M51-A
Vol. 30 No. 11
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Method for Antifungal Disk Diffusion Susceptibility Testing of Nondermatophyte Filamentous Fungi; Approved Guideline

This document describes the guidelines for antifungal susceptibility testing by the disk diffusion method of nondermatophyte filamentous fungi (moulds) that cause invasive disease.

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

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Method for Antifungal Disk Diffusion Susceptibility Testing of Nondermatophyte Filamentous Fungi; Approved Guideline

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Abstract

CLSI broth dilution reference methods are available for susceptibility testing of filamentous fungi (see CLSI document M38)[1] and yeasts (see CLSI documents M27[2] and M44[3]). There still remains, however, a need for an alternative simple, rapid, and cost-effective approach to determine the susceptibility of nondermatophyte filamentous fungi (moulds) to various classes of antifungal agents that would make antifungal susceptibility testing more readily available to clinical microbiology laboratories. The CLSI Subcommittee on Antifungal Susceptibility Testing developed a disk diffusion method for testing filamentous fungi to amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole.[4] Although clinical breakpoints have not been assigned, epidemiological cutoff values (ECVs) have been developed based on a comparison of zone diameters vs minimal inhibitory concentrations (MICs) or minimal effective concentrations (MECs) using the rate bounding method; control parameters for these agents have also been determined.[4] ECVs are not used as clinical breakpoints, but rather to detect those isolates that are likely to have acquired resistance mechanisms or reduced susceptibility to the tested agent as compared with the wild-type distribution. One significant advantage of this method is that qualitative results can usually be determined after only 16 to 48 hours incubation as opposed to 24 to 72 hours with CLSI document M38.[1] There are more antifungal agents and it is expected that this document will further encourage the development of disk diffusion testing for some of these agents.

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Subcommittee on Antifungal Susceptibility Tests

Working Group on Antifungal Disk Diffusion Susceptibility Testing of Filamentous Fungi

Contents

Foreword

Due to the increased incidence of systemic fungal infections and the number of antifungal agents, antifungal susceptibility testing has gained greater recognition. Broth dilution reference methods are now available for susceptibility testing of filamentous fungi (moulds) (see CLSI document M38).[1,5-11] There still remains a need for alternative, simple, rapid, and cost-effective approaches to determine the antifungal susceptibility of these fungi. Disk diffusion methodology has served as an example for yeast testing. A collaborative study has identified parameters for testing the susceptibilities of filamentous fungi to five antifungal agents (amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole) by the disk diffusion method.[4] This method often provides qualitative results 8 to 24 hours sooner than the standard CLSI document M38[1] method. In addition, the use of nonsupplemented Mueller-Hinton agar in lieu of supplemented Mueller-Hinton agar should make antifungal susceptibility testing more readily available to clinical laboratories at a reduced cost. Although clinical breakpoints have not been assigned, tentative epidemiological cutoff values (ECVs) have been developed, based on a comparison of zone diameters vs minimal inhibitory concentrations (MICs) or minimal effective concentrations (MECs) using the rate bounding method.[4] The ECVs are used to detect those isolates with reduced susceptibility to the tested agent as compared with the wild-type distribution. ECVs are not used as clinical breakpoints, but rather to detect those isolates that are likely to have acquired resistance mechanisms.

Key Words

Antifungal, antimicrobial, disk, disk diffusion, Kirby-Bauer method, susceptibility testing

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1 Scope

With a need to make antifungal susceptibility testing more readily available to the clinical laboratory, this CLSI document provides an established method for disk diffusion testing of moulds, zone interpretive criteria, and recommended control ranges for amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole.

The method described in this document is intended for testing moulds that cause invasive disease *(Alternaria* spp., *Aspergillus* spp., *Bipolaris* spp., *Fusarium* spp., *Paecilomyces* spp., *Rhizopus oryzae* [*R. arrhizus*] and other mucoraceous [zygomycetes] mould species, the *Pseudallescheria boydii* species complex, and *Scedosporium prolificans).*[4] This method does not currently encompass the yeast or mould form of endemic dimorphic fungi or the dermatophytes.

The method described herein must be followed exactly to obtain reproducible results. When new problems are recognized or improvements in these criteria are developed, changes will be incorporated into future editions of this guideline and also distributed in periodic informational supplements.

This guideline is intended for use by, but not limited to, health care, academic, government, industry, or independent research organizations that perform antifungal susceptibility testing of filamentous fungi.

2 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 major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all known 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.[12] For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.[13]

3 Terminology

3.1 A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, International Organization for Standardization (ISO), and European Committee for Standardization (CEN) documents; and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. In light of this, CLSI's consensus process for development and revision of standards and guidelines focuses on harmonization of terms to facilitate the global application of standards and guidelines.

Of particular note in CLSI document M51-A are two terms whereby CLSI intends to eliminate confusion over time through its commitment to harmonization. For the most part, in this guideline, the term

"accuracy," in its metrological sense, refers to the closeness of the agreement between the result of a single measurement and a true value of a measurand, thus comprising both random and systematic effects. The term "trueness," usually used to replace the term "accuracy" when referring to the closeness of agreement does not apply in M51-A because it refers to the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

3.2 Definitions

accuracy (measurement) – closeness of agreement between a measured quantity value and a true quantity value of a measurand (ISO/IEC Guide 99).[14]

clinical breakpoint – **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; **2) susceptible clinical breakpoint** – a category that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated; **3) intermediate clinical breakpoint** – a category that includes isolates with antimicrobial agent minimal inhibitory concentrations (MICs) or minimal effective concentrations (MECs) that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates; **4) resistant clinical breakpoint** – a category that includes resistant isolates that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or where clinical efficacy has not been reliable in treatment studies.

epidemiological cutoff value (ECV) – the ECV for each agent is the value obtained by considering the wild-type distribution, the modal MIC/MEC for each distribution, and the inherent variability of the test. Usually, the ECV encompasses at least 95% of isolates in the wild-type distribution[15]; **NOTE:** Organisms with acquired resistance mechanisms may be included among those for which the MICs/MECs are higher than the ECV (for disk testing, those with acquired resistance mechanisms would show a zone diameter smaller than the ECV).

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.

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.

modal MIC/MEC – the most frequent MIC or MEC found within an MIC or MEC distribution.

precision (measurement) – closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions (ISO/IEC Guide 99).[14]

quality control – part of quality management focused on fulfilling quality requirements (ISO 9000)[16]; **NOTE:** This includes operational techniques and activities used to fulfill these requirements.

reproducibility (measurement) – measurement precision (closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions) under reproducibility conditions of measurement (condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects) (ISO/IEC Guide 99).[14]

reproducibility condition (of measurement) – condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects (ISO/IEC Guide 99)[14]; **NOTE 1:** The different measuring systems may use different measurement procedures (ISO/IEC Guide 99)[14]; **NOTE 2:** A specification should give the conditions changed and unchanged, to the extent practical (ISO/IEC Guide 99)[14]; **NOTE 3:** The changed conditions may refer to different lots, runs, time (day), technician, and so on (I/LA21).[17]

trueness (of measurement) – closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO 3534-1).[18]

wild-type organism – a strain that does not harbor any acquired resistance to the particular antimicrobial agent being examined[15]; **NOTE:** The typical MIC/MEC distribution for wild-type organisms covers three to four twofold dilution steps surrounding the modal MIC/MEC.[19] For disk diffusion testing, the wild-type zone diameter profile is established by parallel testing of each antimicrobial agent by both MIC/MEC and disk diffusion methods.

3.3 Abbreviations and Acronyms

ATCC	American Type Culture Collection
ECV	epidemiological cutoff value
ISO	International Organization for Standardization
MEC	minimal effective concentration
MIC	minimal inhibitory concentration

4 Selection of Antimicrobial Agent Disks for Routine Testing and Reporting

Because clinical relevance of testing this group of fungal pathogens remains uncertain and breakpoints with proven clinical relevance have not been identified yet, routine testing is not generally recommended. However, testing may be warranted under certain selected circumstances such as (1) part of periodic batch surveys that establish antibiograms for collections of pathogenic isolates obtained from within an institution; and (2) to aid in the management of invasive infections due to filamentous fungi when the utility of the antifungal agents is uncertain. Some institutions may find it useful to systematically test selected drug-organism combinations (eg, mould isolates from sterile sites). Specimens for culture and other procedures should be obtained before initiation of antifungal therapy.

4.1 Use of Nonproprietary or Generic Names

To minimize confusion, all antifungal agents should be referred to by international nonproprietary (ie, generic) names.

4.2 Number of Agents Tested

To make routine susceptibility tests relevant and practical, the number of antimicrobial agents tested should be limited. Although this is not an immediate issue for antifungal agents, the same principle applies.

4.3 Suggested Guidelines for Selective Reporting

Disk diffusion interpretive criteria with proven clinical relevance are not available yet; the tentative zone diameter epidemiological cutoffs listed in Table 1 (see M51 Informational Supplement[20]) were assigned for the five antifungal agents (amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole), based on *in vitro* data as described below (see Section 8).

5 Equipment/Materials

The following equipment is recommended for performance of antifungal disk diffusion susceptibility testing:

- Incubator set at 35 ± 2 °C with ambient air
- McFarland 0.5 Turbidity Standard (only for testing the reference standard yeast isolate)
- Sterile cotton (not synthetic polyester fiber) swabs
- Sterile physiological (8.5 g/L NaCl; 0.85%) normal saline
- Class IIA or IIB biological safety hood
- Spectrophotometer

6 Test Procedures

6.1 Nonsupplemented Mueller-Hinton Agar Medium (see Appendix B)

Of the many agar media available, nonsupplemented Mueller-Hinton agar without additional calcium or magnesium is recommended for routine susceptibility testing of filamentous fungi for the following reasons:

- It is readily available.

- It has been demonstrated in both collaborative and noncollaborative comparisons[4,21,22] of nonsupplemented and supplemented Mueller-Hinton agar for testing filamentous fungi by disk diffusion that the former agar supported better growth of most mould species, did not interfere with the antifungal activity of any of the drugs evaluated, and produced clear and easy-to-determine zone diameters (see Appendix A). In addition, zone diameters could be determined in a shorter incubation time than on supplemented Mueller-Hinton agar.

The base medium should not be supplemented with either 2% glucose or 0.5% methylene blue dye.

6.1.1 pH of Nonsupplemented Mueller-Hinton Agar

The pH of each batch of prepared Mueller-Hinton agar should be checked. The method used largely depends on the type of equipment available in the laboratory. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. The pH can be checked by one of the following means:

- Macerate a sufficient amount of agar to submerge the tip of a pH electrode.

- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.

- Use a properly calibrated surface electrode.

6.1.2 Moisture of Agar Surface

If excess surface moisture is present, the agar plates should be dried in an incubator or laminar flow hood with the lids ajar until the excess moisture has evaporated (usually 10 to 30 minutes). The surface should be moist, but with no droplets on the agar surface or the Petri dish cover.

6.1.3 Storage of Antimicrobial Disks

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. **NOTE:** Only voriconazole disks are commercially available in the United States. Amphotericin B and itraconazole are commercially available in the United Kingdom. Other antifungal disks may be obtained from the drug manufacturer. Most disks used in the development of this method were provided by the drug manufacturers.

Disks should be stored as follows:

- Refrigerate the containers at 2 to 8 °C or below, or freeze at −15 °C or below in a non-frost-free freezer until needed. The disks may retain greater stability if stored frozen until the day of use. Always refer to instructions in the product insert.

- The disk containers should be removed from the refrigerator or freezer 30 minutes to one hour before use so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.

- Once a cartridge of disks is removed from its sealed packaging, it should be placed in a tightly sealed, desiccation container.

- A disk-dispensing apparatus should be fitted with a tight cover and supplied with an adequate desiccant. The dispenser should be allowed to warm to room temperature before opening. The desiccant should be replaced when the indicator changes color.

- When not in use, the dispensing apparatus containing the disks should always be refrigerated.

- Only disks within their valid shelf life may be used. Disks should be discarded on the expiration date.

7 Procedure for Performing the Disk Diffusion Test

7.1 Inoculum Preparation

When the risk of substantial spatter or aerosolization is present, the isolate handling should be performed in a Class IIA or IIB biological safety cabinet. Details are further outlined in CLSI document M29.[13]

7.1.1 Nondermatophyte Filamentous Fungi

Initial work demonstrated that reliable stock, nongerminated conidial or sporangiospore suspensions (range of approximately 0.4×10^6 to 5×10^6 CFU/mL) could be prepared by a spectrophotometric procedure (see CLSI document M38)[1,5-8] and that concentrations of viable conidial or sporangiospore stock inocula provided the most reproducible data.

(1) To induce conidium and sporangiospore formation, most fungi should be grown on potato dextrose agar for 7 days at 35 ± 2 °C or until good sporulation is obtained; good sporulation may be obtained after 48 hours of incubation for some isolates (eg, mucoraceous [zygomycetes] moulds and *Aspergillus* spp.). *Fusarium* spp. may need to be incubated for 48 hours to 72 hours at 35 ± 2 °C and then until day 7 at 28 to 30 ± 2 °C.

(2) Cover sporulating colonies with approximately 1 mL of sterile 0.85% saline, and prepare a suspension by gently probing the colonies with the tip of a transfer pipette. Adding one drop (approximately 0.01 mL) of Tween 20 may facilitate the preparation of *Aspergillus* inocula, in

particular. The resulting mixture of conidia or sporangiospores and hyphal fragments is withdrawn and transferred to a sterile tube.

(3) After heavy particles are allowed to settle for 3 to 5 minutes, the upper homogeneous suspension is transferred to a sterile tube, the cap is tightened, and it is mixed with a vortex mixer for 15 seconds. **CAUTION:** Remove the cap carefully because liquid adhering to the cap may produce aerosols upon opening.

(4) The densities of the conidial or sporangiospore suspensions are read on a spectrophotometer with a 1-cm light path at 530-nm wavelength (see Appendix C) and adjusted to an optical density that ranges from 0.09 to 0.13 for *Aspergillus* spp., *Paecilomyces lilacinus,* and *Paecilomyces variotii*; 0.15 to 0.17 for *Fusarium* spp., *R. oryzae,* and other mucoraceous mould (zygomycetes) spp., the *Pseudallescheria boydii* species complex, and *S. prolificans*; and 0.25 to 0.3 for *Alternaria* and *Bipolaris* spp.[1,4-8] These suspensions are used undiluted.

7.1.2 Reference *Paecilomyces variotii* ATCC®[a] MYA-3630 Isolate

The inoculum suspension of this mould isolate should be prepared as described above (see Section 7.1.1).

7.1.3 Reference *Candida krusei* ATCC® 6258 Isolate

The yeast isolate must be subcultured onto blood agar or Sabouraud dextrose agar to ensure purity and viability (see CLSI document M27).[2] Throughout the incubation, the temperature must be 35 ± 2 °C. The inoculum is prepared by selecting five distinct colonies of approximately 1 mm in diameter from a 24-hour-old culture. Colonies are suspended in 5 mL of normal saline. The resulting suspension is vortexed for 15 seconds, and its turbidity is adjusted either visually or with a spectrophotometer with a 1-cm light path by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard (see Appendix C) at 530-nm wavelength. This procedure yields the stock suspension of 1×10^6 to 5×10^6 cells per mL needed for the test.

7.2 Inoculum Quantitation

This step can be performed by plating 0.01 mL of a 1:10 dilution of the adjusted mould inoculum or 0.01 mL of the reference yeast isolate inoculum on Sabouraud dextrose agar to determine the viable number of CFU/mL (see CLSI document M38).[1]

(1) The plates are incubated at 28 to 30 ± 2 °C and observed daily for the presence of fungal colonies.

(2) Colonies should be counted as soon as possible after growth becomes visible, especially for isolates of *R. oryzae* and other mucoraceous moulds (zygomycetes). The incubation time ranges from 24 hours or less (eg, mucoraceous moulds) to three days (eg, the *Pseudallescheria boydii* species complex and *S. prolificans*). See Section 7.5 for details.

7.3 Inoculation of Test Plates

(1) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the undiluted inoculum suspension. The swab should be rotated several times and pressed firmly against the inside wall of the tube above the fluid level. This removes excess fluid from the swab.

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

NOTE: If plates cannot be inoculated within 15 minutes, the inoculum suspension may be refrigerated. The inoculum suspension should not be refrigerated for longer than two hours.

(2) Inoculate the dried surface of a sterile **nonsupplemented** Mueller-Hinton agar plate by evenly streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, swab the rim of the agar.

(3) The lid may be left ajar for three to five minutes, but no more than 15 minutes, to allow for absorption of any excess surface moisture before applying the drug-impregnated disks.

NOTE: Variations in inoculum density must be avoided. For streaking plates, never use inoculum suspensions that have not been adjusted for turbidity as described in Section 7.1.1. (Also see Appendix C.)

7.4 Application of Disks to Inoculated Agar Plates

(1) Dispense antimicrobial disks onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure its complete contact with the agar surface. Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so they are no closer than 32 mm from center to center when testing amphotericin B, caspofungin, and itraconazole; and no closer than 55 mm from center to center when testing posaconazole and voriconazole, especially against *Alternaria* spp., *Aspergillus* spp., *Bipolaris* spp., and *Paecilomyces* spp. Ordinarily, no more than four to six disks should be placed per 150-mm plate, nor more than one disk per 100-mm plate. Because the drug diffuses almost instantaneously, a disk should not be moved once it comes into contact with the agar surface. Instead, a new disk is placed in another location on the agar.

(2) Invert the plates and place them in an incubator set to 35 ± 2 °C within 15 minutes after the disks are applied.

7.5 Reading Plates and Interpreting Results

Each plate is examined after 16 to 24 hours of incubation when testing *R. oryzae* and other mucoraceous (zygomycetes) mould isolates; 24 hours (*Aspergillus flavus*, *A. fumigatus,* and *A. niger*) to 48 hours (other *Aspergillus* spp.); and 48 hours to 72 hours when testing *Alternaria* spp., *Bipolaris* spp., *Fusarium* spp., *Paecilomyces* spp., the *Pseudallescheria boydii* species complex, and *S. prolificans*. Plates are read at longer incubation times only when insufficient growth is observed after the incubation times listed above. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth (see Appendix A). The plate is held a few inches above a black, nonreflecting background illuminated with reflected light. The zone diameter is measured to the nearest whole millimeter at the point at which there is a prominent reduction in growth (80%). Slight trailing around the zone edge or hyphal elements extending into the inhibition zone should be ignored when testing the triazoles, but not for amphotericin B. In addition, microcolonies or trailing growth within a well-defined zone of inhibition should be ignored when testing caspofungin (see Appendix A). The measurement of inhibition zones is highly subjective, and experience results in greater accuracy.

8 Interpretation of Disks Diffusion Test Results

8.1 Zone Diameter Epidemiological Cutoff Values

Clinical breakpoints have not been established for mould testing. However, tentative epidemiological cutoff values (ECVs) were developed during a collaborative study to evaluate the performance of the agar

disk diffusion method, in detecting those mould isolates with reduced susceptibility (non–wild type) to amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole.[4] The wild-type distributions for each antifungal agent were derived by determining the MICs/minimal effective concentrations (MECs) for a large collection of clinical isolates, and the tentative ECVs were 1 mcg/mL for all five drugs. Scatter plots of the MICs or MECs and corresponding zone diameter values of each antifungal agent were developed, and by using the error rate bounding method (see CLSI document M23),[23] tentative zone diameter ECVs were assigned. Table 1 (see M51 Informational Supplement)[20] provides these tentative zone diameters and MIC/MEC ECVs. **ECVs can be used as a measure of the emergence of strains with reduced susceptibility to a given agent.[24] They are not clinical breakpoints. Although organisms whose MICs/MECs/zone diameters fall outside the ECV show reduced susceptibility as compared to the wild-type population, and may exhibit one or more acquired resistance mechanisms, they may yet respond to clinical treatment because the MIC/MEC may lie below (zone diameter above) the true (and as yet undetermined) clinical breakpoint.[25]**

The clinical relevance of testing this group of fungal pathogens remains uncertain because clinical breakpoints of proven relevance have not yet been identified or approved by CLSI or any regulatory agency.

Disk diffusion zone diameters correlate inversely with MICs or MECs from standard dilution tests. Table 1 (see M51 Informational Supplement[20]) lists the tentative zone diameter ECVs. These criteria were based on zone diameter vs MIC or MEC comparisons as described above.[4] These MIC/MEC categories are listed in CLSI document M38.[1]

9 Quality Control Procedures

9.1 Purpose

The goals of a quality control program are to monitor the following:

- The precision (reproducibility) and accuracy of the susceptibility test procedure
- The performance of reagents used in the test
- The performance of persons who carry out the tests and read results

These goals are best achieved by, but not limited to, the testing of quality control or reference strains with known susceptibility to the antimicrobial agents being tested.

9.2 Standard Reference Strains for Control

To control the precision (reproducibility) and accuracy of the results obtained with the disk diffusion test procedure, two reference control strains should be obtained from a reliable source. Data for these reference stains were obtained in a collaborative study.[4] The recommended reference strains are

- *Paecilomyces variotii* ATCC® MYA-3630
- *Candida krusei* ATCC® 6258

9.3 Storing Reference Strains

- The quality or reference control strains should be tested by the standard disk diffusion test procedure described herein using the same materials and methods that are used to test clinical isolates.

- Quality control or reference strains are stored in a way that minimizes the possibility of mutation in the organism.

There are several methods for prolonged storage of reference strains. For example, the isolates may be grown on slants of potato dextrose agar and then frozen at −70 °C. Alternatively, strains can be preserved by suspending the mould or the yeast into vials containing 10% glycerol solution for freezing and storing at −70 °C. Commercial storage systems that use a cryogenic solution containing porous beads and that have been demonstrated by the manufacturer to preserve fungi are also available.[26-28]

- Working control cultures are stored on Sabouraud (the yeast reference strain *Candida krusei* ATCC® 6258) or potato dextrose agar (the mould reference strain *Paecilomyces variotii* ATCC® MYA-3630) at 2 to 8 °C and subcultured each week for no more than three successive weeks. New working cultures should be prepared at least monthly from frozen, freeze-dried, or commercial cultures.

- Frozen or freeze-dried cultures should be subcultured at least twice before testing.

- A control strain can be used to monitor the precision (reproducibility) and accuracy of the disk test as long as there is no significant change in the mean zone diameter that cannot be attributed to a faulty methodology. If an unexplained result suggests a change in the organism's inherent susceptibility, a fresh new stock culture of the control strain should be obtained.

- Avoid multiple serial subcultures of quality control organisms over extended periods of time.

9.4 Zone Diameter Reference Control Limits

Zone diameter control limits for the two reference strains are listed in Table 2 (see M51 Informational Supplement[20]). The overall performance of the test system should be monitored using these ranges by testing the appropriate reference strains each day the test is performed or, if satisfactory performance is documented, testing may be done weekly (see Section 9.5.2.1).

9.5 Frequency of Control Testing

9.5.1 Daily Testing (See Appendix D)

When testing is performed daily, for each antimicrobial agent/organism combination, 1 out of every 20 consecutive results may be out of the acceptable range (based on 95% confidence limits, 1 out of 20 random results may be out of range). Any more than 1 out-of-range result in 20 consecutive tests requires corrective action (see Section 9.6).

9.5.2 Weekly Testing (See Appendix D)

9.5.2.1 Demonstrating Satisfactory Performance for Conversion From Daily to Weekly Control Testing

- All applicable control strains are tested for 20 consecutive test days and results are documented.

- To convert from daily to weekly control testing, no more than 1 out of 20 zone diameters for each antimicrobial agent/organism combination may be outside the acceptable zone diameter limits in Table 2 (see M51 Informational Supplement[20]).

9.5.2.2 Implementing Weekly Quality Control Testing

- Weekly quality control testing may be implemented once satisfactory performance is documented (see Section 9.5.2.1).

- Quality control testing is performed once per week and whenever any reagent component of the test (eg, a new lot of agar plates or a new lot of disks from the same or a different manufacturer) is changed.

- If any of the weekly control results are out of the acceptable range, corrective action is required (see Section 9.6).

- If a new antimicrobial agent is added, it must be tested for 20 consecutive test days and satisfactory performance documented before converting to a weekly schedule. In addition, 20 days of consecutive testing are required if there is a major change in the method of reading test results, such as conversion from manual zone measurements to an automated zone reader.

9.6 Corrective Action

9.6.1 Out-of-Range Result Due to an Obvious Error

Obvious reasons for out-of-range results include

- Use of the wrong disk
- Use of the wrong control strain
- Contamination of the strain
- Use of a control strain that has been passaged too many times
- Inadvertent use of the wrong incubation temperature or conditions
- Wrong inoculum density

In such cases, the reason is documented and the strain is retested on the day the error is observed. If the repeated result is within range, no further corrective action is required.

9.6.2 Out-of-Range Results Not Due to an Obvious Error

9.6.2.1 Immediate Corrective Action

If there is no obvious reason for an out-of-range result, immediate corrective action is required.

- The antimicrobial agent/organism combination is tested for a total of five consecutive test days. All results in question are documented.

- If all five zone diameter measurements for the antimicrobial agent/organism combination are within acceptable ranges, as defined in Table 2 (see M51 Informational Supplement[20]), no additional corrective action is necessary.

- If any of the five zone diameter measurements are outside the acceptable range, additional corrective action is required (see Section 9.6.2.2).

- Daily control tests must be continued until final resolution of the problem is achieved.

9.6.2.2 Additional Corrective Action

When immediate corrective action does not resolve the problem, it is likely that the error is due to a systematic vs a random error. The following common sources of error should be investigated to ensure the following:

- Zone diameters were measured and transcribed correctly.
- The turbidity standard has not expired, is stored properly, meets performance requirements (see Appendix C), and was adequately mixed before use.
- All materials used were within their expiration date and stored at the proper temperature.
- The incubator is at the proper temperature and atmosphere.
- Other equipment used (eg, pipettors) is functioning properly.
- Disks are stored desiccated and at the proper temperature.
- The control strain has not changed and is not contaminated.
- Inoculum suspensions were prepared and adjusted correctly.
- Inoculum for the test was prepared from a plate incubated for the correct length of time and in no case was more than 24 hours old (yeast) or 7 days old (mould).

It may be necessary to obtain a new reference strain (either from freezer stock or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. If the problem appears to be related to a commercial product, the manufacturer should be contacted. It is also helpful to exchange reference strains and test materials with another laboratory using the same method. Until the problem is resolved, an alternative test method should be used.

Once the problem is corrected, documentation of satisfactory performance for another 20 consecutive days is required before returning to weekly control testing (see Section 9.5.2.1).

9.7 Reporting Patient Results When Out-of-Range Results Occur

Whenever an out-of-range result occurs or corrective action is necessary, careful assessment of whether to report patient results should be made on an individual basis, taking into account if the source of error, when known, is likely to have affected relevant patient results. Options that may be considered include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternative test method or a reference laboratory until the problem is resolved.

10 Limitations of Disk Diffusion Method

10.1 Application to Various Organism Groups

The disk diffusion method described in this document has been standardized for certain mould species only. For other moulds, consultation with an infectious disease specialist is recommended for guidance in determining the need for susceptibility testing and interpretation of results. Published reports in the medical literature and current consensus recommendations for therapy of uncommon microorganisms may obviate the need for testing. If necessary, a reference dilution method may be the most appropriate alternative testing method, and this may require submitting the organism to a reference laboratory.

10.2 Verification of Patient Results

Multiple test parameters are monitored by using the reference control limits described in this guideline. However, acceptable results derived from testing reference control strains do not guarantee accurate results when testing patient isolates. It is important to review all of the results obtained from all drugs tested on a patient's isolates before reporting the results.

Unusual or inconsistent results should be verified by checking for the following: 1) transcription errors; 2) contamination of the test (recheck purity plates); and 3) previous results on the patient's isolates. If a reason for the unusual or inconsistent result cannot be ascertained, repeat the susceptibility test, verify the species identity, or request a new clinical specimen. Each laboratory must develop its own policies for verification of unusual or inconsistent antimicrobial susceptibility test results.

References

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2 CLSI. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition.* CLSI document M27-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.

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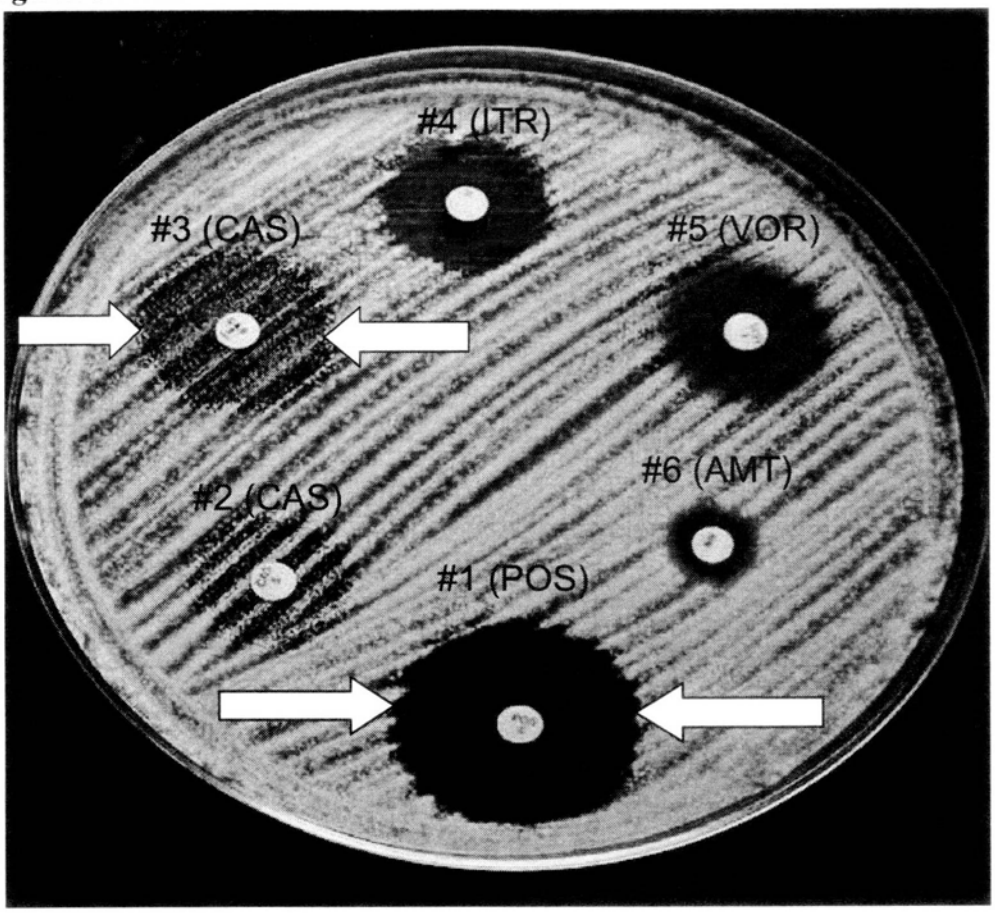
Appendix A. Performance of Nonsupplemented Mueller-Hinton Agar

A1. *Aspergillus terreus*



Shown is the performance of the disk diffusion method using nonsupplemented Mueller-Hinton agar for the determination of the antifungal susceptibility of *A. terreus* to posaconazole (#1-POS), caspofungin (#2 and 3-CAS; two manufacturers), itraconazole (#4-ITR), voriconazole (#5-VOR), and amphotericin B (#6-AMT). The lower arrows show a posaconazole clear zone of inhibition and the upper arrows show a zone with trailing growth usually only seen with caspofungin and other echinocandins. Trailing growth with caspofungin and other echinocandins should be ignored; however, trailing growth with other agents indicates resistance. Also see Section 7.5.

Appendix B. Preparation of Nonsupplemented Mueller-Hinton Agar

The medium can be prepared and poured as the complete commercially prepared Mueller-Hinton plates **(no supplements should be added).** Eliminating the supplements enables the use of routine Mueller-Hinton agar plates from the bacteriology laboratory.

Preparation of Nonsupplemented Mueller-Hinton Agar:

(1) Prepare Mueller-Hinton agar from a commercially available dehydrated Mueller-Hinton agar base according to the manufacturer's instructions.

(2) Autoclave as directed by the manufacturer's instructions.

(3) Immediately after autoclaving, allow the agar solution to cool in a 48 to 50 °C water bath.

(4) Pour the freshly prepared and cooled medium into plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 67 mL to 70 mL of medium for plates with diameters of 150 mm, and 28 mL to 30 mL for plates with a diameter of 100 mm.

(5) Allow the agar medium to cool to room temperature and, unless the plate is used on the same day of preparation, store at refrigerator temperature (2 to 8 °C). The agar medium should have a pH between 7.2 and 7.4 at room temperature (see CLSI document M02[1]).

(6) Use plates within seven days after preparation unless adequate precautions such as wrapping in plastic sleeves have been taken to minimize drying of the agar.

(7) Examine a representative sample of each batch of plates for sterility by incubating at 30 to 35 °C for 24 hours or longer. Plates should undergo quality control testing per Section 9.

Reference for Appendix B

[1]CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Tenth Edition.* CLSI document M02-A10. Wayne, PA: Clinical and Laboratory Standards Institute; 2009.

Appendix C. McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, a $BaSO_4$ turbidity standard is used (0.5 McFarland Standard).

The procedure consists of the following steps:

(1) Prepare this turbidity standard by adding 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot H_2O$) to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.

(2) Verify the correct density of the turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 530 nm should be 0.05 to 0.07 for the 0.5 McFarland standard.

(3) Distribute 4 to 6 mL into screw-cap tubes of the same size as those used in growing or diluting the broth culture inoculum.

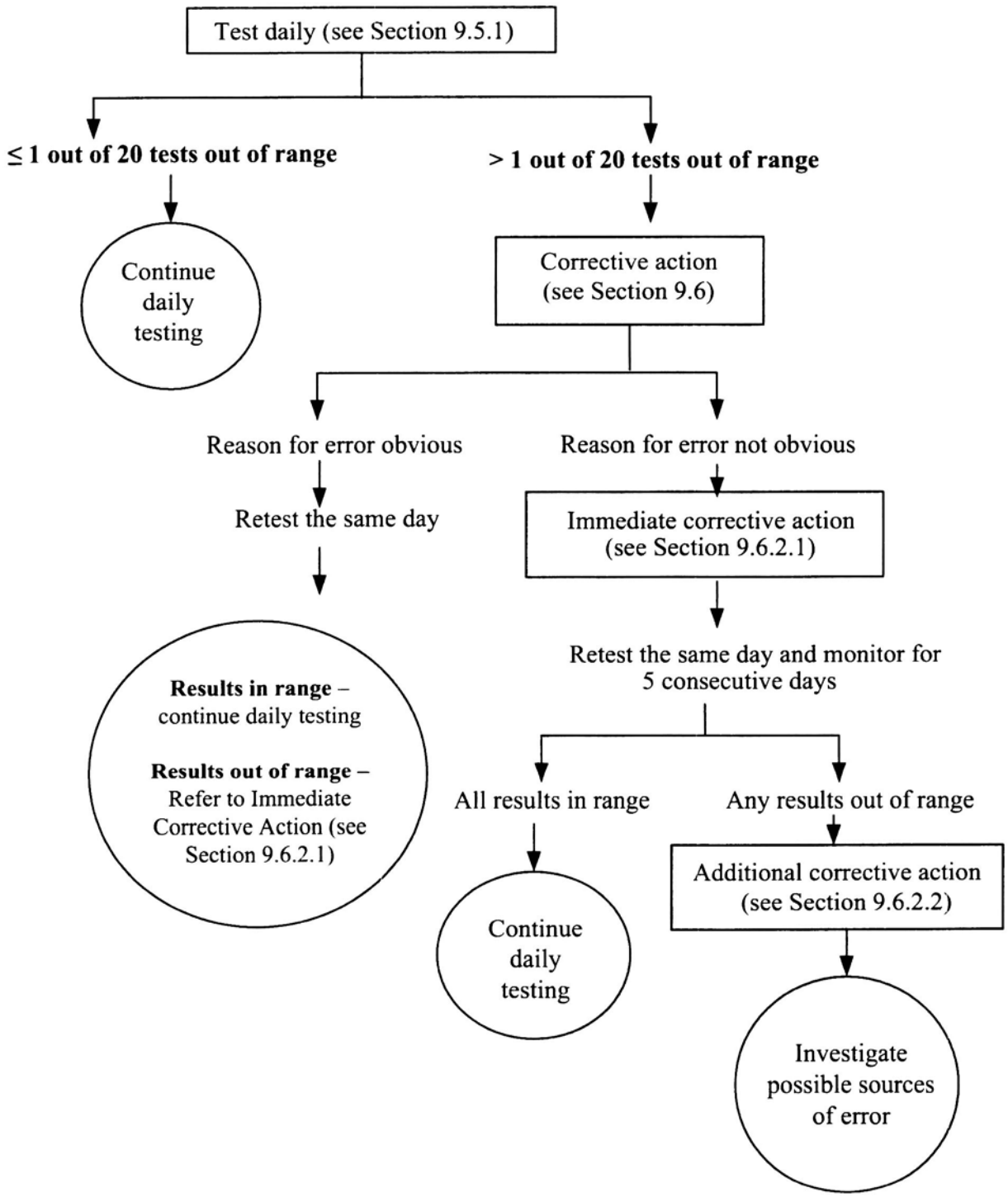
(4) Tightly seal these tubes and store them in the dark at room temperature.

(5) Vigorously agitate this turbidity standard on a mechanical vortex mixer just before use.

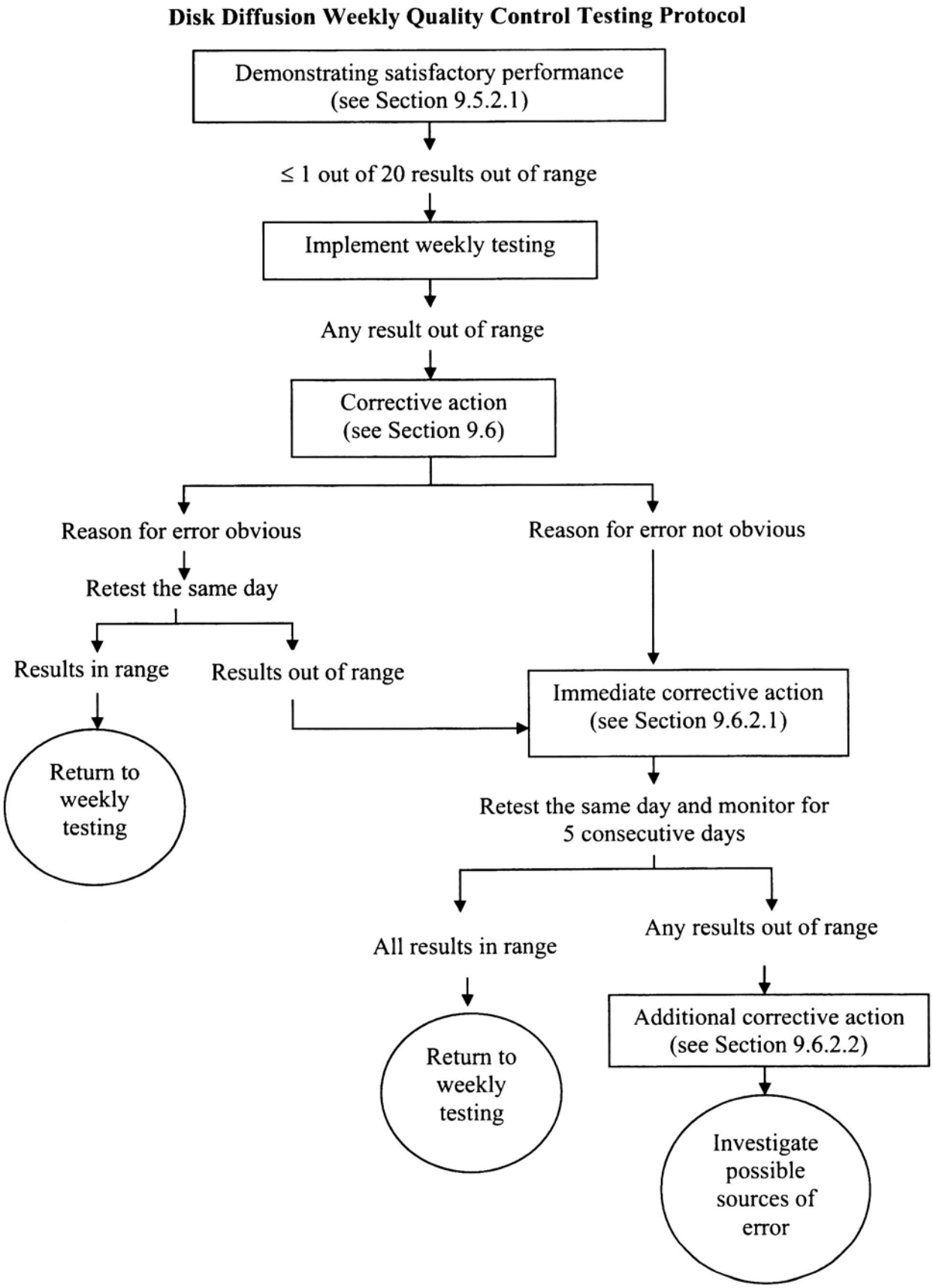
(6) Replace the barium sulfate standards or verify their densities monthly.

Appendix D. Quality Control Protocol Flow Charts

Disk Diffusion Daily Quality Control Testing Protocol



Appendix D. (Continued)



Clinical and Laboratory Standards Institute consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact CLSI or visit our website at www.clsi.org.

Summary of Delegate Comments and Subcommittee Responses

M51-P, *Method for Antifungal Disk Diffusion Susceptibility Testing of Filamentous Fungi; Proposed Guideline*

General

1. As in the anaerobe document, it would be great to have some pictures of results, especially with inner colonies, clear-cut vs not so clear-cut margins of zone.

- **For clarification, the photograph and legend in Appendix A were revised as suggested.**

2. Disk availability – There is no mention of whether or not disks for the mentioned antifungal agents are available commercially.

- **The working group believes that the text regarding antifungal disks is consistent with the text regarding disks in CLSI document M44, *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts.* For clarification, a note was added to Section 6.1.3 that states that only voriconazole disks are commercially available in the United States. Amphotericin B and itraconazole disks are commercially available in the United Kingdom. Other antifungal disks may be obtained from the drug manufacturer. All disks used for the development of this method were provided by the drug manufacturer.**

Area Committee Advisor List

3. Remove "Vice-Chairholder" after Jim Jorgensen's name.

- **The committee list was revised as suggested.**

Foreword

4. Second to last sentence – Add the word "clinical." "...proven clinical relevance..."

- **It appears that the commenter reviewed a previous draft of M51-P. The Foreword currently reads, "Although clinical breakpoints have not been assigned, tentative epidemiological cutoff values (ECVs) have been developed, based on a comparison of zone diameters vs minimal inhibitory concentrations (MICs) or minimal effective concentrations (MECs) using the rate bounding method. The ECVs are used to detect those isolates with reduced susceptibility to the tested agent as compared with the wild-type distribution. ECVs are not used as clinical breakpoints, but rather to detect those isolates that are likely to have acquired resistance mechanisms."**

Invitation for Participation

5. Second paragraph – "Consequently" at the beginning of the second sentence does not seem to fit; would "However" or something similar be a better word choice?

- **The "Invitation for Participation" is boilerplate text included in all proposed-level documents and is removed following the delegate voting period. Therefore, the text in question was deleted. However, the commenter's suggestion will be taken into consideration for incorporation in future proposed-level documents.**

Section 1, Scope

6. Define *S. prolificans*. The *Scedosporium* genus name is not stated in the document.

- ***Scedosporium* was spelled out as suggested.**

Section 3.2, Definitions

7. Epidemiological cutoff value – What is given is not really a definition, but rather how the ECV is obtained. Shouldn't MIC and MEC also be defined? (The abbreviations are identified in Section 3.3, but the terms are not defined.) What is a "modal" MIC/MEC?

- **For clarification, the description of ECV was moved from the definition NOTE to the beginning of the definition. Definitions of MIC and MEC appear directly below the definition for ECV. As suggested, a definition for modal MIC/MEC was added.**

Section 6.1, Nonsupplemented Mueller-Hinton Agar Medium

8. Nonsupplemented Mueller-Hinton agar with or without additional calcium or magnesium?

- **Per the manufacturer, nonsupplemented Mueller-Hinton is used without any additional calcium and magnesium. The first sentence in Section 6.1 was revised to read, "...nonsupplemented Mueller-Hinton agar without additional calcium or magnesium is recommended..."**

Section 6.1.3, Storage of Antimicrobial Disks

9. Third bullet – Remove "," after "sealed."

- **The text was revised as suggested.**

Section 7.1.1, Nondermatophyte Filamentous Fungi

10. Statement #1: It is a little confusing to say grow for 7 days on PDA, and then say 48 hours or 48 to 72 hours at one temperature and then 7 days. List the "usual" time for various moulds because it sounds like that is known for the Aspergillus, Zygomycetes, and Fusarium.

- **The statement is consistent with the recommendations made in CLSI document M38-A2, *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition.* As described in M38-A2, some isolates can show good sporulation before 7 days; therefore, a general recommendation for most isolates was provided.**

11. Statement #4 – Optical densities at 530 nm should be converted to approximate McFarland Standards or percent of transmittance.

- **The percent of transmittance depends on the equipment being used; therefore, each laboratory should determine the optical density range for its equipment. A reference to Appendix C was added for clarification.**

12. Statement #4 – Shouldn't the path length also be specified?

- **As suggested, the statement in #4 was revised to read, "The densities of the conidial or sporangiospore suspensions are read on a spectrophotometer with a 1-cm light path at 530-nm wavelength..."**

13. This paragraph details the procedure for using OD measurements to establish the appropriate conidial concentration for disk diffusion test inoculation, and is almost identical to the procedure described in CLSI document M38-A for broth microdilution. However, M51-P indicates that the recommended OD corresponds to an inoculum of 0.4e6 to 5e6 CFU/mL, whereas M38-A says the same OD corresponds to an inoculum of 0.4e4

to 5.0e4 CFU/mL. Is this a typographical error or does the disk diffusion procedure require a significantly higher inoculum?

- **For clarification, the first paragraph in Section 7.1.1 of M51 was revised to read, "Initial work demonstrated that reliable stock, nongerminated conidial or sporangiospore suspensions (range of approximately 0.4×10^6 to 5×10^6 CFU/mL) could be prepared by a spectrophotometric procedure (see CLSI document M38) and that concentrations of viable conidial or sporangiospore stock inocula provided the most reproducible data."**

Section 7.1.3, Reference *Candida krusei* ATCC® 6258 Isolate

14. Second sentence – Additional clarification is needed in this sentence. What does "throughout" refer to? The agar? The procedure?

- **The text was modified for clarification. It now reads, "Throughout the incubation, the temperature must be 35 ± 2 °C."**

15. The optical path length should be specified for the adjustment of the McFarland standard.

- **The text was modified as suggested. It now reads, "The resulting suspension is vortexed for 15 seconds, and its turbidity is adjusted either visually or with a spectrophotometer with a 1-cm light path by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard (see Appendix C) at 530-nm wavelength."**

16. Why is it not recommended to grow the yeast on PDA or at least offer it as a choice?

- **As described in CLSI document M27-A3, *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition*, PDA medium is not used for growth of yeasts for susceptibility testing; therefore, PDA is not offered as a choice for yeast growth in this procedure.**

Section 7.2, Inoculum Quantitation

17. Perhaps there should be a recommendation for how often the inoculum quantitation should be done—or is it supposed that this needs to be done every time?

- **The subcommittee believes that it is commonly understood that quantitation should be performed every time to ensure that the correct inoculum size is used; therefore, no change was made.**

Section 7.3, Inoculum of Test Plates

18. Statement #1 NOTE: Instead of "maximum of two hours, it should state, "do not refrigerate for longer than two hours."

- **The NOTE was revised to read, "If plates cannot be inoculated within 15 minutes, the inoculum suspension may be refrigerated. The inoculum suspension should not be refrigerated for longer than two hours."**

19. Statement #1 NOTE: The wording makes it sound like if you cannot use the inoculum in 15 minutes, then you most wait for two hours before you can use it. "If…the inoculum preparation must be refrigerated and used within two hours."

- **The NOTE was revised to read, "If plates cannot be inoculated within 15 minutes, the inoculum suspension may be refrigerated. The inoculum suspension should not be refrigerated for longer than two hours."**

20. Statement #3 NOTE: What is a "nonstandardized" vs "standardized" inoculum?

- **For clarification, the NOTE was revised to read, "Variations in inoculum density must be avoided. For streaking plates, never use inoculum suspensions that have not been adjusted for turbidity as described in Section 7.1.1. (Also see Appendix C.)"**

Section 7.5, Reading Plates and Interpreting Results

21. Second sentence – The wording of this sentence is awkward; I suggest that the end be rewritten. Change to "...is observed after the incubation times listed above."

- **As suggested, the text was revised to read, "...when insufficient growth is observed after the incubation times listed above."**

22. Second sentence from the end – I found this sentence confusing, and it did not help to look at Appendix A. Is there a way to describe "microcolonies or trailing growth within a well-defined zone of inhibition"? Could additional photos be added to Appendix A to help make this clearer?

- **For clarification, the photograph and legend in Appendix A that shows trailing growth within a well-defined zone of inhibition were revised. A discrete zone of inhibition and a zone with trailing growth are marked with arrows.**

Section 8.1, Zone Diameter Epidemiological Cutoff Values

23. Bolded statement – It is not the relevance of testing the group of organisms but rather the relevance of the test results. The clinical relevance of the test results for this group of fungal pathogens remains uncertain because breakpoints with proven clinical relevance have yet...agency.

- **It appears that the commenter reviewed a previous draft of M51-P. The document currently reads, "The clinical relevance of testing this group of fungal pathogens remains uncertain because clinical breakpoints of proven relevance have not yet been identified or approved by CLSI or any regulatory agency."**

Section 9.1, Purpose; Section 9.2, Standard Reference Strains for Control; and Section 9.3, Storing Reference Strains

24. Change "repeatability" to reproducibility" where it appears in the document. Any test can be repeated but the reproducibility of the test results is another matter.

- **The text was revised as suggested.**

Section 9.3, Storing Reference Strains

25. Add something about being cognizant of the number of passages your control strains have undergone. Limit the passaging of control strains used in disk diffusion tests. Repeated subculturing can lead to contamination, genetic drift, or mutation as continuously smaller portions of a population are selected. Low-temperature storage greatly reduces phenotypic and genotypic drift and helps to ensure reproducible results in a series of quality control tests using a consistent stock. (Note: We have seen that repeated passaging can affect antimicrobial susceptibility and alter phenotypic and genotypic traits. We have also seen too many clinical laboratories passaging QC strains way too much and then calling to find out why the strains are still not giving good results after 20 to 50 passages. This is becoming a problem as more and more microbiology classes cut laboratories and teaching techniques such as cryopreservation out of their teaching programs.)

- **Section 9.3 of the guideline states that working cultures should be subcultured weekly for no more than three successive weeks and to prepare new working cultures at least monthly. This recommendation is consistent with other CLSI guidelines and standards such as M02, *Performance Standards for Antimicrobial Disk Susceptibility Tests;* M07, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically;* and M44, *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts.* For clarification, the following statement was added to the end of Section 9.3: "Use of a control strain that has been passaged too many times."**

Section 9.6.1, Out-of-Range Result Due to an Obvious Error

26. Add the following to the obvious reasons for out-of-range results: "Control strain has been passaged too many times. Obtain a new reference strain or use a new frozen culture."

- **An additional bulleted item was added that reads as follows: "Use of a control strain that has been passaged too many times."**

Section 9.6.2.2, Additional Corrective Action

27. First sentence – Should it be "systematic" rather than "system"?

- **For clarification, the statement was revised to read, "When immediate corrective action does not resolve the problem, it is likely that the error is due to a systematic vs a random error."**

28. Second bullet – Section 7.1 is Inoculum Preparation, and it says nothing about the performance requirements of the turbidity standard.

- **The reference to Section 7.1 was deleted.**

29. Fifth bullet – Change "are" to "is."

- **As suggested, "are" was replaced with "is."**

Section 10.2, Verification of Patient Results

30. First line – This is a guideline rather than a standard.

- **"Standard" was replaced with "guideline" as recommended.**

References

31. Updated reference for cryopreservation: Cryopreservation Technical Manual, F.P. Simione, Nalge Nunc International, Rochester, New York, 2006.
http://www.atcc.org/Portals/1/Pdf/Cryopreservation_Technical_Manual.pdf

- **The reference was updated as suggested.**

Appendix A, Performance of Nonsupplemented Mueller-Hinton Agar

32. This would be more useful if the results were also stated. Use a full-size picture, and state the measured zone diameter; indicate which is evidence of reduced susceptibility. Is there any significance to the use of two lots of caspofungin?

- **For clarification, the photograph and legend in Appendix A were revised as suggested. Two different lots of caspofungin obtained from different manufacturers were used for the study; therefore, both appear in the photograph.**

33. The testing is not to determine the susceptibility of the antifungal agents but rather the susceptibility of the fungus. Shown is...in determining the antifungal susceptibility of *Aspergillus terreus* to posaconazole...(#6 disk).

- **It appears that the commenter reviewed a previous draft of M51-P. The legend for Figure A1 currently reads, "Shown is the performance of the disk diffusion method using nonsupplemented Mueller-Hinton agar for the determination of antifungal susceptibility of *A. terreus* to posaconazole (#1-POS), caspofungin (#2 and #3-CAS; two manufacturers), itraconazole (#4-ITR), voriconazole (#5-VOR), and amphotericin B (#6-AMT). The lower arrows show a posaconazole clear zone of inhibition and the upper arrows show a zone with trailing growth usually only seen with caspofungin and other echinocandins.**

Trailing growth with caspofungin and other echinocandins should be ignored; however, trailing growth with other agents indicates resistance. Also see Section 7.5."

34. Would CAS be measured as 6 mm or as a larger zone with trailing growth? Please add interpretation to the photo legend.

- **For clarification, the photograph and legend in Appendix A were revised as suggested.**

Appendix B. Preparation of Nonsupplemented Mueller-Hinton Agar

35. Reference for Appendix B – CLSI document M07-A8 is the reference for dilution (broth and agar). Shouldn't the reference be M02-A10, which is the reference for disk diffusion susceptibility testing?

- **The reference to M07-A8, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition*, was replaced by a reference to M02-A10, *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Tenth Edition*.**

Active Membership List

36. Industry Members – Check the list for accuracy. There are two Siemens sites in California that are members (Los Angeles and Sacramento); there is no Illinois site.

- **The member list was verified and updated as suggested. A current membership list will be added to the document before publication at the approved level.**

Clinical and Laboratory Standards Institute consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact CLSI or visit our website at www.clsi.org.

M51-S1, *Performance Standards for Antifungal Disk Diffusion Susceptibility Testing of Filamentous Fungi; Informational Supplement*

Table 1

1. NOTE – Clarify the sentence. "These **interpretive** criteria have not been approved for **reporting test results** for clinical **isolates** but **are** proposed on a pragmatic basis **from the** evaluation of a series **of tests** using putative susceptible and resistant isolates."

- **It appears that the commenter reviewed a previous draft of M51-S1. NOTE 1 under Table 1 currently reads, "These tentative criteria have not been approved for use in clinical testing but were proposed on an epidemiological basis during evaluation of a series of putative susceptible and resistant isolates.[1] As discussed in Section 8 of M51-A, the clinical relevance of testing this group of fungal pathogens remains uncertain, and either MIC or zone diameter breakpoints with proven clinical relevance have not yet been identified or approved by CLSI or any regulatory agency."**

  No additional changes were made.

2. NOTE – Clarify the sentence. As discussed…proven clinical relevance…

- **It appears that the commenter reviewed a previous draft of M51-S1. NOTE 1 under Table 1 currently reads, "These tentative criteria have not been approved for use in clinical testing but were proposed on an epidemiological basis during evaluation of a series of putative susceptible and resistant isolates.[1] As discussed in Section 8 of M51-A, the clinical relevance of testing this group of fungal pathogens remains uncertain, and either MIC or zone diameter breakpoints with proven clinical relevance have not yet been identified or approved by CLSI or any regulatory agency."**

  No additional changes were made.

Table 1. Tentative Zone Diameter Epidemiological Cutoff Values (ECV) and Corresponding Minimal Inhibitory Concentration (MIC) or Minimal Effective Concentration (MEC) for Filamentous Fungi

3. Footnote a – In the table, the designation of units for the Equivalent MIC or MEC ECV is "µg/mL," but in the footnote it is "mcg/mL"; the units should be identified the same way in both places. Use either "µg/mL" or "mcg/mL."

- **As suggested, "mcg/mL" was changed to "µg/mL" in footnote a.**

4. NOTE – Add a comment in bold as follows: "The clinical…agency." This will allow reader to know earlier that the interpretive criteria have not been validated by clinical studies. I also propose revising the bold wording in Section 8.1. "The clinical relevance of the test results for this group of fungal pathogens remains uncertain because breakpoints with proven clinical relevance have yet…agency."

- **It appears that the commenter reviewed a previous draft of M51-S1. NOTE 1 under Table 1 currently reads, "These tentative criteria have not been approved for use in clinical testing but were proposed on an epidemiological basis during evaluation of a series of putative susceptible and resistant isolates.[1] As discussed in Section 8 of M51-A, the clinical relevance of testing this group of fungal pathogens remains uncertain, and either MIC or zone diameter breakpoints with proven clinical relevance have not yet been identified or approved by CLSI or any regulatory agency."**

  No additional changes were made.

5. Close the parentheses on 'µg/mL' in the header of the last column.

- **This change was made as suggested.**

Table 2. Recommended Reference Control Zone Diameter (mm) Ranges

6. Add the same footnote to *C. krusei* that is in the rest of our documents for consistency.

- **A footnote was added for consistency. It reads, "Because *Issatchenkia orientalis* is now known to be the sexual form (the teleomorph) of *C. krusei*, it would be technically correct to use *I. orientalis* as the name for this fungus. However, this change would confuse most users and the far more widely used name *C. krusei* is retained."**

7. Footnote † requires parentheses around "and 48" to signify that the value in parentheses in the table is the 48-hour value: † Reference control ranges obtained at 24 (and 48) hours in the collaborative study.

- **The text in Footnote † was revised as follows: "Reference control ranges obtained at 24 (and 48) hours in the collaborative study."**

8. In the two columns for the zone diameters, identify what the two different sets of numbers represent. Is the first set what was obtained at 24 hours, and the set in parentheses what was seen at 48 hours?

- **The text in Footnote † was revised as follows: "Reference control ranges obtained at 24 (and 48) hours in the collaborative study."**

9. Footnotes – Table 2 needs to define what is in parentheses (48-hour zones?).

- **The text in Footnote † was revised as follows: "Reference control ranges obtained at 24 (and 48) hours in the collaborative study."**

10. It is not clear to the reader what the values in parentheses represent.

- **The text in Footnote † was revised as follows: "Reference control ranges obtained at 24 (and 48) hours in the collaborative study."**

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 CLSI document HS01—*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 as follows:

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

M51-A 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 and Records	Organization	Personnel	Equipment	Purchasing and Inventory	Process Control	Information Management	Occurrence Management	Assessments—External and Internal	Process Improvement	Customer Service	Facilities and Safety
ILA21		ILA21	ILA21		X ILA21 M02 M27 M38 M44	ILA21		ILA21	ILA21		ILA21 M29

Adapted from CLSI document HS01—*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 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.

M51-A 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
				X M02 M27 M38	X M02 M27 M38 M44	X M02 M27 M38 M44	X M02 M27 M38 M44	X M27 M38

Adapted from CLSI document HS01—*A Quality Management System Model for Health Care.*

Related CLSI Reference Materials*

I/LA21-A2 **Clinical Evaluation of Immunoassays; Approved Guideline—Second Edition (2008).** This document addresses the need for clinical evaluation of new immunoassays and new applications of existing assays, as well as multiple assay formats and their uses. As a guide to designing and executing a clinical evaluation, this document will aid developers of "in-house" assays for institutional use, developers of assays used for monitoring pharmacologic effects of new drugs or biologics, and clinical and regulatory personnel responsible for commercializing products.

M02-A10 **Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Tenth Edition (2009).** This document contains the current CLSI-recommended methods for disk susceptibility testing, criteria for quality control testing, and updated tables for interpretive zone diameters.

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.

M27-A3 **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition (2008).** 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.

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.

M38-A2 **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition (2008).** 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.

M44-A2 **Methods for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline—Second Edition (2009).** This document provides newly established methodology for disk diffusion testing of *Candida* spp., criteria for quality control testing, and interpretive criteria.

M51-S1 **Performance Standards for Antifungal Disk Diffusion Susceptibility Testing of Nondermatophyte Filamentous Fungi; Informational Supplement (2010).** These supplemental tables provide zone diameter reference limits for CLSI document M51-A.

* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

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LAC/USC Medical Center (CA)
Lafayette General Medical Center (LA)
Lakeland Regional Medical Center (FL)
Lancaster General Hospital (PA)
Landstuhl Regional Medical Center (APO, AE)
Langley Air Force Base (VA)
Laredo Medical Center (TX)
LeBonheur Children's Medical Center (TN)
Legacy Laboratory Services (OR)
Letherbridge Regional Hospital (Canada)
Lewis-Gale Medical Center (VA)
L'Hotel-Dieu de Quebec (Canada)
Licking Memorial Hospital (OH)
LifeLabs Medical Laboratory Services (Canada)
Loma Linda University Medical (CA)
Long Beach Memorial Medical Center-LBMMC (CA)
Louisiana Office of Public Health Laboratory (LA)
Louisiana State University Medical Ctr. (LA)
Lourdes Hospital (KY)
Lower Columbia Pathologists, P.S. (WA)
Lyndon B. Johnson General Hospital (TX)
Maccabi Medical Care and Health Fund (Israel)
Madigan Army Medical Center (WA)
Mafraq Hospital (United Arab Emirates)
Magnolia Regional Health Center (MS)
Main Line Clinical Laboratories, Inc. (PA)
Makerere University Walter Reed Project Makerere University Medical School (Uganda)
Marquette General Hospital (MI)
Marshfield Clinic (WI)
Martha Jefferson Hospital (VA)
Martin Luther King, Jr.-Harbor Hospital (CA)
Martin Memorial Health Systems (FL)
Mary Hitchcock Memorial Hospital (NH)
Mary Imogene Bassett Hospital (NY)
Mary Washington Hospital (VA)
Massachusetts General Hospital (MA)
Maxwell Air Force Base (AL)
Mayo Clinic (MN)
Mayo Clinic Florida (FL)
MCG Health (GA)
Meadows Regional Medical Center (GA)
Medecin Microbiologiste (Canada)
Medical Center Hospital (TX)
Medical Center of Louisiana at NO-Charity (LA)
Medical Centre Ljubljana (Slovenia)
Medical College of Virginia Hospital (VA)
Medical Univ. of South Carolina (SC)
Memorial Hermann Healthcare System (TX)
Memorial Hospital at Gulfport (MS)
Memorial Medical Center (IL)
Memorial Medical Center (PA)
Memorial Regional Hospital (FL)
Mercy Franciscan Mt. Airy (OH)
Methodist Dallas Medical Center (TX)
Methodist Hospital (Houston, TX)
Methodist Hospital (San Antonio, TX)
Methodist Hospital – Park Nicollet Health Services (MN)
Methodist Hospital Pathology (NE)
MetroHealth Medical Center (OH)
Metropolitan Medical Laboratory, PLC (IA)
The Michener Inst. for Applied Health Sciences (Canada)
Mid Michigan Medical Center – Midland (MI)
Middelheim General Hospital (Belgium)
Mississippi Baptist Medical Center (MS)
Mississippi Public Health Lab (MS)
Monongalia General Hospital (WV)
Montreal General Hospital (Quebec, Canada)
Mt. Carmel Health System (OH)
Mt. Sinai Hospital (Canada)
Mt. Sinai Hospital – New York (NY)
Naples Community Hospital (FL)
Nassau County Medical Center (NY)
National B Virus Resource Laboratory (GA)
National Cancer Center (S. Korea)
National Institutes of Health, Clinical Center (MD)
National Naval Medical Center (MD)
National University Hospital Department of Laboratory Medicine (Singapore)
Nationwide Children's Hospital (OH)
Nationwide Laboratory Services (FL)
Naval Hospital Great Lakes (IL)
The Naval Hospital of Jacksonville (FL)
Naval Medical Center Portsmouth (VA)
NB Department of Health (Canada)
The Nebraska Medical Center (NE)
New England Baptist Hospital (MA)
New Lexington Clinic (KY)
New York City Department of Health and Mental Hygiene (NY)
New York Presbyterian Hospital (NY)
New York University Medical Center (NY)
Newark Beth Israel Medical Center (NJ)
Nor-Lea General Hospital (NM)
North Carolina Baptist Hospital (NC)
North District Hospital (Hong Kong, China)
North Mississippi Medical Center (MS)
North Shore Hospital Laboratory (New Zealand)
North Shore-Long Island Jewish Health System Laboratories (NY)
Northeastern NV Regional Hospital (NV)
Northridge Hospital Medical Center (CA)
Northside Hospital (GA)
Northwest Texas Hospital (TX)
Norton Healthcare (KY)
Ochsner Clinic Foundation (LA)
Ohio State University Hospitals (OH)
Ohio Valley Medical Center (WV)
Onze Lieve Vrouw Ziekenhuis (Belgium)
Ordre Professionel des Technologistes Medicaux du Quebec (Quebec, Canada)
Orebro University Hospital (Sweden)
Orlando Regional Healthcare System (FL)
Ospedale Casa Sollievo Della Sofferenza – IRCCS (Italy)
The Ottawa Hospital (Canada)
Our Lady of Lourdes Reg. Medical Ctr. (LA)
Palmetto Baptist Medical Center (SC)
Parkland Health & Hospital System (TX)
Pathlab (IA)
Pathology and Cytology Laboratories, Inc. (KY)
Pathology Associates Medical Laboratories (WA)
Peace River Regional Health Center (FL)
Penn State Hershey Medical Center (PA)
Pennsylvania Hospital (PA)
The Permanente Medical Group (CA)
Peterborough Regional Health Centre (Canada)

Piedmont Hospital (GA)
Pitt County Memorial Hospital (NC)
Potomac Hospital (VA)
Prairie Lakes Hospital (SD)
Premiere Medical Laboratories, P.A. (FL)
Presbyterian Hospital – Laboratory (NC)
Presbyterian/St. Luke's Medical Center (CO)
Prince County Hospital (Canada)
Princess Margaret Hospital (Hong Kong)
Providence Alaska Medical Center (AK)
Providence Health Care (Canada)
Providence Health Services, Regional Laboratory (OR)
Providence Medford Medical Center (OR)
Provincial Health Services Authority (Vancouver, BC, Canada)
Provincial Laboratory for Public Health (Edmonton, AB, Canada)
Queen Elizabeth Hospital (Canada)
Queen Elizabeth Hospital (China)
Queensland Health Pathology Services (Australia)
Queensway Carleton Hospital (Canada)
Quest Diagnostics, Incorporated (CA)
Quest Diagnostics JV (OH)
Quintiles Laboratories, Ltd. (GA)
Rady Children's Hospital San Diego (CA)
Ramathibodi Hospital (Thailand)
Redington-Fairview General Hospital (ME)
Regions Hospital (MN)
Reid Hospital & Health Care Services (IN)
Renown Regional Medical Center (NV)
Rex Healthcare (NC)
River Valley Health-Chalmers Regional Hospital (NB)
Riverside County Regional Medical Center (CA)
Riverside Health System (VA)
Riverside Methodist Hospital (OH)
Riyadh Armed Forces Hospital, Sulaymainia (Saudi Arabia)
Riyadh National Hospital (Saudi Arabia)
Rockford Memorial Hospital (IL)
Royal Victoria Hospital (Canada)
Sacred Heart Hospital (FL)
Sacred Heart Hospital (WI)
Sahlgrenska Universitetssjukhuset (Sweden)
St. Agnes Healthcare (MD)
St. Anthony Hospital (OK)
St. Barnabas Medical Center (NJ)
St. Christopher's Hospital for Children (PA)
St. Elizabeth Community Hospital (CA)
St. Eustache Hospital (Canada)
St. Francis Hospital (SC)
Saint Francis Hospital & Medical Center (CT)
St. Francis Medical Center (NJ)
St. John Hospital and Medical Center (MI)
St. John's Hospital (IL)
St. John's Hospital & Health Ctr. (CA)
St. John's Mercy Medical Center (MO)
St. John's Regional Health Center (MO)
[illegible]seph Hospital (MI)
[illegible]seph's Medical Center (CA)
St. Joseph's Regional Medical Center (NJ)
St. Jude Children's Research Hospital (TN)
St. Luke's Hospital (IA)
St. Luke's Hospital (PA)
St. Mary Medical Center (CA)
St. Mary's Hospital (WI)
St. Mary of Nazareth Hospital (IL)
Saint Mary's Regional Medical Center (NV)
St. Tammany Parish Hospital (LA)
Saints Memorial Medical Center (MA)
Sampson Regional Medical Center (NC)
Samsung Medical Center (Korea)
San Francisco General Hospital-University of California San Francisco (CA)
Sanford USD Medical Center (SD)
Santa Clara Valley Medical Center (CA)
SARL Laboratoire Caron (France)
Schneck Medical Center (IN)
Scott & White Memorial Hospital (TX)
Seoul National University Hospital (Korea)
Seoul St. Mary's Hospital (Korea)
Seton Medical Center (CA)
Sheik Kalifa Medical City (UAE)
Shiel Medical Laboratory Inc. (NY)
Shore Memorial Hospital (NJ)
Shriners Hospitals for Children (SC)
Singapore General Hospital (Singapore)
South Bend Medical Foundation (IN)
South County Hospital (RI)
South Miami Hospital (FL)
Southern Community Laboratories (New Zealand)
Southern Health Care Network (Australia)
Southern Maine Medical Center (ME)
Spectrum Health – Blodgett Campus (MI)
Stanford Hospital and Clinics (CA)
Stanton Territorial Health Authority (Canada)
State of Connecticut Department of Public Health (CT)
State of Ohio/Corrections Medical Center Laboratory (OH)
State of Washington-Public Health Labs (WA)
Stevens Memorial Hospital (WA)
Stillwater Medical Center (OK)
Stony Brook University Hospital (NY)
Stormont-Vail Regional Medical Ctr. (KS)
Strong Memorial Hospital (NY)
Sudbury Regional Hospital (Canada)
Sunbury Community Hospital (PA)
Sunnybrook Health Sciences Centre (ON, Canada)
Sunrise Hospital and Medical Center (NV)
Sutter Roseville Medical Center (CA)
Swedish Medical Center (CO)
Sydney South West Pathology Service Liverpool Hospital (Australia)
T.J. Samson Community Hospital (KY)
Tai[illegible] (Taiwa
Taipei Ve[illegible]
Taiwan Soc. Medicine (
Tallaght Hospital (
Tartu University Clinics (Estonia)
Temple Univ. Hospital – Parkinson Pav. (PA)
Texas Children's Hospital (TX)
Texas Department of State Health Services (TX)
Texas Health Presbyterian Hospital Dallas (TX)
Thomason Hospital (TX)
Timmins and District Hospital (Canada)
Tokyo Metro. Res. Lab of Public Health (Japan)
The Toledo Hospital (OH)
Ton Yen General Hospital (Taiwan)
Touro Infirmary (LA)
Tri-City Medical Center (CA)
Trident Medical Center (SC)
Tripler Army Medical Center (HI)
Tuen Mun Hospital, Hospital Authority (China)
Tufts Medical Center Hospital (MA)
Tulane Medical Center Hospital & Clinic (LA)
Twin Lakes Regional Medical Center (KY)
UCI Medical Center (CA)
UCLA Medical Center Clinical Laboratories (CA)
UCSD Medical Center (CA)
UCSF Medical Center – China Basin (CA)
UMC of Southern Nevada (NV)
UNC Hospitals (NC)
Union Clinical Laboratory (Taiwan)
United Christian Hospital (Hong Kong)
United Clinical Laboratories (IA)
United Medical Center (DC)
United States Air Force School of Aerospace Medicine/PHE (TX)
Unity HealthCare (IA)
Universita Campus Bio-Medico (Italy)
Universitair Ziekenhuis Antwerpen (Belgium)
University College Hospital (Ireland)
University Hospital (TX)
University Hospital Center Sherbrooke (CHUS) (Canada)
University Medical Center at Princeton (NJ)
University of Alabama Hospital Lab (AL)
University of Chicago Hospitals Laboratories (IL)
University of Colorado Health Sciences Center (CO)
University of Colorado Hospital (CO)
University of Illinois Medical Center (IL)
University of Iowa Hospitals and Clinics (IA)
University of Kentucky Med. Ctr. (KY)
University of Maryland Medical System (MD)
University of Medicine & Dentistry of New Jersey (UMDNJ) (NJ)
University of Missouri Hospital (MO)
University of MN Medical Center – Fairview (MN)
Univ. of Pennsylvania Health System (PA)
University of Pittsburgh Medical Center (PA)
University of So. Alabama Children's and Women's Hospital (AL)
University of Texas Health Center (TX)
The University of Texas Medical Branch (TX)
University of the Ryukyus (Japan)
University of Virginia Medical Center (VA)
UPMC Bedford Memorial (PA)
US Naval Hospital Naples (FPO)
USC University Hospital (CA)
UZ-KUL Medical Center (Belgium)
VA (Asheville) Medical Center (NC)
VA (Bay Pines) Medical Center (FL)
VA Central Texas Veterans Health Care System (TX)
VA (Chillicothe) Medical Center (OH)
VA (Cincinnati) Medical Center (OH)
VA (Dayton) Medical Center (OH)
VA (Decatur) Medical Center (GA)
VA (Durham) Medical Center (NC)
VA (Hampton) Medical Center (VA)
VA (San Diego) Medical Center (CA)
VA (Tampa) Hospital (FL)
Valley Health/Winchester Medical Center (VA)
Vancouver Coastal Health Regional Laboratory (BC, Canada)
Vancouver Island Health Authority (SI) (Canada)
Vanderbilt University Medical Center (TN)
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Walter Reed Army Medical Center (DC)
Warren Hospital (NJ)
Waterbury Hospital (CT)
Waterford Regional Hospital (Ireland)
Wayne Memorial Hospital (NC)
Weirton Medical Center (WV)
West China Second University Hospital, Sichuan University (China)
West Jefferson Medical Center (LA)
West Shore Medical Center (MI)
West Valley Medical Center Laboratory (ID)
Westchester Medical Center (NY)
Western Baptist Hospital (KY)
Western Healthcare Corporation (Canada)
Wheaton Franciscan and Midwest Clinical Laboratories (WI)
Wheeling Hospital (WV)
Whitehorse General Hospital (Canada)
William Beaumont Army Medical Center (TX)
William Beaumont Hospital (MI)
William Osler Health Centre (Canada)
Winchester Hospital (MA)
Winn Army Community Hospital (GA)
Wishard Health Sciences (IN)
Womack Army Medical Center (NC)
York Hospital (PA)

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