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Optimal Testing Conditions for Determining MICs and Minimum Fungicidal Concentrations of New and Established Antifungal Agents for Uncommon Molds: NCCLS Collaborative Study

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This collaborative three-center study evaluated NCCLS M38-A document testing conditions and other testing conditions for the antifungal susceptibility testing of 35 isolates of *Aspergillus nidulans*, *A. terreus*, *Bipolaris hawaiiensis*, *B. spicifera*, *Cladophialophora bantiana*, *Dactylaria constricta*, *Fusarium solani*, *Paecilomyces lilacinus*, *Scedosporium prolificans*, *Trichoderma longibrachiatum*, and *Wangiella dermatitidis* for itraconazole, three new triazoles (voriconazole, posaconazole, and ravuconazole), and amphotericin B. MICs and minimum fungicidal concentrations (MFCs) were determined in each center by using four media (standard RPMI-1640 [RPMI], RPMI with 2% dextrose [RPMI-2%], antibiotic medium 3 [M3], and M3 with 2% dextrose [M3-2%]) and two criteria of MIC determination (complete growth inhibition [MICs-0] and prominent growth inhibition [MICs-2]) at 24, 48 and 72 h. MFCs were defined as the lowest drug concentrations that yielded <3 colonies (approximately 99 to 99.5% killing activity). The reproducibility (within three wells) was higher among MICs-0 (93 to 99%) with either RPMI or M3 media than among all MICs-2 (86 to 95%) for the five agents at 48 to 72 h. The agreement for MFCs was lower (86 to 94%). Based on interlaboratory agreement, the optimal testing conditions were RPMI broth, 48 to 72 h of incubation and 100% growth inhibition (MIC-0); MFCs can be obtained after MIC determination with the above optimal testing parameters. These results warrant consideration for inclusion in the future version of the NCCLS M38 document. However, the role of these in vitro values as predictors of clinical outcome remains to be established in clinical trials.

Although *Aspergillus fumigatus* is responsible for the majority (85 to 90%) of the different clinical manifestations of mold (filamentous fungi) infections (8), other molds have emerged as important etiologic agents of severe infections, especially in the immunocompromised host (2, 5, 20, 24, 32). With the increased incidence of fungal infections and the growing number of new antifungal agents, the laboratory's role in the selection and monitoring of antifungal therapy has been underscored. As a result of collaborative studies (10, 11), the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has developed a reproducible reference procedure for the antifungal susceptibility testing of molds (NCCLS M38-A document [26]). This document describes the following testing conditions: standard RPMI-1640 broth; nongerminated conidial inoculum suspensions of approximately 10^4 CFU/ml, and incubation at 35°C for 24 h (*Rhizopus* spp.), 48 h (*Aspergillus flavus*, *A. fumigatus*, *Fusarium* spp.), and 72 h (*Pseudallescheria boydii*). Because the Subcommittee investigated other conditions for testing *Aspergillus* spp. against established agents and new triazoles, the conventional MIC criterion (complete or 100% growth inhibition) is recommended for itraconazole and three new triazoles. Data from that study indicated that this criterion more easily and reliably differentiated between susceptible and potentially resistant isolates of *Aspergillus* spp. than the less stringent criterion (MIC-2 or ≥50% growth inhibition) described in the older M38 version. The interlaboratory reproducibility of minimum fungicidal concentrations (MFCs) for *Aspergillus* spp. was also examined in a parallel study (16a). However, none of these studies have included other uncommon pathogenic molds. Because of that, the NCCLS Subcommittee conducted this collaborative study (with three centers) to examine the testing conditions evaluated for *Aspergillus* spp. for susceptibility testing with itraconazole, new triazoles (posaconazole, ravuconazole, and voriconazole), and amphotericin B (both MICs and MFCs) against a selected set of 35 uncommon mold pathogens.

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MATERIALS AND METHODS

Study design. Three laboratories participated in the present study, and each laboratory received the same panel of 35 coded (3 isolates each) strains of *Aspergillus nidulans, Aspergillus terreus*, *Bipolaris hawaiiensis*, *Bipolaris spicifera*, *Cladophialophora bantiana*, *Dactylaria constricta*, *Fusarium solani*, *Paecilomyces lilacinus*, *Scedosporium prolificans*, *Trichoderma longibrachiatum*, and *Wangiella dermatitidis* (Table 1) and two control isolates. MICs for each isolate were obtained in each of the three centers with amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole by the broth microdilution method following a standard protocol. This protocol included M38-A and other susceptibility testing conditions to be evaluated: (i) four medium formulations (standard RPMI-1640 [RPMI], RPMI with 2% dextrose [RPMI-2%], antibiotic medium 3 [M3]), and M3 with 2% dextrose [M3-2%]; (ii) three incubation times (24, 48,

TABLE 1. OD range and inoculum sizes for 35 uncommon molds in three laboratories

Species (no. of observations)	Stock inoculum range (10^6 CFU/ml)	OD range
A. nidulans (*Emericella nidulans*) (15)	1.1–2	0.09–0.11
Aspergillus terreus (9)	0.9–5	0.09–0.11
Bipolaris hawaiiensis (9)	0.07–0.4	0.2–0.3
B. spicifera (9)	0.3–3	0.2–0.3
Cladophialophora bantiana (9)	0.4–3.1	0.15–0.17
Dactylaria constricta (9)	0.4–1	0.15–0.17
Fusarium solani (9)	0.5–3.1	0.15–0.17
Paecilomyces lilacinus (9)	0.8–2.3	0.09–0.13
Scedosporium prolificans (9)	0.6–1.7	0.15–0.17
Trichoderma longibrachiatum (9)	0.7–2.3	0.09–0.11
Wangiella dermatitidis (9)	1.2–3.7	0.15–0.17

and 72 h); and (iii) two criteria of MIC determination (100% and ≥50% growth inhibition). Following MIC determination with the four media, MFCs were determined for each isolate-drug-testing medium combination in the three centers.

Isolates. The set of isolates evaluated is documented in Table 1. These 35 isolates belonged to the culture collections of the University of Texas and the Medical College of Virginia Campus, Virginia Commonwealth University. Each isolate was maintained as a suspension in water at approximately 25°C until testing was performed. The reference isolate *A. flavus* ATCC 204304 (11, 16) and the quality control strain *Candida parapsilosis* ATCC 22019 (27) were included as controls each time isolates were tested. For *C. parapsilosis* ATCC 22019, there are well-established microdilution MIC ranges of the five agents evaluated in this study (4); reference MIC ranges also have been established for the isolate *A. flavus* ATCC 204304 (11, 16). MIC ranges for these isolates were within established values in the three centers (4, 11, 16, 26).

Broth dilution susceptibility testing. Standard Excel spreadsheets were developed to serve as recording forms and to enter and analyze data. The standard protocol supplied to each laboratory for the broth microdilution method provided detailed instructions concerning the following testing conditions.

A single lot of each of the four liquid medium formulations was provided ready-to-use to all participants by Trek Diagnostic Systems Inc. (Westlake, Ohio). The same lot of each medium was used for the preparation of the drug dilutions. Each batch of standard RPMI and RPMI-2% was supplemented with 0.3 g of L-glutamine per liter and 0.165 M morpholinepropanesulfonic acid buffer (34.54 g/liter) to pH 7.0 ± 0.1 at 35°C and did not contain sodium bicarbonate. Neither batch of M3 and M3-2% was buffered, and the pHs of both media were 7.0 ± 0.1. The five antifungal agents amphotericin B (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn.), itraconazole (Janssen Pharmaceutica, Titusville, N.J.), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), ravuconazole (Bristol-Myers Squibb Pharmaceutical Research Institute), and voriconazole (Pfizer Pharmaceuticals, New York, N.Y.) were provided by the manufacturers as assay powders. Additive drug dilutions were prepared as described in the NCCLS M38-A document (26) and previous reports (10, 11, 16) in a central facility (Trek Diagnostic Systems) and shipped frozen to each participant. As previously described (9, 26), stock inoculum suspensions were prepared in sterile saline (provided by Trek Diagnostic Systems) containing 1% Tween 80 from 7-day-old colonies grown on potato dextrose agar slants (provided by Remel, Lenexa, Kans.). The resulting mixture of mostly nongerminated conidia was adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.3 (Table 1) and were diluted 1:50 in each of the four media, which corresponded to 2 times the density needed for the test of approximately 0.4×10^4 to 5×10^4 CFU/ml. On the day of the test, each microdilution well containing 100 μl of the diluted (2 times) drug concentrations was inoculated with 100 μl of the diluted (2 times) conidial inoculum suspensions (final volume in each well, 200 μl). All microdilution trays were incubated at 35°C and examined after 21 to 26, 46 to 50, and 70 to 74 h of incubation. Two MIC endpoints were determined, the lowest drug concentrations that showed absence of growth or complete growth inhibition (100% inhibition) and the lowest drug concentrations that showed ≥50% growth inhibition.

MFC determination. The in vitro fungicidal activities (MFCs) were determined for each drug-isolate-medium combination as previously described (13, 14, 16a). After 72 h, 20 μl was subcultured from each well that showed complete inhibition (100% or an optically clear well), from the last positive well (growth similar to that for the growth control well), and from the growth control (drug-free medium) onto Sabouraud dextrose agar plates. The plates were incubated at 35°C until growth was seen in the growth control subculture. The MFC was the lowest drug concentration that showed either no growth or fewer than three colonies (approximately 99 to 99.5% killing activity).

Data analysis. The percentage of inoculum densities that were within the range of 0.4×10^6 to 5×10^6 CFU/ml was calculated, and the geometric means for each species' inoculum sizes were also obtained. Both on-scale and off-scale MIC and MFC values were included in the analysis. MICs and MFCs from the three centers for each drug-isolate-medium-incubation time (the latter for MICs only) combination tested were compared to obtain data regarding reproducibility. As previously analyzed (10, 11, 16, 16a), discrepancies between MIC endpoints of no more than three dilutions (three wells) from the three centers were used for the calculation of the percent values of agreement. A measurement of agreement was then determined as the percentage of endpoints within three dilutions (i.e., 1, 2, and 4 μg/ml) for each combination of drug, isolate, medium, incubation time, and criterion of MIC determination. In addition, each 48- or 72-h MIC was compared to its corresponding MFC to assess the differences between in vitro fungistatic and fungicidal endpoints obtained with each medium for each isolate in each laboratory. Comparisons of proportions were performed by chi-square test for Fisher's exact test as appropriate. A two-tailed P value of <0.05 was considered to be significant.

RESULTS

Reproducibility of spectrophotometric procedure for inoculum preparation. A total of 105 inoculum preparations from the three laboratories were analyzed. The optical density (OD) ranges and actual CFU-per-milliliter ranges for the inoculum size values across the species are shown in Table 1. The CFU-per-milliliter range was within the target range of 0.4×10^6 to 5×10^6 for 9 of the 11 species evaluated (97 of 105 preparations or 92.4%). Substantially lower values (0.07×10^6 to 0.3×10^6 CFU/ml) were reported for 8 of the 18 *Bipolaris* spp. suspensions (five values for *B. hawaiiensis* and three for *B. spicifera)*. With the exception of inocula for *B. hawaiiensis,* the mean inoculum size range was 0.9×10^6 to 1.9×10^6 CFU/ml.

Total MICs and MFCs evaluated. Each isolate had three incubation time readings with the five antifungal agents and by each of the other six testing conditions evaluated (four media and two criteria of MIC determination) in all of the participant centers. A total of 120 readings per isolate in each center, or 360 readings from the three centers, were reported. Since 1,430 readings were scored as 0 (no growth), a total of 11,170 MICs were evaluated for the 35 isolates. In addition, 1,757 MFC values were obtained.

Effect of incubation time on MICs. A total of 3,723 MIC pairs were compared for this evaluation. MICs increased no more than two dilutions between 24 and 48 h or 48 and 72 h for most isolates. A major increase (from 0.06 to 1.0 μg/ml to 1 to 8 μg/ml) in MICs-0 was observed with amphotericin B for *B. hawaiiensis* and two *B. spicifera* isolates (24 to 72 h with M3 media) and with posaconazole for *T. longibrachiatum* (24 to 48 h with RPMI media).

Interlaboratory agreement. (i) Agreement among the four media. The four media supported growth of most of the isolates after 48 h of incubation, and both RPMI broths supported growth for all isolates after 72 h. Insufficient growth precluded MIC determination in two centers for two isolates of *C. bantiana* (four media) and one isolate of *P. lilacinus* (M3 media) at 48 h and for one isolate each of these two species at 72 h (M3 media). Although some inocula of *Bipolaris* spp. were substantially lower, sufficient growth allowed MIC determination at 48 h for all six isolates and for five isolates at 24 h. Table 2

TABLE 2. Interlaboratory agreement of MICs and MFCs for 35 uncommon molds in three laboratories

Antifungal agent and parameter	% Agreement at 48–72 h with medium			
	RPMI	RPMI-2%	M3	M3-2%
Amphotericin B				
MIC-0	94–97	95–94	98–99	90–91
MIC-2	91–92	86–90	93	90–91
MFC	92	89.7	94	92.8
Itraconazole				
MIC-0	97–95	93–94	94	92–95
MIC-2	89–90	89	91–94	89–95
MFC	88.8	85.7	86.6	81
Posaconazole				
MIC-0	87	89	95–93	92–93
MIC-2	89–90	91–92	88–92	91
MFC	86.6	82.4	87.6	75.7
Ravuconazole				
MIC-0	98	95–97	99	93–94
MIC-2	90–91	94–97	90–94	89–93
MFC	84.7	88.4	90.1	92.7
Voriconazole				
MIC-0	98	93–98	99	96
MIC-2	94–95	94	92–95	94–97
MFC	87.6	90.5	91.2	88.6

summarizes the percentages of interlaboratory agreement for MICs and MFCs of the five agents obtained with the four media for the 35 isolates; the agreement is stratified by the two criteria of MIC determination. Overall, either RPMI broth or M3 medium demonstrated the best interlaboratory reproducibility for the five agents. Slightly lower interlaboratory agreement was observed among MICs of most of the agents with either M3-2% or RPMI-2% than those with the other two formulations. In general, the agreement was lower among the laboratories for MFC than for MICs; slightly higher values were seen for MFCs obtained following MIC determination with M3 broth (86.6 to 94%) than with RPMI (84.7 to 92%) medium.

(ii) Agreement for the three incubation times. The agreement among the centers was lower (65 to 95%) when 24-h MICs were compared than for those obtained after 48 and 72 h of incubation (86 to 99%) for all the drugs and other testing parameters evaluated ($P = 0.001$).

(iii). Agreement for the two criteria of MIC determination. Overall, the interlaboratory agreement was higher for MICs-0 (93 to 99%) than for MICs-2 (86 to 95%) for four of the five agents with the four media (Table 2). The exceptions were posaconazole MICs, for which the agreement was more dependent on medium and MIC determination criterion; a better agreement was seen with both RPMI for MIC-2 endpoints (89 to 92%) than for MICs-0 (87 to 89%). On the other hand, agreement values were lower with M3 broths for MICs-2 (88 to 92%) than for MICs-0 (92 to 95%). Although the majority (84 to 98%) of MICs-0 were the same or no more than two dilutions higher than MICs-2, differences were seen with all media among posaconazole MICs for *T. longibrachiatum* and one *B. spicifera* (MICs-2 of 0.5 to 1.0 μg/ml versus MICs-0 of 4 to >8 μg/ml) and with ravuconazole and voriconazole for two *S. prolificans* isolates (MICs-2 of 1 to 4 μg/ml versus MICs-0 of ≥8 μg/ml). A similar trend was observed with voriconazole in RPMI broth for one isolate of *B. spicifera* (MICs-2 of 0.5 to 2 μg/ml versus MICs-0 of 4 to >8 μg/ml) and *F. solani* (MICs-2 of 2 to 4 μg/ml versus MICs-0 of 4 to >8 μg/ml). Other major discrepancies between the two criteria of MIC determination were due to the weaker agreement observed among the laboratories for MICs-2.

Interlaboratory agreement for azole and amphotericin B MICs. For amphotericin B and itraconazole, the agreement was good to excellent (94 to 99%) for MICs-0 with RPMI and M3 media. The agreement decreased 1 to 9% with RPMI-2% and M3-2% (Table 2). Greater interlaboratory agreement (93 to 99%) was observed for voriconazole and ravuconazole MICs-0 at both 48 and 72 h than for posaconazole (87 to 95%).

Optimal testing conditions for MIC determination. Although good to excellent agreement (>90%) was found for the four agents with some of the various combinations of media, MIC determination criteria, and incubation times, the testing conditions that better encompassed the five agents were the following: (i) the 100% growth inhibition criterion (MIC-0) with (ii) the standard RPMI broth, and (iii) after 48 to 72 h of incubation. Although reproducibility was higher for posaconazole MICs with M3 broths than with RPMI media, both M3 media did not support growth of all isolates. RPMI was also superior in distinguishing different patterns of fungistatic and fungicidal activity for some agent and species combinations, especially for amphotericin B.

Antifungal activities (MICs and MFCs) of the five agents. Table 3 depicts 48-h MICs-0 as well as MFC results of the five agents obtained with RPMI for each of the species. MICs for *C. bantiana* and *D. constricta* are 72-h values, because growth for the former species and the agreement for MICs among the centers for the latter species were better at that incubation time. Although the range of amphotericin B MICs was narrow for 6 of the 11 species, wider ranges (0.06 to 4 μg/ml) were observed for *Bipolaris* spp. and *F. solani*; MICs for *Bipolaris* spp. were bimodal. Also, high amphotericin B MICs (>2 μg/ml) were obtained in the three centers for *S. prolificans* and *P. lilacinus*. Most triazole MICs were clustered within a relatively low, narrow range, but lack of or low in vitro antifungal activity was observed for *F. solani*, *S. prolificans*, and *T. longibrachiatum* while the activity was good for the other species. MICs-0 of voriconazole and posaconazole for *S. prolificans* had a bimodal susceptibility pattern (MICs-0 of 0.5 to 1.0 μg/ml for one isolate and of ≥8 μg/ml for the other two isolates); this pattern was not observed for MICs-2. For *Bipolaris* spp., the bimodal pattern was species specific with ravuconazole and voriconazole (MICs-0 of <1.0 μg/ml for *B. hawaiiensis* and MICs-0 of 2 to >8 μg/ml for *B. spicifera* after 48 and 72 h of incubation), while low and high MICs-0 were obtained for both species with the other three agents. The wide itraconazole MIC ranges for *P. lilacinus* reflect the poor agreement among the three centers for this species by all testing conditions.

In contrast to MICs with RPMI, amphotericin B MICs obtained with M3 media were uniformly higher and the range was wide only for *Bipolaris* spp. (Table 4). Although the bimodal pattern with M3 for amphotericin B for *Bipolaris* spp. was similar to that with RPMI, MICs were high for five isolates with M3 but for only one isolate with RPMI. The activity of triazoles with M3 media was similar to that observed with RPMI for most of the species, but a bimodal activity was observed with ravuconazole for *S. prolificans* (Table 4).

MFCs of amphotericin B were either the same or no more

TABLE 3. MICs and MFCs for uncommon molds in three laboratories with standard RPMI[a]

Species (no. tested)	Parameter	Range for antifungal agent				
		A	I	P	R	V
A. nidulans (5)	MIC	0.2–2	0.06–0.2	0.03–2	0.12–2	0.12–2
	MFC	0.5–>8	0.06–8	0.03–8	0.12–>8	0.12–>8
A. terreus (3)	MIC	0.5–2	0.06–0.2	0.03–0.12	0.5–2	0.5–1
	MFC	1–8	0.2–8	0.12–1	2–8	1–8
Bipolaris spp. (6)	MIC	0.06–4	0.03–>8	>0.007–8	0.2–>8	0.12–>8
	MFC	0.12–8	0.06–>8	0.03–>8	1–>8	0.2–>8
C. bantiana (3)*	MIC	0.12–1	<0.007–0.5	<0.007–0.06	0.2–1	0.12–0.5
	MFC	0.12–2	0.03–0.5	0.03–1	0.06–4	0.01–2
D. constricta (3)*	MIC	0.06–0.5	0.12–1	0.03–0.12	8–>8	0.5–2
	MFC	0.06–2	0.12–>8	0.03–>8	4–>8	0.12–>8
F. solani (3)	MIC	0.12–4	>8	>8	>8	4–>8
	MFC	0.5–4	ND	ND	ND	8–>8
P. lilacinus (3)	MIC	>8	1–>8	0.12–0.5	0.2–2	0.2–1
	MFC	ND	0.2–>8	0.2–>8	1–>8	0.12–>8
S. prolificans (3)	MIC	2–>8	>8	0.5–>8	8–>8	0.5–>8
	MFC	8–>8	ND	>8	>8	1–>8
T. longibrachiatum (3)	MIC	0.5–2	>8	>8	>8	2
	MFC	1–>8	ND	ND	ND	2–>8
W. dermatitidis (3)	MIC	0.12–0.5	0.12–1	<0.007–1	1–8	0.12–0.5
	MFC	0.2–1	0.2–1	0.03–8	1–>8	0.12–8

[a] Asterisk, 72-h MICs-0 for these species; 48-h MICs-0 for other species. A, amphotericin B; I, itraconazole; P, posaconazole; R, ravuconazole; V, voriconazole. ND, no data.

than two dilutions higher than MICs for 32 of the 35 isolates when testing with RPMI (Table 3). Amphotericin MFCs were >3 dilutions higher than MICs-0 for one isolate each of *B. hawaiiensis, B. spicifera,* and *A. terreus* (0.06 to 1.0 μg/ml versus 1 to >8 μg/ml). MFCs of 1 to ⩾8 μg/ml for *Bipolaris* spp. only reflected poor interlaboratory agreement for two isolates. Among the triazoles, ravuconazole MFCs in RPMI were substantially higher than MICs (0.5 to 1.0 versus 4 to ⩾8 μg/ml) for one isolate of *B. hawaiiensis* and two isolates of *A. terreus*. The same applied to the other two new triazoles for two isolates of *A. terreus* and for posaconazole with one isolate of *B. spicifera*. With M3, MFC values tended to be higher and differences between fungistatic and fungicidal activities tended to be less distinctive, especially for posaconazole and ravuconazole (Table 4).

DISCUSSION

Our study describes the first collaborative evaluation of testing parameters for testing of susceptibility (fungicidal and fungistatic) of nine species of uncommon dematiaceous and moniliaceous molds to amphotericin B, itraconazole, and three

TABLE 4. MICs and MFCs for uncommon molds in three laboratories with M3 medium[a]

Species (no. tested)	Parameter	Range for antifungal agent				
		A	I	P	R	V
A. nidulans (5)	MIC	2–>8	0.12–4	0.03–2	0.2–4	0.2–4
	MFC	4–>8	0.2–>8	0.06–2	0.2–>8	0.2–4
A. terreus (3)	MIC	4–>8	0.12–0.5	0.06–0.12	1–4	1–8
	MFC	8–>8	0.5–>8	0.06–2	0.5–>8	1–>8
Bipolaris spp. (6)	MIC	0.12–>8	0.03–>8	0.01–>8	0.5–>8	0.5–>8
	MFC	1–>8	0.12–8	0.03–>8	1–>8	0.5–>8
C. bantiana (3)*	MIC	NG–8	NG–0.12	NG–0.06	NG–1	NG–0.06
	MFC	2–>8	0.01–0.5	<0.007–0.5	0.01–0.5	0.12–0.5
D. constricta (3)*	MIC	1–4	0.06–8	0.03–0.12	8–>8	1–2
	MFC	1–4	0.2–8	0.06–>8	4–>8	1–8
F. solani (3)	MIC	8–>8	>8	>8	>8	4–>8
	MFC	>8	ND	ND	ND	8–>8
P. lilacinus (3)	MIC	NG–>8	NG–>8	NG–0.5	NG–2	NG–1
	MFC	ND	0.5–>8	0.06–1	1–>8	0.2–1
S. prolificans (3)	MIC	>8	1–>8	0.2–>8	2–>8	0.5–>8
	MFC	ND	1–>8	0.5–>8	2–>8	1–>8
T. longibrachiatum (3)	MIC	4–>8	>8	1–>8	8–>8	1–4
	MFC	8–>8	ND	2–>8	>8	1–>8
W. dermatitidis (3)	MIC	1–4	0.06–0.12	<0.007–1	0.2–1	0.12–0.5
	MFC	2–>8	0.12–0.2	0.01–0.5	0.5–4	0.12–8

[a] Asterisk, 72-h MICs-0 for these species; 48-h MICs-0 for other species. M3 medium, conventional antibiotic #3 medium; A, amphotericin B; I, itraconazole; P, posaconazole; R, ravuconazole; V, voriconazole; NG, no growth; ND, not determined.

new triazoles (posaconazole, ravuconazole, and voriconazole). Our set of isolates included species that are recognized as resistant or less susceptible to the agents evaluated, e.g., *S. prolificans* and *A. terreus* (7, 20, 33). An agent with fungicidal activity may have a therapeutic advantage over an inhibitory agent. However, only one collaborative study has been conducted to detect optimal testing parameters for MFC determination for three *Aspergillus* spp. (16a). Because of that, we examined the effects of the various parameters on both MIC and MFC values regarding the potential detection of different patterns of susceptibility. We also examined the differences between MIC and MFC endpoints for the same purpose.

As for any antimicrobial procedure, the issue of reliability of the different testing parameters should be addressed. Our study has confirmed the reliability of the spectrophotometric procedure for preparing inoculum suspensions of common molds (10, 11, 16) and expanded its use for other, less common species (Table 1). With the exception of *Bipolaris* spp. inocula, excellent reproducibility was demonstrated for the other preparations from the three laboratories. The conidia of *Bipolaris* spp. and the macroconidia of *Fusarium* spp. are larger (6 to 12 by 16 to 35 μm) than conidia of the other species evaluated (2 to 5 by 2 to 13 μm). However, most *Fusarium* spp. also produce much smaller microconidia. Because inoculum suspensions of both *Bipolaris* spp. were below the desired CFU/ml range, these suspensions may require a 10% lower dilution factor than that used for the other species, or the density of these suspensions should be adjusted by using conventional cell counting with a hemacytometer (conidial size is irrelevant). Reliable conidial suspensions can be prepared by both methods (29), but inoculum quantification is highly recommended for confirmation of actual inoculum size (CFU/ml) by either method.

We examined the combined effect of incubation time and MIC determination criterion on MICs as well as their reliability. As previously described (16), MIC increases ($\geq$2 dilutions) between incubation times occurred more frequently for MICs-0 than for MICs-2. In this study, only posaconazole MICs-0 increased >3 dilutions for *T. longibrachiatum* and the different susceptibilities among isolates to the new triazoles were more obvious with MICs-0. However, the clinical significance of discrepant MICs by both criteria should be determined in clinical trials or animal studies. Although it has been reported (3) that the incubation time had no substantial effect on azole and amphotericin B MICs, the combined effects of incubation time and inoculum have increased azole MICs for *A. fumigatus*, *A. flavus* (18), and *Fusarium* spp. (30). As previously reported (16), 24-h MICs yielded the weaker interlaboratoy agreement in our study and, with the exception of *Aspergillus* isolates, lack of growth precluded 24-h MIC determination for some isolates of most other species. Furthermore, it was the 48-h incubation, in addition to the conventional MIC criterion, that allowed eight laboratories to better distinguish itraconazole-resistant isolates from susceptible isolates of *A. fumigatus* (16); these testing parameters have been introduced in the new version of the NCCLS M38 document.

The standard RPMI broth and the M3 medium consistently yielded $\geq$90% agreement among the laboratories for MICs of the five antifungal agents. Also, interlaboratory agreement of triazole MICs was improved (94 to 99%) over that obtained in the *Aspergillus* spp. study (90 to 100%) (16). RPMI-2% did not increase the growth rate or density of any of the species tested at 24 h as has been documented for yeasts (27), and use of this formulation lowered MIC reproducibility (3 to 8%) (Table 2). Similar results have been reported previously for *Aspergillus* spp. (16). The present study has examined the effect of two formulations of M3 on MICs and MFCs. However, in addition to the lot variability reported for M3 (22), its value as a predictor of amphotericin B resistance in candidemia has been questioned (28). In our study, both M3 media precluded MIC determination of all *C. bantiana* and *P. lilacinus* isolates even after 72 h. Arikan et al. (3) reported that buffered (phosphate) M3-2% had a tendency to yield lower MICs of amphotericin B, itraconazole and voriconazole than those obtained with RPMI. In our study, triazole MICs with both M3 and RPMI were essentially the same, but amphotericin B fungicidal and fungistatic concentrations were substantially higher than those with RPMI (Tables 3 and 4). Because of that, the different susceptibilities of our isolates to amphotericin B were better detected with MICs-0 determined using the RPMI broth. The discrepant results between this and Arikan et al.'s study (3) could be due to either the low reliability of M3 or their use of buffered medium. Due to the low M3 reliability, our data suggest that optimal testing conditions for MIC determination of these five agents for the species evaluated are the combination of standard RPMI broth, 48-h incubation for most species, and the conventional MIC criterion or 100% inhibition. For *C. bantiana* and *D. constricta*, 72 h appears to be the most reliable incubation time. MFCs (<3 colonies or approximately 99 to 99.5% killing activity) can be determined following MIC determination with those parameters.

Overall, our MIC and MFC data generated by the optimal testing conditions are comparable to those obtained in other studies for the five agents (1, 6, 7, 12, 13, 15, 16, 16a, 17, 19, 21, 24, 25, 31, 33). Our amphotericin B MIC and MFC values were high, or ranges were wide, for *Bipolaris* spp., *F. solani*, *P. lilacinus*, and *S. prolificans.* In addition, amphotericin B had fungicidal activity beyond safely achievable serum concentrations (MFC of $\geq$4 μg/ml) for one isolate of *Bipolaris* spp. and *A. terreus*, while MICs ($\leq$1 μg/ml) were within safely achievable serum levels. Both RPMI broths yielded two distinct fungicidal levels among isolates of *A. terreus*, *S. prolificans,* and *B. hawaiiensis*. The low in vitro fungicidal activity of amphotericin B has been demonstrated for the former two species but not for *Bipolaris* spp. (14, 15, 33). However, evaluations of in vitro fungicidal activities have focused on more common molds. With the exception of MFCs for some isolates of the new triazoles (*A. terreus*) and ravuconazole (*B. hawaiiensis*, *S. prolificans*, and *Bipolaris* spp.), other triazole fungicidal levels were no more than two dilutions higher than corresponding MICs. In the *Aspergillus* spp. study, MFCs were also consistently higher (1 to 4 dilutions) than corresponding MICs for *A. terreus* isolates with RPMI media. The potential discriminatory value of amphotericin B and voriconazole MFCs for this latter species has been demonstrated (14, 33).

In conclusion, because standard RPMI is a chemically defined medium and reproducibility was good to excellent, RPMI appears to be a suitable testing medium for determination of MICs of the agents and species evaluated. Their fungicidal

activities (<3 colonies) can be evaluated following determination of MICs-0 (100% growth inhibition) with standard RPMI broth after 48 h of incubation. However, for *C. bantiana* and *D. constricta*, 72 h appears to be the best incubation time for MIC determination. Our data also suggest that these MIC parameters have a superior discriminatory power in recognizing different patterns of in vitro antifungal susceptibility, as was demonstrated for *Aspergillus* spp. (16). Although these data indicate that MFCs could be more useful than MICs for some of the species evaluated, further evaluations with isolates that have well-documented in vivo data must confirm their clinical value. The use of M3 is limited until the problem of lot variation is investigated and resolved. Results of this NCCLS collaborative study warrant their introduction in a more advanced version of the NCCLS M38 document.

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