JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2002, p. 3204–3208 Vol. 40, No. 9
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.9.3204–3208.2002

Testing Conditions for Determination of Minimum Fungicidal Concentrations of New and Established Antifungal Agents for *Aspergillus* spp.: NCCLS Collaborative Study

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Received 7 March 2002/Returned for modification 16 April 2002/Accepted 13 June 2002

Standard conditions are not available for evaluating the minimum fungicidal concentrations (MFCs) of antifungal agents. This multicenter collaborative study investigated the reproducibility in three laboratories of itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B MFCs for 15 selected isolates of *Aspergillus* spp. After MIC determinations for the 15 isolates in each center by the NCCLS M38-A broth microdilution method with four media, standard RPMI 1640 (RPMI), RPMI with 2% dextrose, antibiotic medium 3 (M3), and M3 with 2% dextrose, MFCs were determined for each isolate-medium-drug combination. MFCs were defined as the lowest drug dilutions that yielded <3 colonies (approximately 99 to 99.5% killing activity). The highest reproducibility (96 to 100%) was for amphotericin B MFCs with the four media. Although reproducibility was more variable and medium dependent for the azoles (91 to 98%), agreement was good to excellent for itraconazole, ravuconazole, and voriconazole MFCs with RPMI and M3 (93 to 98%). For posaconazole, the agreement was higher with M3 media (91 to 96%) than with RPMI media (91%). These data extend the refinement of testing guidelines for susceptibility testing of *Aspergillus* spp. and warrant consideration for introduction into future versions of the M38 document. The role of the MFC under these standardized testing conditions as a predictor of clinical outcome needs to be established in clinical trials.

Aspergillus fumigatus and other *Aspergillus* spp. are responsible for the majority (85 to 90%) of clinical manifestations of severe infections caused by the filamentous fungi (moulds), especially in the immunocompromised host (4). The increased incidence of fungal infections and the development of new antifungal agents have underscored the importance of the laboratory's role in the selection and monitoring of antifungal therapy. The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has developed a reproducible reference testing procedure for the antifungal susceptibility testing of moulds (the M38-A document [18]). The recommendations described in the M38-A document for determination of MICs include the use of the standard RPMI 1640 broth (RPMI), which contains 0.2% dextrose (18). However, the document does not describe testing conditions for determination of minimum fungicidal (or lethal) concentrations (MFCs). Whether MICs are the best in vitro predictors of in vivo or clinical response to antifungal therapy is uncertain. Although standard conditions are not available for determination of fungicidal activities for either yeasts or moulds, it has been demonstrated that MFCs may be better predictors than MICs of therapeutic failure of amphotericin B in trichosporonosis (26, 27) and candidemia (20). The fungicidal activities of the new triazoles have also been evaluated during the last few years by nonstandardized methods (3, 7, 11, 13, 14, 16, 17, 21, 24, 25).

The purpose of this collaborative study was to investigate the interlaboratory reproducibility of MFCs following determinations of the MICs of five antifungal agents obtained with four media for each *Aspergillus* isolate (15 isolates) in three laboratories. The drugs evaluated were the conventional agents amphotericin B (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn.) and itraconazole (Janssen Pharmaceutica, Titusville, N.J.) and three new triazoles: voriconazole (Pfizer Pharmaceuticals, New York, N.Y.), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), and ravuconazole (Bristol-Myers Squibb Pharmaceutical Research Institute). The four media evaluated were (i) RPMI, (ii) RPMI with 2% dextrose (RPMI-2%), (iii) conventional antibiotic medium 3 (M3), and (iv) antibiotic medium 3 with 2% dextrose (M3-2%). This study was conducted simultaneously with the NCCLS multicenter (eight-center) study that further investigated optimal testing conditions for the determination of the MICs of itraconazole and three new triazoles for *Aspergillus* spp.; the results of that study have been reported elsewhere (10).

MATERIALS AND METHODS

Study design. Three laboratories participated in this study, and each laboratory received the same panel of 15 coded (isolates 1 to 15) strains of *Aspergillus* spp. (Table 1) and two control isolates. The MICs of amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole for each isolate were obtained in each of the three centers by the broth microdilution method following a standard protocol. The standard protocol included the susceptibility testing guidelines described in the NCCLS M38-A document (18) for MIC determination with four different media (10) and a detailed description of the testing parameters to be evaluated for MFC determination. 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 and the available in vitro and in vivo data are documented in Table 1. These 15 isolates belonged to the culture collections

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TABLE 1. Selected isolates evaluated in this study

Species and study isolate no.	Strain	Source	Reference
A. fumigatus			
1	NCPF 7101 (AF210)[a,b]	D. Denning	5
2	NCPF 7102 (AF294)[a,b]	D. Denning	5
3	NCPF 7098 (AF71)[a,b,c]	D. Denning	5, 9, 22
4	NCPF 7097 (AF65)[a,b]	D. Denning	5, 9, 15
5	NCPF 7100 (AF90)[c,d]	D. Denning	5, 9, 22
6	NCPF 7099 (AF72)[d]	D. Denning	5
7	AZ 606[e]	P. Verweij	NA[f]
8	NIH 4215[a,b,g,h]	T. Walsh	2, 12, 23; abstracts[g,h]
9	A 1604[a,b]	A. Espinel-Ingroff	6, 10
A. flavus			
10	A 1340[a]	A. Espinel-Ingroff	6
11	A 830[a]	A. Espinel-Ingroff	6
12	A 003[a,b]	A. Espinel-Ingroff	6, 10
A. terreus			
13	A 122[a]	A. Espinel-Ingroff	6
14	NIH 961290[a,i]	T. Walsh	Abstract[i]
15	NIH 95644[a,i]	T. Walsh	Abstract[i]

[a] Low itraconazole MICs (≤ 1.0 μg/ml) by M38-P, E-test, or other methods described in corresponding publication(s).
[b] Animals and patients responded to itraconazole treatment (isolates had low itraconazole MICs [< 1.0 μg/ml]).
[c] These two isolates also had either good (isolate 3) or decreased (isolate 5) response to posaconazole in a murine model.
[d] Animals and patients did not respond to itraconazole treatment (isolates had high itraconazole MICs [> 8 μg/ml]).
[e] Voriconazole laboratory mutant.
[f] NA, reference is not available.
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[i] Walsh et al., Prog. Abstr. 14th Congr. Int. Soc. Hum. Anim. Mycol. Isolates of *A. terreus* resistant in vivo to amphotericin B in experimental pulmonary aspergillosis responded to posaconazole and itraconazole in a rabbit model of invasive pulmonary aspergillosis.

of the University of Manchester, Salford, United Kingdom; the University of Texas; the Medical College of Virginia; the National Cancer Institute; and the University Hospital Nijmegen, Nijmegen, The Netherlands. Each isolate was maintained as a suspension in water at approximately 25°C until testing was performed. The reference isolate of *A. flavus* ATCC 204304 (9, 10, 18) and the quality control strain *Candida parapsilosis* ATCC 22019 (19) were included as controls. For *C. parapsilosis* ATCC 22019, there are well-established microdilution MIC ranges of the five agents evaluated in this study (1). Reference MIC ranges also have been established for the isolate of *A. flavus* ATCC 204304 based upon repeated testing in prior studies (9, 10); values for amphotericin B and itraconazole are listed in the M38-A document (18), and those for the new triazoles have been reported elsewhere (9). MIC ranges for the quality control and reference isolates were within established values (1, 9, 10, 18).

Broth microdilution method (M38-A). 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 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). Each batch of standard RPMI and RPMI-2% was supplemented with 0.3 g of L-glutamine per liter and 0.165 M MOPS (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 or M3-2% was buffered, and the pH of both media was 7.0 ± 0.1. The five antifungal agents amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole were provided by the manufacturers as assay powders. Additive drug dilutions were prepared as described in the NCCLS M38-A document (18) and in previous reports (8–10) in a central facility (Trek Diagnostic Systems) and shipped frozen to each participant. As recommended in the M38-A document (18), 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 actual stock inoculum suspensions by inoculum quantification ranged from 0.9×10^6 to 4.5×10^6 CFU/ml for 95% of the inoculum densities evaluated. Low (2.5×10^6 CFU/ml) and high (5.9×10^6 CFU/ml) densities were reported for three isolates. On the day of the test, each microdilution well containing 100 μl of the diluted (twofold) drug concentrations was inoculated with 100 μl of the diluted (two times) conidial inoculum suspensions (final volume in each well, 200 μl). The microdilution trays were incubated at 35°C and examined after 21 to 26, 46 to 50, and 70 to 74 h of incubation. The MIC endpoints were the lowest drug concentrations that showed absence of growth or complete growth inhibition (100% inhibition).

MFC determination. The in vitro fungicidal activities (MFCs) were determined for each drug-isolate-medium combination as previously described (7). After 72 h of incubation, 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 contents of the wells were not agitated prior to removal of the specified volumes. This step was performed by subculturing either one (two laboratories) or more than one (one laboratory) 20-μl volume on each plate. The plates were incubated at 35°C until growth was seen in the growth control subculture (usually before 48 h). The MFC was the lowest drug concentration that showed either no growth or fewer than three colonies to obtain approximately 99 to 99.5% killing activity.

Data analysis. Both on-scale and off-scale MICs and MFCs were included in the analysis. The MICs and MFCs from the three centers for each drug-isolate-medium combination tested were compared to obtain data regarding reproducibility. As previously analyzed (8–10), values were considered in agreement when the differences among the values were within three dilutions (three wells). 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, and medium. In addition, each MIC was compared to its corresponding MFC to assess the differences between the in vitro fungistatic and fungicidal endpoints obtained with each medium for each isolate in each laboratory.

RESULTS AND DISCUSSION

Reproducibility of susceptibility endpoints and detection of in vitro resistance are the main concerns when establishing

TABLE 2. Interlaboratory agreement of azole and amphotericin B MFCs for 15 *Aspergillus* isolates in three laboratories[a]

Antifungal agent	% Agreement with:			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole	93	93	98	93
Posaconazole	91	91	96	91
Ravuconazole	96	93	95	91
Voriconazole	98	91	93	96
Amphotericin B	98	96	100	98

[a] Total of 45 MFCs per agent; *A. fumigatus*, nine isolates; *A. flavus,* three isolates; *A. terreus,* three isolates.

TABLE 3. Amphotericin B MFCs and MICs for 15 *Aspergillus* isolates in three laboratories

Species	No. tested	Parameter	Amphotericin B range (modal)	
			RPMI	M-3
A. fumigatus	9	MFC	0.5–2 (0.5)	4–>8 (4–8)
	9	MIC	0.2–2 (0.2–0.5)	2–>8 (4)
A. flavus	3	MFC	0.5–2 (1–2)	0.2–4 (1–2)
	3	MIC	0.12–2 (1)	0.2–2 (1)
A. terreus	2	MFC	2–>8 (4)	2–>8 (4)
	2	MIC	0.5–2 (0.5–1)	0.5–2 (0.5)
	1	MFC	1–2	1–2
	1	MIC	0.5–2	0.5–1

standard testing guidelines for an antimicrobial susceptibility test. Although a recent NCCLS collaborative study (10) optimized MIC testing guidelines for detection of resistance to itraconazole and perhaps to three new triazoles in *Aspergillus* spp., the clinical relevance of those refined parameters has yet to be determined. Because aspergillosis is a severe and commonly fatal disease, especially in the deeply immunocompromised host (4), an agent with fungicidal activity has therapeutic advantage over an inhibitory agent. The fungicidal activities of three new triazoles against *Aspergillus* spp. and other moulds have been evaluated and compared to those of amphotericin B and itraconazole (3, 7, 11, 13, 14, 16, 17, 21, 24, 25). However, collaborative studies have not been conducted to detect optimal testing parameters for MFC determination. Our study represents the first interlaboratory investigation of testing conditions for determination of the MFCs of amphotericin B, itraconazole, and three new triazoles (voriconazole, posaconazole, and ravuconazole) for three species of *Aspergillus*.

As for any other antimicrobial procedure, the issue of reliability should be addressed first. Table 2 presents the summary of interlaboratory agreement for MFCs that were obtained with four media for the 15 isolates of *Aspergillus* spp. stratified by antifungal agent. Excellent reproducibility (96 to 100%) was demonstrated for amphotericin B MFCs with the four media; 48% of the values were within one dilution, and 20% had the same value. In contrast, for the triazoles, the reproducibility was more variable and dependent on the medium formulation and azole tested. Reproducibility was good to excellent (91 to 98%) with RPMI: 51 (voriconazole) to 6.2% (ravuconazole) of the results had the same value, and 61 (itraconazole) to 29.6% (posaconazole) were within one dilution. Addition of 2% dextrose to either M3 or RPMI medium did not appear to enhance reproducibility. For MIC testing, the other NCCLS study (10) demonstrated that overall interlaboratory reproducibility was higher with RPMI than with the other media for three of the four triazoles. In both studies, differences in agreement among the media were small. The reproducibility of MFC endpoints was also evaluated by repetitive testing (at least three times in each laboratory) of the control isolate of *A. flavus* ATCC 204304; the MFCs of the five antifungal agents for this isolate were consistently within the expected three-well range with the four media.

Table 3 summarizes amphotericin B MIC and MFC endpoints with RPMI and M3. Our amphotericin B values with both M3 media were higher than those routinely obtained. In one of three centers (the University of Texas), ranges for the MIC and the MIC at which 50% of the isolates tested are inhibited (MIC_{50}) of 0.5 to 8 and 1 to 2 μg/ml, respectively, have been obtained with M3 broth for 759 isolates of these three species (with MICs above 2 μg/ml only for 21 *Aspergillus terreus* and 3 *Aspergillus flavus* isolates). The reasons for this discrepancy may be the variability among M3 lots that has been reported when testing *Candida* spp. (19). As previously reported (7, 11, 13, 21), amphotericin B MICs and MFCs were usually the same or the MFCs were no more than one to two dilutions higher than the corresponding MICs for *A. fumigatus* and *A. flavus*. Our amphotericin B MIC and MFC ranges were narrow, which is also one of the disadvantages of amphotericin B MICs for *Candida* spp. Although amphotericin B MICs had a relatively wide range (1 and 2 to >8 μg/ml) with M3 media for *A. fumigatus*, most values were >2 μg/ml or beyond safe concentrations in serum achieved with this agent. Peak levels in plasma that were below the MIC and MFC results in this study correlated with in vivo response in experimental pulmonary aspergillosis caused by isolate 8 (23). This response was directly related to reduced tissue burden (measured in log CFU per gram) in the lungs. Lack of correlation of in vitro results with in vivo response has been reported in a murine model of invasive *A. fumigatus* infection when the animals were infected with isolate 4; this isolate was recovered from a patient who responded incompletely to high-dose amphotericin B (15). In the present study, both media were unable to distinguish this isolate from the others.

Both RPMI broths yielded two distinct amphotericin B fungicidal levels for the three *A. terreus* isolates (Table 3), while with M3, the results were uniformly high for all isolates. Sutton et al. (24) have demonstrated the potential discriminatory value of amphotericin B MFCs for *A. terreus* with M3 broth, and amphotericin B inhibitory activity was found to be superior (geometric mean MICs, 1.7 and 3.37 μg/ml) to its fungicidal activity (geometric mean MFCs, 7.4 and 13.4 μg/ml) for this species in two studies (7, 24). Because M3 is widely used for testing amphotericin B, it is important that laboratory personnel be aware of the problem posed by M3 lot variability as demonstrated in this study for *Aspergillus* testing. In our study, amphotericin B had fungistatic activity beyond safely achievable concentrations in serum for two isolates (modal MFC, 4 μg/ml), while the MFCs (1 to 2 μg/ml) for the third isolate were within safely achievable levels in serum when tested with RPMI. Although amphotericin B had the same inhibitory activity for all three isolates (MIC range, 0.5 to 2 μg/ml), it had fungicidal activity for only one (Table 3). It is noteworthy that

TABLE 4. Azole MFCs and MICs for 15 *Aspergillus* isolates in three laboratories

Medium and species (no. of isolates)[a]	Parameter	Range (MFC_{90}s, MIC_{90}s, or modal values)[b]			
		Itraconazole	Voriconazole	Posaconazole	Ravuconazole
RPMI medium					
A. fumigatus I-Suscep. (6)	MFC	0.2–4 (4)	0.5–4 (4)	0.06–2 (1)	0.5–4 (4)
	MIC	0.12–1 (1)	0.5–1 (1)	0.03–0.5 (0.2)	0.12–1 (1)
I-Res. (1 [isolate no. 5])	MFC	>8	1–2	1–2	2–4
	MIC	8–>8	1	0.2–1	2
I-Res. (1 [isolate no. 6])	MFC	ND[c]	0.5–2	2–>8	2
	MIC	>8	0.2–0.5	0.5–1	0.2
V-Res. (1)	MFC	1–4	2–8	0.2–0.5	4–8
	MIC	0.5–4	2–8	0.2	2–8
A. flavus (3)	MFC	0.12–1 (0.5)	0.5–2 (1)	0.06–0.5 (0.12)	1–4 (2)
	MIC	0.12–0.5 (0.2)	0.12–1 (1)	0.03–0.5 (0.06)	0.5–2 (2)
A. terreus (1)	MFC	0.5–4	2–4	0.2–1	4–8
	MIC	0.12–0.5	0.5–1	0.03–0.5 (0.03)	0.5–1
A. terreus (2)	MFC	0.2–1 (0.5)	1–4 (2)	0.12–0.2 (0.12)	1–4 (4)
	MIC	0.06–0.5 (0.2)	0.5–1 (0.5)	0.03–0.2 (0.03)	0.12–1 (0.5)
M3 medium					
A. fumigatus I-Suscep. (6)	MFC	0.5–1 (1)	0.5–8 (4)	0.06–1 (0.5)	1–4 (4)
	MIC	0.2–1 (0.5)	0.5–4 (2)	0.03–0.12 (0.12)	0.5–2 (2)
I-Res. (isolate no. 5)	MFC	>8	2–4	0.5–2	>8
	MIC	8–>8	1	0.2–1	8
I-Res. (isolate no. 6)	MFC	>8	0.2–0.5	0.5–2	0.5–2
	MIC	4–>8	0.2–0.5	0.5–1	0.2–0.5
V-Res. (1)	MFC	2–>8	8–>8	0.2–0.5	8–>8
	MIC	2–>8	8–>8	0.2–0.5	8–>8
A. flavus (3)	MFC	0.12–0.5 (0.5)	2–4 (4)	0.06–0.5 (0.12)	1–4 (2)
	MIC	0.12–0.5 (0.5)	0.5–4 (2)	0.12–0.2 (0.12)	0.5–4 (2)
A. terreus (1)	MFC	1–2	4–8	0.2–0.5	4–8
	MIC	0.2–1	2–4	0.12–0.2	1–2
A. terreus (2)	MFC	0.5–1 (0.5)	4–8 (4)	0.12–0.5 (0.2)	2–8 (4)
	MIC	0.06–0.5 (0.5)	1–4 (2)	0.03–0.2 (0.2)	0.2–2 (1)

[a] I-Suscep, itraconazole-susceptible isolates; I-Res., itraconazole-resistant isolates; V-Res., voriconazole-resistant laboratory mutant.
[b] MFC_{90}s, MIC_{90}s for six isolates; modal values for three isolates.
[c] ND, not determined.

the E-test can also yield distinctive data for these three isolates (0.5 to 1 and 4 μg/ml).

Table 4 summarizes azole MFCs and MICs that were obtained with RPMI and M3 media for the 15 isolates stratified by species and according to established azole susceptibilities (5, 10). The MFC data with RPMI-2% and M3-2% were similar (data not listed in Table 4). The MFCs of most triazoles were consistently higher (one to four dilutions) than the corresponding MICs for *A. fumigatus* and *A. terreus* isolates with RPMI media. In two previous studies with larger numbers of isolates, a substantial difference was found between voriconazole MICs (geometric means, 0.22 and 0.63 μg/ml) and MFCs (geometric means, 17.4 and 6.8 μg/ml) for *A. terreus* (7, 24). Differences between MICs and MFCs for the three isolates of *A. terreus* were less pronounced in this study. It has been reported (3, 7, 14) that voriconazole MICs and MFCs were comparable for *A. fumigatus* and *A. flavus* (MIC_{90}s of 0.5 to 1.0 μg/ml versus MFCs at which 90% of isolates are killed [MFC_{90}s] of 0.5 to 2 μg/ml), while itraconazole MFCs for *A. fumigatus* tended to be three to four dilutions higher than MICs (MIC_{90}s of 0.5 μg/ml versus MFC_{90}s of 4 μg/ml). The differences between fungicidal and fungistatic activities of the four triazoles against *A. flavus* were less substantial: 4.2 to 8.3% of MFCs were more than two dilutions higher than MICs, and 17 to 33% of MFCs for the other species were higher. Fungicidal data for posaconazole and ravuconazole are more scarce. Our results confirm previous reports (6, 13, 21) in which posaconazole in vitro fungicidal and fungistatic activities appeared to be superior to those of the other triazoles. Posaconazole MICs and MFCs were higher for the two itraconazole-resistant isolates than those for most of the other isolates (Table 4). In a temporarily neutropenic model of invasive aspergillosis (22), there was an ~100-fold difference between the fungal counts in lungs and kidneys of animals infected with isolate 5 (Tables 1 and 4) and those in animals infected with isolate 3 with a lower MIC (0.01 μg/ml). Although these results suggest that posaconazole MICs of ≥0.5 μg/ml may indicate potential in vitro resistance to this agent, the MFCs for these isolates were similar. Ravuconazole MFCs with RPMI for isolates 5 and 6 (Table 4) indicated that that agent had similar fungicidal activities for these isolates but different fungistatic activities. The clinical significance of these discrepant results has yet to be determined.

In conclusion, because standard RPMI is a chemically defined medium and MFC reproducibility is good to excellent for the five agents, RPMI appears to be a suitable testing medium for determination of fungicidal susceptibilities of *Aspergillus* spp. to these five agents. Although the reproducibility of M3 medium was similar to that of RPMI, its use is limited until the problem of lot-to-lot variation is investigated and resolved. The results of this NCCLS collaborative study extend the refinement of testing conditions for susceptibility testing of *As-*

pergillus spp. to amphotericin B and four triazoles. However, the role of the MFC as a predictor of clinical outcome for *Aspergillus* spp. by following this standardized procedure should be established in either clinical trials or experimental infections. The introduction of these optimal testing conditions for MFC determination in a more advanced version of the NCCLS M38 document is warranted.

ACKNOWLEDGMENTS

This work was partially supported by grants from Pfizer Inc., Schering-Plough Research Institute, and Bristol-Myers Squibb.

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