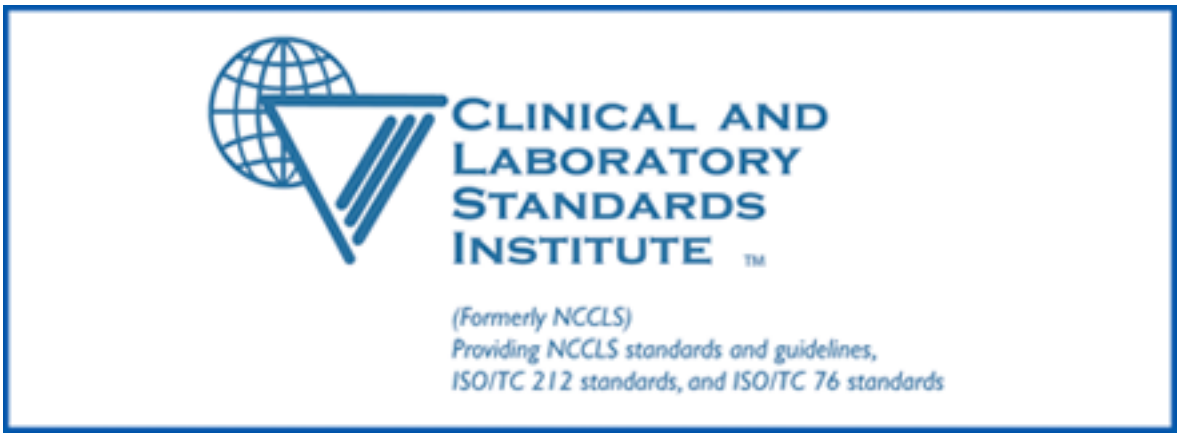
M11-A6
Vol. 24 No. 2
Replaces M11-A5
Vol. 21 No. 2

Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth Edition



This standard provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by agar dilution and broth microdilution.

A standard for global application developed through the NCCLS consensus process.

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NCCLS...

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M11-A6
ISBN 1-56238-517-8
Volume 24 Number 2 ISSN 0273-3099

Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth 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 reliably be predicted from existing antibiograms. Antimicrobial resistance patterns for many anaerobic bacteria have changed significantly over the last 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.

Two endpoint-determining susceptibility testing methods for anaerobic bacteria are described in this standard. The agar dilution method (Wadsworth) remains the reference standard, and is well suited for surveillance testing and research. It is also the standard to which other methods are compared. Broth microdilution is well suited for the clinical laboratory, but is currently limited to testing of *Bacteroides fragilis* group organisms and selected antibiotics. Quality control criteria for each procedure are also described.

The tabular information presented represents the most current information for drug selection, interpretation, and quality control. Users should replace tables published in earlier standards with these new tables. (Changes in the tables since the most recent edition appear in boldface type). 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 as informational supplements.

NCCLS. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth Edition*. NCCLS document M11-A6 (ISBN 1-56238-517-8). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA 2004.

Suggested Citation

(NCCLS. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth Edition.* NCCLS document M11-A6 [ISBN 1-56238-517-8]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2004.)

Proposed Standard
November 1979

Tentative Standard
January 1982

Approved Standard
June 1984

Tentative Standard—Second Edition
April 1989

Approved Standard—Second Edition
August 1990

Approved Standard—Third Edition
December 1993

Approved Standard—Fourth Edition
December 1997

Approved Standard—Fifth Edition
March 2001

Approved Standard—Sixth Edition
January 2004

ISBN 1-56238-517-8
ISSN 0273-3099

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Contents

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Foreword

Antibiotic resistance among anaerobic organisms has increased significantly in recent years.[1,2] Resistance rates vary among species and also from hospital to hospital. Even within the same species, MICs to particular agents may vary significantly.[2-4] For example, among the various members of the *Bacteroides fragilis* group reported resistance rates include clindamycin (15 to 44%), cefotetan (13 to 94%), cefoxitin (3.5 to 41.5%), cefmetazole (7 to 93%), and clinafloxacin (7 to 47%). Frequently, the non-*B. fragilis* members of the *B. fragilis* group are more resistant to many antibiotics. Variations in susceptibility were found to be both species- and hospital-dependent. Virtually all *Bacteroides fragilis* group spp. are resistant to penicillin. Among non-*Bacteroides* anaerobes, significant resistance is also identified in many species, including *Prevotella* spp. (penicillin, 50%; clindamycin, 17%; piperacillin, 11%; cefotetan, 6%), *Peptostreptococcus* spp. (clindamycin, 16%), *Clostridium* spp. (clindamycin, 11%; cefotetan, 12%; cefoxitin 14%), and *Fusobacterium* spp. (ceftizoxime 18%).[1,2] Other anaerobic organisms with known intrinsic resistance include *Sutterella wadsworthensis* and *Bilophila wadsworthia*. Among these non-*Bacteroides* genera, penicillin resistance can be common but is not predictable. The increasing and prevalent antibiotic resistance among anaerobic organisms is correlated with the discovery and characterization of multiple, transferable resistance determinants corresponding to their respective resistance phenotype(s). In addition, heavy use of some antibiotics may result in the selection for, and transfer of, these resistance determinants.[1]

An important question is whether the observed antibiotic resistance correlates with poor clinical outcome. Until recently, studies demonstrating such a correlation are few and retrospective in nature.[5] Factors limiting these studies include the nature of the infection (mixed aerobes and anaerobes), the lack of identification of anaerobes, a lack of clinical data, the use of inaccurate or modified susceptibility testing methods, and the effects of surgical drainage or debridement. However, a recently published prospective study of *Bacteroides* bacteremia clearly demonstrates increased mortality and microbiological persistence for patients receiving ineffective therapy compared with those receiving effective therapy.[6]

The recent and varied trends in antibiotic resistance, the spread of resistance genes, and the potential for poor clinical outcomes when using an ineffective antibiotic argue strongly for more susceptibility testing of anaerobic organisms. The anaerobe working group has carefully considered these significant observations, and has endeavored to develop reliable and reproducible methods that can be used to determine the susceptibility of these important pathogens. M11-A5 included a step-by-step guide to susceptibility testing. This edition also contains a step-by-step guide to susceptibility testing (Appendix B), including guidance on the number and species of organisms to test, how often to test, and selection of appropriate antibiotics (Table 1). Color plates illustrating both agar and broth microdilution endpoint determinations are also included in this edition (Figures 2 and 3).

As a result of rigorous evaluation and comparison among these methods, the working group is confident that susceptibility testing can be reliably performed by the clinical laboratory, or performed at a reference laboratory using these or other comparable methods. Thus, the anaerobe working group recommends (in certain clinical situations) susceptibility testing of anaerobic isolates. At a minimum, susceptibility testing for surveillance purposes should be strongly considered utilizing these or validated equivalent methods when expertise is available, or the isolate should be sent to a reference laboratory.

As a result of the standardization and correlation studies performed by the working group, either of two methods is recommended for testing—agar dilution or broth microdilution. In the fifth edition, changes to the broth microdilution procedure were featured. Earlier versions of this standard have contained recommendations regarding broth microdilution allowing one of several broth media for testing, based upon previous experience and comparison with various other methods.[7,8] Recognizing that while broth microdilution is utilized extensively, limitations exist that include lack of growth or poor growth of many anaerobic species, as well as few extensive comparative studies with the reference standard.[9] Further, the working group recognizes the need for a method that could be utilized without the requirement of an

anaerobic chamber for preparation and incubation. In the fifth edition, a standardized broth microdilution procedure based on multilaboratory evaluations of growth and testing conditions was introduced.[10,11] Based on these studies and established QC ranges, additional antibiotics have been added to this edition as appropriate for use in broth microdilution testing (Table 6). To date, broth microdilution is approved only for testing of *B. fragilis* group isolates. Additionally, QC ranges for a number of agents were added to the QC table (Table 5) for agar dilution. Occasionally, questions arise concerning testing of agents for which QC ranges have not been established. In the case of vancomycin, for example, while QC ranges have not been established for the anaerobic strains, vancomycin is active *in vitro* against many gram-positive anaerobes. *Staphylococcus aureus* ATCC® may be used as a QC strain if vancomycin is tested. The working group plans to establish QC ranges for additional antimicrobials for both agar and broth microdilution testing.

The working group expects that new studies using the methods recommended in this version will result in greater consistency in testing, and will serve as the gold standard for all future comparisons and clinical studies. Some clinical laboratories may choose to perform other, simpler methods such as a concentration gradient agar diffusion method. Should alternative methods be used, laboratories should ensure that the QC values are within acceptable ranges.

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Key Words

Agar dilution, anaerobic bacteria, antimicrobial susceptibility, broth microdilution, minimal inhibitory concentration (MIC)

Summary of Major Changes in This Document

Additions to This Document

Location	Information Added
Section 4.1	Text regarding the reporting of antimicrobial agent concentrations
Section 5.1	Text added regarding *Bacteroides* bacteremia
Section 8.2.1	Text added to clarify suspension and storage of colonies
Section 8.3 (2)	Incubation temperature added
Section 10.5	Text added regarding confirmation of growth and cross-contamination
Table 1	Table headings changed to reflect new groupings of anaerobic bacteria
	Moxifloxacin added to *B. fragilis* group & other BLA-positive anaerobes for Supplemental Choices

Table 1 (continued)	Doxycycline added as supplemental choice to *B. fragilis* Group & other BLA-positive anaerobes; Species of *Clostridium* Other than *C. perfringens* and *Clostridium perfringens,* Gram-positive Cocci, and Non-Sporeforming Gram-positive Bacilli
	New footnotes "d" and "e" added
Table 2 (c)	Text added regarding amoxicillin breakpoints
Table 5	Linezolid MIC criteria for agar dilution added to *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, and *E. lentum* ATCC 43055
Table 6	Amoxicillin-clavulanic acid MIC criteria for broth microdilution added to *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741
	Chloramphenicol MIC criteria for broth microdilution added to *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, and *E. lentum* ATCC 43055
	Doxycycline MIC criteria for broth microdilution added to *B. thetaiotaomicron* ATCC 29741 and *E. lentum* ATCC 43055
	Linezolid MIC criteria for broth microdilution added to *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, and *E. lentum* ATCC 43055
	Meropenem MIC criteria for broth microdilution added to *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, and *E. lentum* ATCC 43055
	Penicillin MIC criteria for broth microdilution added to *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741

Changes to This Document

Location	Information Changed
Table 1	Table headings changed
Table 5	Meropenem MIC criteria for agar dilution changed for *B. fragilis* ATCC 25285 and *E. lentum* ATCC 43055

Deletions from This Document

Location	Information Deleted
Table 1	Clindamycin deleted from *B. fragilis* group & other BLA-positive anaerobes for Primary Choices

NCCLS Subcommittee on Antimicrobial Susceptibility Testing Mission Statement

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

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

- Develop standard reference methods for antimicrobial susceptibility tests
- Provide quality control parameters for standard test methods
- Establish interpretive criteria for the results of standard antimicrobial susceptibility tests
- Provide suggestions for testing and reporting strategies that are clinically relevant and cost-effective
- Continually refine standards and optimize the detection of emerging resistance mechanisms through the development of new or revised methods, interpretive criteria, and quality control parameters
- Educate users through multimedia communication of standards and guidelines
- 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 NCCLS guidelines. The values that guide this mission are quality, accuracy, fairness, timeliness, teamwork, consensus, and trust.

Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth Edition

1 Scope

The methods described in this document are intended for testing commonly isolated anaerobic bacteria. The agar dilution method may be used to test a wide variety of anaerobic organisms. Currently the broth microdilution method using the new medium is only suggested for testing organisms from the *Bacteroides fragilis* group. When other applications have been validated, they will be published in annual updates of NCCLS document M100—*Performance Standards for Antimicrobial Susceptibility Testing of Anaerobic Bacteria.*

Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be 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 any pathogen and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections.*

2 Introduction

Either a broth or an agar dilution method may be used to measure quantitatively the *in vitro* activity of an antimicrobial agent against a given bacterial isolate. This document describes the NCCLS reference agar dilution method, the alternative broth microdilution method, and a method for β-lactamase testing for anaerobic bacteria. The agar dilution method has been studied extensively in collaborative multicenter trials and is the recommended reference method for all anaerobic organisms. The broth microdilution method is useful as a more "user friendly" method that allows testing of multiple antimicrobial agents on one plate. However, recent multilaboratory collaborative studies comparing broth microdilution to agar dilution using the medium recommended in this edition limit its current application to members of the *Bacteroides fragilis* group with some antibiotics. (See Foreword.) For those agents tested to date (Table 2), the methods are considered equivalent. To perform the tests, twofold dilution series of antimicrobial agents are prepared in agar and added to petri plates or prepared in broth and added to wells of a microdilution plate. A standardized suspension of the test organism is then inoculated onto each agar surface or into each well. After incubation for 42 to 48 hours, growth on each plate or in each well is examined and the minimal inhibitory concentration (MIC) is determined. The final result is significantly influenced by methodology, which must be carefully controlled if reproducible results (interlaboratory and intralaboratory) are to be achieved.

Commercial methods (such as the agar plate concentration gradient methods) or systems that are based on either of these standard methods are or may become available and may provide substantially equivalent results to the methods described here. It is the responsibility of the United States Food and Drug

Administration (FDA) to approve or clear commercial devices for use in the United States. NCCLS does not approve or endorse commercial products or devices.

3 Definitions

Agar dilution susceptibility test – An *in vitro* antimicrobial susceptibility test method conducted using serial concentrations of an antimicrobial agent incorporated into an agar growth medium in separate petri dishes.

Antimicrobial susceptibility test interpretive category – A classification based on an *in vitro* response of an organism to an antimicrobial agent. Determination of value considers multiple factors including pharmacokinetic and pharmacodynamic properties, concentrations of the agent corresponding to blood, tissue, or other body fluid levels attainable with usually prescribed doses of that agent, distribution of MIC values for clinical isolates, and, whenever possible, relation to clinical efficacy.

> *Susceptible Antimicrobial Susceptibility Test Interpretive Category* – A category that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species, unless otherwise indicated;
>
> *Intermediate Antimicrobial Susceptibility Test Interpretive Category* – A category that implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used; also indicates a "buffer zone" that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations;
>
> *Resistant Antimicrobial Susceptibility Test Interpretive Category* – Resistant isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or fall in the range where specific microbial resistance mechanisms are likely (e.g., beta-lactamases), and clinical efficacy has not been reliable in treatment studies.

Culture – 1) The intentional growing of microorganisms, such as bacteria, viruses, or tissues, in a controlled environment, for purposes of identification or other scientific study, or for commercial and/or medicinal use; **2)** The product resulting from the intentional growth of microorganisms or tissue.

Culture medium – A substance or preparation used for the cultivation and growth of microorganisms or tissue.

Minimal inhibitory concentration, MIC – The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test. (See Section 10.5.)

4 Indications for Performing Susceptibility Tests on Anaerobic Bacteria

Ideally, clinical laboratories that accept specimens for culture of anaerobic bacteria should be able to recover in pure culture all medically significant anaerobes that may be present. Susceptibility testing of such isolates should be performed:

- to assist in the management of infection in individual patients with serious or life-threatening infections;
- to periodically monitor local and regional resistance patterns so that such information is available as a guide for empiric antimicrobial choice; and

- to determine patterns of susceptibility of anaerobes to new antimicrobial agents as they are approved for treatment of infections involving anaerobes.

The major indications for testing of clinical isolates are situations in which decisions about the selection of agents are critical because of the:

- known resistance of a particular organism or species;
- persistence of the infection despite adequate treatment with an appropriate therapeutic regimen;
- difficulty in making empiric decisions based on precedent; or
- confirmation of appropriate therapy for severe infections or for those that may require long-term therapy.

4.1 Routine Testing

Susceptibility testing may not be necessary for many individual patient isolates. Examples of specific infections from which isolates should be tested include brain abscess, endocarditis, osteomyelitis, joint infection, infection of prosthetic devices or vascular grafts, and bacteremia. Isolates from normally sterile body sites should be tested unless they are believed to be contaminants. Communication with the physician is important in deciding on the need for susceptibility testing. Such testing should always be done upon reasonable request from the physician.

When the nature of the infection is not clear and the specimen contains indigenous anaerobic flora in which the organisms probably bear little relationship to the infectious process being treated, susceptibility testing is probably unnecessary, and the results may be misleading. Determining which anaerobes to test when several are present and which antimicrobial agents to test is more difficult. Organisms recognized as highly virulent or for which susceptibility to the antimicrobial agent(s) commonly used for treatment cannot be predicted, or both, should be considered for testing. These include some species of *Bacteroides*, *Prevotella*, *Fusobacterium*, *Clostridium, Bilophila*, and *Sutterella*.

For each organism to be tested, a suspension of several well-isolated colonies of similar appearance should be removed from nonselective medium after first verifying purity and inability to grow aerobically. Identification procedures are often performed from the same culture. Susceptibility tests should never be performed directly from clinical material.

The MIC obtained using a dilution test may suggest to a physician the concentration of antimicrobial agent needed at the site of infection to inhibit the infecting organism. The MIC, however, does not represent an *absolute* value. The “true” MIC is somewhere between the lowest test concentration that inhibits the organism's growth (that is, the MIC reading) and the next lower or higher test concentration. If, for example, twofold dilutions were used and the MIC was determined to be 16 μg/mL, the “true” MIC would be between 32 and 8 μg/mL. Even under the best of controlled conditions, a dilution test may not yield the same endpoint each time it is performed. Generally, the acceptable reproducibility of the test is within one twofold dilution of the actual endpoint. To avoid even greater variability, the dilution test must be standardized and carefully controlled as described herein.

Antimicrobial agent concentrations used to determine MICs are typically derived from serial twofold dilutions (e.g., 16, 8, 4, 2, 1 μg/mL, etc). Other dilution schemes have also been used, and results should be reported as obtained. When there is inhibition of growth at the lowest concentration tested, the true MIC value cannot be accurately determined and should be reported as less than or equal to the lowest concentration tested. Similarly, if the growth persists at the highest concentration tested, results should be reported as greater than the highest concentration tested.

Whenever MIC results are reported, an interpretive category (i.e., susceptible, intermediate, or resistant) should accompany the MIC result based on the criteria outlined in Table 2. When four or fewer consecutive concentrations are tested, or when nonconsecutive concentrations are tested alone, an interpretive category should be reported. An MIC value may also be reported, if desired, but the MIC testing range must be specified.

4.2 Surveillance Testing

If a hospital laboratory routinely identifies anaerobic bacteria and does not perform susceptibility testing, the local patterns of resistance for the commonly encountered anaerobes should be established and verified periodically. The laboratory, in consultation with infectious disease practitioners, and those individuals responsible for establishing the institutional antibiotic formulary, should strongly consider surveillance testing annually to detect emerging resistance. Surveillance testing to detect emerging resistance should be performed annually either by the hospital laboratory if expertise is available, or by a reference laboratory. Strains to be tested should be collected over several months and stored until a total of 50 to 100 are available for batch testing, if possible. (See Section 8.2.)[12] This number of isolates will make the most economical use of time, materials, and personnel needed to perform the test accurately and will provide internally consistent results. The variety of strains tested should reflect the frequency of isolation over the test period. At least 20 isolates of *Bacteroides* spp. and ten isolates from other frequently isolated genera should be tested. The antimicrobial agents to be tested should be based upon the hospital's formulary; however, laboratories should strongly consider including at least one agent from each appropriate antimicrobial class even if not on the formulary (see Table 1). Results of the most recent test should be published annually, comparing current with previous results so that trends in emerging resistance may be documented.

5 Limitations of Susceptibility Testing of Anaerobic Bacteria

Both the reporting laboratory and the clinician should be cautioned that *in vitro* results may not necessarily predict individual patient response to therapy for anaerobic infections. This should be accomplished by including a comment in the susceptibility report. The procedures described here may not be suitable for all anaerobic bacteria or all antimicrobial agents. It is important to remember the following:

- Susceptibility cannot be reliably determined unless adequate growth is achieved.

- Modifications of methods and of media composition may permit the growth of the more fastidious anaerobic organisms, but appropriate quality control tests must be included and results must conform to published values. If modifications are made, reporting of results should acknowledge changes from standard methods.

- MICs for control organisms should fall within the acceptable range for each antimicrobial agent reported.

- Because many infections involving anaerobes are polymicrobial and successful treatment often involves a combination of surgical intervention and the use of empirical broad-spectrum antimicrobial therapy, the relative importance of the susceptibility of a single organism to predict a favorable clinical outcome is difficult to determine. Susceptibility of the most resistant organisms (usually *B. fragilis* group) should be considered for testing and reporting first.

5.1 Interpretive Criteria (Breakpoints) and Clinical Correlation

No uniform agreement presently exists on breakpoints used to determine susceptibility of anaerobes to most antimicrobial agents. In many cases, existing breakpoints have been determined based on animal

models or on results of clinical trials involving patients with polymicrobial infections containing both aerobes and anaerobes. Because most anaerobic infections take place in closed spaces and involve multiple microorganisms, *in vitro* activity of a single antimicrobial agent against a single microorganism cannot be directly correlated with its *in vivo* efficacy.

In addition to population distribution and clinical efficacy, the susceptible and intermediate breakpoints for interpretation of MIC results are based on serum levels achieved with maximum recommended dosage, because such dosage regimens are generally recommended for treatment of anaerobic infections. The intermediate range was established because of the difficulty in reading endpoints and the clustering of MICs near the breakpoint for several drugs. When maximum dosages are used along with appropriate ancillary therapy, it is believed that organisms with susceptible endpoints are generally amenable to therapy, and those with intermediate endpoints may respond but patient response should be carefully monitored. Ancillary therapy, such as drainage procedures and debridement, are obviously of great importance for the proper management of anaerobic infections. However, increased mortality and microbiological persistence of *Bacteroides* bacteremia for patients receiving ineffective therapy compared with those receiving effective therapy has been clearly demonstrated.

5.2 Organisms

The agar dilution procedure is appropriate for testing many different anaerobic species. The broth microdilution method, however, is currently limited to the testing of isolates of the *Bacteroides fragilis* group. When testing organisms that swarm (e.g., *C. difficile* or *C. tetani*), staggered placement of inoculum on agar testing plates is necessary. For *C. septicum*, only a single isolate can be tested per plate.

6 Antimicrobial Agents

6.1 Source

Antimicrobial standard or reference powders can be obtained directly from the drug manufacturer, from the United States Pharmacopoeia (12601 Twinbrook Parkway, Rockville, Maryland 20852, 800-227-8772), or from certain other commercial sources. Parenteral preparations should not be used for susceptibility testing. Acceptable standard reference powders bear a label that states the drug's generic name, lot number, its assay potency (usually expressed in micrograms [µg] or International Units [IU] per mg of powder), and its expiration date. The powders are to be stored as recommended by the manufacturer or at ≤-20 ºC in a desiccator (preferably in a vacuum). When the desiccator is removed from the refrigerator or freezer, it should be allowed to come to room temperature before being opened, to avoid condensation of water.

6.2 Weighing Antimicrobial Powders

All antimicrobial agents are assayed for standard units of activity. The assay units may differ widely from the actual weight of the powder and often may differ between drug production lots. Thus, a laboratory must standardize its antimicrobial solutions based on assays of the lots of antimicrobial powders that are being used.

The value for potency supplied by the manufacturer should include consideration of measures of purity (usually by HPLC assay), water content (e.g., by Karl Fischer analysis or by weight loss on drying), and the salt/counter-ion fraction (if the compound is supplied as a salt instead of free acid or base). The potency may be expressed as a percentage, or in units of µg/mg (w/w).

In some cases, a certificate of analysis with values for each of these components may be provided with antibiotic powders; in this case, an overall value for potency may not be provided, but can be calculated from HPLC purity, water content, and when applicable, the active fraction for drugs supplied as a salt

(e.g., hydrochloride, mesylate). However, if when testing these calculations, any value is unknown or is not clearly determined from the certificate of analysis, it is advisable that the factors used in this calculation be confirmed with the supplier or the manufacturer. The following demonstrates an example calculation:

Example: meropenem trihydrate

Certificate of analysis data:

Assay purity (by HPLC): 99.8%

Measured water content (by Karl Fischer analysis): 12.1% (w/w)

Active fraction: 100% (supplied as the free acid, and not a salt)

Potency calculation from above data:

Potency = (Assay purity) x (Active fraction) x (1- Water Content)

Potency = (0.998) x (1.0) x (1- 0.121)

Potency = 0.877 µg/mg or 87.7%

Either of the following formulas below may be used to determine the amount of powder or diluent needed for a standard solution:

$$\textit{Weight}\ (\text{mg}) = \frac{\textit{Volume}\ (\text{mL}) \bullet \textit{Concentration}\ (\mu\text{g/mL})}{\textit{Potency}\ (\mu\text{g/mg})} \qquad (1)$$

or

$$\textit{Volume}\ (\text{mL}) = \frac{\textit{Weight}\ (\text{mg}) \bullet \textit{Potency}\ (\mu\text{g/mg})}{\textit{Concentration}\ (\mu\text{g/mL})} \qquad (2)$$

The antimicrobial powder should be weighed on an analytical balance that has been calibrated with National Institute of Standards and Technology (NIST) weights (or other approved reference weights). If possible, more than 100 mg of powder should be weighed. It is advisable to accurately weigh a portion of the antimicrobial agent in excess of that required 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 1,280 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 volume of diluent needed is then as follows:

$$\textit{Volume}\ (\text{mL}) = \frac{182.6\ \text{mg} \bullet 750\ \mu\text{g/mg}}{1280\ \mu\text{g/mL}} = \frac{(\textit{Actual Weight})\quad(\textit{Potency})}{(\textit{Desired Concentration})} = 107.0\ \text{mL} \qquad (3)$$

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

6.3 Preparing Stock Solutions

Antimicrobial agent stock solutions are to be prepared at concentrations of at least 1,000 µg/mL (example: 1,280 µg/mL) or ten times the highest concentration to be tested, whichever is greater. There are some antimicrobial agents, however, of limited solubility that may require lower concentrations. In all cases, consider directions provided by the drug's manufacturer as part of determining solubility.

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 can then be made with water or appropriate diluent as indicated in Table 3.
- For potentially toxic solvents, consult the Material Safety Data Sheets available from the manufacturer (see Table 3.)

Because microbial contamination is extremely rare, solutions that have not been sterilized are generally acceptable. If desired, however, solutions may be sterilized by membrane filtration. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antimicrobial agents, must *not* be used. Whenever filtration is used, it is important that the absence of adsorption be documented by appropriate assay procedures.

Small volumes of the sterile stock solutions may be dispensed into sterile glass, polypropylene, polystyrene, or polyethylene vials, carefully sealed, and stored (preferably at ≤-60 ºC but never at a temperature warmer than -20 ºC and never in a frost-free freezer). Vials may be thawed as needed and used the same day. Any unused drug should be discarded at the end of the day. Stock solutions of most antimicrobial agents can be stored at ≤-60 ºC for six months or more without significant loss of activity. In all cases, directions provided by the drug's manufacturer must be considered in addition to these general recommendations. Any significant deterioration of an antimicrobial agent should be reflected in the results of susceptibility testing using quality control strains.

6.4 Number of Concentrations Tested

The concentrations to be tested for a particular antimicrobial agent should encompass the interpretive breakpoints shown in Table 2, but the actual number of concentrations tested is the decision of the laboratory. Five or more concentrations are recommended for reporting of MIC results. However, it is advisable to choose a range that allows at least one quality control organism to have on-scale values.

7 Selection of Antimicrobial Agents for Routine Testing and Reporting

Selection of the most appropriate antimicrobial agents to test and report routinely is a decision made best by each clinical microbiology laboratory in consultation with the infectious disease practitioners and the pharmacy, as well as the pharmacy and therapeutics and infection control committees of the medical staff. The recommendations in Table 1 comprise agents that are indicated for the treatment of certain infections due to susceptible anaerobic bacteria and that have shown acceptable *in vitro* test performance. Testing of selected agents may be useful for epidemiologic or surveillance purposes, or as an aid in identification.

7.1 Routine Reports

The list of agents in Table 1 constitutes recommendations for testing and reporting that are considered appropriate at present. The list is based on various considerations, including microbiological, clinical, and pharmacological factors, as well as clinical indications and efficacy. To avoid misinterpretation, routine reports to physicians should include only those drugs appropriate for therapeutic use, as suggested in

Table 1. Agents may be added to or removed from this basic list as conditions demand. Drugs other than those appropriate for use in therapy may also be tested to provide taxonomic data and epidemiologic information, but they should not be included on patient reports. However, such results should be available in the laboratory for the use of the infection control practitioner and/or hospital epidemiologist.

7.2 Nonproprietary Names

To minimize confusion, all antimicrobial agents should be referred to by official nonproprietary (i.e., generic) names. To emphasize the relatedness of the many currently available drugs, they may be grouped together by drug class.

7.3 Drug Classes

Descriptions of the drug classes are described in the section on selection of antimicrobial agents for routine testing in NCCLS document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*; however, the applications described in that document apply mainly to infections caused by aerobic or facultatively anaerobic organisms. The antimicrobial agents listed in this document were chosen based on their anti-anaerobic activity and do not reflect activity against aerobic, or facultatively anaerobic, bacteria isolated from mixed aerobic/anaerobic infections.

8 Inoculum Preparation for Dilution Tests

The test organisms should be selected from growth on supplemented Brucella blood agar (or other medium containing blood that supports adequate growth of the organisms to be tested) that has been incubated for sufficient time (24 to 48 hours) to produce colonies of suitable size. Ordinarily, colonies will be at least 1 mm in diameter. Rapid growers such as members of the *B. fragilis* group and *C. perfringens* may be tested after a 24-hour incubation. Most other anaerobes require up to a 48-hour incubation. For slower growing isolates such as *Bilophila* spp., and *C. gracilis*, it may be necessary to prepare the inoculum from several agar plates to ensure proper standardization. The inoculum may be prepared by either the growth method (see Section 8.3) or direct colony suspension (see Section 8.4).

8.1 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, a $BaS0_4$ turbidity standard equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension) should be used. A $BaS0_4$ 0.5 McFarland standard may be prepared as described in Appendix A2.4.

8.2 Storage and Recovery of Isolates for Testing

8.2.1 Freezing Isolates for Later Testing

Most anaerobic bacteria may be stored at ≤-60 °C in 20% glycerol for several years. The anaerobic bacteria should be grown in a low-carbohydrate, reduced medium such as cooked meat medium without added sugar. Transfer four parts from a 48-hour broth culture to a sterile vial containing one part sterile glycerol. Mix thoroughly to obtain a uniform suspension, and freeze.

Alternatively, suspending several colonies from a 48-hour plate culture directly into 20% sterile skim milk (20% powdered skim milk in water) and freezing at -60 °C is also an acceptable method of storage.

8.2.2 Recovering Isolates from Frozen Storage

Frozen isolates must be subcultured by at least two serial transfers on supplemented Brucella blood agar and the purity of the culture verified before being used for susceptibility testing.

8.3 Growth Method

(1) Using growth on supplemented Brucella blood agar that has been incubated for 24 hours or for a sufficient time to produce colonies of suitable size (at least 1 mm in diameter [see Section 8]), portions of five or more well-isolated colonies of similar morphology (or enough to obtain a 3-mm loopful of growth) are inoculated into enriched thioglycollate medium without indicator. If an anaerobic chamber is used for the procedure, an enrichment medium such as supplemented Brucella, supplemented brain-heart infusion, or Schaedler broth may be used instead of the thioglycollate broth.

(2) Incubate the broth for 6 to 24 hours at 35 to 37 °C or until adequate turbidity is obtained.

(3) Adjust turbidity to the density equivalent to a No. 0.5 McFarland standard by addition of Brucella broth or other clear broth that has been reduced, or boiled and cooled, before being used. This results in a suspension containing approximately 1 to 2 x 10^8 CFU/mL for *E. coli* ATCC® 25922 or for *B. fragilis* ATCC® 25285. However, variation in inoculum size has been noted among various anaerobic species.[13]

8.4 Direct Colony Suspension Method

(1) For this method the colonies should be selected from growth on a supplemented Brucella blood agar plate that is 24 to 48 hours old. The plate should not remain in an aerobic atmosphere for more than 30 minutes before making the suspension.

(2) By lightly touching portions of five or more well-isolated colonies of similar morphology, suspend the growth directly into Brucella broth or other clear broth that was reduced, or boiled and cooled, before being used, to achieve a turbidity equivalent to a 0.5 McFarland standard.

Counts from suspensions for some species of anaerobes may be slightly higher when prepared by this method.[13]

9 β-lactamase Testing

Testing for β-lactamase activity using a chromogenic, cephalosporin-based method may be performed on anaerobic organisms other than the *B. fragilis* group prior to susceptibility testing. Since the great majority of *B. fragilis* group isolates are β-lactamase producers, they should be considered resistant to penicillin, amoxicillin, and ampicillin; therefore, β-lactamase tests need not be performed nor results reported for *B. fragilis* group organisms. Any β-lactamase-producing anaerobe should be considered resistant to penicillin and ampicillin and reported as such, regardless of any additional *in vitro* susceptibility testing. It should be remembered that some anaerobes are resistant to β-lactam antimicrobial agents by mechanisms other than β-lactamase production. Therefore, a negative β-lactamase test does not necessarily assure susceptibility to this drug class.

10 Reference Agar Dilution Procedure (Wadsworth Method)

Several test methods have been described to measure the *in vitro* susceptibility of anaerobes, including agar disk diffusion, broth disk elution, broth microdilution, broth macrodilution, agar dilution, and techniques using a drug concentration gradient. The reference agar dilution method has been the focus of numerous studies performed by the NCCLS Subcommittee on Antimicrobial Susceptibility Testing. In the fourth edition of the standard, Brucella agar supplemented with laked sheep blood, hemin, and vitamin K_1 (Wadsworth method) became the recommended reference agar medium, replacing Wilkins-Chalgren agar.[14,15,16] The recommendation for this change was based on results from a multicenter study[10,11]

comparing the ability of various agar preparations to support the growth of medically important and nutritionally fastidious anaerobes. The results showed that supplemented Brucella blood agar supports good growth of essentially all anaerobes in contrast to Wilkins-Chalgren agar. Brucella blood agar supplemented with hemin and vitamin K_1 was superior to both Wilkins-Chalgren agar and to Wilkins-Chalgren agar plus blood for growth of the organisms that were tested. In addition, a second multicenter study comparing Wilkins-Chalgren agar to Brucella blood agar or Wilkins-Chalgren agar plus blood revealed no significant differences in MIC values for six antimicrobial agents evaluated. Values for ATCC® reference strains also demonstrated no statistical differences among all three media tested.

In the reference agar dilution procedure, each test concentration of antimicrobial agent is added to molten agar (cooled to 48 to 50 ºC), mixed, poured into a petri dish, and allowed to solidify. Standardized suspensions of isolates to be tested are inoculated onto the surface of each plate in the concentration series with the use of a replicating device. The inocula are allowed to absorb into the medium, and the plates are placed in an anaerobic atmosphere within 30 minutes of inoculation. After plates have incubated anaerobically for approximately 48 hours, the plates are examined visually. The lowest concentration of each antimicrobial agent that inhibits growth of an organism is reported as the MIC of the antimicrobial agent.

10.1 Reagents and Materials

A detailed list of reagents and materials needed for the agar dilution procedure is included in Appendix B.

10.1.1 Anaerobic Jars and Chambers

Atmospheric conditions appropriate for susceptibility testing of anaerobes may be created using a disposable hydrogen/carbon dioxide generator, an evacuation/replacement method, or an anaerobic chamber. The incubation atmosphere should contain 4 to 7% CO_2. An indicator to confirm successful achievement of anaerobiosis should always be included.

10.1.2 Supplemented Brucella Agar

The medium used is Brucella agar supplemented with 5 µg hemin and 1 µg vitamin K_1 per mL and 5% (v/v) laked sheep blood. Details about the composition and preparation of Brucella agar and the preparation and addition of the supplements are given in Appendix A2.1. It is convenient to prepare tubes of agar in advance and melt them on the day of use. After appropriate dilutions of the antimicrobial agent are prepared, the antibiotics are added along with the laked sheep blood to the molten agar.

10.2 Procedure for Preparing Agar Dilution Plates

A detailed list of supplies and equipment needed for preparing agar dilution plates is provided in Appendix B, along with a daily timetable for the procedure. Before beginning, determine the antimicrobial agents and the concentrations that will be tested. The concentrations to be tested for a particular antimicrobial agent should encompass the interpretive breakpoints shown in Table 2 and include the quality control strain MICs, but the actual number of concentrations tested is the decision of the laboratory. When preparing agar dilution plates, a total of 20 mL agar is used for round 15 x 100 petri dishes and 30 mL for square plates. Preparation of the plates involves addition of one part of a 10x solution (i.e., 10x the desired final concentration) of antimicrobial agent to nine parts of agar. For example, for the preparation of round plates containing 20 mL of agar, it is necessary to add 1 mL of laked sheep blood and 2 mL of 10x antimicrobial agent solution to 17 mL of molten Brucella agar (maintained at 48 to 50 °C) that has been previously supplemented with hemin and vitamin K_1. For square plates, the amounts are 25.5 mL of agar, 1.5 mL of laked blood, and 3 mL of 10x antimicrobial agent solution.

10.2.1 Making Agar Blanks

(1) Either on or before the day of testing, prepare sufficient Brucella agar supplemented with vitamin K_1 and hemin and dispense the appropriate amount into tubes, preparing one for each concentration of antimicrobial agent to be tested.

(2) If tubes are to be used on the day of testing, after dispensing, place them in a water bath at 48 to 50 °C. If tubes have been prepared previously, melt the medium (in a boiling water bath, a microwave oven, or by briefly autoclaving) and place the tubes to cool in the water bath (48 to 50 °C).

10.2.2 Making Dilutions of Antimicrobial Agents

(1) Thaw previously prepared antimicrobial agent stock solutions (see Section 6.3) or prepare fresh on the day of testing.

(2) Prepare dilution blanks by dispensing the designated amount (see Table 4) of sterile, distilled water or buffer into appropriate tubes.

(3) Using the dilution format described in Table 4, prepare the intermediate (10x) antimicrobial agent solutions by making successive 1:2, 1:4, and 1:8 dilutions, rather than making straight serial twofold dilutions. Preparing the dilutions in this manner minimizes dilution errors.

10.2.3 Pouring Agar Dilution Plates

(1) Label one petri plate for each concentration of antimicrobial agent to be prepared, and distribute the plates on a level surface.

(2) For each of the concentrations being prepared, add 1 mL (1.5 mL for square plates) of laked sheep blood and 2 mL (3 mL for square plates) of 10x antimicrobial agent solution to a tube of melted and cooled agar containing 17 mL (25.5 mL for square plates) supplemented Brucella agar. Tighten the cap of the tube and gently invert several times to mix, being careful not to create bubbles. Pour into the petri plates and allow to solidify.

(3) If one antimicrobial agent is being tested, four additional plates with no antibiotic should be prepared, following the same procedure as in (2) above, but substituting sterile, distilled water for the antimicrobial agent solution. For each additional antimicrobial agent tested, prepare one additional plate with no added antimicrobial agent.

(4) After the agar has solidified, dry the plates by placing them with the lids ajar in a laminar flow hood or an incubator for approximately 30 minutes. Alternatively, invert the plates and prop the agar side against the lid.

10.2.4 Storing Agar Dilution Plates

(1) For routine testing, store plates for no longer than seven days in sealed plastic bags at 2 to 8 ºC before testing.

(2) For research and evaluation purposes, storage for not longer than 72 hours is recommended.

(3) Plates containing imipenem, β-lactam/β-lactamase inhibitor combinations containing clavulanate, or any other antimicrobial of known instability, must be prepared on the day of testing.

10.3 Inoculation of Agar Dilution Plates

The most convenient method for applying the inoculum to the surface of the agar is with an inoculum-replicating apparatus.[15,17] Most inoculum replicators deposit approximately 1 to 2 µL onto the agar surface. The final inoculum on the agar will then be approximately 10^5 CFU per spot. All manipulations may be performed in ambient atmosphere, as most anaerobes can tolerate brief exposure to oxygen. For some fastidious isolates, it may be necessary to pre-reduce the plates and to perform all manipulations in an anaerobic environment.

(1) Prepare a standardized inoculum by either growing the microorganisms to a turbidity equivalent to the 0.5 McFarland standard or suspending colonies directly to the same density, as described in Section 8.

(2) Once the inoculum suspensions are prepared and adjusted, arrange the tubes in order in a rack (see Figure 1) and transfer a small volume (approximately 0.5 mL) to the appropriate well of the replicator seed block. The agar plates should be marked for orientation of the inoculum spots.

Pattern for 100-mm Round Plate

	1	2	3	4	
5	6	7	8	9	10
11	12	13	14	15	16
17	18	19	20	21	22
23	24	25	26	27	28
	29	30	31	32	

Pattern for 100-mm Square Plate

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

Figure 1. Suggested Patterns for Arrangement of Isolates in Replicator for Agar Dilution Testing of Antimicrobial Agents

(3) Being careful to avoid splashing, apply the inoculum onto the surface of each plate.

- At the beginning of inoculation, inoculate two control plates with no antibiotic. Label one of these plates "pre-O_2" (to check for aerobic contamination) and one "pre-Ana" (for the beginning anaerobic growth control).
- Inoculate each series of antimicrobial agents from the lowest drug concentration to the highest.
- Between each series of plates, inoculate one control plate with no antimicrobial to use for growth control and as a check for possible contamination that might occur as plates are being stamped.
- At the end of the final series, again inoculate two plates, labeled "post-O_2" and "post-Ana," to verify the final organism viability and purity.

(4) It is advisable to occasionally perform colony counts on inoculum suspensions to ensure that the final inoculum concentration routinely obtained closely approximates 1.5 x 10^8 CFU/mL for *B. fragilis* ATCC® 25285. This can be easily accomplished by removing a 0.01-mL aliquot from the 0.5 McFarland suspension and diluting in 10 mL of 0.9% saline (9 g/L sodium chloride) (1:1,000 dilution); this will result in a suspension of 10^5 CFU/mL. From this tube, remove 0.1 mL and dilute into 10 mL saline, resulting in a suspension of 10^3 CFU/mL. After mixing, a 0.1-mL aliquot is spread over the surface of a suitable agar medium. After incubation, the presence of approximately 150 colonies would indicate an inoculum density of 1.5 x 10^8 CFU/mL or ~10^5 CFU/spot.

10.4 Incubation of Agar Dilution Plates

(1) Once the plates have dried, invert them and place all plates for anaerobic incubation in an anaerobic jar or alternative anaerobic environment at 35 to 37 °C for 42 to 48 hours.

(2) Plates for aerobic incubation (to verify inability to grow in the presence of atmospheric oxygen) should be placed in an atmosphere containing 5% CO_2 at 35 to 37 °C for 42 to 48 hours.

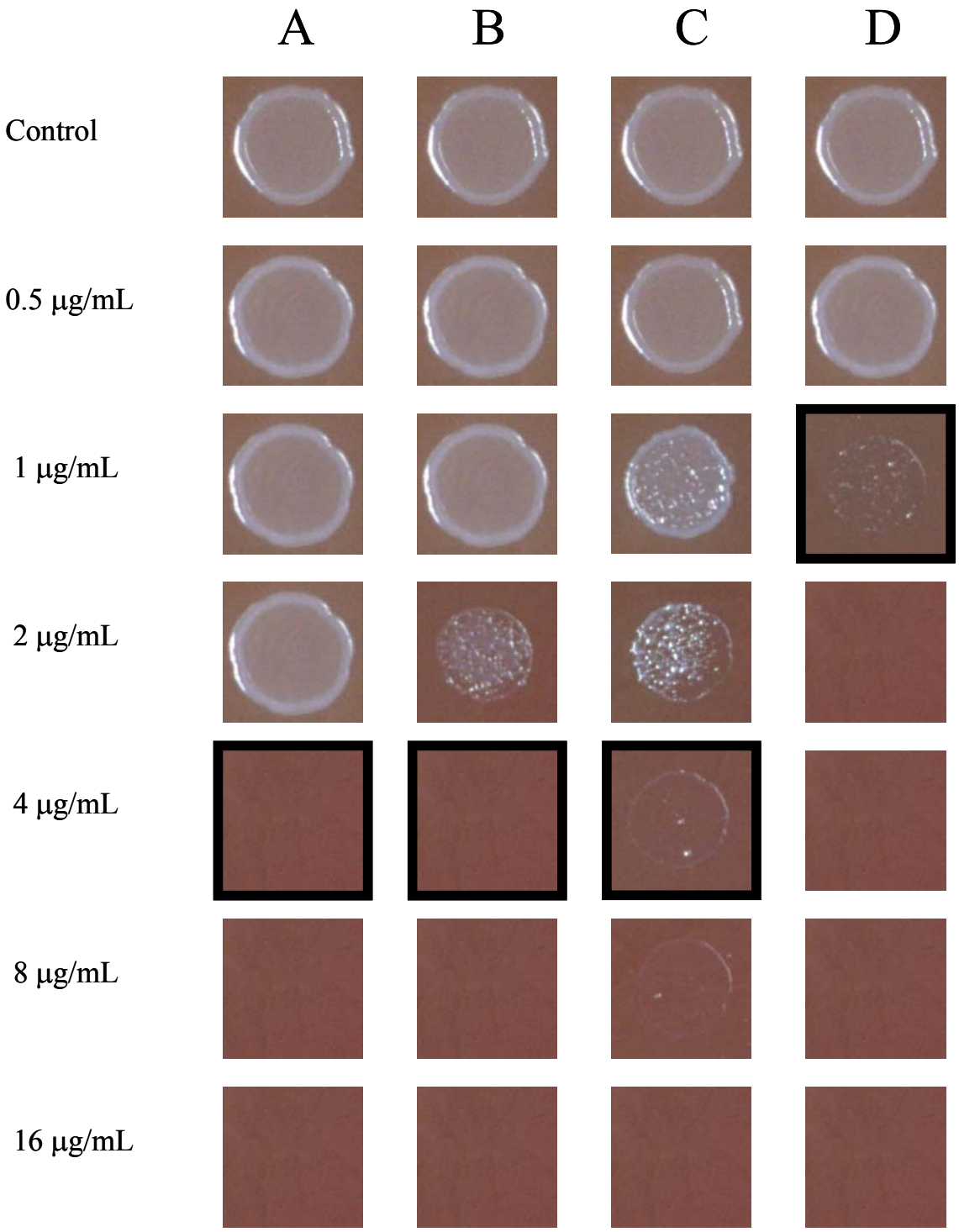

Figure 2. MIC Endpoints – Agar Dilution. Examples of endpoints using reference agar dilution method. Boxes indicate MIC values. Lane A, a marked reduction to no growth (MIC = 4 µg/mL); Lane B, a reduction in growth at 2 µg/mL, and no growth at 4 µg/mL (MIC = 4 µg/mL); Lane C, a marked reduction to light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

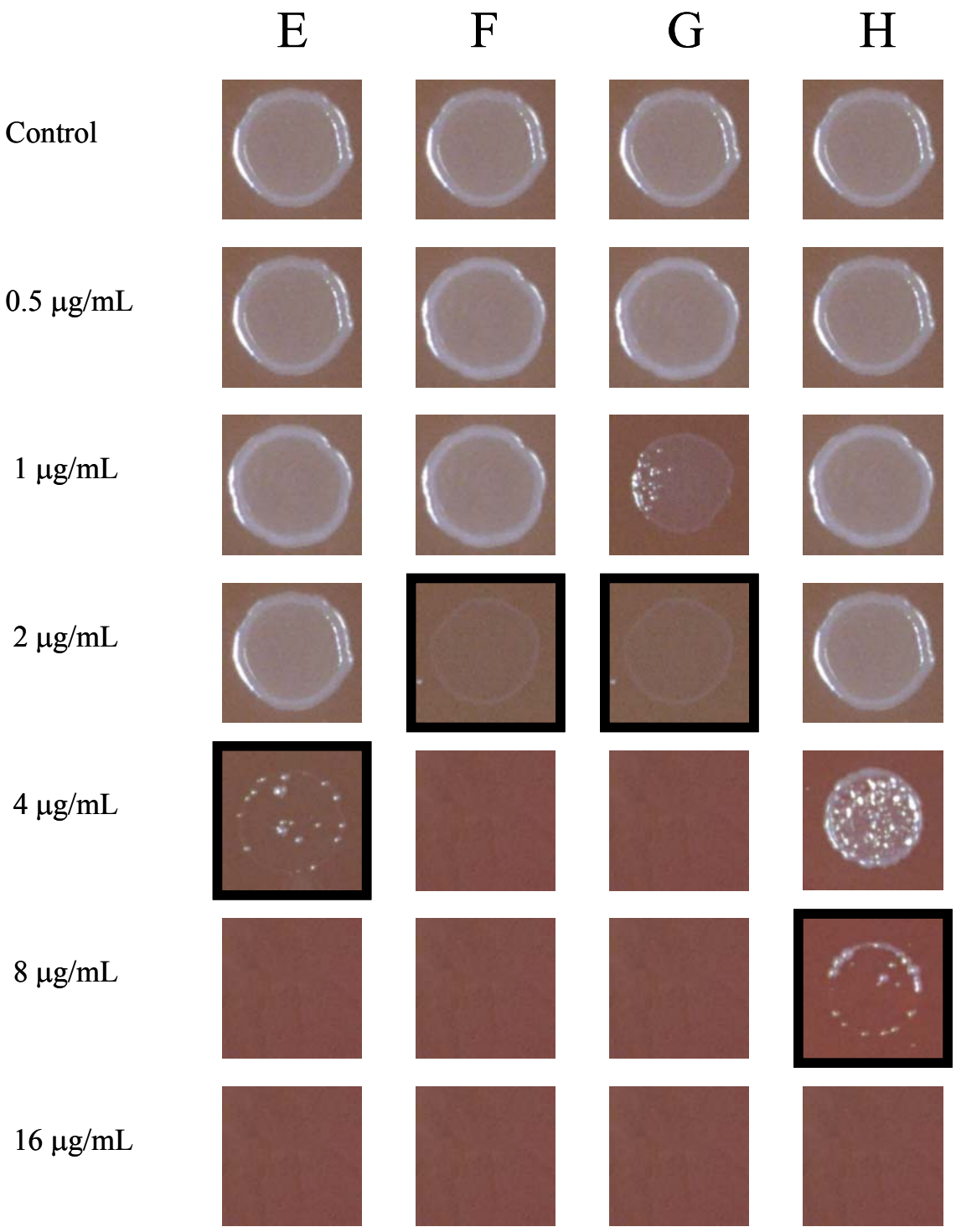

Figure 2 (Continued). Examples of endpoints using reference agar dilution method. Boxes indicate MIC values. Lane E, a marked reduction to several normal-sized colonies (MIC = 4 µg/mL); Lane F, a marked reduction to a haze (MIC = 2 µg/mL); Lane G, a marked reduction to lesser growth at 1 µg/mL, and a haze at 2 µg/mL (MIC = 2 µg/mL); Lane H, reduction in growth to a few large colonies (MIC = 8 µg/mL).

10.5 Reading Agar Dilution Plates

(1) Examine each plate against a dark, nonreflecting background, reading the control plates first. Look for possible aerobic contamination or cross-contamination between wells. Confirm that no growth is seen on aerobic control plates. For example, if a resistant strain splashes, you might see the persistence of several full-size colonies on the inoculum spots of a neighboring strain. If contamination is detected, it may be necessary to retest the strains where this occurred.

(2) Examine the test plates, reading the MIC endpoint at the concentration where a marked reduction occurs in the appearance of growth on the test plate as compared to that of growth on the anaerobic control plates, as illustrated in Figure 2. Examples of a marked change in growth include a change to a haze, multiple tiny colonies, or one to several normal-sized colonies. Illustrated figures should be used as a guide and may be particularly helpful in cases where the endpoint reading is ambiguous.

11 Broth Microdilution Procedure

Until further studies are performed to validate this procedure for testing other organisms, it should be used only for testing members of the *Bacteroides fragilis* group. A limited number of important antimicrobial agents have been validated using this method and appear in Table 2.

11.1 Reagents and Materials

11.1.1 Anaerobic Jars and Chambers

Conditions described in Appendix B for the agar dilution procedure also apply to broth microdilution.

11.1.2 Supplemented Brucella Broth

The medium recommended for the broth microdilution procedure is Brucella broth supplemented with hemin (5 µg/mL), vitamin K_1 (1 µg/mL), and lysed horse blood (5%). Details about its preparation and the preparation of the supplements are given in Appendix A2.2.

11.2 Procedure for Preparing Broth Microdilution Plates

(1) This method is called "microdilution" because it involves the use of small volumes of broth dispensed in sterile, plastic microdilution trays or plates that have round or conical bottom wells. Each well should contain at least 0.1 mL of broth.

(2) To prepare the intermediate (10x) antimicrobial solutions, the concentrated antimicrobial stock solution (see Section 6.3) may be diluted as described in Table 4 or by making serial twofold dilutions. Nine parts broth are then added to one part of the 10x antimicrobial dilutions, resulting in the desired final concentration. These antimicrobial/broth solutions are then dispensed into the plastic microdilution trays. Thawed antimicrobial solutions should be discarded.

(3) The most convenient method for preparing microdilution trays is by use of a dispensing device using antimicrobial dilutions made in at least 10 mL of broth. These dilutions are used to dispense 0.1 (±0.02) mL into each of the 96 wells of a standard tray. As described below, the inoculum may then be added using disposable plastic inoculators or by pipet. If the inoculum is to be added by pipet, the antimicrobial solutions are prepared at twice the final concentration, and the wells are filled with 0.05 mL instead of 0.1 mL. Each tray should contain both a growth control well and sterility well that is uninoculated. Alternatively, an uninoculated plate may be incubated to confirm sterility.[18]

(4) The filled trays should be sealed in plastic bags and immediately placed in a freezer at ≤-20 °C (preferably at ≤-60 °C) until needed. Although the antimicrobial agents in frozen trays usually remain stable for several months, certain agents (e.g., clavulanic acid and imipenem) are more labile than others and must be stored at ≤-60 °C. Trays must not be stored in a self-defrosting freezer; repeated freeze-thaw cycles accelerate the degradation of some antibiotics, particularly β-lactams.

11.3 Inoculation of Broth Microdilution Trays

A standardized inoculum may be prepared by either growing the microorganisms to the turbidity equivalent to a 0.5 McFarland standard or suspending colonies directly to achieve the same density as described in Section 8.

(1) Optimally, within 15 minutes of preparation, the adjusted inoculum suspension should be diluted in water or saline so that, after inoculation, each tube or well contains approximately 1 x 10^6 CFU/mL. The dilution procedure to obtain this final inoculum varies according to the method of delivery of the inoculum to the individual wells, and it must be calculated for each situation. For microdilution tests the exact inoculum volume delivered to the wells must be known to make this calculation. For example, if the volume of medium in the well is 0.1 mL and the inoculum volume is 0.01 mL, then the 0.5 McFarland suspension (~1.5 x 10^8 CFU/mL) should be diluted 1:15 to yield 1 x 10^7 CFU/mL. When 0.01 mL of this suspension is inoculated into the broth, the final test concentration of bacteria will be approximately 1 x 10^6 CFU/mL (or 1 x 10^5 CFU/well).

(2) Within 15 minutes after the inoculum has been standardized as described above, each well of a microdilution tray can be inoculated using an inoculating device that delivers a volume that does not exceed 10% of the volume in the well (e.g., ≤0.01 mL of inoculum in 0.1 mL). Conversely, if a 0.05-mL pipet is used to inoculate the wells, it results in a 1:2 dilution of the contents of each well (containing 0.05 mL).

(3) Laboratories are encouraged to perform colony counts on inoculum suspensions periodically to ensure that the final inoculum concentration routinely obtained closely approximates 1 x 10^6 CFU/mL for *B. fragilis* ATCC® 25285. This can be easily accomplished by removing a 0.01-mL aliquot from the growth control well or tube immediately after inoculation and diluting it in 10 mL of 0.9% saline. After mixing, a 0.1-mL aliquot is spread over the surface of a suitable agar medium. After incubation, the presence of approximately 100 colonies would indicate an inoculum density of 1 x 10^6 CFU/mL in the well or 10^5 CFU/well. An initial inoculum density of 0.5 McFarland, prepared as described in (1), will result in a final inoculum of ~10^6 CFU/mL for *B. fragilis* and cells of similar sizes.

(4) It is advisable to perform a purity check of the inoculum suspension by subculturing an aliquot onto two nonselective supplemented agar plates for simultaneous incubation both aerobically and anaerobically.

11.4 Incubation of Broth Microdilution Trays

(1) To maintain the same temperature for all cultures, the trays should not be stacked more than four high.

(2) Incubate the trays for 46 to 48 hours at 35 to 37 °C in an anaerobic atmosphere, using either a chamber, large jar, or other approved device.

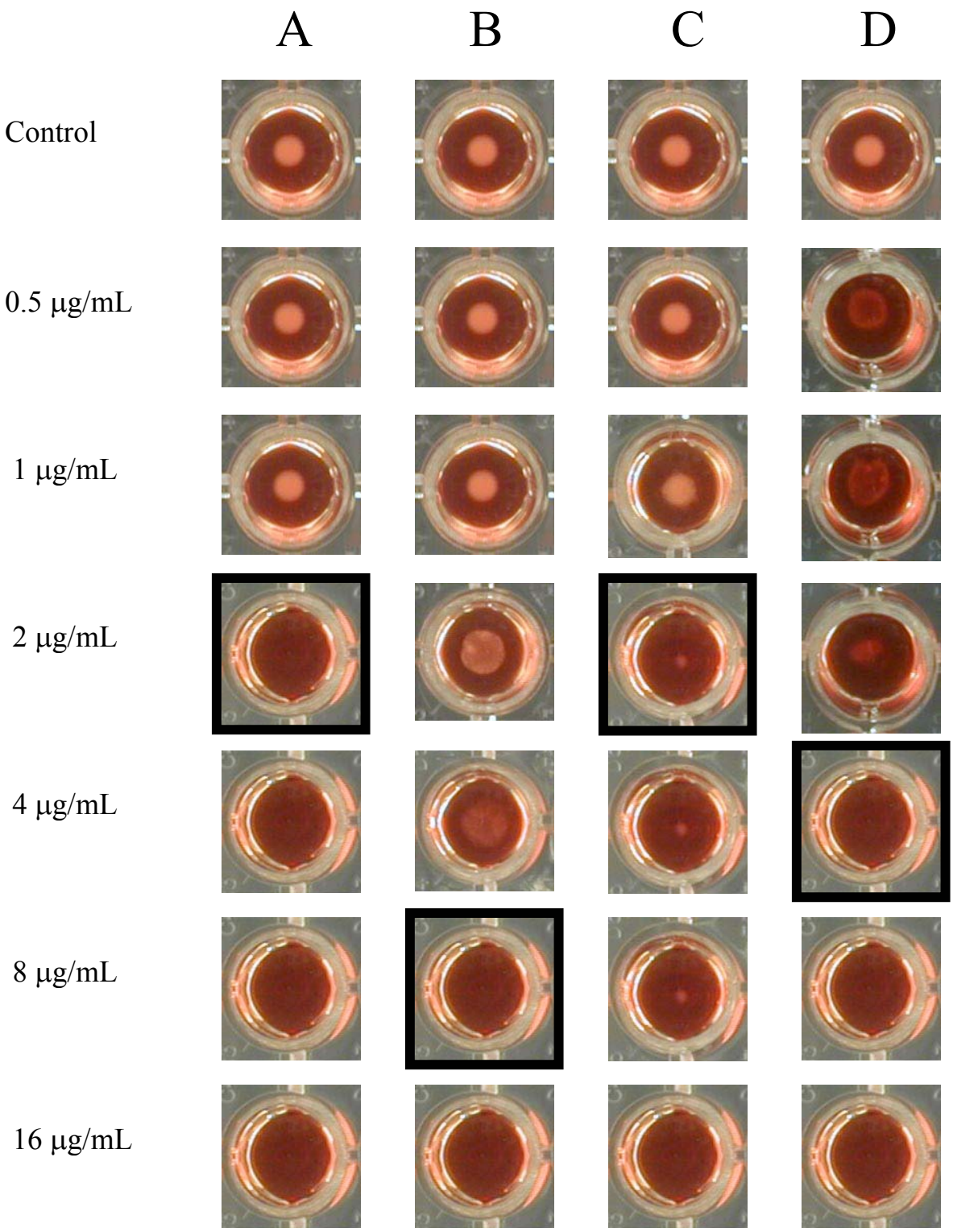

Figure 3. MIC Endpoints – Broth Microdilution. Examples of endpoints using broth microdilution method using recommended supplemented medium. Lane A, a marked reduction to no growth (MIC = 2 μg/mL); Lane B, a marked reduction to a lesser growth at 4 μg/mL and no growth at 8 μg/mL (MIC = 8 μg/mL); Lane C, a marked reduction to pinpoint (MIC = 2 μg/mL); Lane D, a reduction to lesser growth at 0.5 μg/mL, 1 μg/mL, 2 μg/mL, and no growth at 4 μg/mL (MIC = 4 μg/mL).

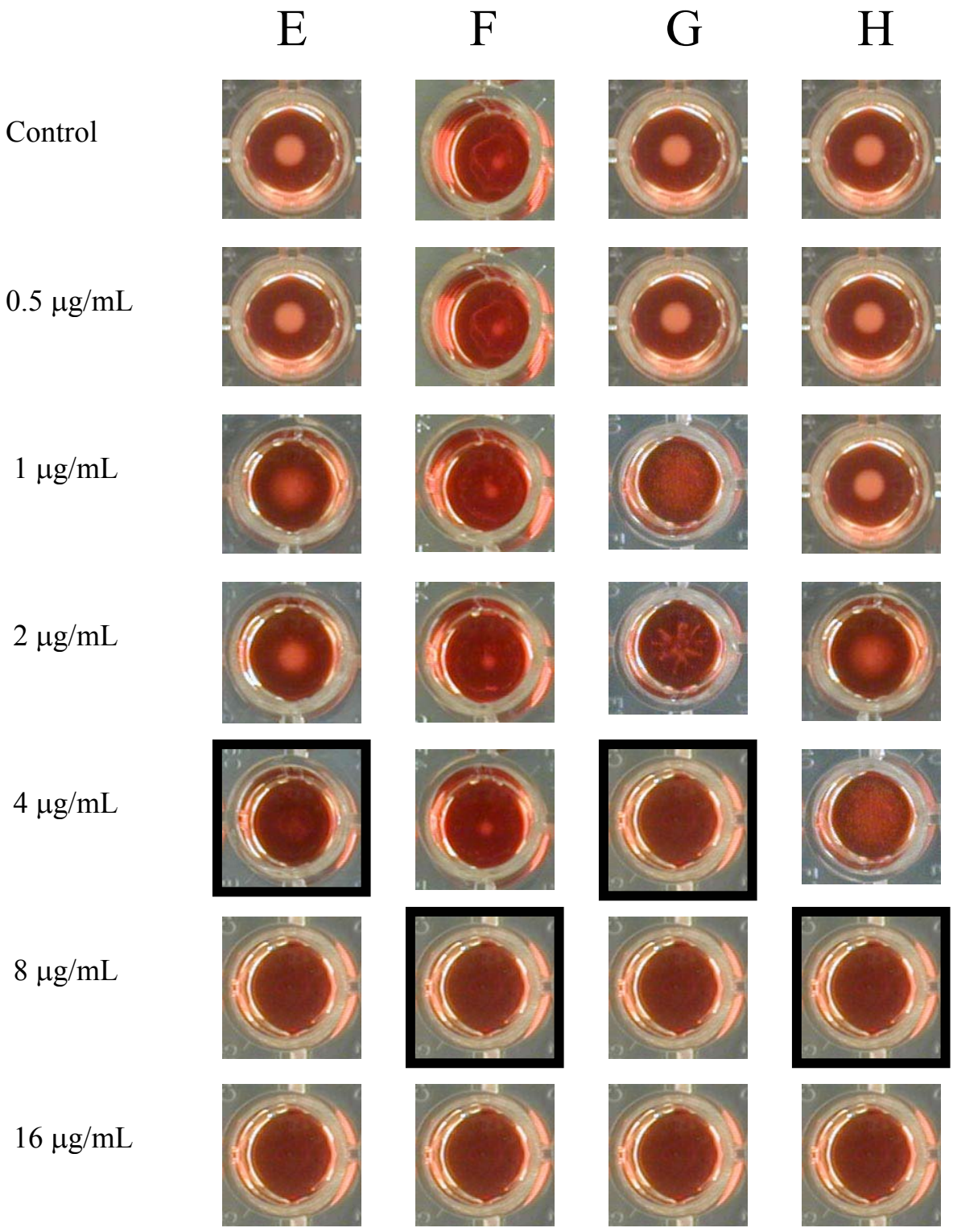

Figure 3 (Continued). Examples of endpoints using broth microdilution method using recommended supplemented medium. Lane E, reduction with diffuse growth at 1 µg/mL and 2 µg/mL, and marked reduction to very little growth at 4 µg/mL (MIC = 4 µg/mL); Lane F, ATCC® 43055 reduction from very little growth to no growth at 8 µg/mL (MIC = 8 µg/mL); Lane G, reduction in growth at 1 µg/mL and 2 µg/mL, no growth at 4 µg/mL (MIC = 4 µg/mL); Lane H, reduction with diffuse growth at 2 µg/mL and 4 µg/mL, no growth at 8 µg/mL (MIC = 8 µg/mL).

(3) Steps should be taken to prevent evaporation of the trays. This may be accomplished by incubating them in a humidified atmosphere with a cover tray or, if desiccant is used in the chamber, by placing them in a self-sealing plastic bag that is left slightly open to allow for gas exchange.

11.5 Reading Broth Microdilution Endpoints

(1) Determine the MIC value of each antimicrobial by viewing the broth microdilution plates from the bottom using a viewing apparatus (such as a stand with a mirror).

(2) If the growth in the growth control well is poor or there is no growth, the test should not be read.

(3) The MIC endpoint should be read as the concentration where no growth, or the most significant reduction of growth, is observed. A trailing effect may be observed for some drug/organism combinations.[19] Multiple examples to guide reading and determination of endpoints are shown in Figure 3. This is a subjective reading, but use of the photographs and multiple opinions during training to perform the test can help develop consistency.

12 Quality Control Guidelines

12.1 Purpose

An effective quality control program is designed to monitor the accuracy of a susceptibility test procedure, the performance of reagents and equipment, and the performance of persons who conduct the tests. These goals are best realized with the use of standard reference strains selected for their genetic stability and for their usefulness in the particular method.

Quality control must be included in order to:

- demonstrate that the test medium has adequately supported the growth of each test organism;
- verify that antimicrobial agents have not deteriorated during storage; and
- confirm that laboratory personnel performing the test have demonstrated their proficiency to perform the test correctly.

The MIC values obtained by testing the recommended quality control strains in parallel with test strains must fall within the acceptable range indicated in Tables 5 and 6 for each antimicrobial agent to be reported. Modifications of the described procedures, such as the use of supplements for enhancement of growth, must be verified with quality control procedures. If supplements are used, they should not affect the endpoints obtained with the control strains.

12.2 Batch or Lot Quality Control

For agar dilution, quality control testing using all three quality control strains should be performed whenever a new lot of a medium, reagent, supplement, or antimicrobial agent is being used for the first time. For broth microdilution plates made in-house, at least one microdilution tray from each batch should be incubated overnight to be sure of medium sterility. (The remainder of the plates are frozen for later use, if desired, to -20 ºC [or preferably ≤-60 ºC].) Quality control (QC) of each batch is then performed using all three NCCLS-recommended QC organisms. For commercially prepared plates, quality control should follow manufacturer's directions.

12.3 Control Organisms and Frequency of Testing

For agar dilution, at least two of the following three control organisms that will provide on-scale values for antimicrobials being reported are recommended for monitoring the susceptibility test procedures each time an anaerobic susceptibility test is performed. For broth microdilution and routine testing of clinical isolates, one or more of the recommended QC organisms that provides on-scale values for antimicrobials being reported are recommended for monitoring testing trays each time an anaerobic test is performed. The overall performance of the test systems should be monitored using the limits listed in Tables 5 and 6.

- *Bacteroides fragilis* ATCC® 25285
- *Bacteroides thetaiotaomicron* ATCC® 29741
- *Eubacterium lentum* ATCC® 43055

12.4 Corrective Action

When the controls are out of the specified range and there is an obvious reason for the out-of-control result (i.e., use of the wrong control strain, obvious contamination of the strain, or inadvertent use of the wrong incubation conditions or temperature), the test must be repeated. If the repeated result is within range, no further corrective action is required.

If the implicated antimicrobial agent/organism continues to test out of the allowable range, it may be necessary to obtain a new quality control strain (either from freezer storage or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. It is also helpful to exchange quality control strains and materials with another laboratory using the same method.

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

12.5 Other Control Procedures

12.5.1 Growth Control

Each agar dilution plate series and microdilution broth tray should include a growth control of basal medium without antimicrobial agent to assess viability of the test organisms and to serve as a visual aid for comparison when determining endpoints.

12.5.2 Purity Control

Before and after each antibiotic set has been stamped, two drug-free control plates (supplemented Brucella agar) are stamped. One plate is incubated anaerobically to serve as a purity and growth control, and one plate is incubated aerobically to detect aerobic contamination. In the case of broth dilution, a sample (approximately a 0.001-mL loop) from each growth control well is streaked on two supplemented Brucella plates, as growth and purity controls (anaerobic incubation) and aerobic contaminant controls (aerobic incubation). Plates maybe divided to accommodate multiple strains.

12.5.3 Inoculum Control

Plate counts are performed with representative inocula periodically to ensure that the 0.5 McFarland standard and the procedures for standardizing and diluting inocula remain within acceptable ranges. Samples for plate counts are removed immediately after inoculation from the growth control well of microdilution trays or from agar dilution from a random reservoir well of the replicator seed block.

12.5.4 Endpoint Interpretation Control

Endpoint interpretation is monitored periodically to minimize variation in the interpretation of MIC endpoints among observers. All laboratory personnel who perform these tests should independently read a selected set of dilution tests. The results are recorded and compared to the results obtained by an experienced reader. All readers should agree within ±1 twofold concentration increment of one another.[19]

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Appendix A. Preparation of Media and Supplements

A1 Supplements

A1.1 Hemin Stock Solution (5 mg/mL)

(1) Dissolve 0.1 g hemin into 2 mL of 1.0 N NaOH (certified ACS).

(2) Bring the volume to 20 mL with distilled water and sterilize at 121 ºC for 15 minutes.

(3) Store at 4 to 8 ºC, protected from light, for no longer than one month.

A1.2 Sodium Hydroxide, 1.0 N

(1) Dissolve 40 g of sodium hydroxide in 1 L of distilled water.

(2) Store at room temperature in a tightly closed container for up to six months.

A1.3 Vitamin K_1 Solutions

A1.3.1 Stock (10 mg/mL)

(1) Add 0.2 mL of vitamin K_1 (3-phytylmenadione) to 20 mL of 95% ethanol.

(2) Store at 4 to 8 ºC in a dark bottle for no longer than one year.

A1.3.2 Working Solution (1 mg/mL)

(1) Add 1 mL of stock solution to 9 mL of sterile distilled water.

(2) Store at 4 to 8 ºC in a dark bottle for no longer than one month.

A1.4 Sodium Bicarbonate Stock Solution (20 mg/mL)

(1) Dissolve 2 g $NaHCO_3$ (reagent grade, ACS) in 100 mL distilled water.

(2) Sterilize by filtration (0.2 µm).

(3) Prepare on day of usage.

A1.5 Laked Sheep Blood

(1) Freeze defibrinated sheep blood at ≤-20 ºC.

(2) Thaw blood. Acceptable methods include rapidly thawing in a waterbath at 35 to 37 °C or slowly thawing overnight at 2 to 8 °C.

(3) Mix thoroughly by inverting the container several times before use.

(4) Warm blood in a 48 to 50 °C waterbath immediately before adding to molten Brucella agar base.

Appendix A. (Continued)

A1.6 Lysed Horse Blood (LHB, 50%)

(1) Aseptically mix equal volumes of defibrinated horse blood and sterile, distilled water (50%).

(2) Freeze (at ≤-20 ºC) and thaw the 50% horse blood until cells are thoroughly lysed, five to seven times.

(3) Clarify the blood by centrifuging at 12,000 × g for 20 minutes.

(4) Decant the supernatant and check for clarity (lack of precipitate that would hinder reading MIC endpoints). To be used in the broth microdilution test, the broth and LHB must be clear. Recentrifuge if necessary.

NOTE: Lysed horse blood prepared for use in broth microdilution is available from commercial sources.

A2 Media

A2.1 Supplemented Brucella Agar

(1) Use Brucella agar dehydrated powder with the following formulation per liter:

Pancreatic digest of casein	10 g
Peptic digest of animal tissue	10 g
Dextrose	1 g
Yeast extract	2 g
Sodium chloride	5 g
Sodium bisulfite	0.1 g
Agar	15 g

(2) Combine in 1,000 mL of distilled water:

Brucella agar powder	43 g
Hemin stock solution	1 mL
Vitamin K_1 working solution	1 mL

(3) Boil to dissolve agar. Autoclave at 121 °C for 15 minutes.

(4) Cool medium to 48 to 50 ºC.

(5) Add 50 mL sterile laked sheep blood.

NOTE: Medium without blood may be refrigerated in smaller volumes in tubes, bottles, or flasks. Immediately prior to testing, melt base medium and cool to 48 to 50 ºC. Add sterile laked sheep blood in the ratio of 5 mL per 100 mL medium (or 1 mL per 20 mL medium).

Appendix A. (Continued)

A2.2 Supplemented Brucella Broth

(1) Use Brucella broth dehydrated powder with the following formulation per liter:

Pancreatic digest of casein	10 g
Peptic digest of animal tissue	10 g
Dextrose	1 g
Yeast extract	2 g
Sodium chloride	5 g
Sodium bisulfite	0.1 g

(2) Combine in 900 mL of distilled water:

Brucella broth powder	28 g
Hemin stock solution	1 mL
Vitamin K_1 working solution	1 mL

(3) Boil to dissolve. Autoclave at 121 °C for 15 minutes.

(4) Cool broth to ≤ 48 to 50 ºC and add 100 mL sterile lysed horse blood (50%).

(5) Store at 4 to 8 °C.

A2.3 Enriched Thioglycollate Broth

(1) Combine in 1,000 mL of distilled water:

Thioglycollate medium without indicator	30 g
Hemin stock solution	1 mL
Vitamin K_1 working solution	1 mL

(2) Boil to dissolve agar (thioglycollate broth contains a small amount of agar). Dispense in 5-mL amounts into tubes.

(3) Autoclave at 121 °C for 15 minutes.

(4) Store at ambient temperature, protected from light, for up to six months.

(5) Just prior to use, boil for five minutes; cool. Add 0.25 mL of filter-sterilized sodium bicarbonate stock solution to each tube.

(6) As an alternate to adding sodium bicarbonate, a marble chip may be added to each tube before autoclaving.

A2.4 McFarland Turbidity Standard (No. 0.5)

(1) Add 0.5-mL aliquot of 0.048 mol/L $BaCl_2$ (1.175 % w/v $BaCl_2 \cdot 2H_2O$) to 99.5 mL of 0.18 mol/L (0.36 N) H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.

Appendix A. (Continued)

(2) Transfer the barium sulfate suspension in 4- to 6-mL aliquots into screw-cap tubes of the same size as those used for growing or diluting the bacterial inoculum.

(3) Seal tubes tightly and store protected from light at room temperature.

(4) Vigorously agitate the suspension on a vortex mixer before each use and inspect for a uniformly turbid appearance. If large particles appear, the standard should be replaced.

(5) Verify correct density before use and reverify or replace monthly. Verify the correct density of the turbidity standard by determining the absorbency using a spectrophotometer with a 1-cm light path and matched cuvettes. The absorbency at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard.

NOTE: McFarland nephelometer standards made from latex particle suspensions are available commercially. When used, they should be mixed by inverting gently (not on a vortex mixer) immediately prior to use.

Appendix B. Media, Reagents, Supplies, Equipment, and Timetable for Agar Dilution Susceptibility Testing of Anaerobic Bacteria

Media and reagents required	• Supplemented Brucella blood agar (to grow the inoculum) • Supplemented Brucella agar (to perform the agar dilution test) • Laked, defibrinated sheep blood, sterile • Enriched thioglycollate broth, or any other enrichment broth, such as supplemented brain heart infusion or Schaedler broth • Brucella broth • Vitamin K_1, stock and working solutions • Hemin stock solution • Stock solutions of antimicrobial agents to be tested • Distilled water, sterile • 0.5 McFarland standard
Supplies and equipment required	• Screw-cap tubes, sterile, in appropriate sizes for diluting antimicrobial agents and for preparing agar blanks • Serological pipettes • Pipetting device • Petri dishes, sterile (round or square) • Water bath, 48 to 50 °C • Replicating device (e.g., Steers replicator) • Vortex mixer • Anaerobic jars or anaerobic chamber
3 to 4 weeks before the test	• Read procedure carefully. • Check supplies. Order or prepare the following as necessary: -Antimicrobial agents -Solvents and diluents -Supplies -Ingredients necessary for preparation of agar and broth media.
2 weeks before the test	• Order or prepare agar and broth media. • Order sheep blood. • Prepare and freeze antimicrobial stock solutions. • Autoclave replicator.
4 to 7 days before the test	• Subculture test isolates and QC organisms from ≤-60 °C freezer to supplemented Brucella blood agar. Check purity of isolates. • Subculture a sampling of test and QC organisms onto test agar to check for adequate growth.
1 to 3 days before the test	• Re-subculture test and QC organisms onto supplemented Brucella blood agar. • List test isolates and number the order in which they will be placed in wells of replicator. • Prepare tubes of Brucella agar base.

Appendix B. (Continued)

≤1 day before the test	• Label dilution tubes, and fill with appropriate volume of water. (See Table 4.) Cap tubes tightly. • Label petri dishes, marking for correct orientation. • Transfer laked blood to refrigerator (to thaw slowly overnight). • Make certain that waterbath is at required temperature and available for the next day. • If using the growth method for inoculum preparation, inoculate test and QC organisms to enriched thioglycollate broth and incubate at 35 to 37 °C for 6 to 24 hours, as needed.
On the day of the test	• Remove antimicrobial stock solutions from freezer and thaw to room temperature. • Remove laked blood from refrigerator and allow to warm to room temperature. • Prepare Brucella agar base and place in 48 to 50 °C waterbath. -If making fresh media, prepare tubes of Brucella agar base. -If using premade base, melt pretubed Brucella agar base. • Prepare intermediate dilutions (10x) of antimicrobial agents from stock solutions. (See Table 4.) • Disinfect area where plates will be poured. • Add blood and antimicrobial solutions to agar. Mix thoroughly and pour plates. • Allow agar to solidify in plates. • Plate surface must be dry. Allow plates to dry with lids ajar in a laminar flow hood or biological safety cabinet (15 to 30 minutes.). Alternatively, dry plates within a low-humidity incubator. • Adjust turbidity of isolates in supplemented thioglycollate broth to that of No. 0.5 McFarland standard or prepare broth suspension directly from cell paste in Brucella broth. • Pipette inoculum suspensions into replicator seed block. • Inoculate plates as follows: 1) Inoculate two growth control plates (labeled 'pre-O_2' and 'pre-Ana'). 2) Inoculate each set of antimicrobial plates from lowest concentration to highest concentration. 3) Inoculate one growth control plate between each series of antimicrobial agent plates, labeling appropriately. 4) After last set of antimicrobial plates has been inoculated, inoculate last two growth control plates (labeled 'post-O_2' and 'post-Ana'). • When inocula have dried, invert plates. • Organize plates by antimicrobial sets. • Place the O_2 plates at 35 to 37 °C in 5 to 7% CO_2 for 48 hours. • Place all plates containing antimicrobial agents and the 'Ana' control plates into an anaerobe jar or chamber. Incubate at 35 to 37 °C for 48 hours. • Sterilize replicator, rinse thoroughly with tap and distilled water. Dry, package, and resterilize.
42 to 48 hours after inoculation	• Remove all anaerobic plates from anaerobic atmosphere and aerobic control plates from CO_2 atmosphere. • Read and record results.

Table 1. Suggested Grouping of Antimicrobial Agents to be Considered for Testing Anaerobes

NOTE: Most anaerobic infections are polymicrobial including both BLA-positive and BLA-negative strains. Susceptibility of the most resistant strain must be considered first and reported. In case of an infection caused by a single BLA-negative strain, the narrower spectrum agents may be appropriate for testing and reporting.

	***Bacteroides fragilis* group & other BLA-positive anaerobes**[a,b,c]	**BLA-negative, Gram-negative anaerobes**	**Species of *Clostridium* other than *C. perfringens***	***Clostridium perfringens*, Gram-positive cocci, and nonspore-forming, gram-positive bacilli**
PRIMARY CHOICES	Ampicillin-sulbactam or amoxicillin-clavulanic acid Piperacillin-tazobactam or ticarcillin-clavulanic acid	Ampicillin or penicillin[d]	Penicillin or ampicillin[c]	Ampicillin or penicillin[c]
		Clindamycin	Amoxicillin-clavulanic acid or ampicillin-sulbactam Piperacillin-tazobactam or ticarcillin-clavulanic acid	Clindamycin
		Metronidazole		Metronidazole
	Cefoxitin Cefotetan		Cefoxitin or cefotetan	
	Ertapenem Imipenem or meropenem		Clindamycin	
	Metronidazole		Ertapenem Imipenem or meropenem	
			Metronidazole	
SUPPLEMENTAL CHOICES	Ceftizoxime Ceftriaxone	Cefotetan or cefoxitin or ceftizoxime Ceftriaxone	Ceftizoxime Ceftriaxone	Cefotetan or ceftizoxime or cefoxitin Ceftriaxone
	Chloramphenicol		Piperacillin or ticarcillin	Piperacillin or ticarcillin
		Piperacillin or ticarcillin[d]	Tetracycline[e] **Doxycycline**[e]	
	Piperacillin	Moxifloxacin[e]		Tetracycline[e] **Doxycycline**[e]
	Doxycycline[e]			
	Moxifloxacin[e]			

NOTE 1: Information in boldface type in considered tentative for one year.

NOTE 2: The boxes in the table designate clusters of classes of agents; however, interpretive results are not necessarily similar. In addition, an "or" designates a related group of agents that has an almost identical spectrum of activity and interpretive results, and for which cross-resistance and susceptibility are nearly complete.

a. Including other species of *Bacteroides, Prevotella, Porphyromonas, Fusobacterium, Veillonella, Sutterella,* and *Bilophila.*

b. BLA = β-lactamase **(activity determined by the chromogenic cephalosporin test).**

c. If β-lactamase positive, report as resistant to penicillin and ampicillin. **Be aware that BLA-negative isolates may be resistant to β-lactams by other mechanisms.**

d. Most anaerobic infections are polymicrobial, including both β-lactamase positive and β-lactamase negative strains. In the case of an infection caused by a single β-lactamase negative strain, the β-lactam, without the inhibitor, may be tested.

e. Not all of the relevant information (i.e., breakpoints [Table 2]) and quality control values [Tables 5 and 6]) are available for these agents at this point; the information will be added to the tables as soon as it is established. Testing may be applicable for accumulation of surveillance data or research purposes.

Table 2. Interpretive Categories and Minimal Inhibitory Concentration (MIC) Correlates[a]

Antimicrobial Agent	MIC (μg/mL) Susceptible	Intermediate	Resistant
Amoxicillin-clavulanic acid [b]	≤4/2	8/4	≥16/8
Ampicillin[c]	≤0.5	1	≥2
Ampicillin-sulbactam[b,d]	≤8/4	16/8	≥32/16
Cefmetazole	≤16	32	≥64
Cefoperazone	≤16	32	≥64
Cefotaxime	≤16	32	≥64
Cefotetan[b]	≤16	32	≥64
Cefoxitin [b,d]	≤16	32	≥64
Ceftizoxime[b]	≤32	64	≥128
Ceftriaxone	≤16	32	≥64
Chloramphenicol	≤8	16	≥32
Clindamycin[b,d]	≤2	4	≥8
Ertapenem[d]	≤4	8	≥16
Imipenem[b]	≤4	8	≥16
Metronidazole[b,d]	≤8	16	≥32
Meropenem[b]	≤4	8	≥16
Mezlocillin	≤32	64	≥128
Penicillin[c]	≤0.5	1	≥2
Piperacillin[b,d]	≤32	64	≥128
Piperacillin-tazobactam[b]	≤32/4	64/4	≥128/4
Tetracycline	≤4	8	≥16
Ticarcillin	≤32	64	≥128
Ticarcillin-clavulanic acid[b]	≤32/2	64/2	≥128/2
Trovafloxacin[b,d]	≤2	4	≥8

a. The intermediate range was established because of the difficulty in reading endpoints and the clustering of MICs at or near breakpoint concentrations. Where data are available, the interpretive guidelines are based on pharmacokinetic data, population distributions of MICs, and studies of clinical efficacy. To achieve the best possible levels of a drug in abscesses and/or poorly perfused tissues, which are encountered commonly in these infections, maximum approved dosages of antimicrobial agents are recommended for therapy of anaerobic infections. When maximum dosages are used along with appropriate ancillary therapy, it is believed that organisms with susceptible endpoints are generally amenable to therapy, and those with intermediate endpoints may respond but **patient response** should be carefully monitored. Ancillary therapy, such as drainage procedures and debridement, are obviously of great importance for the proper management of anaerobic infections.

b. MIC values using either Brucella blood or Wilkins Chalgren agar (former reference medium) are considered equivalent, based upon published *in vitro* literature and a multicenter collaborative trial for these antimicrobial agents.

c. Members of the *Bacteroides fragilis* group are presumed to be resistant. Other gram-negative anaerobes may be screened for β-lactamase activity with a chromogenic cephalosporin and, if positive, reported as resistant to penicillin, ampicillin, and amoxicillin. Higher blood levels are achievable; infection with non-β-lactamase producing organisms with higher MICs might be treatable. Amoxicillin breakpoints are considered equivalent to ampicillin breakpoints. **Limited *in vitro* data indicate that these two agents appear identical in MIC testing against anaerobic bacteria; however, breakpoints for amoxicillin have not been established.**

d. MIC values for agar or broth microdilutions are considered equivalent.

Table 3. Solvents and Diluents for Preparation of Stock Solutions of Antimicrobial Agents That Require Solvents Other Than Water[a,b]

Antimicrobial Agent	Solvent	Diluent
Amoxicillin-clavulanic acid	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ticarcillin-clavulanic acid	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cefotetan	Dimethyl sulfoxide[c]	Water
Chloramphenicol	95% ethanol[c]	Water
Metronidazole	Dimethyl sulfoxide[c]	Water
Ertapenem and imipenem	Phosphate buffer, pH 7.2, 0.01 mol/L	Phosphate buffer, pH 7.2, 0.01 mol/L

NOTE: All stock solutions should be stored at -60 °C or colder. After thawing, they should not be refrozen. Storage in frost-free freezers is not suitable.

a. These solvents and diluents are for making stock solutions of antimicrobial agents that require solvents other than water. They can be diluted further, as necessary, in water, buffer or broth. Except as noted in footnote "b," the products known to be soluble in water are: cefmetazole, cefoperazone, cefotaxime, cefoxitin, ceftizoxime, ceftriaxone, clindamycin, garenoxacin, meropenem, mezlocillin, penicillin, piperacillin, sulbactam, tazobactam, tetracyclines, and trovafloxacin. Because contamination is extremely rare, solutions that have not been sterilized may be used. If, however, sterilization of solutions is desired, they should be filtered through a membrane filter. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antimicrobial agents, should not be used. Whenever filtration is used, it is important to document the absence of adsorption by appropriate assay procedures.

b. All other cephalosporins and cephamycins should be dissolved (unless the manufacturer indicates otherwise) in phosphate buffer, pH 6.0, 0.1 mol/L, and diluted further in sterile distilled water.

c. These compounds are potentially toxic. Consult the Material Safety Data Sheets (MSDS) available from the product manufacturer before using any of these materials.

Table 4. Scheme for Preparing Dilutions of Antimicrobial Agents to be Used in Agar Dilution or Broth Microdilution Susceptibility Tests

Antimicrobial Solution							
Step	**Conc.**	**Source**	**Vol.**	**Diluent**	**Intermediate Concentration (µg/mL)**	**Final Conc. at 1:10 Dilution in Agar or Broth (g/L)**	**Log_2**
	5,120 µg/mL (mg/L)	Stock	-	-	5,120	512	9
1	5,120	Stock	2 mL	2 mL	2,560	256	8
2	5,120	Stock	1	3	1,280	128	7
3	5,120	Stock	1	7	640	64	6
4	640	Step 3	2	2	320	32	5
5	640	Step 3	1	3	160	16	4
6	640	Step 3	1	7	80	8	3
7	80	Step 6	2	2	40	4	2
8	80	Step 6	1	3	20	2	1
9	80	Step 6	1	7	10	1	0
10	10	Step 9	2	2	5	0.5	-1
11	10	Step 9	1	3	2.5	0.25	-2
12	10	Step 9	1	7	1.25	0.125	-3

Table 5. Acceptable Ranges of Minimal Inhibitory Concentrations (MICs) (µg/mL) for Control Strains for Reference Agar Dilution Testing

Antimicrobial Agent	*Bacteroides fragilis* ATCC®[a] 25285	*Bacteroides thetaiotaomicron* ATCC® 29741	*Eubacterium lentum* ATCC® 43055
Amoxicillin-clavulanic acid (2:1)	0.25/0.125-1/0.5	0.5/0.25-2/1	NR[b,c]
Ampicillin	16-64	16-64	-[c]
Ampicillin-sulbactam (2:1)	0.5/0.25-2/1	0.5/0.25-2/1	0.25/0.125-2/1
Cefmetazole	8-32	32-128	4-16
Cefoperazone	32-128	32-128	32-128
Cefotaxime	8-32	16-64	64-256
Cefotetan	4-16	32-128	32-128
Cefoxitin	4-16	8-32	4-16
Ceftizoxime	NR[b]	4-16	16-64
Ceftriaxone	32-128	64-256	NR[b]
Chloramphenicol	2-8	4-16	-[c]
Clinafloxacin	0.03-0.125	0.06-0.5	0.03-0.125
Clindamycin	0.5-2	2-8	0.06-0.25
Ertapenem	0.06-0.25	0.25-1	0.5-2
Garenoxacin	0.06-0.5	0.25-1	1-4
Imipenem	0.03-0.125	0.125-0.5	0.125-0.5
Linezolid	**2-8**	**2-8**	**0.5-2**
Meropenem	**0.03-0.25**	0.125-0.5	**0.125-1**
Metronidazole	0.25-1	0.5-2	-[c]
Mezlocillin	16-64	8-32	8-32
Moxifloxacin	0.125-0.5	1-4	0.125-0.5
Penicillin	8-32 (16-64)[d]	8-32 (16-64)[d]	-[c]
Piperacillin	2-8	8-32	8-32
Piperacillin-tazobactam	0.125/4-0.5/4	4/4-16/4	4/4-16/4
Tetracycline	0.125-0.5	8-32	-[c]
Ticarcillin	16-64	16-64	16-64
Ticarcillin-clavulanate	NR[b]	0.5/2-2/2	16/2-64/2
Trovafloxacin	0.06-0.5	0.25-1	0.25-1

NOTE 1: Information in boldface type is considered tentative for one year.

NOTE 2: Values are in micrograms per milliliter (µg/mL) except for penicillin.

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

b. NR indicates that no minimal inhibitory concentration range is recommended with this organism/antimicrobial combination. In certain cases, attempts to determine a quality control range have indicated that a wide range of values is obtained (amoxicillin-clavulanic acid with *E. lentum*; ceftriaxone with *E. lentum*; ceftizoxime with *B. fragilis*; and ticarcillin-clavulanate with *B. fragilis*). Accordingly, these are not suitable for quality control.

c. "NR" denotes control ranges have not been successfully established despite extensive studies; a hyphen indicates no studies have been performed by current recommended methods.

d. Penicillin values in parentheses are in units/mL.

Table 6. Acceptable Ranges of Minimal Inhibitory Concentrations (MICs) (μg/mL) for Control Strains for Broth Microdilution Testing

Antimicrobial Agent	*Bacteroides fragilis* ATCC®[a] 25285	*Bacteroides thetaiotaomicron* ATCC® 29741	*Eubacterium lentum* ATCC® 43055
Amoxicillin-clavulanic acid (2:1)	**0.25/0.125-1/0.5**	**0.25/0.125-1/0.5**	**NR[b]**
Ampicillin-sulbactam (2:1)	0.5/0.25-2/1	0.5/0.25-2/1	0.5/0.25-2/1
Cefotetan	1-8	16-128	16-64
Cefoxitin	2-8	8-64	2-16
Ceftizoxime	NR	NR	8-32
Chloramphenicol	**4-16**	**8-32**	**4-16**
Clindamycin	0.5-2	2-8	0.06-0.25
Doxycycline	**NR[b]**	**2-8**	**2-16**
Ertapenem	0.06-0.5	0.5-2	0.5-4
Garenoxacin	0.06-0.25	0.25-2	0.5-2
Imipenem	0.03-0.25	0.25-1	0.25-2
Linezolid	**2-8**	**2-8**	**0.5-2**
Meropenem	**0.03-0.25**	**0.06-0.5**	**0.125-1**
Metronidazole	0.25-2	0.5-4	0.125-0.5
Moxifloxacin	0.12-0.5	1.0-8	0.12-0.5
Penicillin	**8-32**	**8-32**	**NR[b]**
Piperacillin	4-16	8-64	8-32
Piperacillin-tazobactam	0.03/4-0.25/4	2/4-16/4	8/4-32/4
Ticarcillin-clavulanic acid	0.06/2-0.5/2	0.5/2-2/2	8/2-32/2
Trovafloxacin	0.125-0.5	0.5-2	0.25-2

NOTE 1: Information in boldface type is considered tentative for one year.

NOTE 2: For four-dilution ranges, results at the extremes of the acceptable range(s) should be suspect. Verify validity of the antimicrobial concentration with data from other control strains.

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

b. "NR" denotes control ranges have not been successfully established despite extensive studies.

NCCLS consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

M11-A5: *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Fifth Edition*

Section 8.1, Turbidity Standard for Inoculum Preparation

1. The current MIC broth method uses McFarland 1.0. This one uses 0.5 but does not explain the change. People will wonder whether or not is a mistake.

- **M11-A5 and M11-A4 both specify 0.5 McFarland for broth microdilution. Some commercial broth dilution panels utilize a 1.0 McFarland preparation (ultimately resulting in the same inoculum size).**

Section 8.2.1, Freezing Isolates for Later Testing

2. Twenty percent sterile skim milk and 80% what? I assume water, but it does not specify.

- **Twenty percent sterile skim milk and 80% water. The text has been revised for clarification.**

Section 8.3 (2), Growth Method

3. Should the incubation temperature be specified?

- **Incubator temperature is specified at 35 to 37 °C.**

Section 10.5 (2), Reading Agar Dilution Plates (Formerly Section 9.5 [2])

4. The examples given are not clear; the sentence should be reworded.

- **The text refers to the pictures in Figure 2, which should be used as a guide.**

Section 11.5, Reading Broth Microdilution Endpoints (Formerly Section 10.5)

5. My techs are concerned about how to standardize the most significant reduction of growth. Add the following statement: "This is subjective reading but use of the photographs and multiple opinions during training to perform the test can help develop consistency."

- **The additional text has been added as suggested.**

Section 12.5.1, Growth Control

6. How does the growth control help to assess the "most significant reduction of growth"?

- **The growth on test plates may be compared to the growth control plate; this is more fully described in Section 10.5, Reading Agar Dilution Plates.**

Section 12.5.2, Purity Control

7. Why do you need the aerobic purity sample?

- **This section has been revised to clarify the need for an aerobic purity sample.**

Table 1, Suggested Grouping of Antimicrobial Agents to be Considered for Testing Anaerobes

8. A footnote explaining whether drugs in the same box are to be considered equivalent and whether only one needs to be tested would be helpful. Should the word "or" be added? This table is not as clear as those for aerobic MICs.

- **Table 1 has been reformatted and explanatory notes added.**

Table 6, Acceptable Ranges of Minimal Inhibitory Concentrations (MICs) (μg/mL) for Control Strains for Broth Microdilution Testing

9. In the note at the bottom of Table 6, what is meant by "control validity?" An example would be helpful.

- **The note has been revised for clarification.**

10. Why are penicillin and/or ampicillin not in Table 6?

- **Penicillin has been added to Table 6. Only drugs which have been tested in a multiple-laboratory test are included in this table.**

Summary of Delegate Comments and Subcommittee Responses

M11-A6: *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Sixth Edition*

Figure 3, MIC Endpoints – Broth Microdilution

1. In reviewing Figure 3, Lanes C and F seem to look exactly the same, yet Lane C is interpreted as MIC = 2 μg/mL and Lane F is interpreted as MIC = 8 μg/mL. Is this interpretation correct?

- **There is actually little similarity between Lanes C and F. In Lane C, the marked reduction is from good growth at 1 μg/mL to a pinpoint at 2 μg/mL. In Lane F, there is very little growth even in the control, and the change may be seen from very little growth at 4 μg/mL to no growth at 8 μg/mL.**

NOTES

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

M11-A6 addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X M7 M23 M39						M29

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

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

M11-A6 addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Preanalytic					**Analytic**		**Postanalytic**	
Patient Assessment	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpretation	Results Report	Post-test Specimen Management
					X M7	X M7	X M7	

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

Related NCCLS Publications*

M6-A **Protocols for Evaluating Dehydrated Mueller-Hinton Agar; Approved Standard (1996).** This standard contains procedures for evaluating production lots of Mueller-Hinton agar, and for the development and application of reference media.

M7-A6 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Sixth Edition (2003).** This revised standard provides updated reference methods for the determination of minimal inhibitory concentrations (MICs) for aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution. This document contains MIC interpretive criteria and quality control parameters tables updated for 2003.

M23-A2 **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Second Edition (2001).** This document addresses the required and recommended data needed for the selection of appropriate interpretative standards and quality control guidelines for new antimicrobial agents.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** This document provides: guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

M39-A **Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data; Approved Guideline (2002).** This document describes methods for the recording and analysis of antimicrobial susceptibility test data, consisting of cumulative and ongoing summaries of susceptibility patterns of epidemiologically significant microorganisms.

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

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