Archived Document

This archived document is no longer being reviewed through the CLSI Consensus Document Development Process. However, this document is technically valid as of September 2016. Because of its value to the laboratory community, it is being retained in CLSI's library.

September 1999

M26-A

Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline

SAMPLE

This document provides procedures for determining the lethal activity of antimicrobial agents.

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

Clinical and Laboratory Standards Institute

Setting the standard for quality in medical laboratory testing around the world.

The Clinical and Laboratory Standards Institute (CLSI) is a not-for-profit membership organization that brings together the varied perspectives and expertise of the worldwide laboratory community for the advancement of a common cause: to foster excellence in laboratory medicine by developing and implementing medical laboratory standards and guidelines that help laboratories fulfill their responsibilities with efficiency, effectiveness, and global applicability.

Consensus Process

Consensus—the substantial agreement by materially affected, competent, and interested parties—is core to the development of all CLSI documents. It does not always connote unanimous agreement, but does mean that the participants in the development of a consensus document have considered and resolved all relevant objections and accept the resulting agreement.

Commenting on Documents

CLSI documents undergo periodic evaluation and modification to keep pace with advancements in technologies, procedures, methods, and protocols affecting the laboratory or health care.

CLSI's consensus process depends on experts who volunteer to serve as contributing authors and/or as participants in the reviewing and commenting process. At the end of each comment period, the committee that developed the document is obligated to review all comments, respond in writing to all substantive comments, and revise the draft document as appropriate.

Comments on published CLSI documents are equally essential, and may be submitted by anyone, at any time, on any document. All comments are managed according to the consensus process by a committee of experts.

Appeals Process

When it is believed that an objection has not been adequately considered and responded to, the process for appeals, documented in the CLSI Standards Development Policies and Processes, is followed.

All comments and responses submitted on draft and published documents are retained on file at CLSI and are available upon request.

Get Involved—Volunteer!

Do you use CLSI documents in your workplace? Do you see room for improvement? Would you like to get involved in the revision process? Or maybe you see a need to develop a new document for an emerging technology? CLSI wants to hear from you. We are always looking for volunteers. By donating your time and talents to improve the standards that affect your own work, you will play an active role in improving public health across the globe.

For additional information on committee participation or to submit comments, contact CLSI.

Clinical and Laboratory Standards Institute
950 West Valley Road, Suite 2500
Wayne, PA 19087 USA
P: +1.610.688.0100
F: +1.610.688.0700
www.clsi.org
standard@clsi.org

SAMPLE

Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline

Abstract

Established laboratory methods that can assess the bactericidal activity of an antimicrobial agent are needed, both because of the increase in the number of patients who do not have completely normal host immune defenses and because of the new classes of antimicrobial agents that have been introduced. Clinical cure depends largely upon host factors. Bactericidal tests can provide a rough prediction of bacterial eradication. It should be noted, however, that other factors (e.g., postantibiotic effect and the growth-inhibitory effects of sub-MIC concentrations of antibiotics) may also impact bacteriologic response of patients. The special susceptibility tests that assess lethal activity are not routinely applied to all microorganisms, but are applied in unusual situations; e.g., endocarditis. Uniform test procedures are thus needed to permit comparison of different datasets.

The methods for bactericidal testing are now evolving, but more work is needed with the methodological aspects and clinical correlations. The techniques described in this document are intended primarily for testing aerobic bacteria that grow after incubation in adjusted Mueller-Hinton broth or adjusted Mueller-Hinton broth supplemented with human serum or an ultrafiltrate thereof.

Clinical and Laboratory Standards Institute (CLSI). *Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline*. CLSI document M26-A (ISBN 1-56238-384-1). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087.

The Clinical and Laboratory Standards Institute consensus process, which is the mechanism for moving a document through two or more levels of review by the health care community, is an ongoing process. Users should expect revised editions of any given document. Because rapid changes in technology may affect the procedures, methods, and protocols in a standard or guideline, users should replace outdated editions with the current editions of CLSI documents. Current editions are listed in the CLSI catalog and posted on our website at www.clsi.org. If your organization is not a member and would like to become one, and to request a copy of the catalog, contact us at: Telephone: 610.688.0100; Fax: 610.688.0700; E-Mail: customerservice@clsi.org; Website: www.clsi.org.

SAMPLE

M26-A
ISBN 1-56238-384-1
ISSN 0273-3099

September 1999

Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline

Volume 19 Number 18

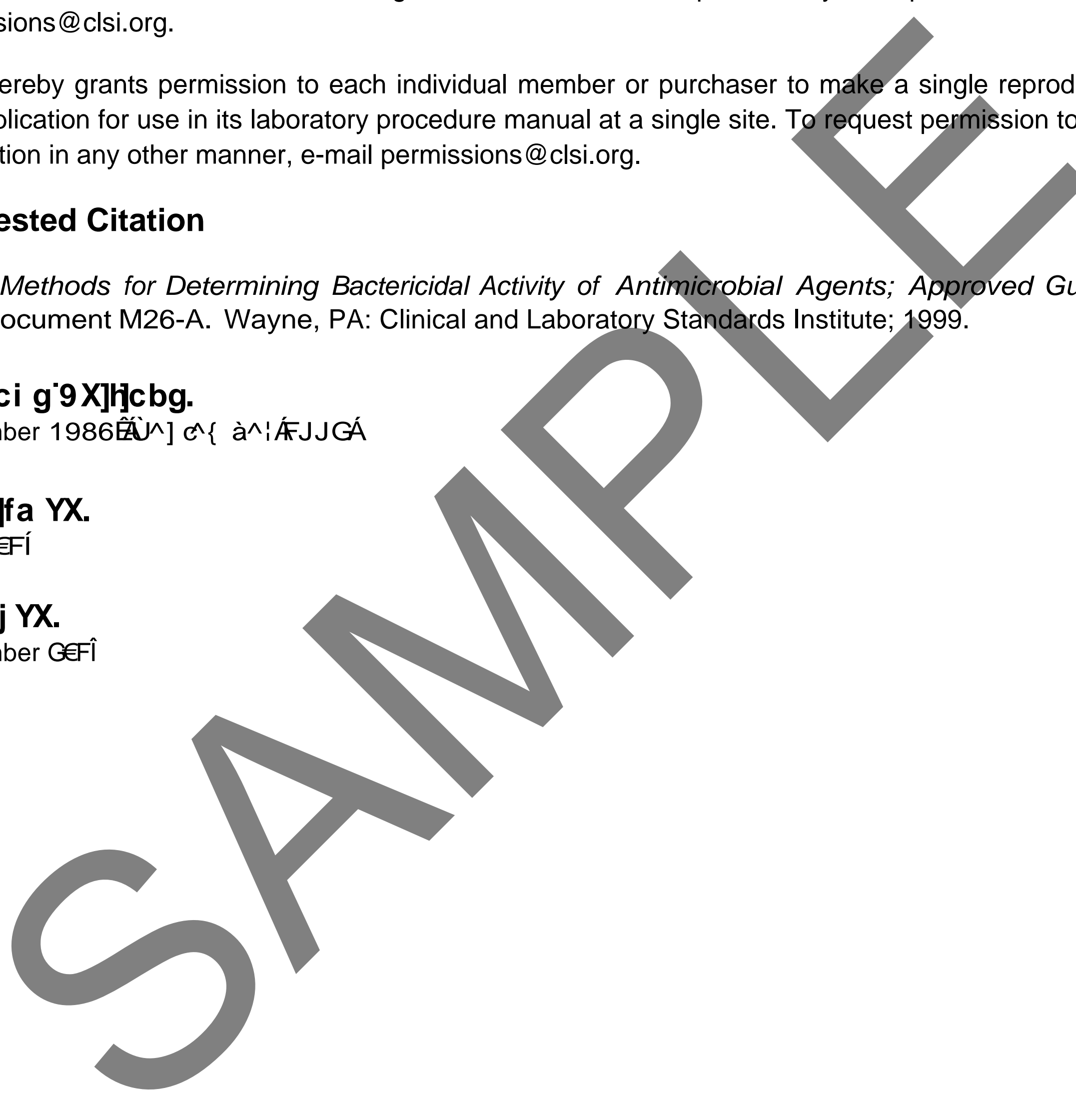
Arthur L. Barry, Ph.D.
William A. Craig, M.D.
Harriette Nadler, Ph.D.
L. Barth Reller, M.D.
Christine C. Sanders, Ph.D.
Jana M. Swenson, M.M.Sc.

SAMPLE

CLINICAL AND LABORATORY STANDARDS INSTITUTE®

Copyright ©1999 Clinical and Laboratory Standards Institute. Except as stated below, any reproduction of content from a CLSI copyrighted standard, guideline, companion product, or other material requires express written consent from CLSI. All rights reserved. Interested parties may send permission requests to permissions@clsi.org.

CLSI hereby grants permission to each individual member or purchaser to make a single reproduction of this publication for use in its laboratory procedure manual at a single site. To request permission to use this publication in any other manner, e-mail permissions@clsi.org.



Suggested Citation

CLSI. *Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline*. CLSI document M26-A. Wayne, PA: Clinical and Laboratory Standards Institute; 1999.

PrYj]ci g˙9X]h]cbg.
September 1986ÊÁÙ^]c^{ à^¦ÁFJJGÁ

FeUZZ]fa YX.
Œ]¦ä GEFÍ

AfW]]j YX.
September GEFÎ

ISBN 1-56238-384-1
ISSN 0273-3099

Contents

Abstract i

Committee Membership v

Active Membership vii

Foreword xv

1 Introduction 1

1.1 Rationale 1
1.2 Methods 1
1.3 Problems 1
1.4 Clinical Relevance 6

2 Test Methods for Determining Bactericidal Activity 8

2.1 Preparation of Antimicrobial Solutions 8
2.2 Broth Medium 8
2.3 Time-Kill Method 9
2.4 Dilution Methods 10
2.5 Preparing Inoculum 12

3 Determining Endpoints 13

3.1 Time-Kill Method 13
3.2 Agar Dilution Plate Count Method 14
3.3 Minimal Bactericidal Concentrations 14

4 Interpretation 15

5 Quality Control 16

5.1 Purpose 16
5.2 Method Control 16
5.3 Selecting Reference Strains 16
5.4 Suggested Quality Control Strains 16
5.5 Batch or Lot Control 16
5.6 Other Control Procedures 16

Table 1. Rejection Value and Calculated Sensitivity and Specificity for Each Initial Concentration on the Basis of Duplicate 0.01-mL Samples 17

Table 2. Rejection Value and Calculated Sensitivity and Specificity for Each Initial Inoculum Concentration on the Basis of a Single 0.01-mL Sample 18

Table 3. Suitable Quality Control Ranges for MICs and MBCs With and Without Human Serum Using ATCC Strains 19

SAMPLE

Contents (Continued)

References 20

Additional References 26

Summary of Comments and Subcommittee Responses 27

Related NCCLS Publications 29

SAMPLE

Foreword

All of the susceptibility test methods commonly performed by clinical microbiology laboratories (e.g., disk diffusion, broth dilution, and agar dilution) measure the inhibitory activity (MIC) of an antimicrobial agent.[1,2,3] In most clinical situations, this is sufficient as the role of the antibiotic is to prevent the spread of bacteria from the focus of infection by preventing microbial replication at new sites; the active participation of the host's defense mechanisms finally achieves bacterial eradication and clinical cure.[4] Antimicrobial assays can provide additional valuable information on the pharmacokinetics of the agent(s) being used and, when combined with the MICs, can allow bacterial eradication to be predicted.

On occasion, it may be necessary to achieve bactericidal activity with an antimicrobial agent. This need has been well documented for endocarditis[5] and has been suggested by some for meningitis,[6] for osteomyelitis,[7] as well as for infections in immunocompromised patients.[8] The clinical occurrence of tolerance[9] may on rare occasion necessitate bactericidal testing.

When assessment of bactericidal activity is deemed appropriate, an *in vitro* test method such as the MBC determination or the use of time-kill kinetic methodology may be useful. Bactericidal activity against the patient's isolate by the antibiotic tested allows eradication to be predicted based upon the usual dosing of this antibiotic or based upon the results of an antimicrobial assay. When clinical experience is lacking and assay methods are not readily available, the serum bactericidal test which integrates both pharmacodynamic and pharmacokinetic properties may be more useful. Depending on certain modifications to the serum bactericidal test, the test can provide a quantitative assessment of bactericidal activity relative to the MBC (the serum bactericidal titer), a dynamic assessment of rapidity of killing over time (the serum bactericidal rate), or both the magnitude of serum bactericidal activity and its duration (the area-under-the-bactericidal-titer-curve). In addition, methods using serum from persons (e.g., volunteers) receiving antibiotics (*ex vivo*) can be used to assess antimicrobial bactericidal activity across drug classes or between members of a class against a wide variety of microorganisms.

Because of the complexity involved with the serum bactericidal test (including the particular method used, the proper collection of timed serum specimens, and the interpretation of results), and the lack of clinical data clearly documenting the usefulness of this test for most infections, it is recommended that consultation with the microbiology laboratory be obtained as a prerequisite for this test. The assistance of the laboratory's director is useful in (1) determining if such a test is needed; (2) selecting NCCLS recommended methodology for testing; and (3) interpreting the results. Techniques for the conduct of the serum bactericidal test may be found in the most current edition of NCCLS document M21—*Methodology for the Serum Bactericidal Test*.

This document describes the details of bactericidal testing and, in particular, the effects of variations in methodology. This information has been obtained largely from published data. Use of these guidelines should result in uniform methodology for bactericidal testing that is sufficiently practical for use in the clinical microbiology laboratory. The methodology described in this document does not imply *per se* that bactericidal testing is clinically relevant, but instead allows such testing to be used as a tool to assess clinical relevance. The techniques described in this document are intended primarily for testing aerobic bacteria that grow well after overnight incubation in either Mueller-Hinton broth or in Mueller-Hinton broth supplemented with human serum as described in Section 2.2.1. Modifications for more fastidious microorganisms such as anaerobes will be described in detail in the future.

SAMPLE

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 new 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, 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 recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

Key Words

Bactericidal activity, minimal bactericidal concentration (MBC), minimal lethal concentration, serum bactericidal concentration, time-kill determination

SAMPLE

Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline

1 Introduction

1.1 Rationale

An increasing number of patients with infections do not have completely normal host immune defenses. In addition, classes of antibiotics that have been considered to be bactericidal can no longer be assumed to kill every clinical isolate due to the possibility of tolerance.[9] Finally, as new classes of antimicrobial agents are introduced, there is a need for established laboratory methods that can assess the bactericidal activity of these agents. Such methods for assessing lethal activity should be considered special susceptibility tests because they are not routinely applied to all microorganisms; rather, they are applied in unusual situations. Because of their specialized nature, complexity, and potential difficulty for interpretation, tests for bactericidal activity should be done in the context of consultation with appropriate persons (such as the microbiology laboratory director) who are aware of the potential problems involved in such testing.

1.2 Methods

The killing effect of an antimicrobial agent on a microorganism can be assessed in several ways:

(1) Lethal activity may be expressed as the rate of killing by a fixed concentration of drug under controlled conditions. This rate is determined by measuring the number of viable bacteria at various time intervals. The resulting graphic depiction is known as the time-kill curve. Bacterial killing rates are, in part, dependent on the class of antibiotic and the concentration of this agent. With certain classes of antibiotics (e.g., aminoglycosides and fluoroquino-lones), the rate of killing increases with increased drug concentrations up to a point of maximum effect.[10] This is termed *concentration-dependent bactericidal acti-vity*. In contrast, the killing rates of β-lactam agents and vancomycin are relatively slow and continue only as long as the concentrations are in excess of the MIC.[10,11,12] This rate of killing is termed *time-dependent bactericidal activity.*

(2) The minimal concentration of drug needed to kill most (≥99.9%) of the viable organisms after incubation for a fixed length of time (generally 24 hours) under a given set of conditions is the most common estimation of bactericidal activity and is known as either the minimal bactericidal concentration (MBC) or the minimal lethal concentration (MLC). It will be referred to in this document as the MBC. Unfortunately, the definition of the MBC (99.9% killing of the final inoculum) is somewhat arbitrary and separates the bacteria into two populations – a segrega-tion which might not have biological relevance.[13] The determination of the MBC, moreover, is so subject to methodologic variables that the clinical relevance of MBCs is nearly impossible to assess, particularly for certain pathogen and drug combinations (e.g., staphylococci and beta-lactam agents).[14]

(3) The serum of a patient receiving an antibiotic may be tested against the infecting microorganism. This can be done using time-kill curve methodology (i.e., serum bactericidal rate) or using dilution methodology (i.e., serum bactericidal titer). The principles of these methods as well as the influence of biological and technical factors are similar.

1.3 Problems

Evaluating *in vitro* lethal effects of an antimicrobial agent is conceptually attractive and appears, at times, to be clinically necessary. However, many biological and technical factors are known to interfere with such *in vitro* measurement of killing.

The biological factors include:

- Persisters
- Paradoxical effect
- Tolerance

SAMPLE

- Phenotypic resistance.

The technical factors include:

- Growth phase of inoculum
- Inoculum size
- Insufficient contact
- Volume transferred
- Antibiotic carryover
- Choice of media.

These variables create uncertainty in the interpretation of bactericidal activity and are a major reason that professional consultation with the microbiology director is needed.

1.3.1 Biological Factors

1.3.1.1 Persisters

For some well-studied antibiotics, e.g., β-lactam agents, a small number (usually <0.1% of the final inoculum) of bacteria were found to survive the lethal effect of an antibiotic.[15] If these persisters are retested, they are just as susceptible as the parent strain and no greater proportion of cells persist. This phenomenon is thought to be due to the fact that some cells are dormant or replicating slowly and consequently are not killed by the antimicrobial agent.[16] The rate of antimicrobial agent-induced killing is strictly proportional to the rate of bacterial growth.[17] Thus, the slower the rate of bacterial growth, the slower the bactericidal effect of the antibiotic. As the growth of a microorganism reaches its maximum, the rate slows and so does the killing effect.

1.3.1.2 Paradoxical Effect

Another factor known as the *paradoxical effect* occurs when the proportion of surviving cells increases significantly as the concentration of the antimicrobial agent increases beyond the MBC.[18,19] This phenomenon is particularly common for cell wall-active agents. It is thought that a high concentration of penicillin inhibits protein synthesis to a degree which prevents the growth necessary for expression of the lethal effect of the drug. Penicillin also has been found to lyse RNA[20] and this might be related to the paradoxical effect.

There is also another mechanism of penicillin-induced cell death which is not related to lysis[21]; the relationship of this mechanism to the paradoxical effect is unknown. A paradoxical effect of aminoglycosides on the growth of gram-negative bacilli also has been described.[22]

The clinical relevance of the paradoxical effect is unclear. However, Eagle and his coworkers[23] infected mice with group B streptococci and demonstrated that the bacteria were killed *in vivo* more slowly by high doses of penicillin. There is at least one reported case[24] where a reduction in dosage of penicillin (peak levels decreasing from 36.7 to 11.3 µg/mL) resulted in a marked increase in bactericidal activity in the patient's serum (peak titers increasing from 1:8 to 1:256) with coincident improvement in clinical status.

1.3.1.3 Tolerance

Tolerance means that the microorganism is able to evade only the lethal action of the antimicrobial agent; there is no change in the MIC.[9,18,25-27] At least four mechanisms have been described which enable clinical isolates to survive during therapy with cell-wall-active agents. Two of these, persisters and the paradoxic effect, have already been described.

Another mechanism is phenotypic tolerance.[16] Phenotypic tolerance is a property of virtually all strains of bacteria and is defined as decreased susceptibility to antimicrobial agents which is manifested only under certain growth conditions.

The last mechanism of tolerance is that in which a microorganism possesses or acquires a unique genetic property, such as a defective autolytic system.[27]

All tolerant isolates, no matter which mechanism is responsible, exhibit unusually high MBCs relative to their MICs, and tolerance has been defined as an MBC-MIC ratio of 1:32 or greater after 24 hours of incubation. However, such a ratio cannot distinguish phenotypic tolerance from tolerance due to a unique genetic property. Instead, a time-kill kinetic study is needed to differentiate these

SAMPLE

Related NCCLS Publications*

M2-A6 **Performance Standards for Antimicrobial Disk Susceptibility Test Sixth Edition; Approved Standard (1997).** This document provides current recommended techniques for disk susceptibility testing, new frequency criteria for quality control testing, and updated tables for interpretive zone diameters.

M7-A4 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically Fourth Edition; Approved Standard (1997).** This document provides reference methods for the determination of minimal inhibitory concentrations (MIC) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

M11-A4 **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard Fourth Edition (1997).** This document provides methods for susceptibility testing of anaerobic bacteria; description of reference agar dilution method, alternative agar methods (Wadsworth and limited dilutions), broth microdilution, and broth (macro) dilution procedures, and quality control criteria for each procedure.

M21-A **Methodology for the Serum Bactericidal Test; Approved Guideline (1999).** This document provides a direct method of antimicrobial susceptibility testing using a patient's serum to measure the activity of the serum against the bacterial pathogen isolated from the patient.

M29-A **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** 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.

SAMPLE

NOTES

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

CLINICAL AND LABORATORY STANDARDS INSTITUTE®

SAMPLE

Explore the Latest Offerings From CLSI!

As we continue to set the global standard for quality in laboratory testing, we are adding products and programs to bring even more value to our members and customers.

By becoming a CLSI member, your laboratory will join 1,600+ other influential organizations all working together to further CLSI's efforts to improve health care outcomes. You can play an active role in raising global laboratory testing standards—in your laboratory, and around the world.

Find out which membership option is best for you at **www.clsi.org/membership.**

Find what your laboratory needs to succeed! CLSI U provides convenient, cost-effective continuing education and training resources to help you advance your professional development. We have a variety of easy-to-use, online educational resources that make *e*Learning stress-free and convenient for you and your staff.

See our current educational offerings at **www.clsi.org/education.**

When laboratory testing quality is critical, standards are needed and there is no time to waste. eCLIPSE™ Ultimate Access, our cloud-based online portal of the complete library of CLSI standards, makes it easy to quickly find the CLSI resources you need.

Learn more and purchase eCLIPSE at **clsi.org/eCLIPSE.**

For more information, visit www.clsi.org today.

SAMPLE

950 West Valley Road, Suite 2500, Wayne, PA 19087 USA
P: +1.610.688.0100 **Toll Free (US):** 877.447.1888 **F:** +1.610.688.0700
E: customerservice@clsi.org **www.clsi.org**

ISBN 1-56238-384-1