disks. Satisfactory media provide essentially clear, distinct zones of inhibition $\geq 20$ mm. Unsatisfactory media produce no zone of inhibition, growth within the zone, or a zone of $<20$ mm.

### 3.1.5 Effects of Variation in Divalent Cations

Variation in divalent cations, principally magnesium and calcium, affects results of aminoglycoside and tetracycline tests with *P. aeruginosa* strains. Excess cation content reduces 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; therefore, disk diffusion testing is not reliable for testing daptomycin. Excess zinc ions may reduce zone sizes of carbapenems. Performance tests with each lot of MHA must conform to the control limits listed in M100[1] Table 4A.

## 3.2 Testing Strains That Fail to Grow Satisfactorily

Only aerobic or facultative bacteria that grow well on unsupplemented MHA should be tested on that medium. Certain fastidious species, such as *Haemophilus* spp., *Neisseria gonorrhoeae, Neisseria meningitidis, Streptococcus pneumoniae,* and viridans and β-hemolytic streptococci do not grow sufficiently on unsupplemented MHA. These organisms require supplements or different media to grow, and they should be tested on the media listed below and described in Appendix B using methods described in this document.

- MHA agar with 5% sheep blood
- *Haemophilus* Test Medium (HTM)
- GC agar base + 1% defined growth supplement

Details for these tests are provided in Subchapter 3.7 and Appendix C.

## 3.3 Antimicrobial Disks

### 3.3.1 Source of Disks and Quality Specifications

Disks should be purchased from a reliable commercial vendor. The disks should be accompanied, at minimum, by a certificate of analysis stating the content of the disks, lot number, expiration date, and assurance that they were tested and performed according to established QC specifications.

### 3.3.2 Storage of Antimicrobial Disks

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Information on disk storage and handling is described below:

| Step | Action | Comment |
|---|---|---|
| 1. | Refrigerate the cartridges at 8°C or below or freeze at −14°C or below until needed. | Do not store 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 one week, at most. Some labile agents (eg, imipenem, cefaclor, and clavulanate combinations) may retain greater stability if stored frozen until the day of use. |
| 2. | Remove the sealed packages containing disk cartridges from the refrigerator or freezer 1–2 hours before use so they may equilibrate to room temperature before opening. | Equilibrating the packages to room temperature minimizes the amount of condensation that occurs when warm air contacts cold disks. |
| 3. | 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 stored in a container 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. |
| 4. | Refrigerate the container with the disk-dispensing apparatus when not in use. | |
| 5. | 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. |

## 3.4 Inoculum Preparation for Disk Diffusion Tests

### 3.4.1 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, use a $BaSO_4$ turbidity standard equivalent to a 0.5 McFarland standard or its optical equivalent (eg, latex particle suspension). Prepare a $BaSO_4$ 0.5 McFarland standard as described in Appendix B. Alternatively, a photometric device can be used.

### 3.4.2 Direct Colony Suspension Method for Inoculum Preparation

The direct colony suspension method is the most convenient method for inoculum preparation. This method can be used with most organisms; it is the recommended method for testing the fastidious organisms, *Haemophilus* spp., *N. gonorrhoeae, N. meningitidis,* and streptococci (see Subchapter 3.7), and for testing cefoxitin and staphylococci to detect methicillin or oxacillin resistance.

| Step | Action | Comment |
|---|---|---|
| 1. | Make a direct broth or saline suspension of isolated colonies selected from an 18- to 24-hour agar plate. | Use a nonselective medium, such as blood agar. |
| 2. | Adjust the suspension to achieve a turbidity equivalent to a 0.5 McFarland standard. This results in a suspension containing approximately 1 to $2 \times 10^8$ CFU/mL for *E. coli* ATCC® 25922. | Use either a photometric device or, if performed visually, use adequate light to compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines. |

Abbreviations: ATCC®, American Type Culture Collection; CFU, colony-forming unit(s).

### 3.4.3 Growth Method for Inoculum Preparation

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.

| Step | Action | Comment |
|---|---|---|
| 1a. | Select at least 3–5 well-isolated colonies of the same morphological type from an agar plate culture. | |
| 1b. | Touch the top of each colony with a loop or sterile swab and transfer the growth into a tube containing 4–5 mL of a suitable broth medium, such as tryptic soy broth. | |
| 2. | Incubate the broth culture at 35°C ± 2°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 2–6 hours). | |
| 3. | Adjust the turbidity of the actively growing broth culture with sterile saline or broth to achieve a turbidity equivalent to that of a 0.5 McFarland standard. This results in a suspension containing approximately 1 to $2 \times 10^8$ CFU/mL for *E. coli* ATCC® 25922. | Use either a photometric device or, if performed visually, use adequate light to compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.<br><br>**NOTE:** Avoid extremes in inoculum density. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates. |

Abbreviations: ATCC®, American Type Culture Collection; CFU, colony-forming unit(s).

### 3.5 Inoculation of Test Plates

| Step | Action | Comment |
|---|---|---|
| 1a. | Dip a sterile cotton swab into the adjusted suspension. | Use inoculum suspension within 15 minutes after adjusting the turbidity to match that of the 0.5 McFarland standard. |
| 1b. | Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level. | Rotating the swab in this manner removes excess fluid from the swab. |
| 2a. | Inoculate the dried surface of an MHA plate by streaking the swab over the entire sterile agar surface. | |
| 2b. | Repeat this procedure by streaking 2 more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. | |
| 2c. | As a final step, swab the rim of the agar. | |
| 3. | Leave the lid ajar for (ideally) 3–5 minutes, but no more than 15 minutes. | Leaving the lid ajar allows any excess surface moisture to be absorbed before applying the drug-impregnated disks. |

Abbreviation: MHA, Mueller-Hinton agar.

### 3.6 Application of Disks to Inoculated Agar Plates

| Step | Action | Comment |
|---|---|---|
| 1a. | Dispense predetermined battery of antimicrobial disks onto the surface of the inoculated agar plate. | Place disks that give predictably small zones (eg, gentamicin, vancomycin) next to those that give larger zones (eg, cephalosporins, penicillins) in an effort to avoid overlapping zones.<br><br>Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so they are no closer than 24 mm from center to center.<br><br>No more than 12 disks should be placed on a 150-mm plate and no more than 6 disks on a 100-mm plate, although some organisms require fewer (see Subchapter 3.7 and Appendix C). |
| 1b. | Press each disk down to ensure complete contact with the agar surface. | 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.<br><br>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. |
| 1c. | If performing the D-zone test for inducible clindamycin resistance, see Subchapter 3.10.1 and M100[1] Table 3G for guidance on disk placement. | |
| 2a. | Invert the plates and place in an incubator set to 35°C ± 2°C within 15 minutes after the disks are applied. | Testing at temperatures above 35°C may not detect MRS. |
| 2b. | Do not incubate the plates in an increased $CO_2$ atmosphere (with the exception of *Haemophilus* spp., *N. gonorrhoeae, N. meningitidis,* and streptococci [see Subchapter 3.7]). | The interpretive standards were developed by using ambient air incubation, and $CO_2$ significantly alters the size of the inhibitory zones of some agents (see M100[1] Table 4D). |

Abbreviation: MRS, methicillin-resistant staphylococci.

### 3.7 Special Considerations for Fastidious Organisms

Mueller-Hinton medium described previously for the rapidly growing aerobic pathogens is not adequate for susceptibility testing of fastidious organisms. If disk diffusion tests are performed with fastidious organisms, the medium, QC procedures, and interpretive criteria must be modified to fit each organism. Disk diffusion tests for *H. influenzae, N. gonorrhoeae, N. meningitidis, S. pneumoniae,* and β-hemolytic and viridans group streptococci have been shown to be accurate for selected agents; they are described in Table 1, below. Other fastidious bacteria may be tested by a dilution or disk diffusion method as described in CLSI document M45.[5] Anaerobic bacteria should not be tested by the disk diffusion test. See CLSI document M11[4] for appropriate anaerobe testing procedures.

**Table 1. Testing Considerations for Fastidious Organisms**

| Step | Action | Organisms (see notes for specific organisms below table) | | | |
|---|---|---|---|---|---|
| | | ***Haemophilus* spp.** | ***N. gonorrhoeae*** | ***N. meningitidis* (see recommended precautions below before testing)*** | ***S. pneumoniae* and Other *Streptococcus* spp.** |
| 1. | Using the direct colony suspension procedure (see Subchapter 3.4.2), prepare a suspension in MHB or saline from organism grown on the source plates indicated and adjust with broth or saline to achieve a turbidity equivalent to a 0.5 McFarland standard. | Chocolate agar plate incubated for 16–18 hours in 5% $CO_2$<br><br>Adjust the suspension using a photometric device, exercising care in preparation (suspension will contain approximately 1 to $4 \times 10^8$ CFU/mL), 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. | Chocolate agar plate incubated for 20–24 hours in 5% $CO_2$ | Chocolate agar plate incubated for 20–22 hours in 5% $CO_2$ | Sheep blood agar plate incubated for 18–20 hours in 5% $CO_2$ |
| 2. | Inoculate plates prepared using the recommended medium within 15 minutes after adjusting the turbidity of the inoculum suspension.<br><br>(See Appendix B for medium preparation or obtain commercially). | HTM<br><br>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.<br><br>Mueller-Hinton chocolate agar is not recommended for routine testing of *Haemophilus* spp. | GC agar to which a 1% defined growth supplement is added after autoclaving<br><br>Cysteine-free growth supplement is not required for disk testing as it is for certain agents with dilution testing (see CLSI document M07[3]).<br><br>Enriched chocolate agar is not recommended for susceptibility testing of *N. gonorrhoeae*. | MHA supplemented with 5% sheep blood<br><br>Enriched chocolate agar is not recommended for susceptibility testing of *N. meningitidis* except as a growth medium for inoculum preparation. | MHA supplemented with 5% sheep blood. |

**Table 1. (Continued)**

| Step | Action | Organisms (see notes for specific organisms below table) | | | |
|---|---|---|---|---|---|
| | | *Haemophilus* spp. | *N. gonorrhoeae* | *N. meningitidis* (see recommended precautions below before testing)[4] | *S. pneumoniae* and Other *Streptococcus* spp. |
| 3. | Dispense antimicrobial disks onto the surface of the inoculated agar plate.<br><br>See M100[1] Table 1B or appropriate portion of M100[1] Table 2 for agents recommended for routine testing and reporting. | 150-mm plate: ≤9 disks<br>100-mm plate: ≤4 disks | 150-mm plate: ≤9 disks<br>100-mm plate: ≤4 disks<br><br>For some agents (eg, fluoroquinolones, cephalosporins) that produce extremely large zones, only 2–3 disks may be tested per 100-mm plate. | 150-mm plate: ≤5 disks<br><br>100-mm plate: ≤2 disks | 150-mm plate: ≤9 disks<br><br>100-mm plate: ≤4 disks |
| 4. | Invert the plates and place in an incubator.<br><br>Incubation conditions and time: | 35°C±2°C; 5% $CO_2$; 16–18 hours | 36°C±1°C (do not exceed 37°C); 5% $CO_2$; 20–24 hours | 35°C±2°C; 5% $CO_2$; 20–24 hours | 35°C±2°C; 5% $CO_2$; 20–24 hours |
| 5. | Read the zones of inhibition (see Subchapter 3.8.1 [2]).<br><br>Specific zone diameter interpretive criteria in M100[1]: | M100[1] Table 2E | M100[1] Table 2F | M100[1] Table 2I | M100[1] Table 2G: *S. pneumoniae*<br><br>M100[1] Table 2H-1: β-hemolytic group *Streptococcus* spp.<br><br>M100[1] Table 2H-2: Viridans group *Streptococcus* spp. |

Abbreviations: CFU, colony-forming unit(s); HTM, *Haemophilus* Test Medium; MHA, Mueller-Hinton agar; MHB, Mueller-Hinton broth.

**NOTES for Table 1 (for specific organisms):**

- ***N. gonorrhoeae:*** *N. gonorrhoeae* with 10-µg penicillin disk zone diameters of ≤ 19 mm generally produce β-lactamase. However, β-lactamase tests (see Subchapter 3.10.2) provide more rapid results than disk diffusion tests and, therefore, are preferred for recognition of this plasmid-mediated penicillin resistance. *N. gonorrhoeae* with plasmid-mediated resistance to tetracycline also has zones of inhibition (30-µg 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 M100[1] Table 2F.

- ***N. meningitidis:*** Disk diffusion testing of *N. meningitidis* has been validated for detection of possible emerging resistance with some antimicrobial agents. To date, resistance has mostly been found in older agents used for therapy (penicillin or ampicillin), or agents used for prophylaxis of case contacts (rifampin, ciprofloxacin). Because resistance to antimicrobial agents such as ceftriaxone or cefotaxime that are often used for therapy of invasive diseases has not been confirmed, routine testing of isolates by clinical laboratories is not necessary. Prophylaxis of close contacts should not be delayed while awaiting susceptibility testing results.

- ***Streptococcus pneumoniae* and other *Streptococcus* spp.:** For nonmeningitis isolates of *S. pneumoniae,* oxacillin zone sizes of ≥20 mm indicate susceptibility to penicillin (oral or parenteral), and other β-lactam agents (see M100[1] Table 2G).

  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.[18] For isolates with oxacillin zones ≤ 19 mm, do not report as resistant without performing a penicillin MIC.

  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 β-hemolytic streptococci only. A penicillin MIC should be determined on isolates of viridans group streptococci from normally sterile body sites (eg, CSF, blood, bone).

  Penicillin and ampicillin disk diffusion tests are not reliable with viridans group streptococci.

  Inducible clindamycin resistance can be identified in *S. pneumoniae* and β-hemolytic streptococci using the method described in Subchapter 3.10.1.

- **Recommended precautions:** Perform all antimicrobial susceptibility testing (AST) of *N. meningitidis* in a biological safety cabinet (BSC).[19-21] Manipulating *N. meningitidis* outside a BSC is associated with increased risk for contracting meningococcal disease. Laboratory-acquired meningococcal disease is associated with a case fatality rate of 50%. Exposure to droplets or aerosols of *N. meningitidis* is the most likely risk for laboratory-acquired infection. Rigorous protection from droplets or aerosols is mandated when microbiological procedures (including AST) are performed on *N. meningitidis* isolates.

  If a BSC is unavailable, manipulation of these isolates should be minimized, limited to Gram staining or serogroup identification using phenolized saline solution, while wearing a laboratory coat and gloves and working behind a full face splash shield. Use Biosafety Level 3 (BSL-3) practices, procedures, and containment equipment for activities with a high potential for droplet or aerosol production and for activities involving production quantities or high concentrations of infectious materials. If Biosafety Level 2 (BSL-2) or BSL-3 facilities are not available, forward isolates to a reference or public health laboratory with a minimum of BSL-2 facilities.

Laboratorians who are exposed routinely to potential aerosols of *N. meningitidis* should consider vaccination according to the current recommendations of the CDC Advisory Committee on Immunization Practices (http://www.cdc.gov/vaccines/acip/index.html). Vaccination decreases but does not eliminate the risk of infection, because it is less than 100% effective.

## 3.8 Reading Plates and Interpreting Results

### 3.8.1 Reading Plates

1. After 16 to 18 hours of incubation (see below, Subchapters 3.7 and 3.9, and Appendix C for exceptions), examine each plate. If the plate was satisfactorily streaked, and the inoculum concentration 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 concentration 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, with the following exceptions:

   - 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 and with the cover removed.

   - For coagulase-negative *Staphylococcus* spp. with cefoxitin, 24 hours of incubation are required before reporting as susceptible; other agents should be read and reported at 16 to 18 hours. If cefoxitin is tested against *Staphylococcus* spp., read the zone diameters with reflected, not transmitted light (plate held up to the light).

   - If testing vancomycin against *Enterococcus* spp., 24 hours of incubation are required before reporting as susceptible; other agents should be read and reported at 16 to 18 hours.

   - If linezolid is tested against *Staphylococcus* spp., read the zone diameters with transmitted light.

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. For *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.

   - For 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.

- For *S. aureus* strains with penicillin zones ≥29 mm, see Subchapter 3.9.1 and M100[1] Table 3D for information on interpretation of the appearance of the zone edge (the penicillin zone-edge test for β-lactamase production).

3. Interpret the sizes of the zones of inhibition by referring to M100[1] Tables 2A through 2I, and report the organisms as susceptible, susceptible-dose dependent, intermediate, or resistant to the agents that have been tested (see Subchapter 1.4.1 for definitions of these interpretive categories). Some agents may be reported only as susceptible or nonsusceptible, because only susceptible breakpoints are given, as no or very few resistant strains have been identified.

## 3.9 Special Considerations for Detecting Resistance

This subchapter discusses organism groups or particular resistance mechanisms for which there are significant testing issues. Testing issues regarding both dilution and disk diffusion testing are discussed here.

### 3.9.1 Staphylococci

#### 3.9.1.1 Penicillin Resistance and β-Lactamase

Most staphylococci are resistant to penicillin, and penicillin is rarely an option for treatment of staphylococcal infections. Penicillin-resistant strains of staphylococci produce β-lactamase and testing penicillin instead of ampicillin is preferred. Penicillin should be used to test the susceptibility of all staphylococci to all penicillinase-labile penicillins, such as amoxicillin, ampicillin, azlocillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin.

Some β-lactamase–producing staphylococcal isolates test susceptible to penicillin. Because staphylococcal β-lactamase is readily inducible, there is a risk of this occurring if penicillin were used to treat such strains. For this reason it is recommended that isolates of *Staphylococcus* with penicillin MICs ≤0.12 µg/mL or zone diameters ≥29 mm be tested for β-lactamase production before reporting the isolate as penicillin susceptible. Several tests for β-lactamase production have been described. These include nitrocefin-based tests or evaluating the zone edge of a penicillin disk diffusion test (ie, a fuzzy zone edge indicates no β-lactamase production, whereas a sharp edge indicates β-lactamase production).[22] The penicillin disk diffusion zone-edge test was more sensitive than nitrocefin-based tests for detection of β-lactamase production in *S. aureus*. The penicillin zone-edge test is recommended if only one test is used for β-lactamase detection in *S. aureus*. However, some laboratories may choose to perform a nitrocefin-based test first, and if this test is positive report the results as positive for β-lactamase (or penicillin resistant). If the nitrocefin test is negative, it is recommended that the penicillin disk diffusion zone-edge test be performed before reporting penicillin susceptible results in cases in which penicillin may be used for therapy for *S. aureus*. For coagulase-negative staphylococci (CoNS), including *Staphylococcus lugdunensis*, only nitrocefin-based tests are recommended. For the most current recommendations to detect β-lactamases in staphylococcal species, see M100[1] Table 3D.

#### 3.9.1.2 Methicillin/Oxacillin Resistance

Historically, resistance to the antistaphylococcal, penicillinase-stable penicillins has been referred to as "methicillin resistance," and the abbreviations "MRSA" (for methicillin-resistant *S. aureus*) or "MRS" (for methicillin-resistant staphylococci) are still commonly used, even though methicillin is no longer available for treatment. In this document, resistance to these agents may be referred to using several terms (eg, "MRS," "methicillin resistance," or "oxacillin resistance"). Most resistance to oxacillin in staphylococci is mediated by the *mecA* gene, which directs the production of a supplemental penicillin-binding protein (PBP), PBP 2a, during bacterial cell replication. Resistance is expressed either homogeneously or heterogeneously. Homogeneous expression of resistance is easily detected with

standard testing methods because nearly all bacterial cell progeny express the resistance phenotype. Heterogeneous expression may be more difficult to detect because only a fraction of the progeny population (eg, 1 in 100 000 cells) expresses resistance. Mechanisms of oxacillin resistance other than *mecA* are rare and include a novel *mecA* homologue, *mecC*.[23] MICs for strains of *S. aureus* with *mecC* are typically in the resistant range for cefoxitin and/or oxacillin; *mecC* resistance cannot be detected by tests directed at *mecA* or PBP 2a.

3.9.1.3 Organism Groups

*S. lugdunensis* are now grouped with *S. aureus* when determining methicillin/oxacillin resistance. Oxacillin-susceptible, *mecA*-negative strains exhibit oxacillin MICs in the range of 0.25 to 1 µg/mL, whereas *mecA*-positive strains usually exhibit MICs ≥ 4 µg/mL, characteristics more like *S. aureus* than other CoNS. Therefore, the presence of *mecA*-mediated resistance in *S. lugdunensis* is detected more accurately using the *S. aureus* rather than the CoNS interpretive criteria and *S. lugdunensis* is grouped with *S. aureus* when describing tests to detect oxacillin resistance in this document and in M100.[1] Oxacillin and cefoxitin testing methods for CoNS do not include *S. lugdunensis*.

3.9.1.4 Methods for Detection of Oxacillin Resistance

Methods recommended for detecting oxacillin resistance in staphylococci are delineated in Table 2, below (also see M100[1] Tables 2C and 3E). Oxacillin disk diffusion methods should not be used. Cefoxitin is used as a surrogate for oxacillin. Cefoxitin tests are equivalent to oxacillin MIC tests in sensitivity and specificity for *S. aureus*, and are better predictors of the presence of *mec*-mediated resistance than oxacillin agar-based methods, including the oxacillin salt agar screening plate. For CoNS, the cefoxitin disk diffusion test has equivalent sensitivity to oxacillin MIC tests but greater specificity (ie, the cefoxitin disk test more accurately identifies oxacillin-susceptible strains than the oxacillin MIC test).

**Table 2. Methods for Detection of Oxacillin Resistance in Staphylococci**

| | Oxacillin MIC | Cefoxitin MIC | Cefoxitin Disk Diffusion | Oxacillin Salt Agar Screening Test |
|---|---|---|---|---|
| *S. aureus* | Yes | Yes | Yes | Yes |
| *S. lugdunensis* | Yes | Yes | Yes | No |
| CoNS (except *S. lugdunensis*) | Yes* | No | Yes | No |

* The oxacillin MIC interpretive criteria listed in M100[1] for CoNS may overcall resistance for some species other than *S. epidermidis*. These isolates display MICs in the 0.5 to 2 µg/mL range but lack *mecA*. For serious infections with CoNS other than *S. epidermidis*, testing for *mecA* or for PBP 2a or with cefoxitin disk diffusion may be appropriate for strains for which the oxacillin MICs are 0.5 to 2 µg/mL.

Abbreviations: CoNS, coagulase-negative staphylococci; MIC, minimal inhibitory concentration; PBP 2a, penicillin-binding protein 2a.

Using the following test conditions will improve the detection of *mecA*-mediated oxacillin resistance in staphylococci, especially heteroresistant MRSA:

- The addition of NaCl (2% w/v; 0.34 mol/L) for both agar and broth dilution testing of oxacillin
- Inoculum preparation using the direct colony suspension method (see Subchapter 3.4.2)
- Incubation at temperatures between 30 and 35°C.
- Incubation of oxacillin MIC tests for a full 24 hours before reporting as susceptible
  - Oxacillin is the preferred antistaphylococcal penicillin to use because it is more resistant to degradation in storage. Do not test oxacillin by disk diffusion.

- Incubation of tests using cefoxitin for 16 to 20 hours for *S. aureus* and *S. lugdunensis* and 24 hours for CoNS

- Reading the zone of inhibition around the cefoxitin disk using reflected light for all staphylococci

For the most current recommendations regarding testing and reporting, refer to M100[1] Tables 2C and 3E.

3.9.1.5 Molecular Detection Methods

Tests for the *mecA* gene or the protein produced by *mecA,* PBP 2a (also called PBP2'), are the most accurate methods for prediction of resistance to oxacillin. *mecC* resistance cannot be detected by tests directed at *mecA* or PBP 2a.

3.9.1.6 Reporting

- Resistance may be reported any time growth is observed after a minimum of 16 hours of incubation.

- If a cefoxitin-based test is used (required for disk diffusion testing), cefoxitin is used as a surrogate for detecting oxacillin resistance. Based on the cefoxitin result, report oxacillin as susceptible or resistant.

- Oxacillin susceptibility test results can be applied to the other penicillinase-stable penicillins.

- In most staphylococcal isolates, oxacillin resistance is mediated by *mecA,* encoding PBP 2a. Isolates that test positive for *mecA* or PBP 2a should be reported as oxacillin resistant.

- Isolates that test resistant by oxacillin MIC, cefoxitin MIC, or cefoxitin disk test or are positive for *mecA* or PBP 2a should be reported as oxacillin resistant.

- Because of the rare occurrence of resistance mechanisms other than *mecA* in *S. aureus,* report isolates that are negative for the *mecA* gene or do not produce PBP 2a, but for which oxacillin MICs are ≥4 µg/mL as oxacillin resistant.

- Oxacillin-resistant staphylococci are considered resistant to all penicillins, cephems (with the exception of the cephalosporins with anti-MRSA activity), β-lactam/β-lactamase inhibitors, and carbapenems. This recommendation is based on the fact that most cases of documented MRS infections have responded poorly to β-lactam therapy, or because convincing clinical data have yet to be presented that document clinical efficacy for those agents in MRS infections.

- Oxacillin-susceptible strains can be considered susceptible to cephems, β-lactam/β-lactamase inhibitor combinations, and carbapenems.

3.9.1.7 Vancomycin Resistance in *Staphylococcus aureus*

In 2006 (M100-S16), the interpretive criteria for vancomycin and *S. aureus* were lowered to ≤2 µg/mL for susceptible, 4 to 8 µg/mL for intermediate, and ≥16 µg/mL for resistant. For CoNS, they remain at ≤4 µg/mL for susceptible, 8 to 16 µg/mL for intermediate, and ≥32 µg/mL for resistant.

The first occurrence of a strain of *S. aureus* with reduced susceptibility to vancomycin (MICs 4 to 16 µg/mL) was reported from Japan in 1997,[24] followed by reports from the United States and France.[25] 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.

Since 2002, *S. aureus* strains for which the vancomycin MICs ranged from 32 to 1024 µg/mL have been reported in the United States. All of these strains contained a *vanA* gene similar to that found in enterococci.[26,27] These strains are reliably detected by the broth microdilution reference method, and the vancomycin agar screen test (see Subchapter 3.9.1.7.1) when the tests are incubated for a full 24 hours at 35°C ± 2°C before reporting as susceptible. The disk diffusion method with vancomycin was removed from M02 and M100[1] in 2009 because it failed to differentiate susceptible strains from strains for which the MICs are 4 to 16 µg/mL.

#### 3.9.1.7.1 Methods for Detection of Nonsusceptibility to Vancomycin

*S. aureus* with vancomycin MICs ≥ 8 µg/mL can be detected by either MIC or the vancomycin agar screen test. In order to recognize strains of staphylococci for which the vancomycin MICs are 4 µg/mL, MIC testing must be performed and the tests incubated for a full 24 hours at 35°C ± 2°C. The vancomycin agar screen test does not consistently detect *S. aureus* with vancomycin MICs of 4 µg/mL. If growth is found on the vancomycin screen agar, perform a vancomycin MIC test to establish the MIC value. Strains with vancomycin MICs <32 µg/mL are not detected by disk diffusion, even with 24-hour incubation.

#### 3.9.1.7.2 Vancomycin Agar Screen

Perform the test using the following procedure by inoculating an isolate of *S. aureus* onto Brain Heart Infusion (BHI) agar containing 6 µg/mL of vancomycin.

| Step | Action | Comment |
|---|---|---|
| 1. | Prepare a direct colony suspension equivalent to a 0.5 McFarland standard as is done for MIC or disk diffusion testing. | See Subchapter 3.4.2. |
| 2. | Use a micropipette to deliver a 10-µL drop to the agar surface. | Alternatively, use a swab from which the excess liquid has been expressed, as is done for the disk diffusion test, and spot an area at least 10–15 mm in diameter. |
| 3. | Incubate the plate at 35°C ± 2°C in ambient air for a full 24 hours. | |
| 4. | Examine the plate carefully, using transmitted light, for evidence of small colonies (> 1 colony) or a film of growth. Greater than 1 colony or a film of growth suggests reduced susceptibility to vancomycin. | Do not reuse plates after incubation. |
| 5. | Confirm results for *S. aureus* that grow on the BHI vancomycin agar screen by repeating identification tests and performing vancomycin MIC tests using a CLSI reference dilution method or other validated MIC method. | For QC, see M100[1] Table 3F. |

Abbreviations: BHI, Brain Heart Infusion; MIC, minimal inhibitory concentration; QC, quality control.

Many *S. aureus* isolates with vancomycin MICs of 4 µg/mL do not grow on this vancomycin agar screen media (see Subchapter 3.9.1.7.1). Also, there are insufficient data to recommend using this agar screen test for CoNS.

3.9.1.7.3 Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus*[28]

When first described in 1997, heteroresistant vancomycin-intermediate *S. aureus* (hVISA) isolates were those *S. aureus* that contained subpopulations of cells (typically 1 in every 100 000 to 1 000 000 cells) for which the vancomycin MICs were 8 to 16 µg/mL, ie, in the intermediate range. Because a standard broth microdilution test uses an inoculum of $5 \times 10^5$ CFU/mL, these resistant subpopulations may go undetected and the vancomycin MICs determined for such isolates would be in the susceptible range (formerly between 1 and 4 µg/mL). Many physicians and microbiologists initially were skeptical that heteroresistance would result in clinical treatment failures with vancomycin, because such strains were susceptible to vancomycin by the standard CLSI broth microdilution reference method. However, after reviewing both clinical and laboratory data, CLSI lowered the susceptible breakpoint for vancomycin (for *S. aureus* isolates only, not CoNS) from ≤4 to ≤2 µg/mL, and the resistant breakpoint from ≥32 to ≥16 µg/mL, to avoid calling some heteroresistant strains susceptible and make the breakpoints more predictive of clinical outcome. Thus, the vancomycin-susceptible breakpoint for *S. aureus* is now ≤ 2 µg/mL, intermediate 4 to 8 µg/mL, and resistant ≥16 µg/mL. These lower breakpoints identify the hVISA strains for which the vancomycin MICs are 4 µg/mL as intermediate. Some susceptible *S. aureus* strains for which the vancomycin MICs are 1 to 2 µg/mL may still be hVISAs.

Determining the population analysis profiles of *S. aureus* isolates (ie, plating a range of dilutions of a standard inoculum of *S. aureus* [$10^1$ to $10^8$ CFU] on a series of agar plates containing a range of vancomycin concentrations, plotting the population curve, dividing the bacterial counts by the area under the curve, and comparing the ratio derived to *S. aureus* control strains Mu3 and Mu50[29,30]) has become the *de facto* best available method for determining hVISA status and investigating the clinical relevance of hVISA strains. Determining population analysis profiles is labor intensive and not suitable for routine clinical laboratory application. Unfortunately, there is no standardized technique at this time that is convenient and reliable for detecting hVISA strains. The inability of both automated commercial systems and standard reference susceptibility testing methods to detect the hVISA phenotype makes it impossible to identify all patients whose infections may not respond to vancomycin therapy because they may be caused by hVISAs.

3.9.1.7.4 Reporting

Vancomycin-susceptible staphylococci should be reported following the laboratory's routine reporting protocols. For strains determined to be vancomycin nonsusceptible (ie, those with MICs ≥4 µg/mL for *S. aureus* and MICs ≥ 8 µg/mL for CoNS), preliminary results should be reported following routine reporting protocols. Send any *S. aureus* with MICs ≥ 8 µg/mL or CoNS with MICs ≥ 32 µg/mL to a reference laboratory; final results should be reported after confirmation by a reference laboratory and public health authorities should be notified according to local recommendations. See M100[1] Table 2C and M100[1] Appendix A for the most current recommendations for testing and reporting.

3.9.1.8 Inducible Clindamycin Resistance

Inducible clindamycin resistance can be identified using the method described in Subchapter 3.10.1.

3.9.1.9 Linezolid Resistance

When testing linezolid by disk diffusion, zones should be examined using transmitted light after incubation for 16 to 18 hours at 35°C±2°C.

3.9.1.10 Mupirocin Resistance

High-level mupirocin resistance (ie, MICs ≥512 µg/mL) occurs in *S. aureus* and is associated with the presence of the plasmid-mediated *mupA* gene.[31-33] Use of mupirocin has been reported to increase rates of

high-level mupirocin resistance in *S. aureus*.[34] High-level mupirocin resistance can be detected using either disk diffusion or broth microdilution tests (see M100[1] Table 3H).[35] For disk diffusion using a 200-µg mupirocin disk, incubate the test a full 24 hours and read carefully for any haze or growth using transmitted light. No zone of inhibition = the presence of high-level mupirocin resistance; any zone of inhibition = the absence of high-level resistance. In a recent study, the majority of *mupA*-negative isolates demonstrated mupirocin 200-µg zone diameters > 18 mm. For broth microdilution testing, an MIC of ≥512 µg/mL = high-level mupirocin resistance; MICs ≤256 µg/mL = the absence of high-level resistance. For dilution testing, a single well containing 256 µg/mL of mupirocin may be tested. For the one-concentration test, growth = high-level mupirocin resistance; no growth = the absence of high-level resistance.

### 3.9.2 Enterococci

#### 3.9.2.1 Penicillin/Ampicillin Resistance

Enterococci may be resistant to penicillin and ampicillin because of production of low-affinity PBPs or, very rarely, because of the production of β-lactamase. Agar or broth dilution MIC testing and disk diffusion testing accurately detect isolates with altered PBPs, but do not reliably detect isolates that produce β-lactamase. β-lactamase–producing strains of enterococci are detected best by using a direct, nitrocefin-based, β-lactamase test (see Subchapter 3.10.2.2). Because of the rarity of β-lactamase-positive enterococci, this test does not need to be performed routinely but can be used in selected cases. A positive β-lactamase test predicts resistance to penicillin, and amino-, carboxy-, and ureidopenicillins.

Strains of enterococci with ampicillin and penicillin MICs ≥ 16 µg/mL are categorized as resistant. However, enterococci with low levels of penicillin (MICs 16 to 64 µg/mL) or ampicillin (MICs 16 to 32 µg/mL) resistance 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, see Subchapter 3.9.2.4) if high doses of penicillin or ampicillin are used. Enterococci possessing higher levels of penicillin (MICs ≥128 µg/mL) or ampicillin (MICs ≥64 µg/mL) resistance may not be susceptible to the synergistic effect.[36,37] Physicians' requests to determine the actual MIC of penicillin or ampicillin for blood and CSF isolates of enterococci should be considered.

#### 3.9.2.2 Vancomycin Resistance

Accurate detection of vancomycin-resistant enterococci (VRE) by the agar or broth dilution MIC testing requires incubation for a full 24 hours (rather than 16 to 20 hours) and careful examination of the plates, tubes, or wells for evidence of faint growth before reporting as susceptible. With disk diffusion, zones should be examined using transmitted light; the presence of a haze or any growth within the zone of inhibition indicates resistance. Organisms with intermediate zones should be tested by an MIC method as described in CLSI document M07.[3] For isolates for which the vancomycin MICs are 8 to 16 µg/mL, perform biochemical tests for identification as listed under the "Vancomycin MIC ≥8 µg/mL" test found in M100[1] Table 3F. A vancomycin agar screen test may also be used, as described in Subchapter 3.9.2.3 and in M100[1] Table 3F.

#### 3.9.2.3 Vancomycin Agar Screen

The vancomycin agar screening-plate procedure can be used in addition to the dilution methods described in Subchapter 3.9.2.2 for the detection of VRE. Perform the test using the following procedure by inoculating an enterococcal isolate onto BHI agar containing 6 µg/mL of vancomycin/mL.[38]

| Step | Action | Comment |
|---|---|---|
| 1. | Prepare a direct colony suspension equivalent to a 0.5 McFarland standard as is done for MIC or disk diffusion testing. | |
| 2. | Inoculate the plate using either a 1- to 10-µL loop or a swab.<br><br>Loop: Spread the inoculum in an area 10–15 mm in diameter.<br><br>Swab: Express as is done for the disk diffusion test and then spot an area at least 10–15 mm in diameter. | |
| 3. | Incubate the plate at 35°C ± 2°C in ambient air for a full 24 hours and examine carefully, using transmitted light, for evidence of growth, including small colonies (> 1 colony) or a film of growth, indicating vancomycin resistance. | Do not reuse plates after incubation.<br><br>Refer to M100[1] Table 3F. |

Abbreviation: MIC, minimal inhibitory concentration.

3.9.2.4 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.[36] Agar or broth high-concentration gentamicin (500 µg/mL) and streptomycin (1000 µg/mL with broth microdilution; 2000 µg/mL with agar) tests or disk diffusion tests using high concentration gentamicin (120 µg) and streptomycin (300 µg) disks can be used to screen for this type of resistance (see M100[1] Table 3I). QC of these tests is also explained in M100[1] Table 3I. Other aminoglycosides do not need to be tested, because their activities against enterococci are not superior to gentamicin or streptomycin. In addition, high-level resistance to both gentamicin and streptomycin implies that resistance will be found for all aminoglycosides, making testing of additional aminoglycosides unnecessary.

### 3.9.3 Gram-Negative Bacilli

The major mechanism of resistance to β-lactam antimicrobial agents in gram-negative bacilli is production of β-lactamase enzymes. Many different types of enzymes have been reported. β-lactamases may be named after the primary substrates that they hydrolyze, the biochemical properties of the β-lactamases, strains of bacteria from which the β-lactamase was detected, a patient from whom a β-lactamase–producing strain was isolated, etc.[39] For example, TEM is an abbreviation for Temoneira, the first patient from whom a TEM β-lactamase–producing strain was reported. β-lactamases may be classified as molecular Class A, B, C, or D enzymes, as shown in Table 3, below.[40]

**Table 3. Enzyme Classification for β-Lactamases**

| Class | Active Site | Examples |
|---|---|---|
| A | Inhibitor-susceptible (rare exceptions) | TEM-1, SHV-1, KPC, OXY, and most ESBLs (including CTX-M) |
| B | Metallo-β-lactamases | Metalloenzymes; VIM, IMP, SPM, NDM |
| C | Inhibitor-resistant β-lactamases | AmpC |
| D | Oxacillin-active β-lactamases that may be inhibitor susceptible | OXA (including rare ESBL and carbapenemase phenotypes) |

Abbreviations: ESBL, extended-spectrum β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase.

β-lactamase enzymes in all four classes inactivate β-lactam antimicrobial agents at different rates. The genes encoding β-lactamases may be located on chromosomes and expressed with or without induction or carried on plasmids in single or multiple copies. An isolate may produce β-lactamases and possess other resistance mechanisms such as porin mutations that restrict antimicrobial access to their active binding sites in the bacterial cell. The variety of β-lactam resistance mechanisms encountered in gram-negative bacteria gives rise to a continuum of antimicrobial activities expressed as a range of MIC values. One would expect the interpretive criteria to be the MIC or zone diameter value that differentiates β-lactamase/other resistance mechanism–negative strains (susceptible) from β-lactamase/other resistance mechanism–positive strains (resistant). However, weak β-lactamase activity or low-level β-lactamase expression may not necessarily mean that the isolate will be refractory to β-lactam therapy. In practice, some isolates that are interpreted as susceptible will produce β-lactamases that have clinically inconsequential enzyme activity. These may be ESBL, AmpC, or carbapenemase-type enzymes as described in Subchapters 3.9.3.1, 3.9.3.2, and 3.9.3.3, respectively.

Identification of a specific β-lactamase resistance mechanism (eg, ESBL, KPC, NDM) is not required or necessary for the determination of a susceptible or resistant interpretation. However, the identification of a specific enzyme may be useful for infection control procedures or epidemiological investigations. M100[1] Table 3A describes tests that can be used to screen for and confirm the presence of ESBLs in *E. coli, K. pneumoniae, Klebsiella oxytoca,* and *P. mirabilis.* M100[1] Tables 3B and 3C describe tests that can be used to screen for and confirm carbapenemase production in *Enterobacteriaceae.*

### 3.9.3.1 Extended-Spectrum β-Lactamases

ESBLs are Class A and D enzymes that hydrolyze expanded-spectrum cephalosporins and monobactams and are inhibited by clavulanate. These enzymes arise by mutations in genes for common plasmid-encoded β-lactamases, such as TEM-1, SHV-1, and OXA-10, or they 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 *K. pneumoniae, K. oxytoca, E. coli, P. mirabilis,*[41] and other genera of the family *Enterobacteriaceae.*[40] β-lactam interpretive criteria are set at MIC and zone diameter values that recognize ESBL activity, in combination with other resistance mechanisms, likely to predict clinical success or failure. Most ESBL-negative gram-negative bacilli will test susceptible; however, some strains that test susceptible may contain ESBL genes producing low amounts of enzyme or enzyme that has poor hydrolytic activity. These strains are categorized correctly as susceptible when using current CLSI interpretive criteria published in M100.[1]

A similar native enzyme, OXY (formerly KOXY or K1), in *K. oxytoca* acts as an extended-spectrum penicillinase, inactivating amino- and carboxypenicillins. Overproduction of OXY enzymes due to promoter mutations results in resistance to ceftriaxone and aztreonam (but not ceftazidime), as well as resistance to all combinations of β-lactams and β-lactamase inhibitors. Although strains producing OXY enzymes may result in a positive ESBL confirmatory test, OXY enzymes are generally not considered ESBLs. MIC and zone diameter interpretive criteria correctly predict susceptibility and resistance.

### 3.9.3.2 AmpC Enzymes

The AmpC β-lactamases are chromosomal or plasmid-encoded enzymes.[42] Isolates that produce AmpC enzymes have a similar antimicrobial susceptibility profile to those that produce ESBLs in that they show reduced susceptibility to penicillins, cephalosporins, and aztreonam. However, in contrast to ESBLs, AmpC β-lactamases also inactivate cephamycins, ie, bacteria expressing AmpC enzyme test as resistant to cefoxitin and cefotetan. In addition, AmpC-producing strains are resistant to the current β-lactamase inhibitor combination agents and may test resistant to carbapenems if accompanied by a porin mutation or in combination with overexpression of specific efflux pumps.

Chromosomal AmpC β-lactamases are found in *Enterobacter, Citrobacter, Serratia,* and some other gram-negative species, and are usually expressed in low amounts but can be induced to produce higher amounts by penicillins, carbapenems, and some cephems such as cefoxitin. The expanded-spectrum cephalosporins (cephalosporin subclasses III and IV) do not induce AmpC enzymes but can be hydrolyzed by them. Use of cephalosporins also may select for stably derepressed chromosomal mutants, which can emerge during therapy.[43]

AmpC enzymes can be carried on plasmids that are transmissible among bacteria. Although plasmid-mediated AmpC enzymes evolved from native chromosomal enzymes among a diverse group of bacteria, they are found primarily in clinical isolates of *K. pneumoniae* and *E. coli.*

There is no CLSI-validated phenotypic test to confirm the presence of plasmid-encoded AmpC β-lactamases in clinical isolates. Strains carrying both ESBLs and plasmid-encoded AmpC β-lactamases are common in some geographical regions. The current interpretive criteria for drugs affected by these combinations of enzymes are the best approach for providing guidance for treatment of these strains.

3.9.3.3 Carbapenemases (Carbapenem-Resistant Gram-Negative Bacilli)

Carbapenemase activity in clinical isolates of gram-negative bacilli occurs as a result of β-lactamase enzymes in Classes A, B, and D. In *Enterobacteriaceae,* KPC- and SME-type enzymes[44,45] within Class A; NDM, VIM, and IMP-type enzymes within Class B; and OXA-type enzymes within Class D represent major families of clinical importance (see Table 4, below). In *Acinetobacter* spp., both Class B and D enzymes occur, but in *P. aeruginosa,* only Class B enzymes are important. NDM-type and other metallo-β-lactamase enzymes require zinc for activity and are inhibited by substances such as EDTA, which binds zinc. *Stenotrophomonas maltophilia, Bacillus anthracis,* and some strains of *Bacteroides fragilis* produce a chromosomal metallo-β-lactamase. Other metalloenzymes may be carried on plasmids and can occur in *Acinetobacter* spp., *P. aeruginosa, Serratia marcescens, K. pneumoniae,* and, increasingly, in other *Enterobacteriaceae.*

The presence of carbapenemase activity in *Enterobacteriaceae* can be confirmed using the modified Hodge test (MHT) as described in M100[1] Tables 3B and 3B-1. The sensitivity and specificity of the MHT for detecting other carbapenemase production can vary. The test is a very sensitive method of detecting KPC-type enzymes but false-negative results or weak reactions can occur with isolates producing an NDM-type enzyme. In addition, MHT-positive results may be encountered in isolates with carbapenem resistance mechanisms other than carbapenemase production (eg, *Enterobacter* spp. with hyperproduction of AmpC and porin loss). Carbapenemase activity can also be detected in *Enterobacteriaceae, P. aeruginosa,* and *Acinetobacter* spp. using the Carba NP colorimetric microtube assay[45-51] as described in M100[1] Tables 3C and 3C-1. Both the MHT and the Carba NP test detect carbapenemase production, but neither identifies which carbapenemase is present.

There is no CLSI-validated phenotypic test to confirm the presence of metallo-β-lactamases in clinical isolates. Current interpretive criteria for drugs affected by these carbapenemases, first published in 2010 in M100-S20-U, are the recommended approach for providing guidance for treatment of infection by *Enterobacteriaceae* containing OXA-, KPC- and NDM-type enzymes. For confirmation of carbapenemase activity in *P. aeruginosa* and *Acinetobacter* spp., the use of the Carba NP or a molecular test is the recommended approach.

Refer to M100[1] Tables 3B and 3C for the most current recommendations for testing and reporting.

Table 4. β-Lactamases With Carbapenemase Activity

| β-Lactamase Class* | Found in | Examples |
|---|---|---|
| A | *K. pneumoniae* and other *Enterobacteriaceae* | KPC, SME |
| B | *Enterobacteriaceae*<br>*P. aeruginosa*<br>*Acinetobacter baumannii* | Metallo-β-lactamases inhibited by EDTA (IMP, VIM, NDM) |
| D | *A. baumannii*<br>*Enterobacteriaceae* | OXA |

*Carbapenemases have not yet been found in Class C.
Abbreviations: EDTA, ethylenediaminetetraacetic acid; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase.

### 3.9.4 *Streptococcus pneumoniae*

#### 3.9.4.1 Penicillin and Third-Generation Cephalosporin Resistance

Penicillin, and cefotaxime, ceftriaxone, or meropenem, should be tested by an MIC method and reported routinely with CSF isolates of *S. pneumoniae.* Such isolates can also be tested against vancomycin using the MIC or disk method. Consult M100[1] Table 2G for reporting of penicillins and third-generation cephalosporins, because there are specific interpretive criteria that must be used depending on the site of infection and the penicillin formulation used for therapy. In M100[1] Table 2G, breakpoints are listed for intravenous penicillin therapy for meningitis and nonmeningitis infections. Separate breakpoints are included for therapy of less severe infections with oral penicillin.

Amoxicillin, ampicillin, cefepime, cefotaxime, ceftriaxone, cefuroxime, ertapenem, imipenem, and meropenem may be used to treat pneumococcal infections; however, reliable disk diffusion susceptibility tests with these agents do not yet exist. Their *in vitro* activity is best determined using an MIC method.

## 3.10 Screening Tests

Screening tests, as described in this document and in M100,[1] characterize an isolate as susceptible or resistant to one or more antimicrobial agents based on a specific resistance mechanism or phenotype. Some screening tests have sufficient sensitivity and specificity such that results of the screen can be reported without additional testing. Others require further testing to confirm the presumptive results obtained with the screen test. The details for each screening test, including test specifications, limitations, and additional tests needed for confirmation, are provided in M100[1] Instructions for Use of Tables, Section VII, and in M100[1] Table 3 locations specified there.

For screening tests, a positive (resistant) and negative (susceptible) QC strain should be tested with each new lot/shipment of disks, or agar plates used for agar dilution, or single wells or tubes used with broth dilution methods. Subsequently, weekly QC testing of the negative control (susceptible strain) is sufficient if the screening test is performed at least once a week and criteria for converting from daily to weekly QC testing have been met (see Subchapter 4.7.2). QC of screening tests with the negative control (susceptible strain) is recommended each day of testing if the test is not performed routinely (ie, at least once a week) or if the antimicrobial agent is labile (eg, oxacillin agar screen for *S. aureus*).

### 3.10.1 Inducible Clindamycin Resistance

Macrolide-resistant isolates of *S. aureus,* coagulase-negative *Staphylococcus* spp., *S. pneumoniae,* and β-hemolytic streptococci may express constitutive or inducible resistance to clindamycin (methylation of the 23S ribosomal RNA 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 *msr*(A) gene in staphylococci or a *mef* gene in streptococci). Infections caused by both staphylococci and streptococci with inducible clindamycin resistance may fail to respond to clindamycin therapy.[52,53] Inducible clindamycin resistance can be detected for all staphylococci, *S. pneumoniae,* and β-hemolytic streptococci using a disk diffusion test.

Using disk diffusion, the test is performed by placing a 2-µg clindamycin disk either 15 to 26 mm away (for staphylococci) or 12 mm away (for streptococci) from the edge of a 15-µg erythromycin disk on a standard blood agar plate used for the inoculum purity check or by using the standard disk diffusion procedure with MHA. Flattening of the clindamycin zone adjacent to the erythromycin disk (referred to as a D-zone) indicates inducible clindamycin resistance. Following incubation (16 to 18 hours for staphylococci and 20 to 24 hours for streptococci), organisms that do not show flattening of the clindamycin zone adjacent to the erythromycin disk in an erythromycin-resistant isolate should be reported as tested (eg, susceptible or intermediate to clindamycin). Organisms that show flattening of the clindamycin zone adjacent to the erythromycin disk (referred to as a D-zone) after incubation have inducible clindamycin resistance. Recommendations for QC and the most recent updates for testing are provided in M100[1] Tables 3G, 4A, and 4B.

Inducible clindamycin resistance can also be detected in these organisms with the broth microdilution test using a combination of erythromycin and clindamycin placed together in a single well (see CLSI document M07[3] Subchapter 3.14.1, and M100[1] Table 3G).

### 3.10.2 β-Lactamase Tests

#### 3.10.2.1 Purpose

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

A positive β-lactamase test result predicts:

- Resistance to penicillin, ampicillin, and amoxicillin among *Haemophilus* spp. and *N. gonorrhoeae*
- 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.

#### 3.10.2.2 Selecting a β-Lactamase Test

Detection of β-lactamase in *S. aureus* is most accurately accomplished by using the penicillin disk diffusion zone-edge test (see Subchapter 3.9.1.1 and M100[1] Table 3D). Nitrocefin-based tests can be used for *S. aureus,* but negative results should be confirmed with the penicillin zone-edge test before reporting penicillin as susceptible. Nitrocefin-based tests are the preferred method for testing CoNS, enterococci, *Haemophilus* spp., *M. catarrhalis,* and *N. gonorrhoeae*[54] but require induction of the enzyme in CoNS including *S. lugdunensis.* Induction can be easily accomplished by testing the growth from the zone margin surrounding a cefoxitin disk test. 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.* 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 (see the manufacturer's recommendations for commercial tests).

## 3.11 Limitations of Disk Diffusion Methods

### 3.11.1 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* complex, and *S. maltophilia,* and they have been modified for testing fastidious organisms such as *H. influenzae* and *H. parainfluenzae* (see M100[1] Table 2E), *N. gonorrhoeae* (see M100[1] Table 2F), *N. meningitidis* (see M100[1] Table 2I), and streptococci (see M100[1] Tables 2G, 2H-1, and 2H-2). For organisms excluded from M100[1] Tables 2A through 2I and not covered in other CLSI guidelines or standards, such as CLSI document M45,[5] 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 diseases 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 is the most appropriate testing method, and this may require submitting the organism to a reference laboratory.

### 3.11.2 Warning

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:

- First- and second-generation cephalosporins, cephamycins, and aminoglycosides against *Salmonella* and *Shigella* spp.
- Penicillins, β-lactam/β-lactamase inhibitor combinations, antistaphylococcal cephems (except for cephalosporins with anti-MRSA activity), and carbapenems against oxacillin-resistant *Staphylococcus* spp.
- Aminoglycosides (except high concentrations), cephalosporins, clindamycin, and trimethoprim-sulfamethoxazole against *Enterococcus* spp.

In addition, some bacterial species have inherent or innate antimicrobial resistance, which is reflected in wild-type antimicrobial patterns of all or almost all representatives of a species. Intrinsic resistance is so common that susceptibility testing is unnecessary. For example, *Citrobacter* species are intrinsically resistant to ampicillin. A small percentage (1% to 3%) may appear susceptible due to method variation, mutation, or low levels of resistance expression. Testing of such species is unnecessary, but, if tested, a "susceptible" result should be viewed with caution. Refer to the intrinsic resistance tables in M100[1] Appendix B for a list of such species and the antimicrobial agents to which they are resistant.

### 3.11.3 Development of Resistance and Testing of Repeat Isolates

Isolates that are initially susceptible may become intermediate or resistant after initiation of therapy. Therefore, subsequent isolates of the same species from a similar body site should be tested in order to detect resistance that may have developed. This can occur within as little as three to four days and has been noted most frequently in *Enterobacter, Citrobacter,* and *Serratia* spp. with third-generation

cephalosporins; in *P. aeruginosa* with all antimicrobial agents; and in staphylococci with quinolones. For *S. aureus*, vancomycin-susceptible isolates may become vancomycin intermediate during the course of prolonged therapy.

In certain circumstances, testing of subsequent isolates to detect resistance that may have developed might be warranted earlier than within three to four days. The decision to do so requires knowledge of the specific situation and the severity of the patient's condition (eg, an isolate of *Enterobacter cloacae* from a blood culture on a premature infant). Laboratory guidelines on when to perform susceptibility testing on repeat isolates should be determined after consultation with the medical staff.

# Chapter 4: Quality Control and Quality Assurance

This chapter includes:

- An overview of the purpose of a QC and a QA program
- Information regarding the responsibility of both the manufacturer and user to ensure testing materials and reagents are maintained properly
- Description of the selection, maintenance, and testing of QC strains
- The recommended frequency for performing QC testing including the amount of testing required to reduce QC frequency from daily to weekly
- Suggestions for troubleshooting out-of-range results with QC strains
- Factors to consider before reporting patient results when out-of-range QC results are observed
- Guidance for confirming noteworthy or uncommon results encountered when testing patient isolates

## 4.1 Purpose

QC includes the procedures to monitor the test system to ensure accurate and reproducible results. This is achieved by, but not limited to, the testing of carefully selected QC strains with known susceptibility to the antimicrobial agents tested. The goals of a QC program are to monitor:

- Precision (reproducibility) and accuracy of susceptibility test procedures
- Performance of reagents used in the tests
- Performance of persons who carry out the tests and report the results

A comprehensive QA program helps to ensure that testing materials and processes consistently provide quality results. QA includes, but is not limited to, monitoring, evaluating, taking corrective actions (if necessary), recordkeeping, calibration and maintenance of equipment, proficiency testing, training, competency assessment, and QC.

## 4.2 Quality Control Responsibilities

Although this subchapter is intended to apply to the standard reference methods, it may be applicable to certain commercially available antimicrobial susceptibility test systems that are based primarily or in part on methods described in M02.

Manufacturers and users of antimicrobial susceptibility tests have a shared responsibility for quality. The primary purpose of QC testing performed by manufacturers (in-house reference methods or commercial methods) is to ensure that the testing materials and reagents have been appropriately manufactured. The primary purpose of QC testing performed by laboratories (users) is to ensure that the testing materials and reagents are maintained properly and testing is performed according to established protocols.

A logical division of responsibility and accountability may be described as follows:

- Manufacturers (in-house or commercial products):
  - Antimicrobial labeling
  - Potency of antimicrobial disks
  - Antimicrobial stability
  - Compliance with good manufacturing practices (eg, quality management system standards)
  - Integrity of product
  - Accountability and traceability to consignee

- Laboratories (users):
  - Storage under the environmental conditions recommended by the manufacturer (to prevent drug deterioration)
  - Proficiency of personnel performing tests
  - Use of current CLSI standards (or manufacturers' instructions for use) and adherence to the established procedure (eg, inoculum preparation, incubation conditions, determination of end points, interpretation of results)

Manufacturers should design and recommend a QC program that allows users to evaluate those variables (eg, inoculum density, storage/shipping conditions) that are most likely to adversely affect test results and to determine that the test results are accurate and reproducible when the test is performed according to established protocols.

**NOTE:** Laboratories in the United States should familiarize themselves with new QC requirements of the Clinical Laboratory Improvement Amendments (http://www.cms.gov). Within these requirements is an option to develop an individualized QC plan, or IQCP. Such a plan is based upon a risk assessment for each laboratory. This risk assessment is described in CLSI document EP23™.[55]

### 4.3 Selection of Strains for Quality Control

Each QC strain should be obtained from a recognized source (eg, ATCC®). All CLSI-recommended QC strains appropriate for the antimicrobial agent and reference method should be evaluated and produce results within the expected ranges listed in M100[1] that were established according to the procedures described in CLSI document M23.[2] Users of commercial systems should follow the QC recommendations in that system's instructions for use.

QC strains and their characteristics are described in Appendix D. Some of these are listed as "routine QC strains" and others as "supplemental QC strains."

QC strains are tested regularly (eg, daily or weekly) to ensure the test system is working and produces results that fall within specified limits listed in M100.[1] The QC strains recommended in Appendix D and in M100[1] should be included if a laboratory performs CLSI reference disk diffusion testing as described herein.

Supplemental QC strains are used to assess particular characteristics of a test or test system in select situations or may represent alternative QC strains. For example, *H. influenzae* ATCC® 10211 is more fastidious than *H. influenzae* ATCC® 49247 or *H. influenzae* ATCC® 49766, and is used to ensure HTM can adequately support the growth of clinical isolates of *H. influenzae* and *H. parainfluenzae*. Supplemental QC strains may possess susceptibility or resistance characteristics specific for one or more special tests listed in CLSI documents M02, M07,[3] and M100.[1] For example, *S. aureus* ATCC® BAA-976 and *S. aureus* ATCC® BAA-977 are used for supplemental QC of tests for inducible clindamycin resistance. Supplemental QC strains can be used to assess a new test, for training new personnel, and for

competency assessment. It is not necessary to include supplemental QC strains in routine daily or weekly AST QC programs.

Expected QC ranges for routine QC strains are listed in M100[1] Tables 4A and 4B. When recommended, acceptable QC ranges for supplemental QC strains are also included in M100[1] Tables 3, which describe screening and confirmatory tests for specific resistance mechanisms.

## 4.4 Maintenance and Testing of Quality Control Strains

Proper organism storage and maintenance is required to ensure acceptable performance of QC strains (also refer to Appendix E).

For long-term storage, maintain stock cultures at −20°C or below (preferably at −60°C or below or in liquid nitrogen) in a suitable stabilizer (eg, 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. **NOTE:** Some QC strains, particularly those with plasmid-mediated resistance (eg, *E. coli* ATCC® 35218), have been shown to lose the plasmid when stored at temperatures above −60°C.

1. Subculture frozen or freeze-dried stock cultures onto appropriate media (eg, tryptic soy or blood agar for nonfastidious QC strains, or enriched chocolate or blood agar for fastidious QC strains) and incubate under the appropriate conditions for the organism. This subculture is designated the F1 subculture. ("F" relates to the "frozen" or "freeze-dried" state of the stock culture; "1" indicates the first passage and "2" the second passage from the stock culture.)

2. From the F1 subculture, prepare an F2 subculture to use for testing QC strains or use the F2 subculture to prepare subsequent subcultures (F3) for testing QC strains as outlined in Appendix E.

3. Store F1 and F2 subcultures at 2 to 8°C or as appropriate for the organism type.

4. Use agar plates (and not broth or agar slants) for preparing the F2 or F3 subcultures that will be used for inoculum suspension preparation. Streak to obtain isolated colonies and always use fresh subcultures (eg, overnight incubation) for inoculum suspension preparation.

5. Prepare a new F2 subculture each week and then prepare F3 subcultures from this F2 subculture for up to seven days; prepare a new F2 subculture on day 8.

6. Prepare F1 subcultures at least monthly from frozen or freeze-dried stock cultures (eg, subculture for F2 subcultures each week for no more than three successive weeks). Some strains may require preparation of a new F1 subculture more frequently (eg, every two weeks).

7. Review the footnotes in Appendix D that highlight special requirements for handling certain QC strains.

If an unexplained QC error occurs that might be due to a change in the organism's inherent susceptibility or resistance, prepare a new F1 subculture or obtain a fresh stock culture of the QC strain from an external source. See Subchapter 4.8 for additional guidance.

Always test the QC strains using the same materials and methods that are used to test clinical isolates.

## 4.5 Batch or Lot Quality Control

1. Test each new batch, shipment, or lot of MHA or supplemented MHA plates or disks with the appropriate QC strains before or concurrent with their first use for testing patient isolates. If zone

diameter measurements do not fall within the acceptable ranges (see M100[1] Tables 4A and 4B), proceed with corrective action.

2. For MHA or supplemented MHA plates prepared in-house, incubate at least one uninoculated agar plate from each batch or lot overnight to confirm sterility of the medium.

Maintain records to include, at a minimum, the lot numbers, expiration dates, and date of use of all materials and reagents used in performing susceptibility tests.

## 4.6 Zone Diameter Quality Control Ranges

Acceptable zone diameter QC ranges for a single QC test (single-drug/single-organism combination) are listed in M100[1] Tables 4A and 4B. These ranges are developed following specific protocols outlined in CLSI document M23[2].

## 4.7 Frequency of Quality Control Testing (also refer to Appendix A and M100[1] Table 4C)

Test the appropriate QC strains each day the test is performed on patient isolates. Alternatively, adopt one of the two plans to demonstrate acceptable performance in order to reduce the frequency of disk diffusion QC tests to weekly. Either plan allows a laboratory to perform weekly QC testing once satisfactory performance with daily testing of QC strains is documented (see Subchapters 4.7.2.1 and 4.7.2.2). The weekly QC testing options are not applicable when disk diffusion tests are performed less than once a week.

### 4.7.1 Daily Quality Control Testing

A laboratory can perform QC testing daily. Daily (vs weekly) QC testing must be performed each day patient isolates are tested if disk diffusion tests are performed less than once a week.

"Daily QC testing" or testing on "consecutive test days" means testing of QC strains each day disk diffusion tests are performed on patient isolates. It does not refer to calendar days.

### 4.7.2 Performance Criteria for Reducing Quality Control Frequency to Weekly

Two plans are available to demonstrate satisfactory performance with daily QC testing before going to weekly QC testing. These include: 1) the 20- or 30-day plan or 2) the 15-replicate (3 × 5 day) plan.

4.7.2.1 The 20- or 30-Day Plan

- Test all applicable QC strains for 20 or 30 consecutive test days and document results.
- Follow recommended actions as described in Appendix A.
- If no more than one out of 20 or three out of 30 zone diameter measurements for each antimicrobial agent/organism combination are outside the acceptable zone diameter QC range listed in M100[1] Tables 4A and 4B, it is acceptable to go to weekly QC testing.
- If completion of the 20- or 30-day plan is unsuccessful, take corrective action as appropriate, and continue daily QC testing.

- If a laboratory is routinely testing QC strains each day of use and desires to convert to a weekly QC plan, it is acceptable to retrospectively analyze QC data from consecutive tests available during the previous two years, providing no aspects of the test system have changed.

4.7.2.2 The 15-Replicate (3 × 5 Day) Plan

- Test three replicates of each applicable QC strain using individual inoculum preparations for five consecutive test days and document results.
- Follow recommended actions as described in Appendix A and Table 5, below.
- Upon successful completion of the 15-replicate (3 × 5 day) plan, it is acceptable to go to weekly QC testing.
- If completion of the 15-replicate (3 × 5 day) plan is unsuccessful, take corrective action as appropriate, and continue daily QC testing.

**Table 5. 15-Replicate (3 × 5 Day) Plan: Acceptance Criteria and Recommended Action***

| Number Out of Range With Initial Testing (Based on 15 Replicates) | Conclusion From Initial Testing (Based on 15 Replicates) | Number Out of Range After Repeat Testing (Based on All 30 Replicates) | Conclusion After Repeat Testing |
|---|---|---|---|
| 0–1 | Plan is successful. Convert to weekly QC testing. | N/A | N/A |
| 2–3 | Test another 3 replicates for 5 days. | 2–3 | Plan is successful. Convert to weekly QC testing. |
| ≥4 | Plan fails. Investigate and take corrective action as appropriate. Continue QC each test day. | ≥4 | Plan fails. Investigate and take corrective action as appropriate. Continue QC each test day. |

*Assess each QC strain/antimicrobial agent combination separately.
Abbreviations: N/A, not applicable; QC, quality control.

For background information that supports the 3 × 5 day plan, refer to the CLSI AST Subcommittee webpage at www.clsi.org for Statisticians' Summary for Alternative QC Frequency Testing Proposal.

4.7.2.3 Implementing Weekly Quality Control Testing

- Weekly QC testing may be implemented once satisfactory performance with daily QC testing has been documented (see Subchapters 4.7.2.1 and 4.7.2.2).
- Perform QC testing once per week and whenever any reagent component of the test (eg, 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 QC results are out of range, take corrective action.
- Refer to M100[1] Table 4C for guidance on QC frequency with new materials or test modifications.

## 4.8 Out-of-Range Results With Quality Control Strains and Corrective Action

Out-of-range QC results can be categorized into those that are 1) random, 2) identifiable, or 3) system related.

QC ranges are established to include ≥95% of results obtained from routine testing of QC strains. A small number of (random) out-of-range QC results may be obtained even when the test method is performed correctly and materials are maintained according to recommended protocols. Such occurrences are due to chance.

Out-of-range results with QC strains due to random or identifiable errors can usually be resolved by a single repeat of the QC test. However, out-of-range QC results that are due to a problem with the test system usually do not correct when the QC test is repeated and may indicate a serious problem that can adversely affect patient results. Every out-of-range QC result must be investigated.

**NOTE:** See disk diffusion Troubleshooting Guide in M100[1] Table 4D for troubleshooting and corrective action for out-of-range results with QC strains.

### 4.8.1 Daily or Weekly Quality Control Testing – Out-of-Range Result Due to Identifiable Error

If the reason for an out-of-range result can be identified and easily corrected, correct the problem, document the reason, and retest the QC strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.

Identifiable reasons for the out-of-control results may include, but are not limited to:

- QC strain
  - Use of the wrong QC strain
  - Improper storage
  - Inadequate maintenance (eg, use of the same F2 subculture for > 1 month)
  - Contamination
  - Nonviability
  - Changes in the organism (eg, mutation, loss of plasmid)

- Testing supplies
  - Improper storage or shipping conditions
  - Contamination
  - Use of a defective agar plate (eg, too thick or too thin)
  - Use of damaged (eg, cracked) plates
  - Use of expired materials

- Testing process
  - Inoculum suspensions incorrectly prepared or adjusted
  - Inoculum prepared from a plate incubated for the incorrect length of time
  - Inoculum prepared from differential or selective media containing antimicrobial agents or other growth-inhibiting compounds
  - Use of the wrong incubation temperature or conditions
  - Use of wrong disk, ancillary supplies
  - Improper disk placement (eg, inadequate contact with the agar) or disk falls off agar
  - Incorrect reading or interpretation of test results
  - Transcription error

- Equipment
  - Not functioning properly or out of calibration (eg, pipettes)

### 4.8.2 Daily Quality Control Testing – Out-of-Range Result Not Due to Identifiable Error

Perform corrective action if results for a QC strain/antimicrobial agent combination are out of range and the error is not identifiable on two consecutive days of testing or if more than three results for a QC strain/antimicrobial agent combination are out of range during 30 consecutive days of testing (see QC ranges listed in M100[1] Tables 4A and 4B).

### 4.8.3 Weekly Quality Control Testing – Out-of-Range Result Not Due to Identifiable Error

If the reason for the out-of-range result with the QC strain cannot be identified, perform corrective action, as follows, to determine if the error is random.

- Test the out-of-range antimicrobial agent/organism combination on the day the error is observed or as soon as an F2 or F3 subculture of the QC strain is available.

- If the repeat results are in range, evaluate all QC results available for the antimicrobial agent/organism combination when using the same lot numbers of materials that were used when the out-of-range QC result was observed. If five acceptable QC results are available, no additional days of QC testing are needed. The following tables illustrate two scenarios that might be encountered and suggested actions:

**Scenario #1:**

Ampicillin *E. coli* ATCC® 25922; acceptable range: 15 to 22 mm

| Week | Day | Lot Number (Disks) | Lot Number (MHA) | Result | Action |
|---|---|---|---|---|---|
| 1 | 1 | 3564 | 16481 | 18 | |
| 2 | 1 | 3564 | 16481 | 19 | |
| 3 | 1 | 3564 | 16481 | 18 | |
| 4 | 1 | 3564 | 16481 | 19 | |
| 5 | 1 | 3564 | 16481 | 14 | Out of range. Repeat QC same day. |
| 5 | 2 | 3564 | 16481 | 17 | In range. Five acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 3564 and MHA lot 16481. Resume weekly QC testing. |

Abbreviations: ATCC®, American Type Culture Collection; MHA, Mueller-Hinton agar; QC, quality control.

**Conclusion:** Random QC error.

**Scenario #2:**

Ampicillin *E. coli* ATCC® 25922; acceptable range: 15 to 22 mm

| Week | Day | Lot Number (Disks) | Lot Number (MHA) | Result | Action |
|---|---|---|---|---|---|
| 1 | 1 | 9661 | 16922 | 18 | |
| 2 | 1 | 9661 | 16922 | 19 | |
| 3 | 1 | 9661 | 16922 | 14 | Out of range. Repeat QC same day. |
| 3 | 2 | 9661 | 16922 | 18 | In range. Three acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 9661 and MHA lot 16922. Repeat QC 2 more consecutive days. |
| 3 | 3 | 9661 | 16922 | 18 | In range. |
| 3 | 4 | 9661 | 16922 | 17 | In range. Five acceptable in-range QC tests for *E. coli* ATCC® 25922 with ampicillin disks lot 9661 and MHA lot 16922. Resume weekly QC testing. |

Abbreviations: ATCC®, American Type Culture Collection; MHA, Mueller-Hinton agar; QC, quality control.

**Conclusion:** Random QC error.

4.8.3.1 Additional Corrective Action

- If repeat results with QC strains are still out of range, additional corrective action is required. It is possible that the problem is due to a system error rather than a random error (see Subchapter 4.8.1 and M100[1] Tables 4A and 4B).

- Daily QC tests must be continued until final resolution of the problem is achieved.

- If necessary, obtain a new QC strain (either from stock cultures 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 and lot numbers of materials used. It may be helpful to exchange QC strains and materials with another laboratory using the same method in order to determine the root cause of out-of-range QC results where the reason is not identifiable. Until the problem is resolved, it may be necessary to use an alternative test method.

## 4.9 Reporting Patient Results When Out-of-Range Quality Control Results Are Observed

When an out-of-range result occurs when testing QC strains or when corrective action is necessary, each patient test result must be carefully examined to determine if it can be reliably reported. Factors to consider may include, but are not limited to:

- Size and direction of QC strain error (eg, slightly or significantly increased or decreased zone size)

- Actual patient result and its proximity to the interpretive breakpoint

- Results with other QC organisms

- Results with other antimicrobial agents

- Usefulness of the particular QC strain/antimicrobial agent as an indicator for a procedural or storage issue (eg, inoculum dependent, heat labile) (refer to M100[1] Table 4D, Troubleshooting Guide)

Options to consider for patient results include:

- Suppressing the results for an individual antimicrobial agent
- Reviewing individual patient or cumulative data for unusual patterns
- Using an alternative test method or a reference laboratory until the problem is resolved

## 4.10 Confirmation of Results When Testing Patient Isolates

Multiple test parameters are monitored by following the QC recommendations described in this standard. However, acceptable results derived from testing QC 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 before reporting the results. This should include ensuring that:

- The antimicrobial susceptibility results are consistent with the identification of the isolate.

- The results from individual antimicrobial agents within a specific drug class follow the established hierarchy of activity rules (eg, third-generation cephalosporins are more active than first- or second-generation cephalosporins against *Enterobacteriaceae*).

- The isolate is susceptible to those antimicrobial agents for which resistance has not been documented (eg, vancomycin and *Streptococcus* spp.) and for which only "susceptible" interpretive criteria exist in M100.[1]

Unusual or inconsistent results should be confirmed by checking for:

- Previous results on the patient (eg, did the patient previously have the same isolate with an unusual antibiogram?)

- Previous QC performance (eg, is there a similar trend or observation with recent QC testing?)

- Problems with the testing supplies, process, or equipment (see Subchapter 4.8.1 and M100[1] Table 4D, Troubleshooting Guide)

If a reason for the unusual or inconsistent result for the patient's isolate cannot be ascertained, a repeat of the susceptibility test or the identification, or both, may be needed. 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 M100[1] Appendix A. Each laboratory must develop its own policy for confirmation of unusual or inconsistent antimicrobial susceptibility test results. This policy should emphasize those results that may significantly impact patient care.

## 4.11 End-Point Interpretation Control

Monitor end-point interpretation periodically to minimize variation in the interpretation of zone sizes among observers. All laboratory personnel who perform these tests should independently read a selected set of tests. Record the results and compare to the results obtained by an experienced reader; or, when using QC strains, compare to the expected results from M100[1] Tables 4A and 4B. Generally, zone measurement readings from several individuals should not vary more than ±2 mm.

## Chapter 5: Conclusion

This document presents a process workflow for standard agar disk diffusion techniques, which includes preparation of agar plates, testing conditions, interpretation of results, limitations of disk diffusion methods, and QC procedures. Use of the methods within this document along with the corresponding M100[1] supplement enables laboratories to assist the clinician in the selection of appropriate antimicrobial therapy for patient care.

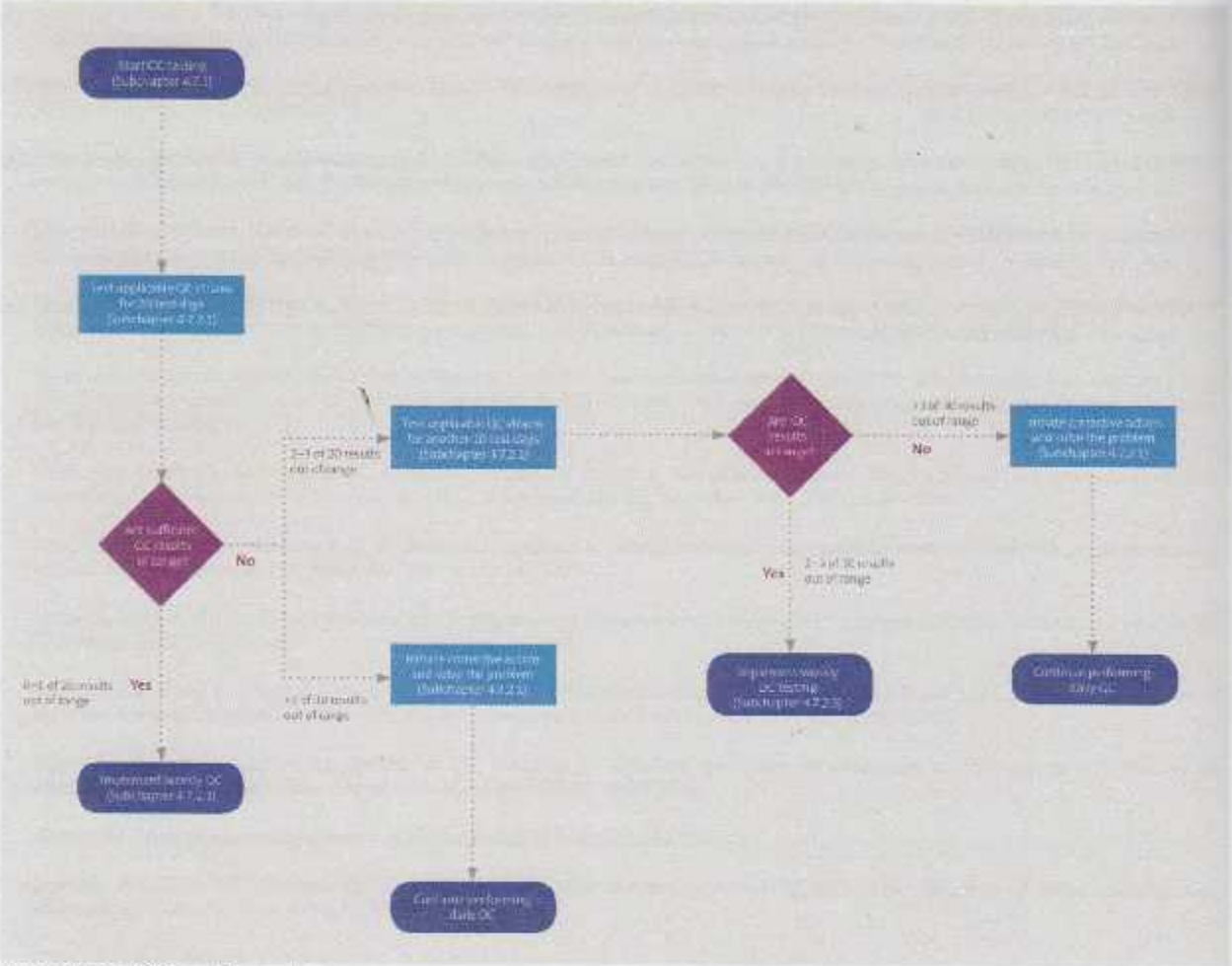
## Chapter 6: Supplemental Information

This chapter includes:

- References
- Appendixes
- The Quality Management System Approach
- Related CLSI Reference Materials

## Appendix A. Quality Control Protocol Flow Charts

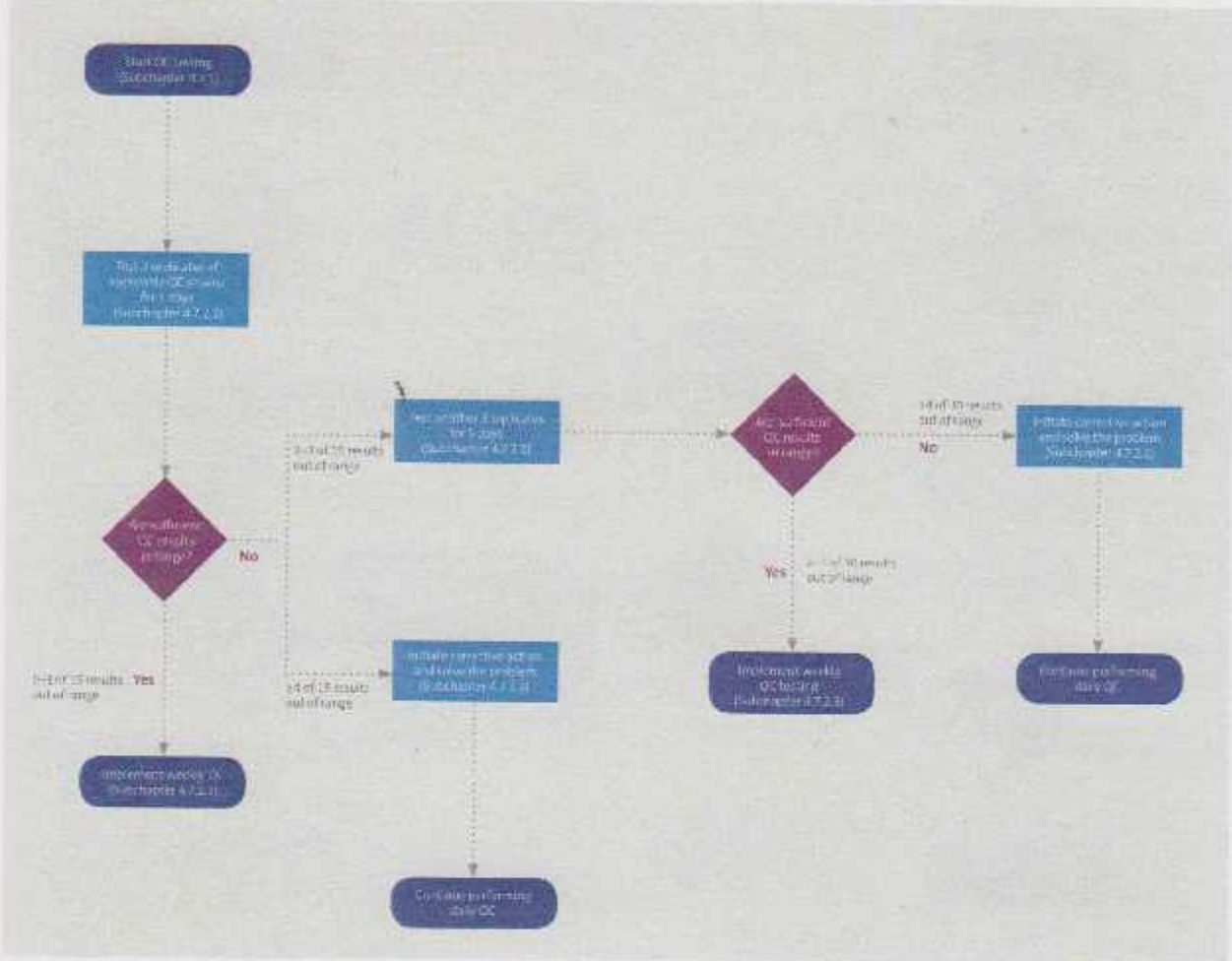
### A1 Quality Control Protocol: Conversion From Daily to Weekly Testing (20- or 30-Day Plan)




Abbreviation: QC, quality control.

## Appendix A. (Continued)

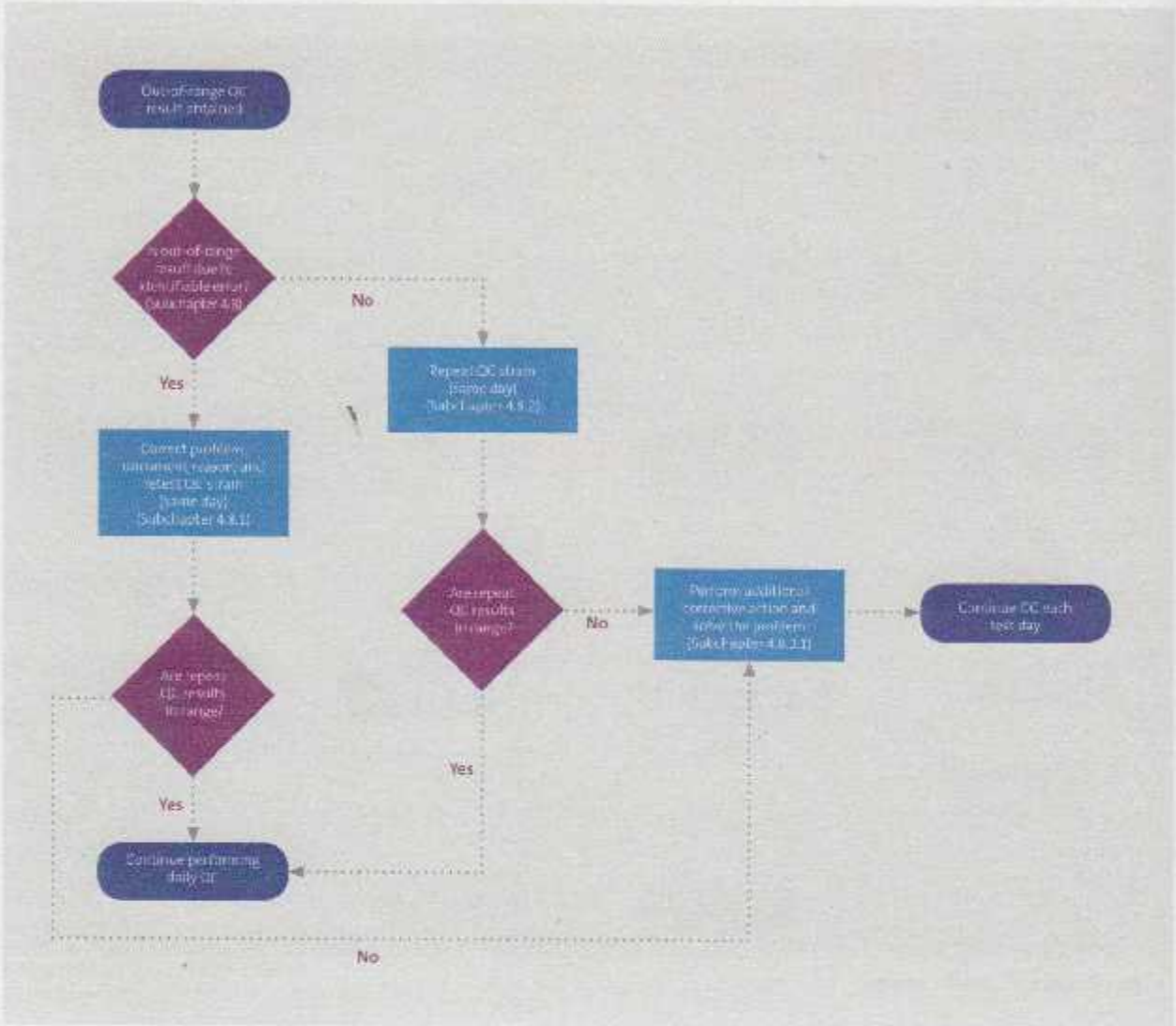
### A2 Quality Control Protocol: Conversion From Daily to Weekly Testing (15-Replicate 3×5 Day Plan)




Abbreviation: QC, quality control.

## Appendix A. (Continued)

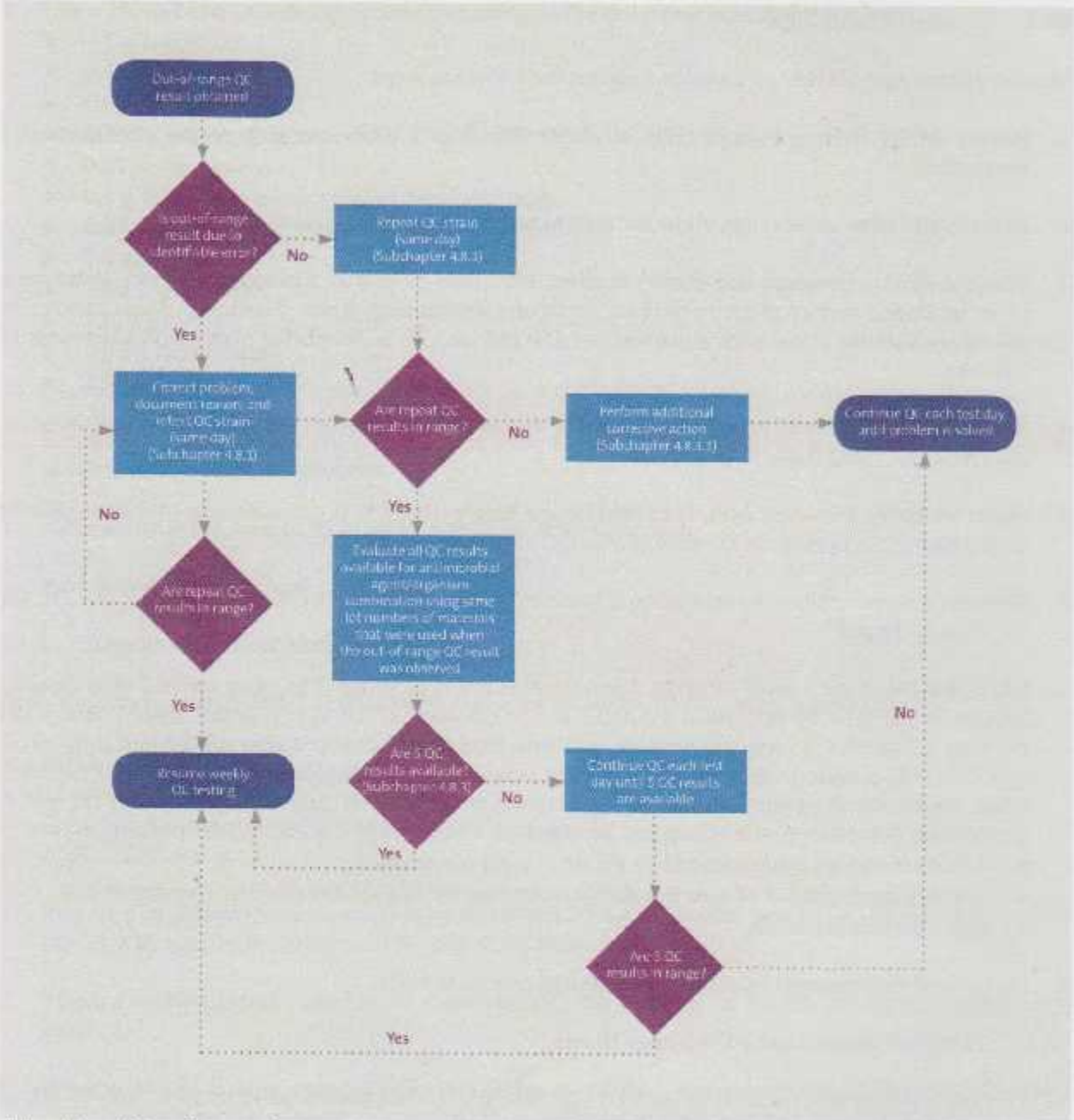
### A3 Quality Control Protocol: Daily Quality Control Testing – Corrective Action




Abbreviation: QC, quality control.

## Appendix A. (Continued)

### A4 Quality Control Protocol: Weekly Quality Control Testing – Corrective Action




Abbreviation: QC, quality control.

# Appendix B. Preparation of Media and Reagents

## B1 Agar Media

### B1.1 Mueller-Hinton Agar

Mueller-Hinton agar (MHA) preparation includes the following steps:

1. Prepare MHA 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 plates 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. Plates are stable for seven days, but could have a longer shelf life if precautions are taken to prevent drying and QC is in range at the time of testing.

6. Examine a representative sample of each batch of plates for sterility by incubating at 35°C ± 2°C for 24 hours or longer.

7. Check the pH of each batch of MHA when the medium is prepared. The exact method used depends 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, and must therefore be checked after solidifying. If the pH is less than 7.2, certain drugs will appear to lose potency (eg, aminoglycosides, macrolides), whereas other antimicrobial agents may appear to have excessive activity (eg, 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.

8. Do not add supplemental calcium or magnesium cations to MHA.

### B1.2 Mueller-Hinton Agar + 5% Sheep Blood

1. Prepare MHA as described above in B1.1 (2). When MHA has cooled to 45 to 50°C, add 50 mL of defibrinated sheep blood to 1 L of MHA. Continue as described in B1.1.

2. Check the pH after aseptic addition of the blood to the autoclaved and cooled medium. The final pH should be the same as unsupplemented MHA, pH 7.2 to 7.4.

## Appendix B. (Continued)

### B1.3 GC Agar+1% Defined Growth Supplement

1. Use a 1% defined growth supplement that contains the following ingredients per liter:
   - 1.1 g L-cystine
   - 0.03 g guanine HCl
   - 0.003 g thiamine HCl
   - 0.013 g *p*-aminobenzoic acid
   - 0.01 g vitamin $B_{12}$
   - 0.1 g thiamine pyrophosphate (cocarboxylase)
   - 0.25 g nicotinamide adenine dinucleotide (NAD)
   - 1 g adenine
   - 10 g L-glutamine
   - 100 g glucose
   - 0.02 g ferric nitrate
   - 25.9 g L-cysteine HCl
2. Prepare 1 L of single strength GC agar base from a commercially available dehydrated base according to the manufacturer's instructions.
3. After autoclaving, cool to 45 to 50°C in a 45 to 50°C water bath.
4. Add 10 mL of 1% defined growth supplement.

### B1.4 *Haemophilus* Test Medium Agar

In its agar form, *Haemophilus* Test Medium (HTM) consists of the following ingredients:

- MHA
- 15 µg/mL β-NAD
- 15 µg/mL bovine or porcine hematin
- 5 g/L yeast extract

1. Prepare a fresh hematin stock solution by dissolving 50 mg of hematin powder in 100 mL of 0.01 mol/L NaOH with heat, and stirring until the powder is thoroughly dissolved.
2. Prepare an NAD stock solution by dissolving 50 mg of NAD in 10 mL of distilled water; filter sterilize.
3. Prepare MHA from a commercially available dehydrated base according to the manufacturer's directions, adding 5 g of yeast extract and 30 mL of hematin stock solution to 1 L of MHA.
4. After autoclaving, cool to 45 to 50°C.
5. Aseptically add 3 mL of the NAD stock solution.
6. Verify that the pH is 7.2 to 7.4.

## Appendix B. (Continued)

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

### B2 Reagents

#### B2.1 0.5 McFarland Turbidity Standard

1. Prepare a 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) stock solution.

2. Prepare a 0.18 mol/L (0.36 N) $H_2SO_4$ (1% v/v) stock solution.

3. With constant stirring to maintain a suspension, add 0.5 mL of the $BaCl_2$ solution to 99.5 mL of the $H_2SO_4$ stock solution.

4. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvettes. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.

5. Transfer the barium sulfate suspension in 4- to 6-mL aliquots into screw-cap tubes of the same size as those used for standardizing the bacterial inoculum.

6. Tightly seal the tubes and store in the dark at room temperature.

7. Vigorously agitate the barium sulfate turbidity standard on a vortex mixer before each use and inspect for a uniformly turbid appearance. Replace the standard if large particles appear. **NOTE:** McFarland standards made from latex particle suspension are commercially available. When used, they should be mixed by inverting gently (not on a vortex mixer) immediately before use.

8. The barium sulfate standards should be replaced or their densities verified monthly.

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

## Appendix C. Conditions for Disk Diffusion Antimicrobial Susceptibility Tests

**Table C1. Conditions for Disk Diffusion Antimicrobial Susceptibility Tests for Nonfastidious Organisms**

| Organism/Organism Group | M100[1] Table | Medium | 0.5 McFarland Inoculum | Incubation | Incubation Time | Minimal QC[a] | Comments/Modifications |
|---|---|---|---|---|---|---|---|
| *Enterobacteriaceae* | 2A | MHA | Direct colony suspension in broth or saline, or growth method | 35°C ± 2°C; ambient air | 16–18 hours | *Escherichia coli* ATCC®[b] 25922<br><br>*P. aeruginosa* ATCC® 27853 for carbapenems<br><br>*E. coli* ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations) | |
| *Pseudomonas aeruginosa* | 2B-1 | MHA | Direct colony suspension in broth or saline, or growth method | 35°C ± 2°C; ambient air | 16–18 hours | *P. aeruginosa* ATCC® 27853<br><br>*E. coli* ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations) | |
| *Acinetobacter* spp. | 2B-2 | MHA | Direct colony suspension in broth or saline, or growth method | 35°C ± 2°C; ambient air | 20–24 hours | *P. aeruginosa* ATCC® 27853<br><br>*E. coli* ATCC® 25922 for tetracyclines and trimethoprim-sulfamethoxazole<br><br>*E. coli* ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations) | |
| *Burkholderia cepacia* complex | 2B-3 | MHA | Direct colony suspension in broth or saline, or growth method | 35°C ± 2°C; ambient air | 20–24 hours | *P. aeruginosa* ATCC® 27853<br><br>*E. coli* ATCC® 25922 for minocycline and trimethoprim-sulfamethoxazole | |

## Appendix C. (Continued)

**Table C1. (Continued)**

| Organism/Organism Group | M100[1] Table | Medium | 0.5 McFarland Inoculum | Incubation | Incubation Time | Minimal QC[a] | Comments/Modifications |
|---|---|---|---|---|---|---|---|
| *Stenotrophomonas maltophilia* | 2B-4 | MHA | Direct colony suspension in broth or saline, or growth method | 35°C+2°C; ambient air | 20–24 hours | *P. aeruginosa* ATCC® 27853<br><br>*E. coli* ATCC® 25922 for minocycline and trimethoprim-sulfamethoxazole | |
| *Staphylococcus* spp. | 2C | MHA | Direct colony suspension in broth or saline | 35°C±2°C; ambient air (Testing at temperatures above 35°C may not detect MRS.) | 16–18 hours; 24 hours for cefoxitin with CoNS | *Staphylococcus aureus* ATCC® 25923 | Direct colony suspension only. Examine linezolid zones carefully with transmitted light.<br><br>Vancomycin disk diffusion testing is not recommended for *S. aureus* or CoNS. |
| *Enterococcus spp.* | 2D | MHA | Direct colony suspension in broth or saline, or growth method | 35°C ± 2°C; ambient air | 16–18 hours; 24 hours for vancomycin | *S. aureus* ATCC® 25923 | Examine vancomycin zones carefully with transmitted light for small colonies or haze inside the zone of inhibition; any growth = resistance. |

Abbreviations: ATCC®, American Type Culture Collection; CoNS, coagulase-negative staphylococci; MHA, Mueller-Hinton agar; QC, quality control; MRS, methicillin-resistant staphylococci.

## Appendix C. (Continued)

**Table C2. Conditions for Disk Diffusion Antimicrobial Susceptibility Tests for Fastidious Organisms**

| Organism/Organism Group | M100[1] Table | Medium | 0.5 McFarland Inoculum | Incubation | Incubation Time | Minimal QC[2] | Comments/Modifications |
|---|---|---|---|---|---|---|---|
| *Haemophilus influenzae* and *Haemophilus parainfluenzae* | 2E | HTM | Direct colony suspension in broth or saline prepared from an overnight (16- to 18-hour) chocolate agar plate[c] | 35°C ± 2°C; 5% $CO_2$ | 16–18 hours | *H. influenzae* ATCC® 49247<br><br>and/or<br><br>*H. influenzae* ATCC® 49766<br><br>*E. coli* ATCC® 35218 (for amoxicillin-clavulanate) | Test a maximum of 9 disks on a 150-mm plate and 4 disks on a 100-mm plate. |
| *Neisseria gonorrhoeae* | 2F | GC agar base with 1% defined growth supplement | Direct colony suspension in broth or 0.9% phosphate-buffered saline, pH 7.0, prepared from overnight chocolate agar plate incubated in 5% $CO_2$ | 36°C ± 1°C (do not exceed 37°C); 5% $CO_2$ | 20–24 hours | *N. gonorrhoeae* ATCC® 49226 | Test a maximum of 9 disks on a 150-mm plate and 4 disks on a 100-mm plate. For some antimicrobial agents, eg, fluoroquinolones or cephalosporins, only 2–3 disks may be tested per plate. |
| *Streptococcus pneumoniae* | 2G | MHA with 5% sheep blood | Direct colony suspension in broth or saline using colonies from an overnight (18- to 20-hour) sheep blood agar plate | 35°C ± 2°C; 5% $CO_2$ | 20–24 hours | *S. pneumoniae* ATCC® 49619 | Test a maximum of 9 disks on a 150-mm plate and 4 disks on a 100-mm plate.<br><br>Measure the zone of growth inhibition, not the zone of inhibition of hemolysis. |
| *Streptococcus* spp. | 2H-1<br>2H-2 | MHA with 5% sheep blood | Direct colony suspension in broth or saline | 35°C ± 2°C; 5% $CO_2$ | 20–24 hours | *S. pneumoniae* ATCC® 49619 | Test a maximum of 9 disks on a 150-mm plate and 4 disks on a 100-mm plate.<br><br>Measure the zone of growth inhibition, not the zone of inhibition of hemolysis. |
| *Neisseria meningitidis* | 2I | MHA with 5% sheep blood[d] | Direct colony suspension in broth or saline prepared from a 20- to 24-hour chocolate agar plate incubated in 5% $CO_2$ | 35°C ± 2°C; 5% $CO_2$ | 20–24 hours | *S. pneumoniae* ATCC® 49619 (5% $CO_2$)<br><br>*E. coli* ATCC® 25922 (ambient air or 5% $CO_2$; for ciprofloxacin, nalidixic acid, minocycline) | Test a maximum of 5 disks on a 150-mm plate and 2 disks on a 100-mm plate.<br><br>**Caution: Perform all testing in a BSC.** |

## Appendix C. (Continued)

Abbreviations: ATCC®, American Type Culture Collection; BSC, biological safety cabinet; HTM, *Haemophilus* Test Medium; MHA, Mueller-Hinton agar; QC, quality control.

**Footnotes**

[a] See specific M100[1] Tables 3A through 3I for additional QC recommendations for screening and confirmatory tests.

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

[c] This suspension will contain approximately 1 to 4 × $10^8$ colony-forming units (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.

[d] Enriched chocolate agar is not recommended for susceptibility testing of *N. meningitidis*.

### Reference for Appendix C

[1] CLSI. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement.* CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.

## Appendix D. Quality Control Strains for Antimicrobial Susceptibility Tests (refer to current edition of M100[1] for the most current version of this table)

| Routine QC Strains | Organism Characteristics | Disk Diffusion Tests | MIC Tests[c] | Screening Tests | Other |
|---|---|---|---|---|---|
| *Bacteroides fragilis* ATCC®[a] 25285 | • β-lactamase positive | | • All anaerobes | | |
| *Bacteroides thetaiotaomicron* ATCC® 29741 | • β-lactamase positive | | • All anaerobes | | |
| *Clostridium difficile* ATCC® 700057 | • β-lactamase negative | | • Gram-positive anaerobes | | |
| *Enterococcus faecalis* ATCC® 29212 | | | • Nonfastidious gram-positive bacteria | • Vancomycin agar<br>• HLAR<br>• High-level mupirocin resistance MIC test | • Assess suitability of medium for sulfonamide or trimethoprim MIC tests.[e]<br>• Assess suitability of cation content in each batch/lot of MHB for daptomycin broth microdilution. |
| *E. faecalis* ATCC® 51299 | • Resistant to vancomycin (*vanB*) and high-level aminoglycosides | | | • Vancomycin agar<br>• HLAR | |
| *Escherichia coli* ATCC® 25922 | • β-lactamase negative | • Nonfastidious gram-negative bacteria<br>• *Neisseria meningitidis* | • Nonfastidious gram-negative bacteria<br>• *N. meningitidis* | | |
| *E. coli* ATCC® 35218 | • Contains plasmid-encoded TEM-1 β-lactamase (non-ESBL)[e,f,g] | • β-lactam/β-lactamase inhibitor combinations | • β-lactam/β-lactamase inhibitor combinations | | |
| *Eggerthella lenta* ATCC® 43055 (formerly *Eubacterium lentum*) | | | • All anaerobes | | • Growth on Brucella media not optimum |
| *Haemophilus influenzae* ATCC® 49247 | • BLNAR | • *Haemophilus* spp. | • *Haemophilus* spp. | | |
| *H. influenzae* ATCC® 49766 | • Ampicillin susceptible | • *Haemophilus* spp. (more reproducible with selected β-lactams) | • *Haemophilus* spp. (more reproducible with selected β-lactams) | | |
| *Klebsiella pneumoniae* ATCC® 700603 | • Contains SHV-18 ESBL[e,f,g] | • ESBL screen and confirmatory tests<br>• β-lactam/β-lactamase inhibitor combinations | • ESBL screen and confirmatory tests<br>• β-lactam/β-lactamase inhibitor combinations | | |
| *Neisseria gonorrhoeae* ATCC® 49226 | • CMRNG | • *N. gonorrhoeae* | • *N. gonorrhoeae* | | |

## Appendix D. (Continued)

| Routine QC Strains | Organism Characteristics | Disk Diffusion Tests | MIC Tests | Screening Tests | Other |
|---|---|---|---|---|---|
| *Pseudomonas aeruginosa* ATCC® 27853[a] | • Contains inducible AmpC β-lactamase | • Nonfastidious gram-negative bacteria | • Nonfastidious gram-negative bacteria | | • Assess suitability of cation content in each batch/lot of Mueller-Hinton for gentamicin MIC and disk diffusion. |
| *Staphylococcus aureus* ATCC® 25923 | • β-lactamase negative<br>• *mecA* negative<br>• Little value in MIC testing due to its extreme susceptibility to most drugs | • Nonfastidious gram-positive bacteria | | • High-level mupirocin resistance disk diffusion test<br>• Inducible clindamycin resistance disk diffusion test (D-zone test) | |
| *S. aureus* ATCC® 29213 | • Weak β-lactamase–producing strain<br>• *mecA* negative | | • Nonfastidious gram-positive bacteria | • Oxacillin agar<br>• High-level mupirocin resistance MIC test<br>• Inducible clindamycin resistance MIC test | • Assess suitability of cation content in each batch/lot of MHB for daptomycin broth microdilution. |
| *S. aureus* ATCC® 43300 | • Oxacillin-resistant, *mecA* positive | • Cefoxitin disk diffusion testing | • Cefoxitin MIC testing | • Oxacillin agar | |
| *S. aureus* ATCC® BAA-1708 | • High-level mupirocin resistance mediated by the *mupA* gene | | | • High-level mupirocin resistance test | |
| *Streptococcus pneumoniae* ATCC® 49619 | • Penicillin intermediate by altered penicillin-binding protein | • *S. pneumoniae*<br>• *Streptococcus* spp.<br>• *N. meningitidis* | • *S. pneumoniae*<br>• *Streptococcus* spp.<br>• *N. meningitidis* | • Inducible clindamycin resistance MIC test | |

## Appendix D. (Continued)

| Supplemental QC Strains[b] | Organism Characteristics | Disk Diffusion Tests | MIC Tests | Screening Tests | Other |
|---|---|---|---|---|---|
| *E. faecalis* ATCC® 29212 | | | • Ceftaroline MIC testing | | |
| *E. faecalis* ATCC® 33186 | | | | | • Alternative to *E. faecalis* ATCC® 29212 to assess suitability of medium for sulfonamide or trimethoprim MIC and disk diffusion tests.[c] End points are the same as for *E. faecalis* ATCC® 29212. |
| *H. influenzae* ATCC® 10211 | | | | | • Assess each batch/lot for growth capabilities of HTM. |
| *K. pneumoniae* ATCC® BAA-1705 | • KPC-producing strain[c]<br>• MHT positive | • Phenotypic confirmatory test for carbapenemase production (MHT) | | | |
| *K. pneumoniae* ATCC® BAA-1706 | • Resistant to carbapenems by mechanisms other than carbapenemase<br>• MHT negative | • Phenotypic confirmatory test for carbapenemase production (MHT) | | | |
| *S. aureus* ATCC® 29213 | • Weak β-lactamase–producing strain<br>• *mecA* negative | | | • Penicillin zone-edge test | |
| *S. aureus* ATCC® BAA-976 | • Contains *msr*(A)-mediated macrolide-only resistance | • Assess disk approximation tests with erythromycin and clindamycin (D-zone test negative). | | | |
| *S. aureus* ATCC® BAA-977 | • Contains inducible *erm*(A)-mediated resistance | • Assess disk approximation tests with erythromycin and clindamycin (D-zone test positive). | | | |

Abbreviations: ATCC®, American Type Culture Collection; BLNAR, β-lactamase negative, ampicillin resistant; CMRNG, chromosomally mediated penicillin-resistant *Neisseria gonorrhoeae;* ESBL, extended-spectrum β-lactamase; HLAR, high-level aminoglycoside resistance; HTM, *Haemophilus* Test Medium; KPC, *Klebsiella pneumoniae* carbapenemase; MHB, Mueller-Hinton broth; MHT, modified Hodge test; MIC, minimal inhibitory concentration; QC, quality control.

## Appendix D. (Continued)

**Footnotes**

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

[b] *E. coli* ATCC® 35218 is recommended only as a control organism for β-lactamase inhibitor combinations, such as those containing clavulanate, sulbactam, or tazobactam. This strain contains a plasmid-encoded β-lactamase (non-ESBL); subsequently, 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 QC strain for the QC test to be valid; however, the plasmid may be lost during storage at refrigerator or freezer temperatures. To ensure the plasmid is present, test the strain with a β-lactam agent alone (either ampicillin, amoxicillin, piperacillin, or ticarcillin) in addition to a β-lactam/β-lactamase inhibitor agent (eg, amoxicillin-clavulanate). If the strain loses the plasmid, it will be susceptible to the β-lactam agent when tested alone, indicating that the QC test is invalid and a new culture of *E. coli* ATCC® 35218 must be used.

[c] Careful attention to organism maintenance (eg, minimal subcultures) and storage (eg, −60°C or below) is especially important for QC strains *E. coli* ATCC® 35218, *K. pneumoniae* ATCC® 700603, and *K. pneumoniae* ATCC® BAA-1705 because spontaneous loss of the plasmid encoding the β-lactamase or carbapenemase has been documented. Plasmid loss leads to QC results outside the acceptable limit, such as decreased MICs for *E. coli* ATCC® 35218 with enzyme-labile penicillins (eg, ampicillin, piperacillin, ticarcillin), decreased MICs for *K. pneumoniae* ATCC® 700603 with cephalosporins and aztreonam, and false-negative MHT with *K. pneumoniae* ATCC® BAA-1705.

[d] Develops resistance to β-lactam antimicrobial agents after repeated transfers onto laboratory media. Minimize by removing new culture from storage at least monthly or whenever the strain begins to demonstrate results above the acceptable range.

[e] End points should be easy to read (as 80% or greater reduction in growth as compared to the control) if media have acceptable levels of thymidine.

[f] Rasheed JK, Anderson GJ, Yigit H, et al. Characterization of the extended-spectrum beta-lactamase reference strain, *Klebsiella pneumoniae* K6 (ATCC® 700603), which produces the novel enzyme SHV-18. *Antimicrob Agents Chemother.* 2000;44(9):2382-2388.

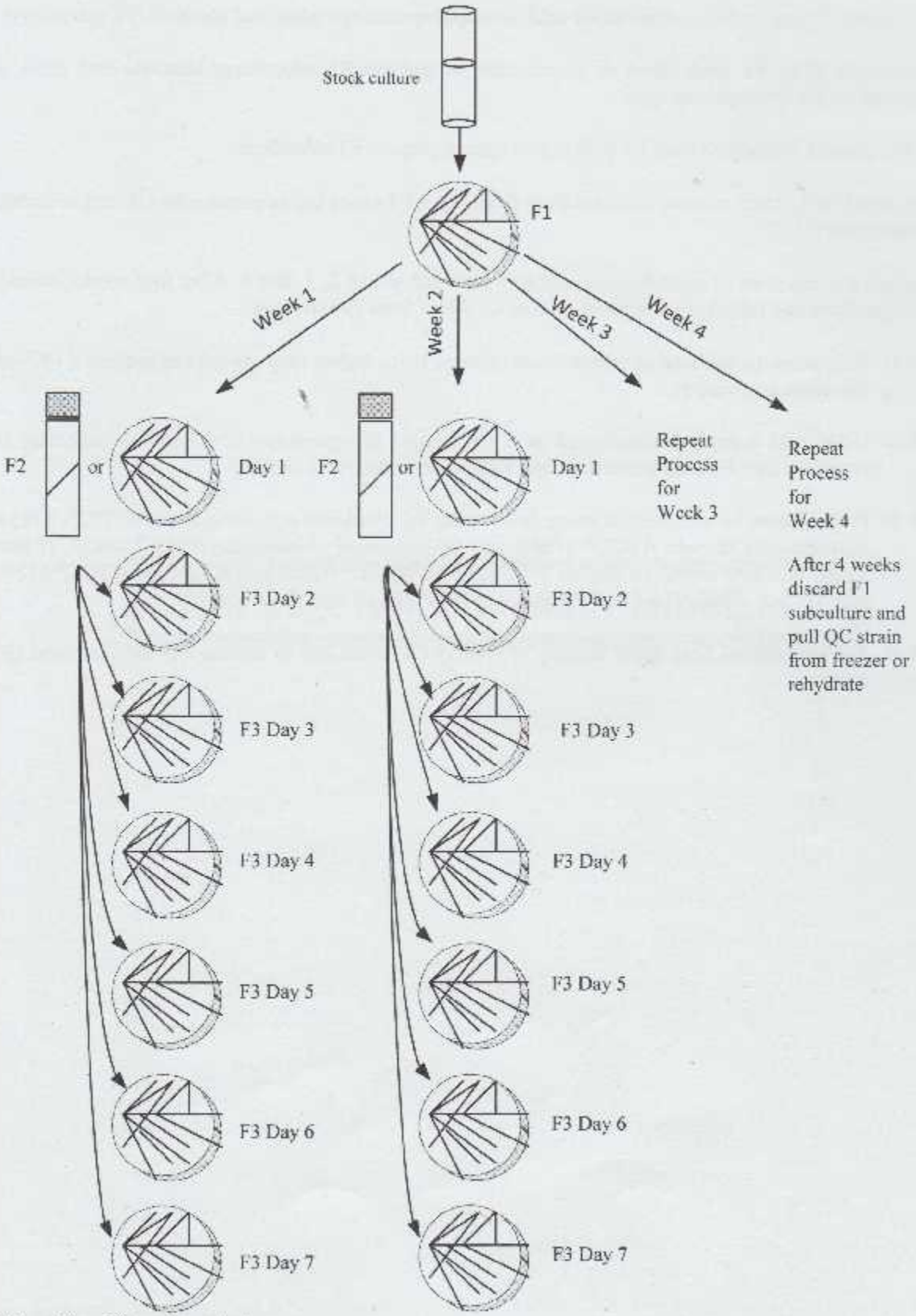
[g] Queenan AM, Foleno B, Gownley C, Wira E, Bush K. Effects of inoculum and beta-lactamase activity in AmpC- and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates tested by using NCCLS ESBL methodology. *J Clin Microbiol.* 2004;42(1):269-275.

[h] See Subchapter 4.4 of this document.

### Reference for Appendix D

[1] CLSI. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement.* CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.

## Appendix E. Quality Control Strain Maintenance (also refer to Subchapter 4.4)




Abbreviation: QC, quality control.

## Appendix E. (Continued)

1. Subculture frozen or freeze-dried stock culture to appropriate agar plate and incubate (F1 subculture).

2. Subculture from F1 onto tubed or plated agar to prepare F2 subculture; incubate and store as appropriate for the organism type.

3. Subsequently, subculture from F2 onto plated agar to prepare F3 subcultures.

4. On days 1 to 7, select isolated colonies from fresh F2 or F3 agar plate to prepare the QC test inoculum suspension.

5. Repeat, starting with F1 subculture on the agar plate for weeks 2, 3, and 4. After four weeks, discard F1 subculture and rehydrate new stock culture or obtain from frozen stock.

**NOTE 1:** Subculture lyophilized or frozen stock cultures twice before they are used to prepare a QC test inoculum suspension.

**NOTE 2:** If QC test appears contaminated or if QC results are questionable, it may be necessary to prepare a new F1 subculture from lyophilized or frozen stock culture.

**NOTE 3:** Prepare new F1 subcultures every two weeks for *Pseudomonas aeruginosa* ATCC®[a] 27853, *Enterococcus faecalis* ATCC® 51299, and *Streptococcus pneumoniae* ATCC® 49619. If held longer than two weeks, results for *P. aeruginosa* ATCC® 27853 and *E. faecalis* ATCC® 51299 may fall out of range and *S. pneumoniae* ATCC® 49619 may die.

**NOTE 4:** The suggestions here apply to daily or weekly QC plans and to routine and supplemental QC strains.

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

## The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system (QMS) 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 QMS approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any health care service's path of workflow (ie, 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 QSEs are as follows:

| | | | |
|---|---|---|---|
| Organization | Personnel | Process Management | Nonconforming Event Management |
| Customer Focus | Purchasing and Inventory | Documents and Records | Assessments |
| Facilities and Safety | Equipment | Information Management | Continual Improvement |

M02-A12 addresses the QSE indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

| Organization | Customer Focus | Facilities and Safety | Personnel | Purchasing and Inventory | Equipment | Process Management | Documents and Records | Information Management | Nonconforming Event Management | Assessments | Continual Improvement |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M29 | | | | X<br>EP23<br>M06<br>M07<br>M11<br>M23<br>M27<br>M27-S4<br>M45 | M07 | | | | |

### Path of Workflow

A path of workflow is the description of the necessary processes to deliver the particular product or service that the organization or entity provides. A laboratory path of workflow consists of the sequential processes: preexamination, examination, and postexamination and their respective sequential subprocesses. All laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

M02-A12 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 Reference Materials 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<br>EP23<br>M07<br>M11<br>M27<br>M27-S4 | X<br>EP23<br>M07<br>M11<br>M27<br>M27-S4<br>M100 | X<br>EP23<br>M07<br>M11<br>M27<br>M27-S4<br>M100 | X<br>M07<br>M11<br>M27<br>M27-S4<br>M100 | M27<br>M27-S4 |

## Related CLSI Reference Materials*

**EP23-A™** **Laboratory Quality Control Based on Risk Management; Approved Guideline (2011).** This document provides guidance based on risk management for laboratories to develop quality control plans tailored to the particular combination of measuring system, laboratory setting, and clinical application of the test.

**M06-A2** **Protocols for Evaluating Dehydrated Mueller-Hinton Agar; Approved Standard—Second Edition (2006).** This document provides procedures for evaluating production lots of dehydrated Mueller-Hinton agar, and for developing and applying reference media.

**M07-A10** **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Tenth Edition (2015).** This standard addresses reference methods for the determination of minimal inhibitory concentrations of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

**M11-A8** **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Eighth Edition (2012).** This standard provides reference methods for the determination of minimal inhibitory concentrations of anaerobic bacteria by agar dilution and broth microdilution.

**M23-A3** **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Third Edition (2008).** This document addresses the required and recommended data needed for the selection of appropriate interpretive criteria and quality control ranges for antimicrobial agents.

**M27-A3** **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition (2008).** This document addresses the selection and preparation of antifungal agents; implementation and interpretation of test procedures; and quality control requirements for susceptibility testing of yeasts that cause invasive fungal infections.

**M27-S4** **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Fourth Informational Supplement (2012).** This document provides updated tables for the CLSI antimicrobial susceptibility testing standard M27-A3.

**M29-A4** **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Fourth Edition (2014).** Based on US 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.

**M45-A2** **Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline—Second Edition (2010).** 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 M02 or M07. 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.

**M100-S25** **Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement (2015).** This document provides updated tables for the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M02-A12, M07-A10, and M11-A8.

* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

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CLINICAL AND LABORATORY STANDARDS INSTITUTE®

12 January 2016

To: Recipients of M100S, 26th ed.

From: Jennifer K. Adams, MT(ASCP), MSHA

Subject: Errors in Tables 2D and 2H-2 for Antimicrobial Agent Telavancin in CLSI Document M100S

This notification is to inform you of errors in Tables 2D and 2H-2 for antimicrobial agent telavancin in CLSI document M100S, 26th ed., *Performance Standards for Antimicrobial Susceptibility Testing.*

The susceptible minimal inhibitory concentration (MIC) interpretive criteria for telavancin in Tables 2D and 2H-2 is incorrect and has been corrected as shown highlighted below:

**Table 2D. Zone Diameter and Minimal Inhibitory Concentration Interpretive Standards for *Enterococcus* spp.**

| Test/Report Group | Antimicrobial Agent | Disk Content | Zone Diameter Interpretive Criteria (nearest whole mm) | | | MIC Interpretive Criteria (µg/mL) | | | Comments |
|---|---|---|---|---|---|---|---|---|---|
| | | | S | I | R | S | I | R | |
| LIPOGLYCOPEPTIDES | | | | | | | | | |
| C | Oritavancin | – | – | – | – | ≤0.12 | – | – | (10) For reporting against vancomycin-susceptible *E. faecalis*. |
| C | Telavancin | 30 µg | ≥15 | – | – | ≤0.25 | – | – | See comment (10). |

Abbreviations: I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible.

**Table 2H-2. Zone Diameter and Minimal Inhibitory Concentration Interpretive Standards for *Streptococcus* spp. Viridans Group**

| Test/Report Group | Antimicrobial Agent | Disk Content | Zone Diameter Interpretive Criteria (nearest whole mm) | | | MIC Interpretive Criteria (µg/mL) | | | Comments |
|---|---|---|---|---|---|---|---|---|---|
| | | | S | I | R | S | I | R | |
| LIPOGLYCOPEPTIDES | | | | | | | | | |
| C | Oritavancin | – | – | – | – | ≤0.25 | – | – | |
| C | Telavancin | 30 µg | ≥15 | – | – | ≤0.06 | – | – | |

Abbreviations: I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible.

If you require any additional clarification regarding this correction, please contact CLSI Customer Service (customerservice@clsi.org).

We appreciate your commitment to CLSI, and regret any inconvenience.

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|---|---|---|---|---|---|---|---|---|---|
| | | | S | I | R | S | I | R | |
| LIPOGLYCOPEPTIDES | | | | | | | | | |
| C | Oritavancin | – | – | – | – | ≤0.12 | – | – | (10) For reporting against vancomycin-susceptible *E. faecalis*. |
| C | Telavancin | 30 µg | ≥15 | – | – | ≤0.25 | – | – | See comment (10). |

Abbreviations: I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible.

**Table 2H-2. Zone Diameter and Minimal Inhibitory Concentration Interpretive Standards for *Streptococcus* spp. Viridans Group**

| Test/Report Group | Antimicrobial Agent | Disk Content | Zone Diameter Interpretive Criteria (nearest whole mm) | | | MIC Interpretive Criteria (µg/mL) | | | Comments |
|---|---|---|---|---|---|---|---|---|---|
| | | | S | I | R | S | I | R | |
| LIPOGLYCOPEPTIDES | | | | | | | | | |
| C | Oritavancin | – | – | – | – | ≤0.25 | – | – | |
| C | Telavancin | 30 µg | ≥15 | – | – | ≤0.06 | – | – | |

Abbreviations: I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible.

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