



M27-A3

Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition

This document addresses the selection and preparation of antifungal agents; implementation and interpretation of test procedures; and quality control requirements for susceptibility testing of yeasts that cause invasive fungal infections.

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

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Abstract

Clinical and Laboratory Standards Institute document M27-A3—*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition* describes a method for testing the susceptibility of antifungal agents to yeast that cause invasive fungal infections, including *Candida* spp. (and *Candida glabrata*), and *Cryptococcus neoformans*. Selection and preparation of antifungal agents, implementation and interpretation of test procedures, and the purpose and implementation of quality control procedures are discussed. A careful examination of the responsibilities of the manufacturer and the user in quality control is also presented.

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Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory aids to guide in the selection of antifungal therapy have gained greater attention. In 1982, the CLSI Area Committee for Microbiology formed the Subcommittee on Antifungal Susceptibility Testing. In 1985, this subcommittee published its first report¹ in which the results of a questionnaire and a small collaborative study were presented. These results are summarized as follows:

- Approximately 20% of the responding CLSI membership whose hospitals had greater than 200 beds was performing antifungal testing.
- Most testing involved broth dilution methodology.
- Most strains tested were *Candida albicans* or other species of yeasts.
- Most centers tested only a few isolates per year.
- Agreement in minimal inhibitory concentration (MIC) results among several laboratories that participated in a collaborative study was unacceptably low.

Based on these findings, the subcommittee concluded that it would be useful to work toward a more reproducible reference testing procedure.

Agreement already existed regarding several elements of the procedure. To facilitate further analysis of various test conditions, the reference method should be a broth macrodilution procedure. Because of examples of drug antagonism by some complex media for certain antifungals, the subcommittee restricted its interest only to fully defined synthetic media. Drug stock solution preparation and dilution procedures previously developed for antibacterial testing procedures were adopted with minor modifications.

Despite agreement in some areas, other factors required additional data to be resolved. These included inoculum preparation; inoculum size; choice among several synthetic media; temperature of incubation; duration of incubation; and end-point definition. These factors were the focus of a series of collaborative studies.²⁻⁵ As a result, agreement within the subcommittee was achieved on all of the factors and led to the publication of M27-P in 1992. In the next four years (1992-1996), reference MIC ranges were established for two quality control strains for the available antifungal agents,^{6,7} and broth microdilution procedures paralleling the broth macrodilution reference procedure became available.^{5,8-10} This information was included in a revised standard in 1995 (M27-T). In further revising the document, the subcommittee focused its attention on developing relevant breakpoints for available antifungal agents,¹¹ included in M27-A (1997). Since then, the subcommittee has developed 24- and 48-hour reference MIC ranges for microdilution testing of both established and newly introduced antifungal agents.¹² The results of these studies are included in the current M27-A3 and M27-S3 (Informational Supplement)¹³ documents.

Key Words

antifungal, broth macrodilution, broth microdilution, susceptibility testing, yeasts

Updated Information in This Edition

Definitions (Section 4)

Modified definition:

Minimal inhibitory concentration (MIC)

Added definition:

Antimicrobial susceptibility test interpretive category

Quality control

Additional Section

Indications for performing susceptibility tests (Section 5)

Time of reading (Section 7.8.1)

Data Inclusion/Exclusion

Established numerical scale criteria for visual comparison of the amount of growth in the control tubes (Section 7.6)

Established guide for reading and interpretation of results of Echinocandin antifungals (Sections 7.6.3 and 7.7.8)

Expanded recommendations and explanations on acceptable time of reading for antifungal agents when growth is adequate (Sections 7.8.1 and 7.9)

Tables

All related tables were updated and compiled separately as M27-S3, Informational Supplement instead of a document Appendix. Updates on each table include:

Table 1: Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida* spp.

Added new column on “nonsusceptible (NS)” criteria for interpretive guidelines.

Added breakpoints criteria for the following antifungal agents:

Anidulafungin

Caspofungin

Micafungin

Voriconazole (first added in M27-S2, published February 2006)

Provided additional footnote information for Flucytosine, Anidulafungin, Caspofungin, and Micafungin.

Table 2: Solvents and Diluents for Preparation of Stock Solutions of Antifungal Agents

Added solvents and diluents recommendations for the following antifungal agents:

Anidulafungin

Caspofungin

Micafungin

Table 5: Recommended 48-Hour MIC Limits for Two Quality Control and Four Reference Strains for Broth Macrodilution Procedures

Added information on *Issatchenkia orientalis* as the known sexual form of *Candida krusei*.

Table 6: Recommended 24- and 48-Hour MIC Limits for Two Quality Control Strains for Broth Microdilution

Added the following antifungal agents:

Anidulafungin (first added in M27-S2, published February 2006)

Caspofungin (first added in M27-S2, published February 2006)

Micafungin

Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition

1 Scope

This document describes a method for testing the susceptibility to antifungal agents of yeasts, including *Candida* spp. and *Cryptococcus neoformans*, that cause infections. This method has not been extensively validated for the yeast forms of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum* variety *capsulatum*.

The subcommittee has focused on developing relevant breakpoints for available antifungal agents,¹¹ and reference MIC ranges for microdilution testing of both established and newly introduced antifungal agents.¹² Interpretive minimal inhibitory concentration (MIC) breakpoints and MIC ranges for quality control (QC) isolates are summarized in an Informational Supplement¹³ to this document.

2 Introduction

The broth macrodilution method described in this document is intended for testing yeasts that cause invasive infections. These yeasts encompass *Candida* spp. (including *Candida glabrata*) and *C. neoformans*. The method has not been used in studies of the yeast forms of dimorphic fungi, such as *B. dermatitidis* and/or *H. capsulatum* variety *capsulatum*. Moreover, testing filamentous fungi (moulds) introduces several additional problems in standardization not addressed by the current procedure. A reference method for broth dilution antifungal susceptibility testing of filamentous fungi has been developed and is now available as CLSI document M38.¹⁴⁻¹⁶

M27-A3 is a “reference” standard developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of yeasts to antifungal agents. An important use of a reference method is to provide a standard basis from which other methods can be developed, which also will result in interlaboratory agreement within specified ranges. For example, broth microdilution methods, described in this document, have been configured to produce results paralleling those obtained by the broth macrodilution reference method. Such methods might have particular advantages, such as ease of performance, economy, or more rapid results; therefore, their development could be highly desirable. To the extent that any method produces concordant results with this reference method, it would be considered to be in conformity with M27-A3.

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention.¹⁷ For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to CLSI document M29.¹⁸

4 Definitions

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

antimicrobial susceptibility test interpretive category – 1) a classification based on an *in vitro* response of an organism to an antimicrobial agent at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent; **2) susceptible antimicrobial susceptibility test interpretive category** – a category that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection; **3) susceptible-dose dependent antimicrobial susceptibility test interpretive category** – the “susceptible-dose dependent” category implies clinical efficacy when higher than normal dosage of a drug can be used and maximal possible blood level achieved; **4) intermediate antimicrobial susceptibility test interpretive category** – the “intermediate” category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates and/or available data do not permit them to be clearly categorized as either “susceptible” or “resistant.” This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations; **5) resistant antimicrobial susceptibility test interpretive category** – a category that implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules; **6) nonsusceptible** – this category is used for organisms that currently have only a susceptible interpretive category, but not intermediate or resistant interpretive categories (ie, susceptible-only interpretive category) and is often given to new antimicrobial agents for which no resistant isolates have yet been encountered.

minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that causes a specified reduction in visible growth in an agar or broth dilution susceptibility test. The magnitude of reduction in visible growth is assessed using the following numerical scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in visible growth; 3, slight reduction in visible growth; and 4, no reduction in visible growth.

quality control – the operational techniques that are used to ensure accuracy and reproducibility.

5 Indications for Performing Susceptibility Tests¹⁹⁻²¹

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. Susceptibility tests are most often indicated when the causative organism is thought to belong to a species capable of exhibiting resistance to commonly used antimicrobial agents. Mechanisms of resistance include alteration of drug targets, altered drug uptake or efflux, and absence of microbial enzymes to metabolize drug to active form. Some organisms have predictable susceptibility to antimicrobial agents, and empiric therapy for these organisms is widely accepted. Susceptibility tests are also important in studies of the epidemiology of resistance and in studies of new antimicrobial agents.

Isolated colonies of each type of organism that may be pathogenic should be selected from primary agar plates and tested for susceptibility. Identification procedures are often performed at the same time. Mixtures of different types of microorganisms should not be tested on the same susceptibility test plate or panel. The practice of conducting susceptibility tests directly with clinical material (eg, normally sterile body fluids and urine) should be avoided except in clinical emergencies when the direct gram stain suggests a single pathogen. When testing has been carried out directly with the clinical material, results should be reported as preliminary, and the susceptibility test must be repeated using the standardized methodology.

When the nature of the infection is not clear and the specimen contains mixed growth or normal flora, in which the organisms probably bear little relationship to the infectious process being treated, susceptibility tests are often unnecessary, and the results may be misleading.

The MIC obtained using a dilution test may tell a physician the concentration of antimicrobial agent required at the site of infection to inhibit the infecting organism. The MIC, however, does not represent an

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 approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*. 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:

- | | | | |
|----------------------------------|--|--|--|
| Documents & Records Organization | Equipment Purchasing & Inventory Process Control | Information Management Occurrence Management Assessments—External & Internal | Process Improvement Customer Service Facilities & Safety |
|----------------------------------|--|--|--|

M27-A3 addresses the QSEs 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.

| Documents & Records | Organization | Personnel | Equipment | Purchasing & Inventory | Process Control | Information Management | Occurrence Management | Assessment—External & Internal | Process Improvement | Customer Service | Facilities & Safety |
|---------------------|--------------|-----------|-----------|------------------------|--|------------------------|-----------------------|--------------------------------|---------------------|------------------|---------------------|
| M7 | | | | | X M2 M7 M11 M23 M24 M29 M38 | | | | | | M29 |

Adapted from CLSI/NCCLS document HS1—*A Quality Management 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, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow, which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

M27-A3 addresses the clinical laboratory path of workflow steps 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.

| 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 |
| | | | M24 | X M24 M38 | X M2 M7 M11 M24 M38 | X M2 M7 M11 M24 M38 | X M2 M7 M11 M24 M38 | X M24 M38 |

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

Related CLSI Reference Materials*

- M2-A9** **Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition (2006).** This document contains the current Clinical and Laboratory Standards Institute-recommended methods for disk susceptibility testing, criteria for quality control testing, and updated tables for interpretive zone diameters.
- M7-A7** **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition (2006).** This document addresses reference methods for the determination of minimal inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.
- M11-A7** **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Seventh Edition (2007).** This standard provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by agar dilution and broth microdilution.
- 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.
- M24-A** **Antimycobacterial Susceptibility Testing; Approved Standard (2003).** This standard provides protocols and related quality control parameters and interpretive criteria for the susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.
- M29-A3** **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on U.S. 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.
- M38-A** **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard (2002).** This document addresses the selection of antifungal agents; preparation of antifungal stock solutions and dilutions for testing; implementation and interpretation of test procedures; and quality control requirements for susceptibility testing of filamentous fungi (moulds) that cause invasive fungal infections.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most current editions.



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