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Multicenter Evaluation of Proposed Standardized Procedure for Antifungal Susceptibility Testing of Filamentous Fungi

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A multicenter study was conducted to expand the generation and analysis of data that supports the proposal of a reference method for the antifungal susceptibility testing of filamentous fungi. Broth microdilution MICs of amphotericin B and itraconazole were determined in 11 centers against 30 coded duplicate pairs of *Aspergillus* spp., *Fusarium* spp., *Pseudallescheria boydii*, and *Rhizopus arrhizus*. The effect of inoculum density (approximately 10^3 and 10^4 CFU/ml), incubation time (24, 48, and 72 h), and procedure of MIC determination (conventional and colorimetric [Alamar Blue] evaluation of growth inhibition) on intra- and interlaboratory agreement was analyzed. Based on intra- (97 to 100%) and interlaboratory (94 to 95%) agreement for both drugs, the overall optimal testing conditions identified were determination of colorimetric MICs after 48 to 72 h of incubation with an inoculum density of approximately 10^4 CFU/ml. These testing conditions are proposed as guidelines for a reference broth microdilution method.

The last 15 years have witnessed marked increases in the frequency of severe fungal infections (2, 18, 20) as well as in the number of available antifungal agents. The number of invasive infections caused by the filamentous fungi is lower than those caused by the yeasts. However, the emergence of new and less susceptible mold pathogens (1, 3, 17, 18) warrants the evaluation of their in vitro antifungal susceptibilities to both established and investigational antifungal agents. Work on development of meaningful and standardized procedures for testing of yeasts (6, 10) has led to the publication of the document M27-T (tentative standard) (12) by the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing. The endeavors of the NCCLS Subcommittee have recently been directed towards developing standard guidelines for the antifungal susceptibility testing of filamentous fungi (7). Initial work demonstrated that reliable conidial suspensions could be prepared by a spectrophotometric procedure (5). Based on these results, the NCCLS Subcommittee adapted other testing variables of the M27-T method to the filamentous fungi. In a first study focusing on intra- and interlaboratory comparisons of broth macro- and microdilution methods performed by using buffered (MOPS [morpholinepropanesulfonic acid]) RPMI 1640 medium and standardized inoculum densities (0.4×10^4 to 3.4×10^4 CFU/ml), 91 to 97% and 83 to 100% interlaboratory agreement was demonstrated by both micro- and macrodilution methods, respectively (7).

In this second multicenter (11-center) study, the NCCLS Subcommittee investigated in a microdilution system the effect of inoculum density, incubation time, and procedure of MIC determination on the antifungal susceptibilities of six species of conidium-forming (size range, 2 to 7 μm) filamentous fungi. The objective was the development of a reference method for the antifungal susceptibility testing of these fungi. The intralaboratory comparison of matching pairs of MICs, the interlaboratory agreement of MICs among the 11 centers, and the comparison between two methods of MIC determination were evaluated. MICs of amphotericin B and itraconazole were determined in each center by using 30 duplicate pairs of *Aspergillus* spp., *Fusarium* spp., *Pseudallescheria boydii*, and *Rhizopus arrhizus*.

MATERIALS AND METHODS

Study design. Each of the 11 laboratories received the same panel of 30 coded pairs of filamentous fungi and two quality control (QC) isolates. Each isolate was tested in each of the participating centers with amphotericin B and itraconazole by the broth microdilution antifungal susceptibility method following a standard protocol. Inoculum suspensions were adjusted by a spectrophotometric procedure in each laboratory as detailed in Table 1. The protocol included other preliminary NCCLS recommendations for the antifungal susceptibility testing of this group of fungi (7, 8) and a detailed description of the additional testing parameters to be evaluated: (i) two inoculum densities (approximately 10^3 and 10^4 CFU/ml), (ii) three incubation times (24, 48, and 72 h), and (iii) two methods of MIC determination (conventional and colorimetric evaluation of growth inhibition). The objectives of the study were (i) to determine the intralaboratory

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agreement between broth microdilution MICs for duplicate isolates tested in a blinded fashion in each center, (ii) to determine the interlaboratory reproducibly of the broth microdilution test, and (iii) to determine the variability of the broth microdilution test when two different inoculum densities, three different incubation times, and two methods of MIC determination were evaluated.

Antifungal drugs. The MICs of amphotericin B (E. R. Squibb & Sons, Princeton, N.J.) and itraconazole (Janssen Research Foundation, Beerse, Belgium) were determined by a broth microdilution method. The antifungal drugs were provided by the manufacturers as assay powders.

Isolates. A set of 30 well-characterized clinical isolates of selected conidium- and sporangiospore-forming (size range, 2 to 7 μm) filamentous fungi were tested. The duplicate coded isolates included five pairs each of *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Fusarium solani*, *R. arrhizus*, and *P. boydii* from the culture collections of the University of Texas, the Medical College of Virginia, the National Cancer Institute, and the Columbia Presbyterian Medical Center. Each isolate originated from a different patient and was maintained as a suspension in water at approximately 25°C until testing was performed. An isolate of *Candida parapsilosis* ATCC 22019 and *Paecilomyces variotii* ATCC 22319 were included as control isolates. *C. parapsilosis* ATCC 22019 is a QC strain for the M27-T method and has well-established MIC ranges for amphotericin B (0.25 to 1 μg/ml) and itraconazole (0.064 to 0.25 μg/ml) (13, 15). Reference MIC ranges, for the *P. variotii* isolate, of amphotericin B (0.25 to 1 μg/ml) and itraconazole (0.03 to 1 μg/ml) were established based upon repeated testing in a prior study (7).

Antifungal susceptibility testing. Standard recording forms were used as working sheets. The standard protocol supplied to each laboratory for the broth microdilution method provided detailed instructions concerning the following test conditions.

(i) Medium. A single lot of liquid RPMI 1640 medium (Whittaker Bioproducts, Inc., Walkersville, Md.) supplemented with both 0.3 g of L-glutamine per liter and 0.165 M MOPS buffer (34.54 g/liter) and without sodium bicarbonate was provided ready for use to all participants by Alamar Bioscience (now Sensititre/Alamar, Westlake, Ohio). The same lot of medium was used for the preparation of the drug dilutions. The pH of the medium was 7.0 ± 0.1 at 35°C, and each batch was checked for sterility and pH prior to use in each laboratory.

(ii) Drug dilutions. The drug dilutions were prepared following the additive twofold drug dilution scheme described in the NCCLS M27-T method (12). Briefly, stock drug solutions were first diluted to 100× the final concentrations in 100% dimethyl sulfoxide (e.g., 3,200 to 3 μg/ml for amphotericin B and 1,600 to 1.5 μg/ml for itraconazole) and further diluted 1:50 in 2× medium to obtain the 2× drug concentrations. The final drug concentrations were 32 to 0.03 μg/ml for amphotericin B and 16 to 0.015 μg/ml for itraconazole. Microdilution plates (96 U-shaped wells; Dynatech Laboratories, Inc., Alexandria, Va.) containing antifungal dilutions were prepared in a central facility (Sensitre/Alamar) and shipped frozen to each participant. Rows 2 to 12 contained the series of drug dilutions in 100-μl volumes; well 1 contained 100 μl of 2× drug-free medium and served as the growth control.

QC of the prepared microdilution trays was performed prior to initiation of the study by testing one to two isolates of each species evaluated and the two NCCLS QC isolates *C. parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 in one of the participant laboratories (first author's) and in the central facility with the latter two isolates.

(iii) Inoculum preparation. In a modification of a previously described procedure (5, 7), test inocula of approximately 0.4×10^4 to 5×10^4 (high inoculum) and 0.4×10^3 to 5×10^3 (low inoculum) CFU/ml were evaluated. To induce conidium and sporangiospore formation, fungi were grown on potato dextrose agar slants (Remel, Lenexa, Kans.) at 35°C for 7 days (*Aspergillus* spp., *P. boydii*, and *R. arrhizus*) or at 35°C for 48 to 72 h and then at 25 to 28°C until day 7 (*Fusarium* spp.). Seven-day-old colonies were covered with approximately 1 ml of sterile 0.85% saline, and the suspensions were made by gently probing the colonies with the tip of a Pasteur pipette. The resulting mixture of conidia or sporangiospores and hyphal fragments was withdrawn and transferred to a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogeneous suspensions were collected and mixed with a vortex mixer for 15 s. The densities of the conidial suspensions were read and adjusted to an optical density (OD) that ranged from 0.09 to 0.11 (80 to 82% transmittance) for *Aspergillus* spp. and 0.15 to 0.17 (68 to 70% transmittance) for *Fusarium* spp., *P. boydii*, and *R. arrhizus* (5, 7) (Table 1). These suspensions were diluted 1:50 and 1:500 in sterile distilled water and a second time in sterile distilled water containing a 2× concentration of the colorimetric oxidation-reduction indicator Alamar Blue (Sensititre/Alamar). The 1:50 inoculum dilutions corresponded to 2× the density needed for the high inoculum size of approximately 0.4×10^4 to 5×10^4 CFU/ml, and the 1:500 dilutions corresponded to 2× the density needed for the low inoculum size of approximately 0.4×10^3 to 5×10^3 CFU/ml. The test inoculum was made in sufficient volume to directly inoculate each well with 100 μl of the corresponding diluted inoculum suspension.

Inoculum quantitation for each isolate was performed by each laboratory by plating 0.01 ml of a 1:100 dilution of the adjusted inoculum on modified Sabouraud glucose agar (Difco Laboratories, Detroit, Mich.) to determine the viable number of CFU per milliliter. The plates were incubated at 28 to 30°C and were observed daily for the presence of fungal colonies. Colonies were counted as soon as possible after growth became visible, especially for isolates of *R. arrhizus*. The incubation times ranged from 24 h or less (*R. arrhizus*) to 5 days (*P. boydii*) in the different laboratories.

(iv) Broth microdilution test. Broth microdilution testing was performed as described previously (7). Each well was inoculated on the day of the test with 100 μl of the 2× conidial inoculum suspension. This step diluted the drug concentrations, inoculum densities, Alamar Blue indicator (if present), and the RPMI medium to the final desired test concentrations. The growth control wells contained 100 μl of the corresponding diluted inoculum suspension and 100 μl of the 2× sterile drug-free medium and dimethyl sulfoxide mixture. Both QC isolates were included each time that a set of isolates was tested in each laboratory with each drug.

(v) Incubation and MIC determination. All microdilution trays were incubated at 35°C and examined after 21 to 26, 46 to 50, and 70 to 74 h of incubation. Both the conventional and colorimetric procedures of MIC determination required visual examination of growth inhibition. The conventional procedure involved the determination of MICs by using medium without Alamar Blue, and the colorimetric procedure used medium supplemented with Alamar Blue. For the conventional procedure, the growth in each MIC well was compared with that of the growth control with the aid of a reading mirror. The microdilution wells were then given a numerical score as follows: 4, no reduction in growth; 3, slight reduction in growth or approximately 75% of the growth control; 2, prominent reduction in growth or approximately 50% of the growth control; 1, slight growth or approximately 25% of the growth control; and 0, optically clear or the absence of growth. For the colorimetric procedure, the wells were examined for a change in color from blue (indicating no growth) to purple (indicating partial inhibition) to red (indicating growth). The wells were given a numerical score as follows: 2, the drug concentrations that showed a slight color change from blue to purple; and 0, the drug concentration that showed no color change or the first well that remained blue.

Analysis of the data. For each species, the overall mean and standard errors of the mean for the inoculum sizes were obtained. In addition, the percentages of inoculum densities that were within the range of 0.4×10^6 to 5×10^6 CFU/ml were calculated. MIC endpoints were determined in two ways with the aid of a computer program as follows: the lowest drug concentration which had a score of 2 for itraconazole and the lowest concentration which had a score of 0 for amphotericin B by each method of MIC determination. Each of the 30 duplicate isolates was evaluated in a blinded fashion by using MIC data from the seven testing conditions.

Two analyses of the MIC data were performed. In the first, only on-scale MICs, values that were within the range(s) of drug concentrations tested, were evaluated. In the second, off-scale MICs were included by converting off-scale MICs of >32 and >16 μg/ml to the next highest concentration of either 64 or 32 μg/ml and off-scale MICs of <0.03 and <0.015 μg/ml to the next lowest concentration of either 0.015 or 0.007 μg/ml (data not shown in Tables 3, 4, and 5). When skips (uneven patterns) were present, the MIC endpoint was the higher drug concentration. Discrepancies between MIC endpoints of no more than 3 dilutions (three wells) were used for calculation of the percent values of agreement. A measure of agreement was then determined as follows. The percentage of MIC endpoints within 3 dilutions was determined for each combination of isolate, drug, inoculum size, incubation time, and method of MIC determination. The differences in these percentages for each variable and with each drug-isolate combination as well as the median percentage of intralaboratory agreement between the two methods of MIC determination and between matching MIC pairs were calculated. The corresponding confidence intervals were also determined.

RESULTS

Spectrophotometric procedure. Six hundred fifteen inoculum preparations from the 11 laboratories were analyzed. The OD ranges, actual CFU-per-milliliter ranges, overall mean, and standard errors of the mean 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 87% of *P. boydii* inoculum preparations and for >92% of the inocula for all of the other species. Higher values (6.4×10^6 to 8.2×10^6 CFU/ml) were obtained in laboratories 4, 6, and 10 with certain isolates of *F. oxysporum* and in laboratories 4 and 9 with some isolates of *Aspergillus* spp. Lower values (0.3×10^5 CFU/ml) were reported by laboratories 8 (all isolates) and 4 (one isolate) for *P. boydii*.

Antifungal susceptibility. All species produced clearly detected growth in medium supplemented and nonsupplemented with Alamar Blue after 48 h of incubation with the high inoculum size (approximately 10^4 CFU/ml). Although growth was evident at 24 h with *Aspergillus* spp. isolates, some laboratories were unable to detect color change, which precluded MIC

TABLE 1. OD range and mean inoculum sizes for filamentous fungi[a]

Fungus (no. of observations)	Range of OD (530 nm)	Inoculum (CFU/ml) (10^6)		
		Range (% in bounds)	Mean	SE
Aspergillus flavus (105)	0.09–0.11	0.4–4.0 (95)	1.6	0.10
Aspergillus fumigatus (104)	0.09–0.11	0.6–5.0 (95)	2.7	0.15
Fusarium oxysporum (105)	0.15–0.17	0.8–5.0 (92)	3.0	0.16
Fusarium solani (103)	0.15–0.17	0.5–3.9 (100)	1.8	0.09
Pseudallescheria boydii (99)	0.15–0.17	0.4–3.2 (87)	1.0	0.07
Rhizopus arrhizus (99)	0.15–0.17	0.4–2.6 (98)	1.3	0.09

[a] Target inoculum size, 0.5×10^6 to 4×10^6 CFU/ml.

determination at that incubation time. In contrast, MICs for *R. arrhizus* were readily determined at 24 h by both MIC determination procedures. Table 2 shows the amphotericin B and itraconazole modal MIC endpoints (most frequent MIC in the 11 centers) for each isolate tested obtained by colorimetric microdilution after 48 h of incubation with the high inoculum. Except for *R. arrhizus*, modal MICs at 48 and 72 h determined by conventional examination of growth inhibition as well as colorimetric modal MICs determined at 72 h were similar (±1 dilution) to the values shown in Table 2 (data not shown). Variable results were noted by the two procedures of MIC determination for itraconazole against *R. arrhizus* after 48 h of incubation (Table 2).

Interlaboratory agreement of microdilution MICs. Of the 7,560 expected MICs for the 30 filamentous isolates tested, a total of 6,552 amphotericin B and 6,410 itraconazole MICs were available for analysis. Two laboratories did not report a complete set of amphotericin B (1.6% of total) and itraconazole (0.9% of total) MICs, and insufficient growth precluded MIC determination with low inoculum and after 24 h of incubation. Table 3 summarizes the statistics for interlaboratory agreement for broth microdilution on-scale MICs. The results are stratified by antifungal agents and testing condition. For each set of MICs from the 11 centers, MICs were considered to be in agreement when they belonged in the largest MIC subset with a range of no greater than 3 dilutions. The interlaboratory comparison of MIC endpoints demonstrated 93 to 97% agreement among the 11 laboratories with amphotericin B after 48 and 72 h of incubation. The agreement was slightly lower and more dependent on the MIC determination procedure and inoculum size with itraconazole; colorimetric MICs showed higher levels of agreement (95%) than did MICs obtained by the conventional method (89 to 92%) with the high inoculum. Analyses using the off-scale MICs gave similar values of agreement (data not shown).

Comparison of conventional and colorimetric procedures of MIC determination. Table 4 summarizes the results of the comparison of MIC pairs determined by the two procedures of MIC determination. For each combination of test conditions (e.g., inoculum density and incubation time and drug), MICs

TABLE 2. Modal MICs for 30 filamentous fungi in 11 centers[a]

Sp.	Testing condition[b]	Drug[c]	Modal MIC (μg/ml) for isolate no.:				
			1	2	3	4	5
Aspergillus fumigatus	H 48	A	1	2	1	2	2
		I	0.25	0.5	0.25	0.25	0.25
Aspergillus flavus	H 48	A	1	1	1	1	1
		I	1.0	0.5	0.5	0.5	0.5
Fusarium oxysporum	H 48	A	2	2	2	2	2
		I	>16	>16	>16	>16	>16
Fusarium solani	H 48	A	1	2	1	1	2
		I	>16	>16	>16	>16	>16
Pseudallescheria boydii	H 48	A	2	2	2	4	4
		I	1.0	1.0	0.5	1.0	1.0
Rhizopus arrhizus							
	H 24-COL[d]	A	0.2	0.2	0.5	0.2	0.2
	H 48-COL[d]	I	4	>16	>16	>16	>16
	H 24-CONV[d]	A	0.2	0.2	0.5	0.2	0.5
	H 48-CONV[d]	I	4	>16	8	2	>16

[a] Modal MIC, most frequent broth microdilution MIC among the 21 values from the 11 centers.
[b] H, inoculum density, approximately 10^4 CFU/ml; 24 and 48, hours of incubation.
[c] A, amphotericin B; I, itraconazole.
[d] CONV and COL, MICs determined by conventional and colorimetric (Alamar Blue) evaluation of growth inhibition, respectively.

TABLE 3. Interlaboratory agreement for broth microdilution antifungal test for 30 filamentous fungi[a]

Antifungal agent and testing condition[b]	% Agreement (no. of MICs)[c]	98% CI[d]
Amphotericin B		
CONV		
H 48	96 (617)	94,97
H 72	97 (615)	95,98
L 48	94 (609)	92,96
L 72	96 (616)	94,97
COL		
H 48	94 (605)	92,96
H 72	95 (606)	93,97
L 48	94 (593)	92,96
L 72	93 (608)	90,95
Itraconazole		
CONV		
H 48	89 (406)	86,92
H 72	92 (365)	89,95
L 48	88 (438)	84,92
L 72	93 (396)	88,95
COL		
H 48	95 (354)	92,97
H 72	95 (336)	92,97
L 48	91 (392)	89,94
L 72	95 (345)	93,97

[a] Agreement (within 3 dilutions) of MICs among 11 centers.
[b] CONV and COL, MICs determined by conventional and colorimetric (Alamar Blue) evaluation of growth inhibition, respectively; H and L, inoculum densities, approximately 10^4 and 10^3 CFU/ml, respectively; 48 and 72, hours of incubation.
[c] Number of on-scale MICs.
[d] CI, confidence interval.

TABLE 4. Agreement between visual and colorimetric procedures of MIC endpoint determination for broth microdilution testing of filamentous fungi[a]

Antifungal agent	Testing condition[b]	% Agreement	98% CI[c]	No. of observations[d]
Amphotericin B	H48	98	97, 100	600
	H72	100	97, 100	599
Itraconazole	H48	94	88, 96	350
	H72	100	79, 100	326

[a] Agreement (within 3 dilutions) of on-scale MICs determined in 11 centers by conventional and colorimetric (Alamar Blue) evaluations of growth inhibition.
[b] H, inoculum density, approximately 10^4 CFU/ml; 48 and 72, hours of incubation.
[c] CI, confidence interval.
[d] Number of on-scale MIC pairs.

TABLE 5. Intralaboratory agreement for 30 pairs of broth microdilution MICs[a]

Antifungal agent	Testing condition[b]	% Agreement		98% CI[c]		No. of observations[d] (CONV/COL)
		CONV	COL	CONV	COL	
Amphotericin B	H48	97	100	97, 100	93, 100	287/280
	H72	100	97	97, 100	96, 100	284/279
Itraconazole	H48	90	100	88, 100	91, 100	171/152
	H72	100	100	93, 100	100, 100	156/145

[a] Agreement (±1 dilution) between matching on-scale MIC pairs in 11 centers; CONV and COL, MICs determined by conventional and colorimetric (Alamar Blue) evaluation of growth inhibition, respectively.
[b] H, inoculum density, approximately 10^4 CFU/ml; 48 and 72, hours of incubation.
[c] CI, confidence interval.
[d] Number of on-scale MIC pairs.

from each laboratory determined by colorimetric and conventional examination of growth inhibition were considered to be in agreement when the differences between the two procedures were not greater than 3 dilutions. The confidence intervals and number of on-scale observations (MIC pairs) included in each calculation also are listed. The comparison of MICs determined by visual and colorimetric evaluations of growth inhibition demonstrated excellent (98 to 100%) agreement with amphotericin B after 48 and 72 h of incubation. Dependency on the length of incubation period was also noted with itraconazole, and the agreement levels were slightly lower (94 to 100%) than with amphotericin B. Although the itraconazole 72-h MIC pairs were more in agreement than the 48-h pairs, the best confidence interval corresponded to the 48-h readings with the high inoculum. Data with off-scale MICs yielded similar results (data not shown).

Intralaboratory comparison of duplicate MIC determinations. Each of the 30 duplicate pairs of isolates was tested in each center in a blinded fashion. The comparison of amphotericin B and itraconazole on-scale MIC pairs for the 30 isolates is represented in Table 5. The median percentages of agreement were obtained in the same manner described above for the comparison between the two procedures of MIC determination. The intralaboratory agreement was higher by colorimetric (97 to 100%) than by conventional (90 to 100%) determination of MICs with both drugs and after 48 to 72 h of incubation.

DISCUSSION

This present study was the second evaluation conducted by the NCCLS Subcommittee on Antifungal Susceptibility Testing. It was designed to continue the generation and analysis of data that will support the proposal of a standard method for susceptibility testing of filamentous fungi. The augmented frequency of fungal infections by established and emergent filamentous fungi in the immunocompromised host has triggered an interest in testing these pathogens in the clinical laboratory. In order to provide meaningful and reliable MIC data, clinical laboratories should follow standard testing guidelines. The development of a standard method for the antifungal susceptibility testing of filamentous fungi is then essential to achieve interlaboratory consensus of MICs as well as for establishment of their clinical relevance and application to patient responses to therapeutic antifungal management. Based on a prior study, antifungal susceptibility testing of these fungi can be performed by either the macro- or microdilution test methods (7). This second multicenter (11-center) project focused on the evaluation of two inoculum densities, three incubation times, and two procedures of MIC determination by the more convenient microdilution test. We evaluated amphotericin B and itraconazole, because they are the two drugs, among the six available antifungal agents, that are applicable to the treatment of infections caused by the six species included in the study.

Our study confirmed prior results and demonstrated an improvement in the reliability of the spectrophotometric procedure for preparation of conidium and sporangiospore suspensions. Over 92% of the inoculum sizes ranged between 0.4×10^6 and 5×10^6 CFU/ml, for all tested species except *P. boydii* (Table 1). Inoculum suspensions of *P. boydii* may require an approximately 50% lower dilution factor than those used for conidial and sporangiospore suspensions of the other five species. When antifungal activity is evaluated by using inoculum suspensions prepared by this method, the effect on conidium and sporangiospore germination rather than on cell growth is detected. However, preparation of hyphal suspensions is difficult, and the limited data suggest that most discrepancies, when present, between MICs determined with either hyphal, sporangiospore, or conidial suspensions of these species were within 3 dilutions (9). The exception was the comparison of MICs determined with germinated versus ungerminated sporangiospore suspensions of *R. arrhizus*. The former suspensions yielded higher (>3 dilutions) amphotericin B MICs.

As previously reported (7), RPMI 1640 medium supported adequate growth of the filamentous fungi evaluated in this study. However, the impact of such modifications as addition of glucose (16) as well as the suitability of RPMI 1640 for all organism-drug combinations will need to be assessed. Amphotericin B-resistant *Candida* isolates are more readily detected in Antibiotic Medium 3 (14), and MICs determined in this medium for the isolates evaluated in this study were higher for *P. boydii* (0.25 to 32 versus 0.5 to 4 μg/ml) and *F. solani* (1 to 8 versus 0.5 to 1 μg/ml) but lower for *R. arrhizus* isolates (<0.03 to 0.06 versus 0.25 to 1 μg/ml) (4). Although these elevated amphotericin B MICs are consistent with the well-known clinical and in vivo resistance of infections due to *Fusarium* spp. (1) and *P. boydii* (11, 19), variability among different lots of Antibiotic Medium 3 precluded its evaluation in the present study. The reproducibility of MICs determined with this or similar medium formulations is under further investigation by the NCCLS Subcommittee.

Our evaluation demonstrated higher percentages of interlaboratory agreement among the 11 laboratories with both drugs than those observed during the first multicenter study (six centers) (7). The percentage of interlaboratory agreement with amphotericin B ranged from 93 to 97% under all testing conditions in the present study, while the first study yielded 87 to 91% levels of agreement. Improvement of interlaboratory reproducibility was more evident with itraconazole and rose from 58 to 80% in the first study (7) to 89 to 95% under the same test conditions (high inoculum) in the present study (Table 3). The interlaboratory comparison indicated that the optimal procedure for evaluation of the antifungal activity of itraconazole is the colorimetric method, because it showed a higher percentage of agreement in comparison to the conventional method (Table 3).

In our two sets of intralaboratory comparisons, the percent agreement ranged from 97 to 100% with amphotericin B and 90 to 100% with itraconazole after 48 to 72 h of incubation between the two procedures of MIC determination (Table 4) and between matching MIC pairs for each isolate from the 11 centers (Table 5). These results were either similar (amphotericin B) or superior (itraconazole) to previous NCCLS in-

tralaboratory evaluations that involved comparisons of macro- and microdilution methods for the testing of yeasts (6, 10) or filamentous fungi (7). In addition, there was no evidence that data from a particular laboratory or any of the species were more in disagreement than the others, except with amphotericin B MICs for *P. boydii*. Higher intra- and interlaboratory agreement was shown at 72 h than at 48 h (9.3 to 95% versus 89%, respectively). However, this phenomenon was evident only when MICs were determined by the conventional method.

Because intra- and interlaboratory agreement of amphotericin B MICs was not dependent on any of the testing parameters evaluated, the optimal testing conditions for evaluating the antifungal activity of amphotericin B and itraconazole against five of the six species evaluated are determination of colorimetric MIC endpoints between 48 to 72 h of incubation with inoculum densities of approximately 10^4 CFU/ml. Examination of intra- and interlaboratory agreement across the species indicated that the exception was *R. arrhizus*. For this species, the optimal incubation time was 24 h, which reflects the rapid growth rate of many zygomycetes of medical importance. These identified optimal conditions are proposed as guidelines for a broth microdilution reference method for the antifungal susceptibility testing of this group of filamentous fungi. As for the yeasts, evaluation of the relationship between these in vitro results and outcome in vivo is vital, and the NCCLS Subcommittee is currently conducting such studies in animal models with a selected set of the isolates evaluated in this study.

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