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M43-A

Methods for Antimicrobial Susceptibility Testing for Human Mycoplasmas; Approved Guideline

SAMPLE

This document provides guidelines for the performance and quality control of agar and broth microdilution antimicrobial susceptibility tests on human mycoplasmas and ureaplasmas.

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

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Methods for Antimicrobial Susceptibility Testing for Human Mycoplasmas; Approved Guideline

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Abstract

Antimicrobial susceptibility testing is indicated for any organism that contributes to an infectious process warranting antimicrobial chemotherapy, if its susceptibility cannot be reliably predicted from knowledge of the organism's identity. Standardized *in vitro* antimicrobial susceptibility tests are also needed in order to evaluate new antimicrobials against specific groups of organisms in comparison with existing agents. Acquired resistance to one or more classes of antimicrobial agents has now emerged in the major mycoplasmal and ureaplasma species that infect humans, hence the need to establish accurate and reproducible methods to measure antimicrobial activities *in vitro* with these organisms.

This document provides guidelines for performance, interpretation, and quality control of *in vitro* broth microdilution and agar dilution susceptibility tests for several antimicrobial agents suitable for use against *Mycoplasma pneumoniae* (*M. pneumoniae*), *Mycoplasma hominis* (*M. hominis*), and *Ureaplasma* species (*Ureaplasma* spp). Information in this document includes designated reference strains and the expected minimal inhibitory concentration ranges for specific drugs that should be obtained when they are tested.

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Contents

Abstract.....	i
Committee Membership.....	iii
Foreword.....	vii
1 Scope.....	1
2 Introduction.....	1
2.1 Development of Minimal Inhibitory Concentration Interpretive Criteria or Breakpoints.....	2
3 Standard Precautions.....	3
4 Terminology.....	3
4.1 A Note on Terminology.....	3
4.2 Definitions.....	3
4.3 Abbreviations and Acronyms.....	4
5 Indications for Performing Susceptibility Tests on Human Mycoplasmas and Ureaplasmas ...	4
6 Antimicrobial Agents Useful for Treatment of Human Mycoplasma and Ureaplasma Infections	5
7 Consensus Method for Broth Microdilution Assay	6
7.1 Media.....	6
7.2 Supplies.....	6
7.3 Equipment.....	6
7.4 Preparation of Antimicrobial Agents.....	6
7.5 Preparation of Inoculum for Clinical Isolates and Quality Control Strains.....	7
7.6 Performance of Broth Microdilution Assay.....	7
7.7 Incubation.....	8
7.8 Determining Minimal Inhibitory Concentration End Points and Assay Validation	8
8 Consensus Method for Agar Dilution Assay	9
8.1 Media.....	9
8.2 Supplies.....	9
8.3 Equipment.....	9
8.4 Preparing Agar Dilution Plates Containing Antimicrobial Agents.....	9
8.5 Preparation of Inoculum for Clinical Isolates and Quality Control Strains.....	10
8.6 Performance of Agar Dilution Assay.....	10
8.7 Incubation.....	10
8.8 Determining Minimal Inhibitory Concentration End Points and Assay Validation ...	11
9 Quality Control and Quality Assurance Procedures	11
9.1 Purpose.....	11
9.2 Quality Control Responsibilities.....	11
9.3 Reference Strains for Quality Control	12
9.4 Preparing and Storing Quality Control Strains	12

Contents (Continued)

9.5	Frequency of Quality Control Testing.....	13
9.6	Corrective Action.....	13
9.7	Interpretation and Reporting Results.....	14
9.8	Verification of Patient Test Results.....	14
9.9	Broth Microdilution Quality Control Limits.....	15
9.10	Agar Dilution Quality Control Limits.....	16
	References.....	17
	Table 1. Minimal Inhibitory Concentration ($\mu\text{g}/\text{mL}$): Quality Control Ranges for <i>Mycoplasma hominis</i> , <i>Mycoplasma pneumoniae</i> , and <i>Ureaplasma urealyticum</i> (Broth Microdilution Method).....	19
	Table 2. Minimal Inhibitory Concentration ($\mu\text{g}/\text{mL}$): Quality Control Ranges for <i>Mycoplasma hominis</i> , <i>Mycoplasma pneumoniae</i> , and <i>Ureaplasma urealyticum</i> (Agar Dilution Method).....	19
	Table 3. <i>Mycoplasma hominis</i> Information and Minimal Inhibitory Concentration Interpretive Criteria for Broth Microdilution and Agar Dilution.....	20
	Table 4. <i>Mycoplasma pneumoniae</i> Information and Minimal Inhibitory Concentration (MIC) Interpretive Criteria for Broth Microdilution and Agar Dilution.....	21
	Table 5. <i>Ureaplasma urealyticum</i> Information and Minimal Inhibitory Concentration (MIC) Interpretive Criteria for Broth Microdilution and Agar Dilution.....	22
	Appendix A. Media Formulations.....	24
	Appendix B. Preparing Stock Solutions of Antimicrobial Agents.....	29
	Appendix C. Solvents and Diluents for Preparation of Stock Solutions of Antimicrobial Agents.....	32
	Appendix D. Determining Organism Inocula for Broth Microdilution and Agar Dilution Assays for Clinical Isolates and Quality Control Strains.....	33
	The Quality Management System Approach.....	34
	Related CLSI Reference Materials.....	35

Foreword

Methods for *in vitro* susceptibility testing of mycoplasmas were first described in the 1960s. Despite numerous publications over four decades that have reported activities of antimicrobial agents against these organisms using broth- and agar-based methodologies, there has been no universally accepted standardized reference method for testing conditions, media, or quality control (QC) minimal inhibitory concentration (MIC) reference ranges for antimicrobial agents. Lack of a consensus method for MIC determination and the complex *in vitro* growth conditions required by these fastidious organisms has led to considerable confusion and misinformation regarding antimicrobial activities of various drugs.

The need for standardized antimicrobial susceptibility testing (AST) methods and designated QC parameters for human mycoplasmas is not primarily related to a need for diagnostic laboratories to perform testing for every individual clinical specimen submitted for mycoplasma or ureaplasma culture. Conversely, it is needed because such culture-based testing is not routinely performed; susceptibilities may vary geographically and in response to selective antimicrobial pressure; and clinically significant acquired drug resistance potentially affecting multiple antimicrobial classes occurs in all of the most important mycoplasmal and ureaplasma human pathogens. Most mycoplasmal and ureaplasma infections are treated empirically. Thus, standardized AST methods are needed for surveillance of clinical isolates for resistance to currently available drugs due to potential development of resistance, because treatment is usually empirical and individual clinical isolates may need to be tested in special circumstances. Standardized AST methods are also useful to pharmaceutical companies that perform their own testing during drug development, and to reference laboratories that assist in drug development by performing AST. Such testing is required during the initial evaluation of any investigational drug for which an indication for treating infections that may be caused by these organisms is anticipated.

During the past several years, method descriptions and direct comparisons of agar- and broth-based *in vitro* AST methods for testing human mycoplasmas and ureaplasmas were published.^{1,2} The Chemotherapy Working Team of the International Research Program on Comparative Mycoplasmaology attempted to optimize media selection and testing conditions, and at least one multilaboratory investigation was undertaken to compare results. Many aspects of these procedures were incorporated directly into the protocols described in this document. However, five important factors were lacking in these earlier attempts to develop *in vitro* AST methods: 1) there was no organizing infrastructure to coordinate multilaboratory testing; 2) no attempt was made to determine intralaboratory reproducibility of testing results; 3) no standardized medium or testing protocol was adopted by all participating laboratories; 4) there were no designated readily available reference strains used; and 5) testing was limited to *Ureaplasma* spp. These deficiencies were addressed in the work that led to M43.

This guideline is the first publication under the direction of CLSI to describe standardized methods for broth microdilution- and agar dilution-based susceptibility testing of human mycoplasmas and ureaplasmas; the first to designate QC reference strains with defined MIC ranges for various antimicrobial agents; and the first document from any organization to propose interpretive breakpoints for selected antimicrobial agents for use against human mycoplasmas and ureaplasmas. The document was developed using data obtained from six academic microbiology laboratories in the United States, Canada, and France; two US pharmaceutical company microbiology laboratories; a microbiology reference laboratory in the United States; and the Centers for Disease Control and Prevention.

In addition to providing guidelines for performing *in vitro* AST and listing acceptable MIC ranges for specified reference strains for agar- and broth-based test methods, this document also includes recommendations regarding selection of antimicrobials for testing against mycoplasmas and ureaplasmas as well as recommendations for MIC interpretive criteria for a limited number of drugs. However, this document does not endorse or recommend the use of any specific antimicrobial agent for treatment of mycoplasmal or ureaplasma infections.

All of the methodology and MIC reference ranges in this document were reviewed and approved by the Antimicrobial Susceptibility Testing Quality Control Working Group and subjected to the CLSI consensus process before finalization and publication. The document development committee expects that this document will provide a valuable educational resource for researchers, clinical microbiologists, and the pharmaceutical industry in the United States and in other countries.

Ken B. Waites, MD

Chairholder, Document Development Committee on Antimicrobial Susceptibility Testing of Human Mycoplasmas

Note that the trade names IsoVitaleX[®] and Select agar[®] are included in Appendix A. It is the Clinical and Laboratory Standards Institute's policy to avoid using a trade name unless the product identified is the only one available, or it serves solely as an illustrative example of the procedure, practice, or material described. In this case, the document development committee and the consensus committee believe the trade names are used to provide instructions for preparation of the agars used for the dilution method for minimal inhibitory concentration assays, because some commercial broths may require special order to ensure they do not contain other antimicrobial agents routinely incorporated to prevent bacterial overgrowth. Because these trade names are important descriptive adjuncts to the document, it is acceptable to use the products' trade names, as long as the words "or the equivalent" are added to the references. It should be understood that information on these products in this guideline also applies to any equivalent products. Please include in your comments any information that relates to this aspect of M43.

Key Words

Agar dilution, antimicrobial susceptibility testing, broth microdilution, minimal inhibitory concentration, *Mycoplasma*, *Ureaplasma*

Methods for Antimicrobial Susceptibility Testing for Human Mycoplasmas; Approved Guideline

1 Scope

This document contains standardized protocols for broth microdilution and agar dilution *in vitro* susceptibility testing for isolates of *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma* spp. It describes the optimum media formulations for use in broth microdilution and agar dilution assays for each species; provides minimal inhibitory concentration (MIC) quality control (QC) reference ranges for ATCC^{®a} type strains; and offers recommendations for selection of antimicrobials for routine testing and MIC interpretive criteria for a limited number of drugs.

This guideline is intended for use by hospital clinical laboratories; reference microbiology laboratories; and government, industry, and academic research organizations that perform diagnostic testing and/or conduct research in mycoplasmal diseases that affect humans.

2 Introduction

Various methods of antimicrobial susceptibility testing (AST) used for conventional bacteria have been employed for testing mycoplasmas and ureaplasmas. Agar dilution has been used extensively as a reference method. It has the advantages of a relatively stable end point over time, and it allows detection of mixed cultures. However, this technique is not practical for testing small numbers of strains or occasional isolates that may be encountered in diagnostic laboratories.^{1,3} Agar disk diffusion is not useful for testing mycoplasmas because there has been no correlation between inhibitory zones and MICs, and the relatively slow growth of some of these organisms further limits this technology.³ Broth microdilution is the most widely used method to determine MICs for mycoplasmas and ureaplasmas. It allows several antimicrobials to be tested in the same microdilution plates, but, in addition to being labor intensive, it has a shifting end point over the time required for growth of some *Mycoplasma* spp.³ Studies using the agar gradient diffusion technique for detection of tetracycline resistance in *M. hominis* yielded results comparable to broth microdilution.² Additional comparative studies have also evaluated this method for determination of *in vitro* susceptibilities of *M. hominis* to fluoroquinolones and susceptibilities of ureaplasmas to various other antimicrobials.^{4,5} Agar gradient diffusion has the advantages of simplicity of agar-based testing, has an end point that does not shift over time, does not have a large inoculum effect, and can easily be adapted for testing single isolates.³

Irrespective of methodology, there have been no universally accepted standards for pH, media composition, incubation conditions, or duration of incubation for performing mycoplasmal or ureaplasma susceptibility tests. Because of inherent differences in their cultivation requirements and growth rates, no single procedure or medium can be considered sufficient for testing all of the clinically important species. No QC organisms, QC interpretive criteria for antimicrobial agents, or MIC breakpoints for use with clinical isolates of *Mycoplasma* spp. and *Ureaplasma* spp. have been endorsed by any agency or organization to date. Specific challenges that have hampered previous attempts to develop standardized assays and demonstrate reproducibility of various methods for determining *in vitro* susceptibilities of these organisms include the difficulty in measuring the concentration of organisms in the inoculum and the detection of growth in liquid medium because their small size does not result in visible turbidity; the low pH necessary for optimum growth of ureaplasmas and generation of an end point in MIC assays; the limited availability (from commercial sources) of complex media formulations necessary to support growth *in vitro*; the relatively slow growth for some species; and the tendency for broth dilution end points to shift over time.

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

Commercial MIC panels and kits, which have been available in Europe for several years, consist of microwells containing dried antimicrobials, generally in two concentrations, corresponding to thresholds proposed for conventional bacteria to classify a strain as susceptible, intermediate, or resistant. These kits give results comparable to those obtained by conventional methods of MIC determination.⁶ Some of these products are now sold in the United States, but they have not yet gained widespread use, have not been rigorously compared to nonproprietary methods for MIC determination, and do not necessarily follow recommendations included in this document.

Procedures for agar- and broth-based *in vitro* susceptibility assays and the corresponding QC procedures, reference strains, and MIC ranges relevant to both techniques are included in this document. This was done because individual laboratories may have a preference for one or the other assay format, depending on their test volume, frequency, numbers of drugs to test, and other individual needs. Because of the complexity and time-consuming nature of *in vitro* cultivation of human mycoplasmas and ureaplasmas, and the relative infrequent need and impracticality for performing AST on clinical isolates, the document development committee does not believe that the procedures described in this guideline will be widely used in hospital-based clinical laboratories. However, large-volume regional or national reference laboratories, government public health laboratories that perform surveillance for antimicrobial resistance, and pharmaceutical company laboratories will benefit from having these guidelines for use, should the need arise to evaluate *in vitro* susceptibilities of human mycoplasmas for existing or investigational antimicrobial agents.

The three separate and distinct procedures for agar and broth microdilution described in this guideline are individualized for *M. hominis*, *M. pneumoniae*, and *Ureaplasma* spp. (*Ureaplasma* spp. includes both *U. parvum* and *U. urealyticum*, which behave in similar manners and for which the same procedures, QC, and interpretive criteria apply.) There has been no attempt to generalize these methods for application to other mycoplasmal species of human or animal origin, which may have very different growth and testing requirements. Therefore, these procedures should be limited to testing only the organisms for which they are described. Despite the efforts of the laboratories participating in this multicenter project to determine MIC ranges for reference strains for all currently available antimicrobial agents relevant for testing against these organisms, there was lack of consensus for assignment of ranges for some drugs by agar and/or broth methods, so these were omitted from Tables 1 and 2 in this document after careful examination of the data by the Document Development Committee on Antimicrobial Susceptibility Testing of Human Mycoplasmas and the Antimicrobial Susceptibility Testing Quality Control Working Group.

2.1 Development of Minimal Inhibitory Concentration Interpretive Criteria or Breakpoints

Even though *in vitro* susceptibility data of mycoplasmas and ureaplasmas have been reported many times since the 1960s, and various publications have provided interpretations of MIC values, this has been done without authorization of any regulatory or advisory organizations in the United States or other countries. The Document Development Committee on Antimicrobial Susceptibility Testing of Human Mycoplasmas recognizes the fact that if there is a published guideline for performance and QC of *in vitro* AST for these organisms, it will be more valuable to users if there is some guidance regarding the meaning of the MIC values that are obtained when using the recommended procedures. The process of establishing MIC interpretive criteria or breakpoints for a drug for conventional bacteria is complex, and when such criteria are recognized and approved by the US Food and Drug Administration and/or CLSI, considerable data must be presented and analyzed. This includes evaluation of *in vitro* MIC determinations that include organisms with and without well-characterized resistance mechanisms that affect the activities of the drug, pharmacokinetic and pharmacodynamic parameters, and clinical and bacteriological outcomes of patients enrolled in large clinical trials. The process of establishing MIC breakpoints is described in CLSI document M23.⁷ As was the case for fastidious and uncommon bacteria described in CLSI document M45,⁸ clinical and microbiological data are not available for human

The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The quality management system approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any health care service’s path of workflow (ie, operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The QSEs are as follows:

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

M43-A addresses the QSE indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Organization	Customer Focus	Facilities and Safety	Personnel	Purchasing and Inventory	Equipment	Process Management	Documents and Records	Information Management	Nonconforming Event Management	Assessments	Continual Improvement
		M29				X C03 M23 M45					

Path of Workflow

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

M43-A addresses the clinical laboratory path of workflow steps indicated by an “X.”

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

Related CLSI Reference Materials*

- C03-A4** **Preparation and Testing of Reagent Water in the Clinical Laboratory; Approved Guideline—Fourth Edition (2006).** This document provides guidelines on water purified for clinical laboratory use; methods for monitoring water quality and testing for specific contaminants; and water system design considerations.
- M23-A3** **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Third Edition (2008).** This document addresses the required and recommended data needed for the selection of appropriate interpretive criteria and quality control ranges for antimicrobial agents.
- M29-A3** **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.
- M45-A2** **Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline—Second Edition (2010).** This document provides guidance to clinical microbiology laboratories for standardized susceptibility testing of infrequently isolated or fastidious bacteria that are not presently included in CLSI documents M02 or M07. The tabular information in this document presents the most current information for drug selection, interpretation, and quality control for the infrequently isolated or fastidious bacterial pathogens included in this guideline.

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