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Methods for Antimicrobial Susceptibility Testing for Human Mycoplasmas; Approved Guideline

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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Clinical and Laboratory Standards Institute
950 West Valley Road, Suite 2500
Wayne, PA 19087 USA
P: 610.688.0100
F: 610.688.0700
www.clsi.org
standard@clsi.org

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Ken B. Waites, MD
Donald J. Bade, BS
Cecile Bébéar, MD, PhD
Steven D. Brown, PhD, ABMM
Maureen K. Davidson, PhD
Lynn B. Duffy, MT(ASCP)
George Kenny, PhD
Anne Matlow, MD
Dee Shortridge, PhD
Deborah Talkington, PhD
Patricia A. Totten, PhD
Jeffrey L. Watts, PhD, RM(NRCM)
Xiaotian Zheng, MD, PhD, D(ABMM)

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 ureaplasmal 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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Committee Membership

Consensus Committee on Microbiology

John H. Rex, MD, FACP
Chairholder
AstraZeneca Pharmaceuticals
Waltham, Massachusetts, USA

Mary Jane Ferraro, PhD, MPH
Vice-Chairholder
Massachusetts General Hospital
Boston, Massachusetts, USA

Nancy L. Anderson, MMSc, MT(ASCP)
Centers for Disease Control and Prevention
Atlanta, Georgia, USA

Barbara Ann Body, PhD, D(ABMM)
Laboratory Corporation of America
Burlington, North Carolina, USA

Betty (Betz) A. Forbes, PhD, D(ABMM)
Medical College of Virginia Campus
Richmond, Virginia, USA

Thomas R. Fritsche, MD, PhD
Marshfield Clinic
Marshfield, Wisconsin, USA

Freddie Mae Poole, MS, MT
FDA Center for Devices and Radiological Health
Silver Spring, Maryland, USA

Fred C. Tenover, PhD, D(ABMM)
Cepheid
Sunnyvale, California, USA

John D. Turnidge, MD
SA Pathology At Women's and Children's Hospital
North Adelaide, Australia

Document Development Committee on Antimicrobial Susceptibility Testing of Human Mycoplasmas

Ken B. Waites, MD
Chairholder
University of Alabama Hospital Laboratory
Birmingham, Alabama, USA

Donald J. Bade, BS
Microbial Research, Inc.
Fort Collins, Colorado, USA

Cecile Bébéar, MD, PhD
Université Victor Segalen Bordeaux 2
Bordeaux, France

Steven D. Brown, PhD, ABMM
The Clinical Microbiology Institute
Wilsonville, Oregon, USA

Maureen K. Davidson, PhD
FDA Center for Veterinary Medicine
Laurel, Maryland, USA

George Kenny, PhD
University of Washington
Seattle, Washington, USA

Anne Matlow, MD
The Hospital for Sick Children
Toronto, Ontario, Canada

Dee Shortridge, PhD
bioMérieux, Inc.
Hazelwood, Missouri, USA

Deborah Talkington, PhD
Centers for Disease Control and Prevention
Atlanta, Georgia, USA

Jeffrey L. Watts, PhD, RM(NRCM)
Pfizer Animal Health
Kalamazoo, Michigan, USA

Tonya Wilbon, BS, M(ASCP)
FDA Center for Devices and Radiological Health
Rockville, Maryland, USA

Xiaotian Zheng, MD, PhD, D(ABMM)
Children's Memorial Hospital
Chicago, Illinois, USA

Staff

Clinical and Laboratory Standards Institute
Wayne, Pennsylvania, USA

Luann Ochs, MS
Vice President, Standards Development

Tracy A. Dooley, BS, MLT(ASCP)
Staff Liaison

Megan P. Larrisey, MA
Assistant Editor

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Lynn Duffy, MT(ASCP)
University of Alabama Hospital Laboratory
Birmingham, Alabama, USA

Patricia A. Totten, PhD
Harborview Medical Center
Seattle, Washington, USA

Antimicrobial Susceptibility Testing Quality Control Working Group

Steven D. Brown, PhD, ABMM
Co-Chairholder
The Clinical Microbiology Institute
Wilsonville, Oregon, USA

Sharon K. Cullen, BS, RAC
Co-Chairholder
Siemens Healthcare Diagnostics, Inc.
W. Sacramento, California, USA

William B. Brasso
BD Diagnostic Systems
Sparks, Maryland, USA

Stephen Hawser, PhD
IHMA Europe Sàrl
Epalinges, Switzerland

Janet A. Hindler, MCLS, MT(ASCP)
UCLA Medical Center
Los Angeles, California, USA

Michael D. Huband
AstraZeneca Pharmaceuticals
Waltham, Massachusetts, USA

Ronald N. Jones, MD
JMI Laboratories
North Liberty, Iowa, USA

Ann Macone
Paratek Pharmaceuticals, Inc.
Boston, Massachusetts, USA

Ross Mulder, MT(ASCP)
bioMérieux, Inc.
Hazelwood, Missouri, USA

Susan D. Munro, MT(ASCP)
Campbell, California, USA

Paul E. Oefinger, PhD, D(ABMM)
Covance Central Laboratory Services Inc.
Indianapolis, Indiana, USA

Jean B. Patel, PhD, D(ABMM)
Centers for Disease Control and Prevention
Atlanta, Georgia, USA

Robert P. Rennie, PhD
University of Alberta Hospital
Edmonton, Alberta, Canada

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Donna Crabb
University of Alabama at Birmingham
Birmingham, Alabama, USA

Dena Hensey-Rudloff
Abbott Laboratories
Chicago, Illinois, USA

M. Jeanette Jones, MSc
AstraZeneca
Waltham, Massachusetts, USA

Bill Kabat
Children's Memorial Hospital
Chicago, Illinois, USA

Danuta Kovach
The Hospital for Sick Children
Toronto, Ontario, Canada

Hélène Renaudin
Université Victor Segalen
Bordeaux, France

Lanier Thacker
Centers for Disease Control and Prevention
Atlanta, Georgia, USA

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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 ureaplasmal human pathogens. Most mycoplasmal and ureaplasmal 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 Mycoplasmology 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 ureaplasmal 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 ureaplasmal 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.