CLINICAL AND LABORATORY STANDARDS INSTITUTE®

9th Edition

M11

Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria

This standard provides reference methods for determining minimal inhibitory concentrations of anaerobic bacteria by agar dilution and broth microdilution.

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

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Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria

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Abstract

Clinical and Laboratory Standards Institute standard M11—*Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* describes the reference standard agar dilution method (Wadsworth) and the alternative broth microdilution method. Antimicrobial resistance patterns for many anaerobic bacteria have changed significantly over the past several years, resulting in a lack of predictability for many species. Susceptibility testing of anaerobes is recommended for surveillance purposes and for specific clinical situations. The agar dilution method is well suited for surveillance testing and research. It is also the standard with which other methods are compared. The alternative method, broth microdilution, is well suited for the medical laboratory but is currently limited to testing *Bacteroides* spp. and *Parabacteroides* spp. organisms and selected antimicrobial agents. QC criteria for each procedure are also described. This standardized procedure, when used in conjunction with the M100[1] tables, includes the most current information for drug selection, interpretation, QC, and antibiogram reports. When new problems are recognized or improvements in these criteria are made, changes will be incorporated into future editions of this standard and in M100.[1]

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Foreword

High-level antimicrobial agent resistance among anaerobic organisms is continually reported.[2-6] Resistance rates vary among species and from institution to institution.[5] Even within the same species, minimal inhibitory concentrations (MICs) to particular agents may vary significantly.[7] Among *Bacteroides* spp., resistance to some commonly used antimicrobial agents (eg, clindamycin, moxifloxacin) can be high. Significant variability can occur among isolates from hospitalized patients in multiple institutions in the same geographic region. In addition, resistance has been reported among the most active drugs, such as imipenem, piperacillin-tazobactam, tigecycline, ampicillin-sulbactam, and metronidazole.[6,8-11]

Significant resistance rates are also identified in many non-*Bacteroides* anaerobe species, including *Prevotella* spp., *Peptostreptococcus* spp., *Clostridium* spp., and *Fusobacterium* spp.[12,13] Other anaerobic organisms with known intrinsic resistance include *Fusobacterium canifelinum,* which is intrinsically resistant to quinolones. Penicillin resistance can be common but is not predictable among these non-*Bacteroides* genera. To date, resistance to approved agents for *Clostridioides* (formerly *Clostridium*) *difficile* is rare; however, antimicrobial susceptibility testing (AST) is not often used in the clinical setting, because results do not imply efficacy for treating intraluminal infections.[10] For *C. difficile,* AST and typing may be useful for epidemiological purposes.[14-16] Refer to CLSI document M100[1] for a current antibiogram representing a four-year average from several laboratories.

Antimicrobial agent resistance among anaerobes correlates with the discovery and characterization of multiple, transferable resistance determinants corresponding to respective resistance phenotype(s),[17] and horizontal gene transfer is considered a major cause of rapid spread of resistance.[18-20] *Bacteroides fragilis,* in particular, is known as a reservoir for antimicrobial resistance determinants.[16] In addition, heavy use of some antimicrobial agents may result in the selection for and transfer of resistance determinants.[17] An important question is whether the observed antimicrobial agent resistance correlates with a poor clinical outcome. Factors limiting the ability to answer this question include:

- Nature of the infection (mixed aerobes and anaerobes)
- Lack of anaerobe identifications
- Lack of clinical data
- Use of inaccurate or modified susceptibility testing methods
- Effects of surgical drainage or debridement

However, studies on *Bacteroides* spp. bacteremia clearly demonstrate increased mortality and microbiological persistence for patients receiving ineffective therapy compared with those receiving effective therapy.[21-24] Furthermore, reports indicate that the incidence of anaerobic bacteremia is increasing.[25,26] The recent and varied trends in antimicrobial agent resistance, the spread of resistance genes, and the potential for poor clinical outcomes when using an ineffective antimicrobial agent indicate the need for increased AST on anaerobic organisms. The Working Group on Antimicrobial Susceptibility Testing of Anaerobic Bacteria has carefully considered these significant observations and has endeavored to develop reliable and reproducible methods for use in determining the antimicrobial susceptibility of these important pathogens. M11 includes guidance on:

- Number and species of organisms to test
- Testing frequency
- Selection of appropriate antimicrobial agents (see M100[1] Table 1C)

For the most current breakpoints, interpretive categories, and QC recommendations, refer to M100[1] Table 2J. Color plates illustrating both agar and broth microdilution end-point determinations are also included in M11 (see Subchapters 3.5.6 and 3.6.5). After rigorously evaluating and comparing methods, the working group is confident that AST can be reliably performed by the medical laboratory or performed at a referral laboratory using these or other comparable methods. Thus, in certain clinical situations, AST of anaerobic

isolates is recommended. At a minimum, AST for surveillance purposes should be strongly considered when expertise is available, or the isolate should be sent to a referral laboratory.

Because of standardization and correlation studies, agar dilution or broth microdilution are recommended for testing.[27,28] Although broth microdilution is used extensively for aerobic bacteria, limitations for anaerobic bacteria include lack of growth or poor growth of many species.[29] Because of poor strain growth due at least in part to excessive exposure to oxygen during set-up procedures, testing more fastidious anaerobes by this method provides inconsistent and unreliable results. Therefore, broth microdilution is recommended only for testing *Bacteroides* spp. and *Parabacteroides* spp. organisms.

The broth microdilution method has been evaluated with bacterial species other than *Bacteroides* spp. and *Parabacteroides* spp. Several requests for data comparing broth microdilution and agar dilution methods have been made. Based on data available at the time of this standard's publication, variable correlation was found between agar dilution MIC values and broth microdilution MIC values for *Clostridium* spp.[30] and gram-negative anaerobes other than *Bacteroides* spp. and *Parabacteroides* spp. Therefore, the recommendation for using the broth microdilution method for *Bacteroides* spp. and *Parabacteroides* spp. only is retained.[30]

MIC variability with some agents has been reported with *Eggerthella lenta* (*E. lentum*) ATCC® 43055. Therefore, QC ranges may not have been established for all antimicrobial agents with this organism. If MIC result variability is documented in early drug development studies (see CLSI document M23[31] QC tier 1[a]), this organism does not need to be included in CLSI document M23[31] QC tier 2 studies. Because of the problems associated with *E. lenta* ATCC® 43055, another QC organism has been established for testing agents active against gram-positive anaerobes. *C. difficile* ATCC® 700057 is a nontoxigenic strain, and QC values for relevant drugs are included in M100[1] Tables 5D and 5E. Furthermore, some QC ranges for the broth microdilution method were established before the method was restricted to *Bacteroides* spp. and *Parabacteroides* spp., and the QC ranges established following CLSI document M23[31] guidelines are still valid. Therefore, for historical and/or reference purposes, these ranges are still available in M100[1] Table 5E.

It is expected that new studies using the methods recommended in this edition will result in greater testing consistency and will serve as the reference standard for all future comparisons and clinical studies.

Overview of Changes

This standard replaces the previous edition of the approved standard, M11-A8, published in 2012. Several changes were made in this edition, including:

- **General:**
  - Reorganized to fit the CLSI quality management system and path of workflow format
  - Made minor text revisions throughout for improved clarity and consistency with other CLSI documents
  - To align with the International Organization for Standardization, changed the name of the inoculum preparation method from growth method to broth culture method and changed direct colony suspension to colony suspension
  - Updated nomenclature for *Bacteroides fragilis* to *Bacteroides* spp. and *Parabacteroides* spp. per current standards

[a] When referring to tier 1, 2, or 3 QC studies, the guidelines from CLSI document M23[31] are implied.

Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria

Chapter 1: Introduction

This chapter includes:

- Standard's scope and applicable exclusions
- Background information pertinent to the standard's content
- Standard precautions information
- "Note on Terminology" that highlights particular use and/or variation in use of terms and/or definitions
- Terms and definitions used in the standard
- Abbreviations and acronyms used in the standard

1.1 Scope

This standard describes the CLSI antimicrobial susceptibility testing (AST) reference agar dilution method as well as the alternative broth microdilution method for *Bacteroides* spp. and *Parabacteroides* spp. organisms used to determine *in vitro* susceptibility to antimicrobial agents of bacteria that grow anaerobically. A method for β-lactamase testing on anaerobic bacteria is also described. This standard includes:

- Preparation of broth and agar dilution tests
- Testing conditions (including inoculum preparation and standardization, incubation time, and incubation temperature)
- Reporting minimal inhibitory concentration (MIC) results
- QC procedures
- Limitations of the dilution test methods
- A step-by-step guide to AST (see Appendix A)

To assist the medical laboratory, suggestions are provided for selecting antimicrobial agents for routine testing and reporting.

The disk diffusion method has not been standardized for use with anaerobic organisms and is not included. Methods for culturing and identifying anaerobic bacteria are not discussed. See CLSI document M56[32] for information on methods for culturing and identifying anaerobic bacteria. Methods for AST of aerobic bacterial species are also not discussed. The AST 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

The agar dilution method is the recommended reference method for all anaerobic organisms. The broth microdilution procedure is a more user-friendly method that enables testing of multiple antimicrobial agents on one microdilution tray for one isolate. However, multilaboratory collaborative studies comparing broth microdilution with agar dilution using the recommended medium limit the current application of broth microdilution to *Bacteroides* spp. and *Parabacteroides* spp. for some antimicrobial agents (see M100[1]). To date, for antimicrobial agents tested, the methods are considered equivalent.

Briefly, the tests are performed by preparing twofold dilution series of antimicrobial agents in either agar plates or broth (added to wells of a microtiter plate). A standardized suspension of the test organism is then inoculated onto each agar surface or into each well. After incubation, the growth on each plate or in each well is examined and the MIC is determined. Careful adherence to the described methodology is essential to achieving reproducible (interlaboratory and intralaboratory) results.

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. Published guidelines are available that discuss the daily operations of diagnostic medicine in humans and animals while encouraging a culture of safety in the laboratory.[33] 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.[34]

1.4 Terminology

1.4.1 A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization whenever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in different countries and regions and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. CLSI recognizes its important role in these efforts, and its consensus process focuses on harmonization of terms to facilitate the global application of standards and guidelines.

1.4.2 Definitions

antibiogram – overall profile of antimicrobial susceptibility testing results of a microbial species to a battery of antimicrobial agents.

breakpoint – minimal inhibitory concentration (MIC) value used to categorize an organism as susceptible, intermediate, resistant, or nonsusceptible; **NOTE 1:** MIC values generated by a susceptibility test can be interpreted based upon established breakpoints; **NOTE 2:** See **interpretive category.**

Either formula can be used to determine the amount of powder (1) or diluent (2) needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (µg/mL)}}{\text{Assay Potency (µg/mg)}} \quad (1)$$

or

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Assay Potency (µg/mg)}}{\text{Concentration (µg/mL)}} \quad (2)$$

The antimicrobial agent powder should be weighed on an analytical balance that has been calibrated by approved reference weights from a national metrology organization. If possible, more than 10 mg of powder should be weighed. It is advisable to accurately weigh a portion of the antimicrobial agent in excess of that needed and to calculate the volume of diluent needed to obtain the final concentration desired as in formula (2) above.

Example: To prepare 100 mL of a stock solution containing 1280 µg/mL of antimicrobial agent with antimicrobial powder that has a potency of 750 µg/mg, 170 to 200 mg of the antimicrobial powder should be accurately weighed. If the actual weight is 182.6 mg, the diluent volume needed is:

$$\text{Volume (mL)} = \frac{\text{(Actual Powder Weight)(Assay Potency)}}{\text{(Desired Concentration)}} = \frac{182.6 \text{ mg} \cdot 750 \text{ µg/mg}}{1280 \text{ µg/mL}} = 107.0 \text{ mL} \quad (3)$$

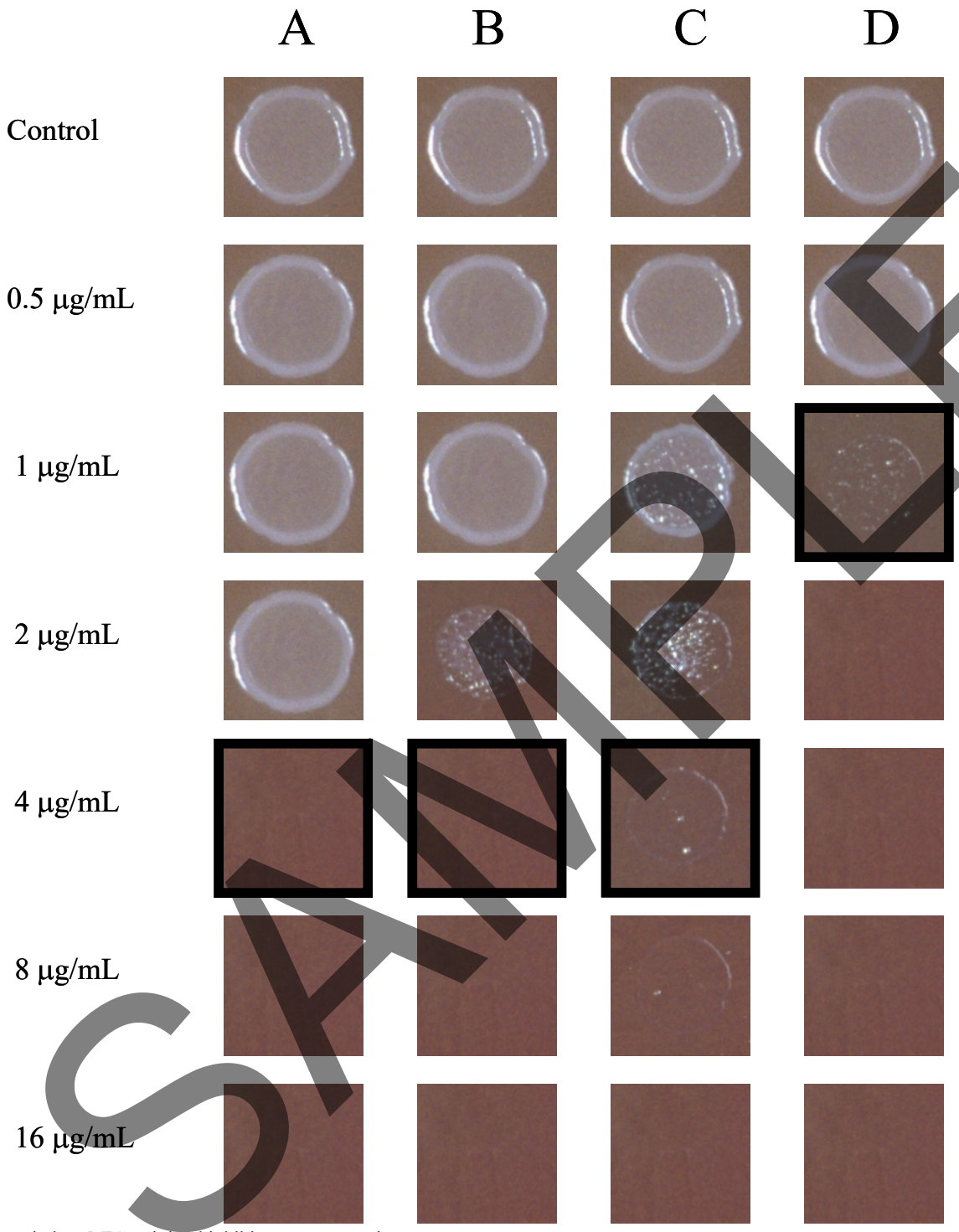
Therefore, 182.6 mg of antimicrobial powder is dissolved in 107.0 mL of diluent.

3.2.3 Preparing Stock Solutions

Antimicrobial agent stock solutions should be prepared at concentrations of at least 1000 µg/mL or 10 times the highest concentration to be tested (eg, 1280 µg/mL for a test plate of 128 µg/mL), whichever is greater. Some antimicrobial agents with limited solubility may need lower concentrations. In all cases, directions provided by the drug's manufacturer to determine solubility should be considered. Some drugs must be dissolved in solvents other than water. In such cases:

- A minimum amount of solvent should be used to solubilize the antimicrobial powder.
- The final stock concentration dilution should be completed with water or the appropriate diluent, as indicated in M100[1] Table 6A.
- For potentially toxic solvents, the material safety data sheets available from the manufacturer should be consulted (see M100[1] Table 6A).

Because microbial contamination is extremely rare, solutions that have been prepared aseptically but not filter sterilized are generally acceptable. Small volumes of sterile stock solutions should be dispensed into sterile glass, polypropylene, polystyrene, or polyethylene vials, carefully sealed, and stored (preferably at ≤ −60°C but never at a temperature warmer than −20°C and never in a self-defrosting freezer). Vials may be thawed as needed and used the same day. Any unused stock solution should be discarded at the end of



Abbreviation: MIC, minimal inhibitory concentration.

Figure 3. MIC End Points: Agar Dilution. Examples of end points using the reference agar dilution method. Boxes indicate MIC values. Lane A, marked reduction to no growth (MIC = 4 µg/mL); lane B, reduction in growth at 2 µg/mL and no growth at 4 µg/mL (MIC = 4 µg/mL); lane C, marked reduction to light growth or a haze (MIC = 4 µg/mL); lane D, marked reduction to multiple, tiny colonies (MIC = 1 µg/mL).