JOURNAL OF CLINICAL MICROBIOLOGY, June 2007, p. 1811–1820 Vol. 45, No. 6
0095-1137/07/$08.00+0 doi:10.1128/JCM.00134-07

Multicenter Evaluation of a New Disk Agar Diffusion Method for Susceptibility Testing of Filamentous Fungi with Voriconazole, Posaconazole, Itraconazole, Amphotericin B, and Caspofungin▽

A. Espinel-Ingroff,[1]* B. Arthington-Skaggs,[2] N. Iqbal,[2] D. Ellis,[3] M. A. Pfaller,[4] S. Messer,[4] M. Rinaldi,[5] A. Fothergill,[5] D. L. Gibbs,[6] and A. Wang[6]

Virginia Commonwealth University Medical Center, Richmond, Virginia[1]; Centers for Disease Control, Atlanta, Georgia[2]; Women's and Children's Hospital, North Adelaide, South Australia, Australia[3]; University of Iowa, Iowa City, Iowa[4]; University of Texas, San Antonio, Texas[5]; and Giles Scientific Inc., Santa Barbara, California[6]

Received 18 January 2007/Returned for modification 9 March 2007/Accepted 2 April 2007

The purpose of this study was to correlate inhibition zone diameters, in millimeters (agar diffusion disk method), with the broth dilution MICs or minimum effective concentrations (MECs) (CLSI M38-A method) of five antifungal agents to identify optimal testing guidelines for disk mold testing. The following disk diffusion testing parameters were evaluated for 555 isolates of the molds *Absidia corymbifera*, *Aspergillus* sp. (five species), *Alternaria* sp., *Bipolaris spicifera*, *Fusarium* sp. (three species), *Mucor* sp. (two species), *Paecilomyces lilacinus*, *Rhizopus* sp. (two species), and *Scedosporium* sp. (two species): (i) two media (supplemented Mueller-Hinton agar [2% dextrose and 0.5 μg/ml methylene blue] and plain Mueller-Hinton [MH] agar), (ii) three incubation times (16 to 24, 48, and 72 h), and (iii) seven disks (amphotericin B and itraconazole 10-μg disks, voriconazole 1- and 10-μg disks, two sources of caspofungin 5-μg disks [BBL and Oxoid], and posaconazole 5-μg disks). MH agar supported better growth of all of the species tested (24 to 48 h). The reproducibility of zone diameters and their correlation with either MICs or MECs (caspofungin) were superior on MH agar (91 to 100% versus 82 to 100%; *R*, 0.71 to 0.93 versus 0.53 to 0.96 for four of the five agents). Based on these results, the optimal testing conditions for mold disk diffusion testing were (i) plain MH agar; (ii) incubation times of 16 to 24 h (zygomycetes), 24 h (*Aspergillus fumigatus*, *A. flavus*, and *A. niger*), and 48 h (other species); and (iii) the posaconazole 5-μg disk, voriconazole 1-μg disk, itraconazole 10-μg disk (for all except zygomycetes), BBL caspofungin 5-μg disk, and amphotericin B 10-μg (zygomycetes only).

The Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) Subcommittee on Antifungal Susceptibility Tests has developed reproducible procedures for antifungal susceptibility testing of molds by the broth microdilution method (M38-A document) (3). An agar diffusion method has been developed for yeasts by disk diffusion methodology (CLSI M44-A document for fluconazole and voriconazole) (2, 4, 6). Reference guidelines are not available for mold disk diffusion testing. However, although infections caused by molds are not as common as yeast infections, an increased incidence of systemic infections caused by *Aspergillus* and more recently the zygomycetes and other species (*Aspergillus*, *Fusarium*, and *Scedosporium*) has been documented (16). Therefore, there is a need for an easier and more economical standard method to test the susceptibility of mold isolates to available antifungal agents.

The overall objective of this study was to identify standard testing guidelines for disk testing of molds (i) by determining the correlation between zone diameters in millimeters by a disk diffusion method that were read at each of three incubation times with broth microdilution reference MICs (CLSI M38-A method) or MECs (minimum effective concentrations, caspofungin) (3), (ii) by determining the reproducibility of replicate zone diameters obtained on 3 different days and under different testing conditions by the disk diffusion method, and (iii) by determining the performance of the disk diffusion method in identifying resistant isolates. This study evaluated the following 18 mold species (555 isolates): *Absidia corymbifera*, *Aspergillus* sp. (five species), *Alternaria* sp., *Bipolaris spicifera*, *Fusarium* sp. (three species), *Mucor* sp. (two species), *Paecilomyces lilacinus*, *Rhizopus* sp. (two species), and *Scedosporium* sp. (two species). Because MIC or MEC breakpoints are not available for mold testing, isolates were grouped as susceptible (MIC or MEC, ≤1 μg/ml), intermediate (MIC or MEC, 2 μg/ml) and resistant (MIC or MEC, ≥4 μg/ml) to determine the performance of the disk diffusion method for identifying resistant isolates. These categorical breakpoints were chosen to enable this determination; supporting clinical data are not available. These breakpoints have not been approved by the CLSI, the FDA, or the pharmaceutical companies.

MATERIALS AND METHODS

Study design. Two of the five laboratories received the same panel of 72 isolates, including 4 isolates of each of the 18 species evaluated in the study and 2 quality control (QC) isolates. Each isolate was tested with amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole by both the broth microdilution M38-A and disk diffusion assays on 3 different days to obtain reproducibility data. In addition, three of the five laboratories tested a total of 483 isolates, including 5 to 56 isolates of each of the same 18 species with the same antifungal agents by both the broth microdilution and disk diffusion procedures. The study protocol included CLSI M38-A broth microdilution testing conditions (3) and a detailed description of the disk testing parameters to be evaluated: (i) two media, supplemented Mueller-Hinton agar (2% dextrose and

* Corresponding author. Mailing address: Virginia Commonwealth University Medical Center, Richmond, VA 23298-0049. Phone: (804) 828-5743. Fax: (804) 828-3097. E-mail: avingrof@vcu.edu.

▽ Published ahead of print on 11 April 2007.

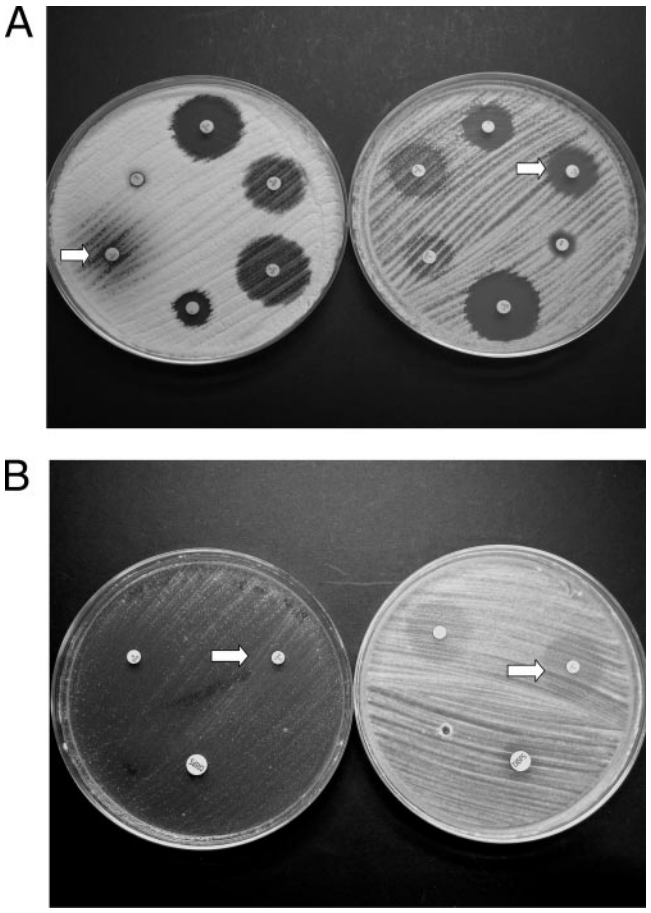

FIG. 1. Interference of MGM agar with voriconazole disks for *A. terreus* (A) and with caspofungin disks for *P. lilacinus* (B), where low MICs corresponded to very small or no inhibition zone diameters. These conflicting results were not observed on MH agar.

0.5 μg/ml methylene blue [MGM]) and plain Mueller-Hinton (MH) agar; (ii) three incubation times (16 to 24, 48, and 72 h); and (iii) seven disks (amphotericin B and itraconazole 10-μg disks; voriconazole 1- and 10-μg disks; two sources of caspofungin 5-μg disks [BBL and Oxoid]; and posaconazole 5-μg disks).

Isolates. A total of 483 isolates were evaluated at three of the five centers; isolates for which the MICs of the five antifungal agents are high and low were included. The set of 483 isolates included 15 *Absidia corymbifera*, 23 *Alternaria* sp., 56 *A. fumigatus*, 38 *A. flavus*, 31 *A. nidulans*, 38 *A. niger*, 40 *A. terreus*, 18 *Bipolaris spicifera*, 28 *Fusarium moniliforme*, 20 *F. oxysporum*, 29 *F. solani*, 17 *Mucor circinelloides*, 5 *M. ramosissimus*, 37 *Rhizopus arrhizus*, 10 *R. microsporus* var. *rhizopodiformis*, 24 *Paecilomyces lilacinus*, 28 *Scedosporium apiospermum*, and 26 *S. prolificans* isolates. In addition, two centers evaluated another set of 72 isolates on 3 different days to obtain reproducibility data; these isolates included four isolates of each of the 18 species listed above. The 72 isolates were shipped to each of these two centers from the University of Texas Health Science Center, San Antonio. All of the isolates evaluated were clinical isolates submitted to the Virginia Commonwealth University Medical Center, Richmond; the Centers for Disease Control, Atlanta, GA; and the University of Texas Health Science Center.

The CLSI QC isolates *Candida krusei* ATCC 6258 and *Paecilomyces variotii* ATCC MYA-3630 were tested each time a set of isolates was evaluated in each of the five centers with each antifungal agent and by both methods. MICs for *C. krusei* ATCC 6258 were within the established MIC limits of the five antifungal agents (2, 5); the same applied for *Paecilomyces variotii* ATCC MYA-3630 with amphotericin B, itraconazole, and voriconazole (14); three posaconazole MICs were below the established MIC range from one laboratory for this QC isolate

TABLE 1. Species and numbers of mold isolates tested at three of five centers

Species	No. of isolates
Absidia corymbifera	15
Alternaria sp.	23
Bipolaris spicifera	18
Aspergillus fumigatus	56
A. flavus	38
A. niger	38
A. terreus	40
A. nidulans	31
Fusarium solani	29
F. moniliforme	28
F. oxysporum	20
Mucor circinelloides	17
Mucor ramosissimus	5
Rhizopus arrhizus	37
Rhizopus microsporus var. *rhizopodiformis*	10
Paecilomyces lilacinus	24
Scedosporium apiospermum	28
S. prolificans	26
Total	483[a]

[a] Another set of 72 isolates was tested at another two centers to obtain reproducibility data.

TABLE 2. Reproducibility of inhibition zone diameters, in millimeters, obtained by the disk diffusion method with five antifungal agents for 72 mold isolates at two of the five centers[a]

Antifungal agent and medium[b]	% within 2-SD zone at:		
	24 h	48 h	72 h
Itra			
MH	96	91	88
MGM	87	82	82
Posa			
MH	97	95	92
MGM	96	96	94
Vori-1			
MH	97	94	93
MGM	97	95	90
Vori-10			
MH	92	93	92
MGM	92	93	92
Cas-BBL			
MH	100	96	95
MGM	96	94	87
Cas-Oxoid			
MH	100	97	97
MGM	100	100	97
Amb			
MH	95	95	95
MGM	100	95	91

[a] Percentages of replicate zone diameters within 2 standard deviations of the mean; each of the 72 isolates was tested on 3 different days in two of the five laboratories with each antifungal agent. Inhibition zone diameters, in millimeters, on both MH and MGM agar were read at 16 to 24, 48, and 72 h.

[b] Itra, itraconazole 10-μg disk; Posa, posaconazole 5-μg disk; Cas-BBL and Cas-Oxoid, caspofungin 5-μg disks from two sources; Vori-1 and Vori-10, voriconazole 1-μg and 10-μg disks, respectively; Amb, amphotericin B 10-μg disk.

TABLE 3. Inhibition zone diameters obtained on MH agar by the disk diffusion method for two QC isolates[a]

QC isolate[b] and antifungal agent	Zone range in millimeters (% agreement) at:		
	24 h	48 h	72 h
Paecilomyces variotii ATCC 3630			
Itra	25–31 (99)	23–31 (94)	24–31 (97)
Posa	35–46 (100)	37–45 (97)	37–44 (97)
Vori-1	40–53 (94)	41–53 (94)	41–53 (100)
Vori-10	56–64 (97)	60–71 (86)	61–73 (89)
Cas-BBL	23–29 (100)	23–30 (97)	24–30 (97)
Cas-Oxoid	23–34 (100)	23–34 (97)	22–33 (89)
Amb	13–22 (94)	9–19 (83)	10–19 (89)
Candida krusei ATCC 6258			
Itra	21–30 (100)	20–29 (100)	20–28 (100)
Posa	31–43 (97)	30–41 (97)	30–41 (97)
Vori-1	29–38 (100)	25–35 (100)	24–36 (97)
Vori-10	47–59 (92)	46–60 (97)	53–61 (92)
Cas-BBL	14–24 (100)	18–27 (97)	17–25 (97)
Cas-Oxoid	21–29 (100)	21–30 (100)	21–32 (100)
Amb	19–27 (100)	15–25 (97)	13–25 (97)

[a] Tests were performed 5 to 16 times for each isolate in each of the five laboratories for each disk and incubation time on MH agar.

[b] Itra, itraconazole 10-μg disk; Posa, posaconazole 5-μg disk; Cas-BBL and Cas-Oxoid, caspofungin 5-μg disks from two sources; Vori-1 and Vori-10, voriconazole 1-μg and 10-μg disks, respectively; Amb, amphotericin B 10-μg disk.

TABLE 4. Correlation between inhibition zone diameters (in millimeters) obtained by the disk diffusion method and reference M38-A MICs (amphotericin B and triazoles) and MECs (caspofungin) for 483 mold isolates at three of the five centers[a]

Antifungal agent and incubation time (h)[b]	Correlation coefficient (R value)[c] for:	
	MH	MGM
Itra		
24	0.79	ND
48	0.75	0.58
72	0.74	0.67
Posa		
24	0.77	ND
48	0.71	0.65
72	0.71	0.71
Vori-1		
24	0.85	ND
48	0.83	0.87
72	0.82	0.53
Vori-10		
24	0.83	ND
48	0.80	0.85
72	0.83	0.58
Cas-BBL		
24	0.92	ND
48	0.88	0.96
72	0.90	0.87
Cas-Oxoid		
24	0.93	ND
48	0.89	0.88
72	0.90	0.87
Amb		
24	0.68	ND
48	0.43	0.32
72	0.31	0.54

[a] MICs were read at 24 h (zygomycetes), 72 h (*Scedosporium* sp.), and 48 h (other species) as recommended in the CLSI M38-A document. Caspofungin MECs were read at 24 h (all species except for *Scedosporium* sp., which were read at 48 h). Each MIC or MEC was plotted against inhibition zone diameters in millimeters obtained on both MH and MGM agars at each of the three incubation times; results from three centers were combined for linear regression analysis.

[b] Itra, itraconazole 10-μg disk; Posa, posaconazole 5-μg disk; Cas-BBL and Cas-Oxoid, caspofungin 5-μg disks from two sources; Vori-1 and Vori-10, voriconazole 1-μg and 10-μg disks, respectively; Amb, amphotericin B 10-μg disk.

[c] ND, not determined. The 48-h results for MGM medium encompassed 24- and 48-h zones of inhibition; 71% of the 24-h results were reported as no growth.

(14). Caspofungin QC MIC limits have not been established for molds, but our results were within a 3-dilution range (0.015 to 0.06 μg/ml) at the five centers for *P. variotii* ATCC MYA-3630.

(i) CLSI broth microdilution procedure (M38-A2). MICs or MECs (caspofungin only) were determined by the CLSI broth microdilution procedure (M38-A document) at each center for each set of isolates (483 and 72 isolates). Amphotericin B (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT), itraconazole (Janssen, Beerse, Belgium), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), voriconazole (Pfizer Central Research, New York, NY), and caspofungin (Merck Research Laboratories, Rahway, NJ) were provided by the manufacturers as standard powders for the preparation of CLSI microdilution trays. Drug dilutions were prepared at 100 times the final concentration in polyethylene glycol (posaconazole), dimethyl sulfoxide (amphotericin B, itraconazole, and voriconazole), and sterile distilled water (caspofungin) and then diluted in the standard RPMI 1640 medium to yield the final twofold drug concentrations of 0.01 to 32 μg/ml for CLSI methods (2, 3). MIC panels containing the diluted twofold drug concentrations in standard RPMI 1640 medium were shipped frozen to each laboratory from TREK Diagnostics (Westlake, OH).

Mold stock inoculum suspensions were prepared from 7-day cultures grown on potato dextrose agar (Remel, Lenexa, KS) and adjusted spectrophotometrically at a 530-nm wavelength to optical densities that ranged from 0.09 to 0.3 (3, 13); the stock suspension for the QC yeast isolate was adjusted to the optical density of a 0.5 McFarland standard (2). Stock inoculum suspensions were further diluted in RPMI 1640 medium to achieve twofold final concentrations as follows: 1:25 (*Alternaria* sp., *Scedosporium* sp., and *B. spicifera*), 1:50 (other mold species), and 1:1,000 (QC yeast strain) (2, 3, 13). On the day of the test, each microdilution well containing 100 μl was inoculated with 100 μl of the diluted twofold inoculum suspension (the final volume in each well was 200 μl). The twofold test inoculum concentrations ranged from 0.1×10^4 to 5.5×10^4 CFU/ml as demonstrated by colony counts at each center. Growth (drug free) and sterility controls were included for each isolate tested. Microdilution trays were incubated in ambient air at 35°C and examined for MIC or MEC determination at 24 h (zygomycetes), 48 h (*Alternaria* sp., *B. spicifera*, *Aspergillus* sp., *Fusarium* sp., and *P. lilacinus*), and 72 h (*Scedosporium* sp.) (3). By visual examination, reference MICs were defined as the lowest drug concentrations that showed 100% (amphotericin B and triazoles) growth inhibition compared to the growth control well (3). Caspofungin MECs were defined as the lowest drug concentrations that produced growth of small, rounded, compact colonies compared to the hyphal growth seen in the growth control well (9, 18). QC isolates were tested in the same manner.

(ii) Disk diffusion procedure. The following agar disk diffusion procedure was evaluated for determination of zones of inhibition in millimeters at each center. The entire surfaces of MGM and MH agar (Hardy Diagnostics, Santa Maria, CA) agar plates (150 mm) were inoculated simultaneously in three directions with a nontoxic cotton swab dipped in the undiluted mold stock inoculum suspensions. The inoculated agar was allowed to dry for 15 to 30 min. Amphotericin B 10-μg disks, itraconazole 10-μg disks, voriconazole 10-μg disks (Abtek Biologicals Ltd., Liverpool, United Kingdom), posaconazole 5-μg and voriconazole 1-μg disks (Becton Dickson and Company, Sparks, MD), and caspofungin 5-μg disks from two sources (Becton Dickson and Oxoid Limited, Basingstoke, England) were applied to the inoculated agar with a pair of forceps. The plates were incubated in ambient air at 35°C within 15 min after the disks were applied to the inoculated agar. QC isolates were tested in the same manner.

TABLE 5. Agreement of inhibition zone diameters (disk diffusion method) with MICs or MECs (M38-A method) according to assigned susceptible, intermediate, and resistant categories for 483 mold isolates with five antifungal agents as obtained in three laboratories

Antifungal agent, method, and medium[a]	% of MICs by interpretive category[b]			% Error			% Agreement[c]
	S	I	R	Minor	Major	Very major	
Posaconazole							
BMD	78	9	13				
Disk, 5 μg	83	4	13	8.1	0.2	0.2	92
Itraconazole							
BMD	54	11	35				
Disk, 10 μg	52	8	40	12.8	2.5	1.8	83
Voriconazole							
BMD	59	9	32				
Disk, 1 μg	56	1	43	9.7	4.6	0.2	86
Caspofungin							
BMD	51	3	46				
Disk, 5 μg, BBL	50	1	49	3.3	1	0.2	96
Amphotericin B							
BMD	49	24	27				
Disk, 10 μg	58	12	30	23.1	2.7	1.7	73

[a] BMD, MICs and MECs, in micrograms per milliliter, were determined at the recommended incubation times (24 to 48 h for caspofungin and 24 to 72 h for the other agents) by the CLSI M38-A broth microdilution method. Disk inhibition zone diameters, in millimeters, were determined with the optimal medium (MH agar) and incubation times (16 to 24 h and 48 h) by the disk diffusion method.

[b] Percentages of MICs or MECs and inhibition zone diameters, in millimeters, that were within the assigned posaconazole, itraconazole, voriconazole, and caspofungin categories (S [susceptible], MIC or MEC of ≤1 μg/ml, ≥17 mm; I [intermediate], MIC or MEC of 2 μg/ml, 14 to 16 mm; R [resistant], MIC or MEC of ≥4 μg/ml, ≤13 mm) and amphotericin B MIC and zone diameter categories (susceptible, MIC of ≤1 μg/ml, >15 mm; intermediate, MIC of 2 μg/ml, 13 to 14 mm; resistant, MIC of ≥4 μg/ml, 12 mm).

[c] Percent categorical agreement between inhibition zone diameters, in millimeters, and MIC or MEC results.

Zone diameters in the disk diffusion assay were measured to the nearest whole millimeter at the point where there was a prominent reduction of growth (80%) after 16 to 24 h for zygomycetes and after 24, 48, and 72 h for the other species. Microcolonies inside the zone of inhibition were ignored when testing caspofungin and hyphal filaments bending over the inhibition zones and slight trailing around the edges when testing the triazoles, but not for amphotericin B (Fig. 1).

Data analysis. MICs or MECs determined by the reference methods (at the recommended incubation times of 24 h for zygomycetes; 48 h for *Aspergillus* sp., *B. spicifera*, *Alternaria* sp., *Fusarium* sp., and *Paecilomyces* sp.; and 72 h for *Scedosporium* sp. [M38-A document]) were correlated with inhibition zone diameters (in millimeters) around disks; zones were obtained at three incubation times (16 to 24, 48, and 72 h). To obtain correlation results (*R* values), a linear regression analysis by the least-squares method (Pearson's correlation coefficient; MS Excel software) was performed by plotting zone diameters against their respective MIC or MEC endpoints (after log transformation). The reproducibility of zone diameters obtained on 3 different days with each of four isolates of the 18 species was evaluated by calculating the percentage of replicate zone diameters that were within 2 standard deviations of the mean (8). Reproducibility values were not obtained as a range of ± millimeter zone diameter measurements because millimeter variations in large zones tend to yield larger values than the variations in smaller zones. Breakpoints are not available for any antifungal agent versus molds. However, MICs below 1 μg/ml are usually reported for most *Aspergillus* sp. isolates with the five agents, for *Scedosporium apiospermum* and *P. lilacinus* with posaconazole and voriconazole, for *Alternaria* sp. and *B. spicifera* with the three triazoles, and for some zygomycete isolates with posaconazole and amphotericin B (7, 12, 19, 21). Because of that, we grouped as susceptible isolates for which the MICs or MECs were ≤1 μg/ml, as intermediate isolates for which the MICs or MECs were 2 μg/ml, and as resistant isolates for which the MICs or MECs were ≥4 μg/ml to analyze the performance of the disk method in identifying isolates with decreased susceptibility to the five antifungal agents evaluated. Scattergram plots of the MICs or MECs and corresponding disk results of each antifungal agent were developed, and by using the error rate bounding method (17) the following tentative zone diameter categories were assigned: susceptible, ≥17 mm (triazoles and caspofungin) and ≥15 mm (amphotericin B); intermediate, 14 to 16 mm (triazoles and caspofungin) and 13 to 14 mm (amphotericin B); resistant, ≤13 mm (triazoles and caspofungin) and ≤12 mm (amphotericin B). These tentative breakpoints were used to determine the categorical agreement between the disk diffusion and MIC endpoints of each drug. Major errors were identified when the isolate was resistant by the disk method but susceptible by the MIC or MEC result, while minor errors were identified when there were shifts between susceptible and susceptible dose dependent or between susceptible dose dependent and resistant. Very major errors were identified when the MIC or MEC result showed resistance and the disk result showed susceptibility.

RESULTS AND DISCUSSION

Table 1 lists the number of isolates of each species that were evaluated in the study to identify optimal testing guidelines for disk diffusion testing of molds. These species are more commonly associated with severe fungal infections in the immunocompromised host. The antifungal agents included are the most important agents that have been licensed for the treatment or prevention of mold infections. The evaluation was to be conducted with MGM agar, which is the medium that has been selected by the CLSI for testing yeasts with voriconazole and fluconazole by disk diffusion methodology (M44-A) (4). However, it was demonstrated early in this study that MGM medium did not support good growth of most *Aspergillus* sp. at 24 h, including *A. fumigatus* (60%), while zones of inhibition could be read at 24 h for most *Aspergillus* isolates and at 48 for most of the other species on MH agar (88%); similar results have been previously reported (8). In addition, interference with either caspofungin for *P. lilacinus* or voriconazole for *A. terreus* (Fig. 1) was observed, where low MICs corresponded to very small or no inhibition zone diameters. These conflicting results were not observed when disk diffusion tests were per-

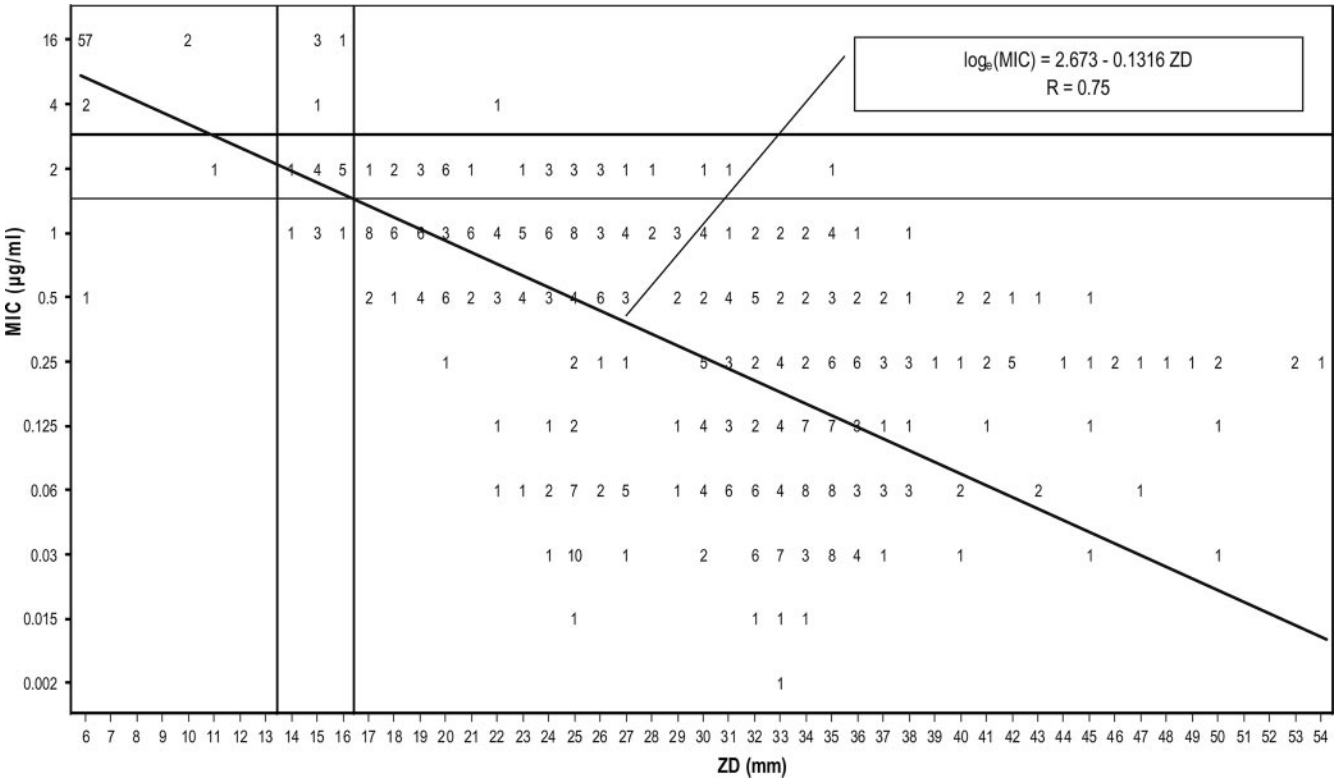

FIG. 2. Correlation of broth microdilution MICs and posaconazole 5-μg disk diffusion zone diameters on MH agar for 483 mold isolates in three laboratories. MICs were determined at the recommended times (24 to 72 h), and zone diameters, in millimeters, were determined at the optimal incubation times (16 to 24 h and 48 h). ZD, zone diameter.

formed on MH agar. MGM agar also did not support the growth of most *P. lilacinus* and *Alternaria* isolates, even after 72 h to 5 days of incubation, and slowed the determination of zones from 48 to 72 h for *B. spicifera* and *S. apiospermum*. In general, zones were better defined and easier to read on MH agar than on MGM agar; inhibition zones could be determined at 16 to 24 h for zygomycetes, at 24 h for most *Aspergillus* isolates (17 of 31 *A. nidulans* isolates [one center] and a few *A. fumigatus* and *A. terreus* isolates needed 48 h of incubation), and at 48 h for the other species. As discussed below, correlation and reproducibility results (*R* values) were superior at these incubation times.

For the 555 isolates and both QC isolates, 95% of the inocula were within the target range of 0.4×10^4 to 5×10^4 CFU/ml. Lower inoculum densities (0.1×10^4 to 0.3×10^4 CFU/ml) were mostly obtained with 22 isolates of *A. flavus*, *A. niger*, *F. solani*, and *P. lilacinus* and higher values (5.1×10^6 to 7×10^6 CFU/ml) were obtained for three isolates of *F. moniliforme* and *P. lilacinus* at two centers. Similar reproducibility results for inoculum suspensions have been reported in prior collaborative studies (92 to 95%) (10, 11). The reproducibility of inoculum suspensions for *Alternaria* isolates has not been previously evaluated, but our results were within the target range (optical density = 0.25 to 0.3).

Table 2 depicts the reproducibility of zone diameters (in millimeters) obtained with the 72 isolates on 3 different days with each antifungal agent on both media. More than 15,000 zone endpoints were analyzed. Overall reproducibility was good with both media and at the three times of incubation, as demonstrated by the high percentages (overall, 82 to 100%) of replicate zone diameters that were within 2 standard deviations of the mean. The best reproducibility was observed with the 24- and 48-h results (91 to 100% on MH agar). By genus, the lowest reproducibility was observed for *P. lilacinus* with itraconazole, voriconazole, and amphotericin B (78 to 84%), for *Fusarium* sp. with itraconazole (83%), for *Mucor* sp. with posaconazole (86%), for *Rhizopus* sp. with itraconazole (89%), and for *B. spicifera* with caspofungin (81%); other reproducibility values ranged from 90 to 100%. Results were lower on MGM than on MH agar; the exception was the results for the zygomycetes (70 to 86% on MH agar versus 91 to 95% on MGM), but not all isolates could be evaluated at 24 h due to lack of growth. Overall, our reproducibility results are similar to those obtained for an evaluation of posaconazole for *Aspergillus* sp. and the zygomycetes (8). Reproducibility results also were comparable or superior to those obtained in other collaborative studies for the development of the M38-A document (3, 10, 11).

Table 3 summarizes the zones of inhibition (in millimeters) obtained on MH agar with the two QC controls at the five centers; reference diameter ranges are not available for either QC isolate versus any antifungal on MH agar. In this study, most of the intervals between the high and low zone diameter limits were within 6 to 11 mm; the reproducibility was 97 to 100% at 24 to 72 h for *C. krusei* ATCC 6258 and at 24 h for *P. variotii* ATCC 3630 with most of the antifungal disks tested.

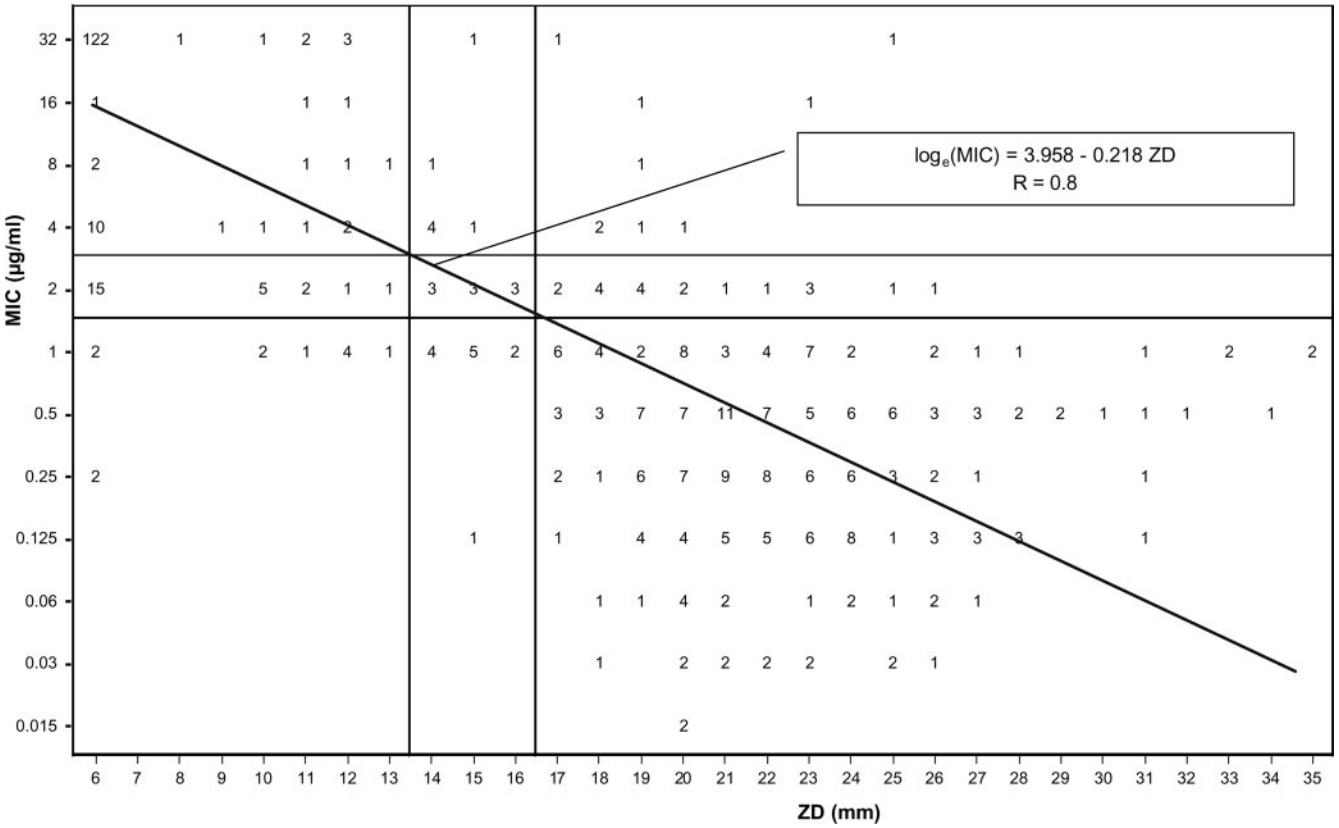

FIG. 3. Correlation of broth microdilution MICs and itraconazole 10-μg disk diffusion zone diameters on MH agar for 483 mold isolates in three laboratories. MICs were determined at the recommended times (24 to 72 h), and zone diameters, in millimeters, were determined at the optimal incubation times (16 to 24 h and 48 h). ZD, zone diameter.

The CLSI zone diameter limit for *C. krusei* ATCC 6258 with a voriconazole 1-μg disk on the recommended MGM medium is 16 to 25 mm (4); our range for that strain on MH agar was consistently larger (29 to 38 mm, 100%). Based on collaborative studies, tentative QC data for posaconazole (23 to 31 mm) and caspofungin (13 to 25 mm) on MGM agar are also available for this QC strain. Our results were within the target zone diameter range for caspofungin (14 to 25 mm, 100%) with the BBL caspofungin disk on both agars, but a larger zone range was obtained with the Oxoid disk on MH agar (21 to 29 mm, 100%). Similar results (larger zone ranges) were obtained with posaconazole for the QC yeast (31 to 43 mm at 24 to 72 h, 97%). It appears that the yeast QC isolate could serve as a control isolate until a QC isolate(s) is selected and standard zone diameter limits are established for mold disk testing.

Table 4 summarizes the coefficients of correlation (linear regression analysis) between either MIC or MEC results and the corresponding inhibition zone diameters, in millimeters (obtained with the seven disks), for the 483 isolates evaluated at three of the five centers (Table 1). The correlation was superior for disks of both caspofungin and voriconazole; similar results were obtained for these agents on both the MGM and MH agars. However, for itraconazole and posaconazole, the correlation was higher on MH agar than on MGM agar; suitable *R* values (>0.7) were observed on MH agar compared to the low *R* values (<0.70) at some incubation times on MGM agar. Our correlation results were comparable to those reported for *Aspergillus* sp. (8, 15, 20) and zygomycete (8, 15) isolates with voriconazole (*R*, 0.79) and posaconazole (*R*, 0.72 to 0.82) disks. The lowest correlation results were obtained for amphotericin B, as previously reported (8), where zones were consistently small for isolates for which the amphotericin B MICs were correspondingly low and high.

The evaluation of a new method requires the determination of breakpoint category agreement between the new and reference methods. Since interpretive breakpoints are not available for molds, this type of analysis was performed with the tentative breakpoints assigned in this study; these breakpoints were useful for illustration or comparison only. We grouped isolates as resistant (MIC or MEC, ≥4 μg/ml), intermediate (MIC or MEC, 2 μg/ml), or susceptible (MIC or MEC, ≤1 μg/ml) based on reported in vitro data obtained with large numbers of isolates (1, 7, 8, 12, 19, 21). Tentative zone diameter breakpoints were assigned by the error rate bounding method (17). Table 5 depicts the results of the breakpoint category analysis agreement for amphotericin B, itraconazole, posaconazole, and voriconazole (1-μg disk results only) inhibition zone diameters obtained at the optimal incubation times of 16 to 24 h for zygomycetes, *A. fumigatus*, *A. flavus*, and *A. niger* and 48 h for the other species on MH agar versus MICs obtained at 24 h (zygomycetes), 72 h (*Scedosporium* sp.), and 48 h (other species). Table 5 also provides the results of the comparison of caspofungin MECs from the first reading (16 to 24 h [zygomycetes], 48 h [*Scedosporium* sp.], and 24 h [other species]) with

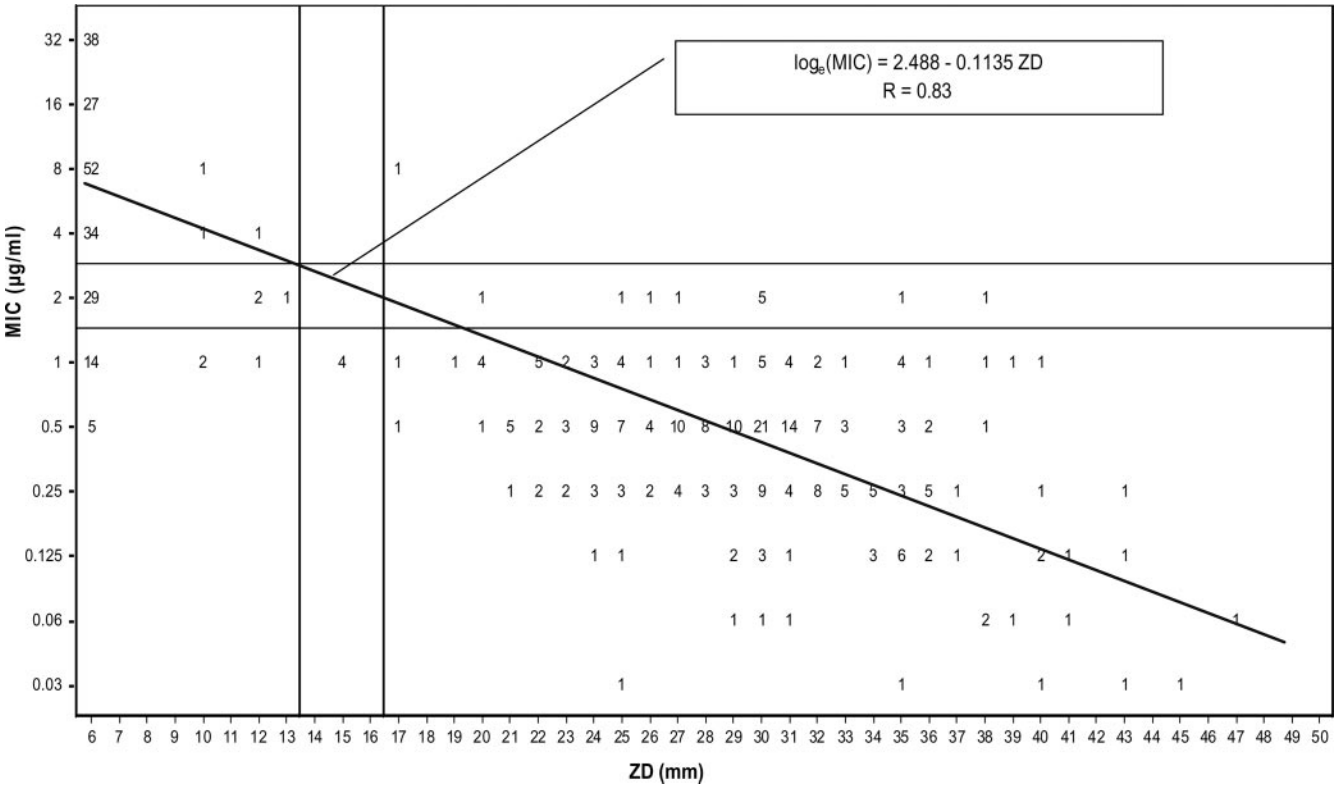

FIG. 4. Correlation of broth microdilution MICs and voriconazole 1-μg disk diffusion zone diameters on MH agar for 483 mold isolates in three laboratories. MICs were determined at the recommended times (24 to 72 h), and zone diameters, in millimeters, were determined at the optimal incubation times (16 to 24 h and 48 h). ZD, zone diameter.

zone diameters (BBL disk results only) also obtained on MH agar at the same incubation times as for the other four antifungal agents. Figures 2 to 6 depict the results of the regression analysis between either MICs or MECs and zone diameters for the same five disks and by the same optimal testing conditions described above and in Table 5. Among the three laboratories, the percentage ranges of major and very major errors were 0.2 to 6.4% and 0 to 2.7%, respectively, for the five disks; the percentage range of overall categorical agreement was 81 to 96% for posaconazole, caspofungin, and voriconazole 1-μg disks and 65 to 88% for amphotericin B and itraconazole disks (data not shown in Table 5).

The categorical evaluation of the posaconazole 5-μg disk indicated that the percentages of major, very major, and minor errors were low (0.2 and 8.1%) (Table 5). One susceptible isolate was categorized as resistant (≤13-mm diameter, major error), and one resistant isolate was categorized as susceptible (≥17-mm diameter, very major error) (Fig. 2). Of the 39 minor errors, 28 (72%) were among isolates for which the MIC was 2 μg/ml (intermediate category); these 28 isolates (16 *Fusarium* sp. and 9 *S. apiospermum* isolates) were categorized as susceptible (≥17-mm diameter). Agreement was higher with the posaconazole disk (92%) than with the itraconazole 10-μg disk (83%). Nine very major errors (1.8% false-susceptible values, ≥17-mm zone diameters) were observed with the itraconazole disk (four *A. corymbifera* isolates, two *A. niger* isolates, two *P. lilacinus* isolates, and one *S. apiospermum* isolate) (Fig. 3). Of the 52 isolates for which the itraconazole MICs were 2 μg/ml (intermediate category), 24 were categorized as resistant (≤13 mm; 19 zygomycete isolates), 9 were categorized as intermediate (14- to 16-mm diameters), and 19 were categorized as susceptible (≥17-mm diameters; mostly *A. corymbifera* and *P. lilacinus*). It is noteworthy that only two major errors were observed among the 203 *Aspergillus* isolates with itraconazole and none were observed with posaconazole. Therefore, it appears that the posaconazole disk (*R* range among the centers, 0.71 to 0.78) is more suitable than the itraconazole disk (*R* range among the centers, 0.7 to 0.8), despite the latter's higher *R* values, for testing of zygomycete isolates; both disks are suitable for testing of the other species.

Two voriconazole disks were evaluated, which provided different susceptible and resistant zone diameter thresholds (≥17 mm and ≥28 mm as susceptible and ≤13 mm and ≤25 mm as resistant, 1-μg and 10-μg disks, respectively). According to our assigned categories, the performance of the voriconazole 1-μg disk was superior (86% overall agreement; Table 5) to the 10-μg disk (80% overall agreement; data not shown in Table 5). Discrepancies with the 1-μg disk were mostly due to major errors (one very major error and 4.6% zone diameters of ≤13 mm) (Fig. 4 and Table 5), but those with the 10-μg disk were due to very major errors (9% zone diameters of ≥28 mm). Major errors with the 1-μg disk were observed for 13 *Alternaria* sp. isolates (at one of the three centers) and 4 *F. moniliforme* isolates. Among the 43 isolates for which the voriconazole MIC was 2 μg/ml (intermediate category), 11 were categorized as susceptible (≥17-mm zone diameters for *Aspergillus* sp. and *S.*

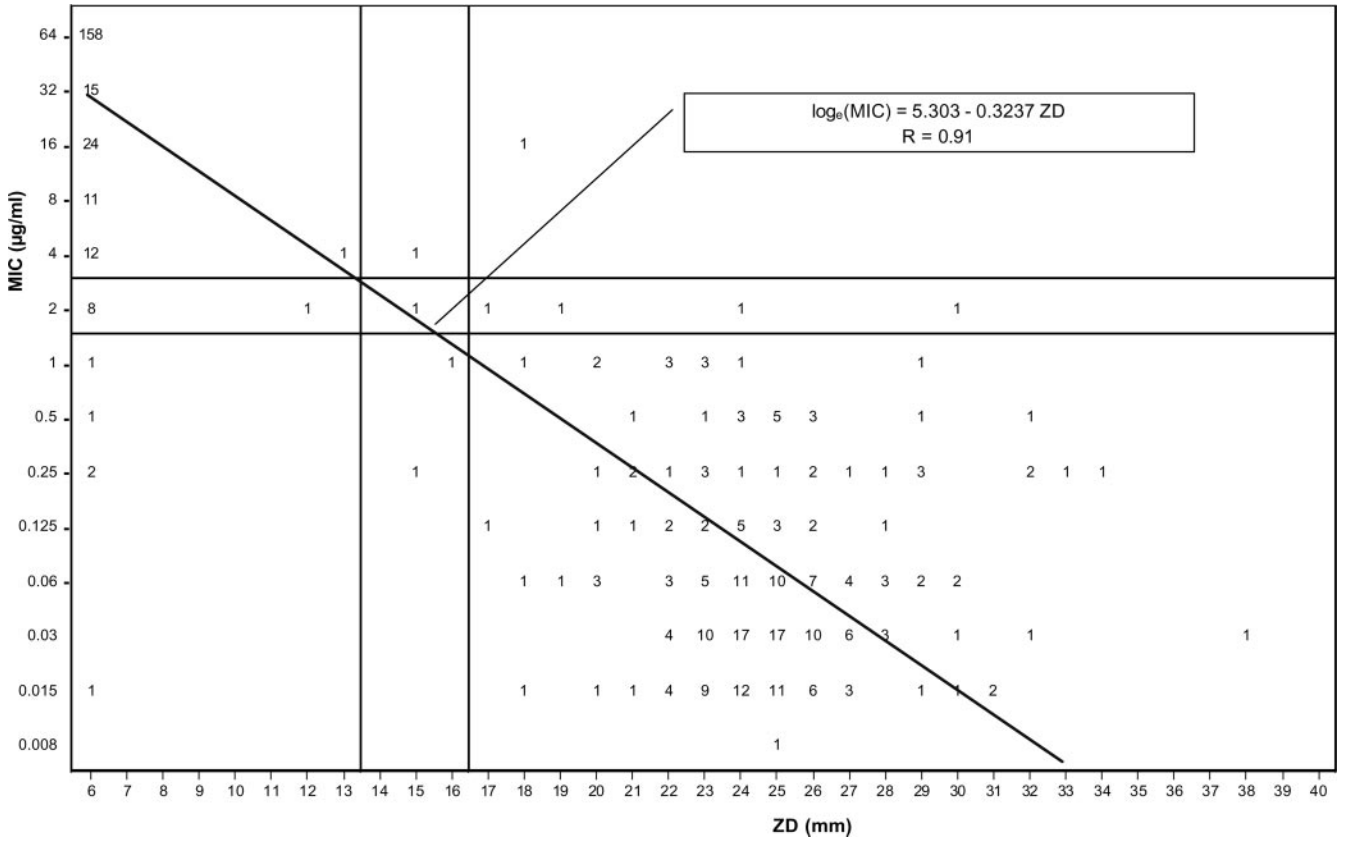

FIG. 5. Correlation of broth microdilution MECs and caspofungin 5-μg disk (BBL) diffusion zone diameters on MH agar for 483 mold isolates in three laboratories. MECs were determined at the recommended times (24 to 48 h), and zone diameters, in millimeters, were determined at the optimal incubation times (16 to 24 h and 48 h). ZD, zone diameter.

apiospermum) and 32 isolates were categorized as resistant (≤13 mm for 30 *Fusarium* isolates, 1 *B. spicifera* isolate, and 1 *Alternaria* sp. isolate) with the 1-μg disk. In contrast, 95% of these 43 isolates were categorized as susceptible (≥28-mm zone diameters) with the 10-μg disk (data not shown in Table 5). In addition, the 1-μg disk was able to identify as resistant all 26 *S. prolificans* and 84 zygomycete isolates, but the 10-μg disk missed 19 of these isolates. Also, from a practical standpoint, only one voriconazole 10-μg disk could be tested per plate to avoid zone overlap (>70-mm zone diameters), while three or four disks could be tested with the 1-μg disk (150-mm plate). Therefore, these results suggest that the 1-μg disk (*R* range among the three centers, 0.77 to 0.88) should be the choice for testing the susceptibilities of most mold isolates to voriconazole. Since all zygomycete and *S. prolificans* isolates are resistant to voriconazole in this and other studies (12, 19), there is no reason to test these isolates.

Two sources of caspofungin 5-μg disks were evaluated (*R* range among the three centers with both Oxoid and BBL, 0.87 to 0.94); the Oxoid disk provided larger zone diameters than the BBL disk. Therefore, the threshold limits between resistant and susceptible results were different (≥19 mm versus ≥17 mm as susceptible and ≤15 mm versus ≤13 mm as resistant, respectively). Table 5 depicts the categorical analysis for the BBL disk. The overall categorical agreement (93 and 96%, Oxoid and BBL, respectively) was similar with both disks on MH agar. However, one very major error (0.2%, Table 5 and Fig. 5) was observed with the BBL disk and six (1.2%) were observed with the Oxoid disk; fewer major and minor errors were also observed with the BBL disk. Among the overall discrepant results, two to five major (BBL and Oxoid, respectively) and five minor errors were observed among *A. nidulans* isolates. Since the BBL disk appears to better identify the resistant isolates, it could be the best choice for testing the susceptibilities of mold isolates to caspofungin. However, as for voriconazole, there is no reason to test zygomycete, *Fusarium* sp., and *S. prolificans* isolates.

The amphotericin B 10-μg disk provided the lowest correlation (*R* range among the three centers, 0.28 to 0.74) between MICs and inhibition zone diameters. The percentage of very major errors was similar to that obtained with the itraconazole disk (Fig. 3 and 6), but the percentage of minor errors was much higher (Table 5). In contrast to itraconazole data, both major (false resistant, ≤12-mm zone diameters) and minor errors were obtained for 65 *A. flavus*, *A. nidulans*, and *A. terreus* and 36 *Fusarium* sp. isolates (data not shown in Table 5); such conflicting results for *Aspergillus* sp. were absent or very few with the three triazole disks and caspofungin disks. One *R. arrhizus* isolate was categorized as resistant among the 2-μg/ml MIC group (intermediate category), but as with posaconazole, all other zygomycetes were categorized as susceptible. There-

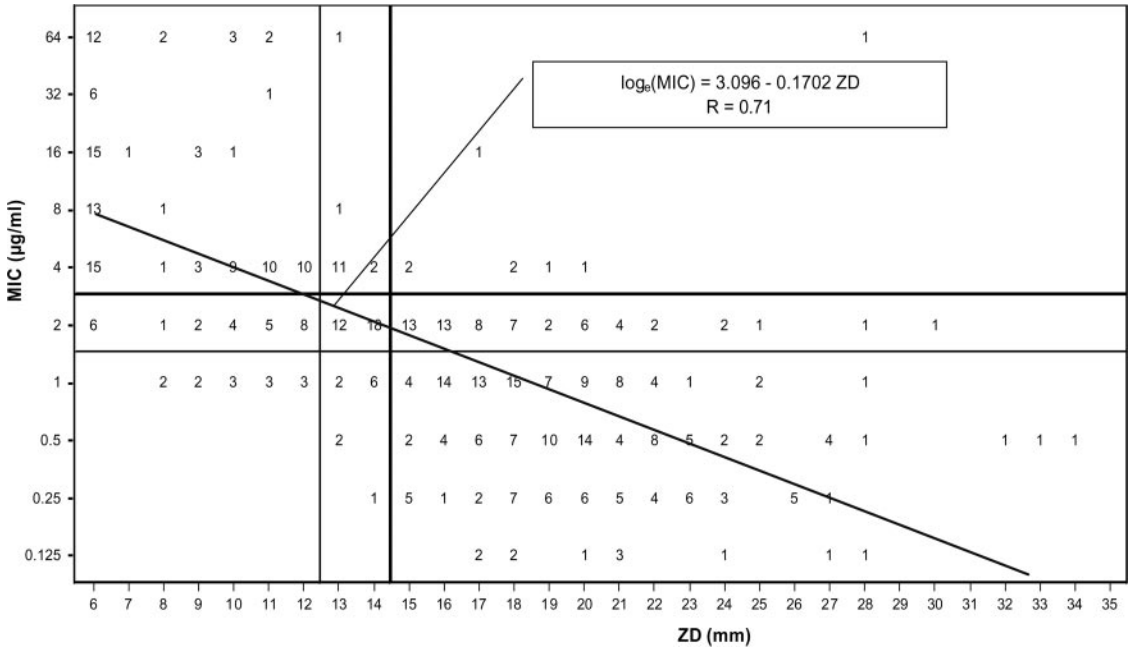

FIG. 6. Correlation of broth microdilution MICs and amphotericin B 10-μg disk diffusion zone diameters on MH agar for 483 mold isolates in three laboratories. MICs were determined at the recommended times (24 to 72 h), and zone diameters, in millimeters, were determined at the optimal incubation times (16 to 24 h and 48 h). ZD, zone diameter.

fore, the amphotericin B disk appears to be more suitable for testing of zygomycetes than for testing of the other species.

In conclusion, overall reproducibility and correlation results were lower when testing was performed on MGM agar than when it was performed on MH agar (Tables 2 and 4) and categorical agreement was suitable with most disks on MH agar (Table 5); the disk was not able to differentiate intermediate from susceptible and resistant values for some species-antifungal combinations. Based on the correlation of inhibition zone diameters with either MICs or MECs, reproducibility data, and the ability of each disk to identify resistant isolates or those for which the MICs or MECs were high (≥4 μg/ml), the optimal conditions identified in this study for testing the susceptibilities of molds to the five agents evaluated were (i) plain MH agar; (ii) incubation times of 16 to 24 h (zygomycetes), 24 h (*A. fumigatus*, *A. flavus*, and *A. niger*), and 48 h (other species); and (iii) posaconazole 5-μg disks, voriconazole 1-μg disks, itraconazole 10-μg disks (for all except zygomycetes), BBL caspofungin 5-μg disks, and amphotericin B 10-μg disks (zygomycetes only). However, the clinical relevance of these in vitro results has yet to be determined since breakpoints for mold testing are not available.

ACKNOWLEDGMENTS

Pfizer, Merck, and Schering-Plough provided voriconazole (1-μg disks), caspofungin (BBL and Oxoid), and posaconazole disks, respectively, and partially sponsored this study. The two types of MH agar used were donated by Hardy Scientific.

REFERENCES

1. **Arikan, S., V. Paetznick, and J. H. Rex.** 2002. Comparative evaluation of disk diffusion with microdilution assay in susceptibility testing of caspofungin against *Aspergillus* and *Fusarium* isolates. Antimicrob. Agents Chemother. **46:**3084–3087.
2. **Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution susceptibility testing of yeasts. Approved standard, 2nd ed. Document M27-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard. Document M38-A. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards.** 2004. Method for antifungal disk diffusion susceptibility testing of yeasts. Approved guideline. Document M44-A. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2006. Quality control minimal inhibitory concentration (MIC) limits for broth microdilution and MIC interpretive breakpoints. Supplement M27-S2. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Clinical and Laboratory Standards Institute.** 2006. Zone diameter interpretive standards and corresponding minimal inhibitory concentration (MIC) interpretive breakpoints. Supplement M44-S1. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Dannaoui, E., J. Meletiadis, J. W. Mouton, J. F. G. M. Meis, P. E. Verweij, and the Eurofong Network.** 2003. In vitro susceptibilities of zygomycetes to conventional and new antifungals. J. Antimicrob. Chemother. **51:**45–52.
8. **Espinel-Ingroff, A.** 2006. Comparison of three commercial assays and a modified disk diffusion assay with two broth microdilution reference assays for testing zygomycetes, *Aspergillus* spp., *Candida* spp., and *Cryptococcus neoformans* with posaconazole and amphotericin B. J. Clin. Microbiol. **44:**3616–3622.
9. **Espinel-Ingroff, A.** 2003. Evaluation of broth microdilution testing parameters and agar diffusion Etest procedure for testing susceptibilities of *Aspergillus* spp. to caspofungin acetate (MK-0991). J. Clin. Microbiol. **41:**403–409.
10. **Espinel-Ingroff, A., M. Bartlett, R. Bowden, N. X. Chin, C. Cooper, Jr., A. Fothergill, M. R. McGinnis, P. Menezes, S. A. Messer, P. W. Nelson, F. C. Odds, L. Pasarell, J. Peter, M. A. Pfaller, J. H. Rex, M. G. Rinaldi, G. S. Shankland, T. J. Walsh, and I. Weitzman.** 1997. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. J. Clin. Microbiol. **35:**139–143.
11. **Espinel-Ingroff, A., M. Bartlett, V. Chaturvedi, M. Ghannoum, K. C. Hazen, M. A. Pfaller, M. Rinaldi, and T. J. Walsh.** 2001. Optimal susceptibility testing conditions for detection of azole resistance in *Aspergillus* spp.: NCCLS collaborative evaluation. Antimicrob. Agents Chemother. **45:**1828–1835.
12. **Espinel-Ingroff, A., K. Boyle, and D. J. Sheehan.** 2001. In vitro antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. Mycopathologia **38:**293–304.
13. **Espinel-Ingroff, A., V. Chaturvedi, A. Fothergill, and M. G. Rinaldi.** 2002.

Optimal testing conditions for determining MICs and minimum fungicidal concentrations of new and established antifungal agents for uncommon moulds: NCCLS collaborative study. J. Clin. Microbiol. **40:**3776–3781.

14. **Espinel-Ingroff, A., A. Fothergill, M. Ghannoum, E. Manavathu, L. Ostrosky-Zeichner, M. A. Pfaller, M. Rinaldi, W. Schell, and T. Walsh.** 2005. Quality control and reference guidelines for CLSI broth microdilution susceptibility method (M38-A document) for amphotericin B, itraconazole, posaconazole, and voriconazole. J. Clin. Microbiol. **43:**5243–5246.
15. **López-Oviedo, E., A. I. Aller, C. Martin, C. Castro, M. Ramirez, J. M. Peman, E. Canton, C. Almeida, and E. Martin-Mazuelos.** 2006. Evaluation of disk diffusion method for determining posaconazole susceptibility of filamentous fungi: comparison with CLSI broth microdilution method. Antimicrob. Agents Chemother. **50:**1108–1111.
16. **Marr, K. A., R. A. Carter, F. Crippa, A. Wald, and L. Corey.** 2002. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. **34:**909–917.
17. **Metzler, D. M., and R. M. DeHaan.** 1974. Susceptibility tests of anaerobic bacteria: statistical and clinical considerations. J. Infect. Dis. **130:**588–594.
18. **Odds, F. C., M. Motyl, R. Andrade, J. Bille, E. Canton, M. Cuenca-Estrella, A. Davidson, C. Durussel, D. Ellis, E. Foraker, A. W. Fothergill, M. A. Ghannoum, R. A. Giacobbe, M. Gobernado, R. Handke, M. Laverdiere, W. Lee-Yang, W. G. Merz, L. Ostrosky-Zeichner, J. Peman, S. Perea, J. R. Perfect, M. A. Pfaller, L. Proia, J. H. Rex, M. G. Rinaldi, J.-L. Rodriguez-Tudela, W. A. Schell, C. Shields, D. A. Sutton, P. E. Verweij, and D. W. Warnock.** 2004. Interlaboratory comparison of results of susceptibility testing with caspofungin against *Candida* and *Aspergillus* species. J. Clin. Microbiol. **42:**3475–3482.
19. **Sabatelli, F., R. Patel, P. A. Mann, C. A. Mendrick, C. C. Norris, R. Hare, D. Loebenberg, T. A. Black, and P. M. McNicholas.** 2006. In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. Antimicrob. Agents Chemother. **50:**2009–2015.
20. **Serrano, M. C., M. Ramirez, D. Morilla, A. Valverde, M. Chavez, A. Espinel-Ingroff, R. Claro, A. Fernandez, C. Almeida, and E. Martin-Mazuelos.** 2004. A comparative study of the disc diffusion method with the broth microdilution and Etest methods for voriconazole susceptibility testing of *Aspergillus* spp. J. Antimicrob. Chemother. **53:**739–742.
21. **Sun, Q. N., A. W. Fothergill, D. I. McCarthy, M. G. Rinaldi, and J. R. Graybill.** 2002. In vitro activities of posaconazole, itraconazole, voriconazole, amphotericin B, and fluconazole against isolates of zygomycetes. Antimicrob. Agents Chemother. **46.5:**1581–1582.