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 and cutaneous fungal infections.

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Abstract

Clinical and Laboratory Standards Institute document M38-A2—Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition describes a method for testing the susceptibility of filamentous fungi (moulds) that cause invasive (Aspergillus spp., Fusarium spp., Rhizopus oryzae (R. arrhizus), Pseudallescheria boydii [Scedosporium apiospermum], S. prolificans, Sporothrix schenckii, and other opportunistic pathogenic moulds) and cutaneous (dermatophyte, Trichophyton, Microsporum, and Epidermophyton spp.) fungal infections to antifungal agents. Selection of antifungal agents, preparation of antifungal stock solutions and dilutions for testing, 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.

Contents

Abstract .................................................................................................................................................... i
Committee Membership ........................................................................................................................ iii
Foreword .............................................................................................................................................. vii
Updated Information in This Edition ................................................................................................ viii
1 Scope .......................................................................................................................................... 1
2 Introduction ................................................................................................................................ 1
3 Standard Precautions .................................................................................................................. 1
4 Definitions ...................................................................................................................................... 2
5 Antifungal Agents ...................................................................................................................... 2
  5.1 Source ................................................................................................................................... 2
  5.2 Weighing Antifungal Powders .............................................................................................. 2
  5.3 Preparing Stock Solutions .................................................................................................... 3
  5.4 Number of Concentrations Tested ...................................................................................... 4
  5.5 Selection of Antifungal Agents for Routine Testing and Reporting ..................................... 4
6 Test Procedures .......................................................................................................................... 5
  6.1 Broth Medium ....................................................................................................................... 5
  6.2 Preparing Diluted Antifungal Agents ................................................................................... 5
  6.3 Inoculum Preparation ......................................................................................................... 6
  6.4 Inoculating RPMI-1640 Medium ......................................................................................... 7
  6.5 Incubation ........................................................................................................................... 7
  6.6 MIC and MEC Reading Results ........................................................................................ 7
  6.7 Interpretation of Results ...................................................................................................... 9
  6.8 Broth Macrodilution Modifications ...................................................................................... 10
  6.9 Other Modifications ............................................................................................................ 11
7 QC ............................................................................................................................................ 11
  7.1 Purpose ............................................................................................................................... 11
  7.2 QC Responsibilities ............................................................................................................. 11
  7.3 Selecting Reference Strains .................................................................................................. 12
  7.4 Storing Reference Strains .................................................................................................... 12
  7.5 Routine Use of Reference Strains ........................................................................................ 13
  7.6 Batch of Medium and Lot of Plasticware Control ................................................................ 14
  7.7 QC Frequency .................................................................................................................... 14
  7.8 Other Control Procedures ................................................................................................... 15
  7.9 QC Strains .......................................................................................................................... 15
Table 1. Solvents and Diluents for Preparation of Stock Solutions of Antifungal Agents ................... 16
Table 2. Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests for Nondermatophyte Isolates ......................... 17
Contents (Continued)

Table 2A. Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests for Dermatophyte Isolates ................................. 18

Table 3. Scheme for Preparing Dilutions of Water-Soluble Antifungal Agents to Be Used in Broth Dilution Susceptibility Tests ................................................................. 19

Table 4. Recommended MIC or MEC Limits for QC and Reference Strains for Broth Dilution Procedures.................................................................................................................. 20

Table 5. Composition of RPMI-1640 Medium ........................................................................ 23

References .................................................................................................................................. 24

Appendix A. MECs of Caspofungin and Anidulafungin .............................................................. 26

Appendix B. RPMI-1640 Medium ......................................................................................... 28

Appendix C. McFarland 0.5 Barium Sulfate Turbidity Standard .............................................. 29

Appendix D. Oatmeal Agar ........................................................................................................ 30

Summary of Delegate Comments and Subcommittee Responses ............................................. 31

The Quality Management System Approach ........................................................................ 34

Related CLSI Reference Materials ....................................................................................... 35
Foreword

With the increased incidence of systemic fungal infections and the growing number of antifungal agents, laboratory methods to guide the selection of antifungal therapy have gained greater attention. The CLSI Area Committee on Microbiology formed the Subcommittee on Antifungal Susceptibility Testing, and data for testing filamentous fungi were collected in a series of collaborative studies. As a result, CLSI document M271 was published with the establishment of quality control MIC ranges and the development of breakpoints.

Based on these achievements, the subcommittee concluded that it would be useful to work toward a reproducible reference testing procedure for the antifungal susceptibility testing of filamentous fungi (moulds). A working group on filamentous fungi was formed and charged with the responsibility of carrying out studies to collect data and to refine the methodology to perform susceptibility testing of these fungal species. As a result of several collaborative studies, agreement within the subcommittee was achieved regarding testing conditions for the nondermatophyte moulds that included inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria for MIC determination.2-5 This consensus method was published in 2002 as M38-A.

In M38-A2, supplemental material (QC data for mould isolates as well as echinocandin testing guidelines) has been incorporated.6-9 In addition, methods for testing dermatophyte moulds are provided, based on a series of consensus studies.10,11

Because of its suitability for antifungal susceptibility testing of yeasts, synthetic RPMI-1640 medium was the test medium that the subcommittee evaluated as the potential reference medium for moulds including the dermatophytes.2,3,10,12 The subcommittee has evaluated other media formulations, but the standard RPMI medium facilitated more consistent identification of itraconazole resistance in Aspergillus spp. in eight laboratories.5 Drug stock solution preparation and dilution previously developed for antifungal testing of yeasts procedures (CLSI document M27)1 also were adopted.

Key Words
antifungal, broth microdilution, dermatophytes, filamentous fungi or moulds, susceptibility testing

1 Scope

This document describes a method for testing the susceptibility of filamentous fungi (moulds) that cause invasive (Aspergillus spp., Fusarium spp., Rhizopus oryzae [R. arrhizus], Pseudallescheria boydii [Scedosporium apiospermum], Sporothrix schenckii, and other pathogenic moulds) and cutaneous (the dermatophytes Trichophyton, Microsporum, and Epidermophyton spp.) fungal infections to antifungal agents. Addressed in this document are testing conditions including inoculum preparation and inoculum size, incubation time and temperature, medium formulation, and criteria end-point determination. Quality control (QC) reference ranges are also provided.

This standard focuses on the fully defined synthetic medium RPMI-1640 for testing of moulds because of the suitability of this test medium for antifungal susceptibility testing of yeasts.

Refer to CLSI document M27 for drug stock solution preparation and dilution procedures.

2 Introduction

The method described in this document is intended for testing common filamentous fungi or moulds, including the dermatophytes, which cause invasive and cutaneous infections, respectively. These moulds encompass Aspergillus spp., Fusarium spp., Rhizopus spp., P. boydii (S. apiospermum), S. prolificans, the mycelial form of S. schenckii, other Zygomycetes and opportunistic monilaceous and dematiaceous moulds, as well as the dermatophyte Trichophyton, Microsporum, and Epidermophyton spp. Caution should be used when interpreting the minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) results for any mould/drug combination. The method has not been used in studies of the yeast or mould form of dimorphic fungi, such as Blastomyces dermatitidis, Coccidioides immitis, Coccidioides posadasii, Histoplasma capsulatum variety capsulatum, Penicillium marneffei, or S. schenckii. The method also has not been used in studies of dermatophytes with the echinocandins or nondermatophyte moulds with ciclopirox, griseofulvin, or terbinafine.

This document is a “reference” standard developed through a consensus process to facilitate agreement among laboratories in measuring the susceptibility of moulds to antifungal agents. It is emphasized that the relationship between in vitro vs in vivo data has only been evaluated in animal models. 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. 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 M38-A2.

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. 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 M29.14

4 Definitions

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

minimal effective concentration (MEC) – the lowest concentration of an antimicrobial agent that leads
to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the
growth control well; NOTE: This terminology is currently used only with respect to testing of the
echinocandin antifungal agents (see Appendix A).

minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that
causes a specified reduction in visible growth of a microorganism in an agar or broth dilution
susceptibility test.

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

5 Antifungal Agents

5.1 Source

Antifungal standards or reference powders can be obtained commercially, directly from the
drug manufacturer. Pharmacy stock or other clinical preparations should not be used. Acceptable powders
bear a label that states the drug’s generic name, its assay potency (usually expressed in micrograms [μg]
or International Units per mg of powder), and its expiration date. Store the powders as recommended by
the manufacturers, or at −20 °C or below (never in a self-defrosting freezer), in a desiccator, preferably in
a vacuum. When the desiccator is removed from the freezer, allow it to come to room temperature before
opening (to avoid condensation of water).

5.2 Weighing Antifungal Powders

Assay all antifungal agents for standard units of activity. The assay units can differ widely from the actual
weight of the powder and often differ within a drug production lot. Thus, a laboratory must standardize its
antifungal solutions based on assays of the lots of antifungal powders used.

Use either of the following formulas to determine the amount of powder or diluent needed for a standard
solution:

\[
\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (μg/mL)}}{\text{Potency (μg/mg)}} \quad (1)
\]

or

\[
\text{Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Potency (μg/mg)}}{\text{Concentration (μg/mL)}} \quad (2)
\]

The antifungal powder should be weighed on an analytical balance that has been calibrated by approved
reference weights from a national metrology organization. Usually, it is advisable to accurately weigh a
portion of the antifungal agent in excess of that required and to calculate the volume of diluent needed to
obtain the concentration desired.

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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
- Personnel
- Equipment
- Purchasing & Inventory
- Process Control
- Information Management
- Occurrence Management
- Assessments—External & Internal
- Process Improvement
- Customer Service
- Facilities & Safety

M38-A2 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.

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.

M38-A2 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.
Related CLSI Reference Materials


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 interpretive standards and quality control guidelines for new antimicrobial agents.

M24-A  Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; 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 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.

* 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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