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Comparative and Collaborative Evaluation of Standardization of Antifungal Susceptibility Testing for Filamentous Fungi

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The purpose of the study was to evaluate the interlaboratory agreement of broth dilution susceptibility methods for five species of conidium-forming (size range, 2 to 7 μm) filamentous fungi. The methods used included both macro- and microdilution methods that were adaptations of the proposed reference method of the National Committee for Clinical Laboratory Standards for yeasts (m27-P). The MICs of amphotericin B, fluconazole, itraconazole, miconazole, and ketoconazole were determined in six centers by both macro- and microdilution tests for 25 isolates of *Aspergillus flavus*, *Aspergillus fumigatus*, *Pseudallescheria boydii*, *Rhizopus arrhizus*, and *Sporothrix schenckii*. All isolates produced clearly detectable growth within 1 to 4 days at 35°C in the RPMI 1640 medium. Colony counts of 0.4×10^6 to 3.3×10^6 CFU/ml (mean, 1.4×10^6 CFU/ml) were demonstrated in 90% of the 148 inoculum preparations. Overall, good intralaboratory agreement was demonstrated with amphotericin B, fluconazole, and ketoconazole MICs (90 to 97%). The agreement was lower with itraconazole MICs (59 to 79% median). Interlaboratory reproducibility demonstrated similar results: 90 to 100% agreement with amphotericin B, fluconazole, miconazole, and ketoconazole MICs and 59 to 91% with itraconazole MICs. Among the species tested, the MICs for *S. schenckii* showed the highest variability. The results of the study imply that it may be possible to develop a reference method for antifungal susceptibility testing of filamentous fungi.

Despite the lower volume of serious infections caused by filamentous fungi compared with that of serious infections caused by yeasts, the performance of antifungal susceptibility testing for these opportunistic pathogens is important in the clinical laboratory (8). Progress has been made in developing guidelines for the antifungal susceptibility testing of yeasts to deal with the standardization of different testing parameters such as inoculum preparation, medium composition and pH, length of incubation, and endpoint criteria (3, 5, 6, 9–12). The standardization of these antifungal susceptibility testing steps has led to increased interlaboratory reproducibility which has opened the possibility of developing standards by adopting and tailoring these steps for antifungal susceptibility tests for the filamentous fungi. The first priority of the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests was to develop a standard for the preparation of inoculum suspensions. Among four procedures evaluated, a spectrophotometric method was recommended as the procedure that gives the least variable results for the preparation of inoculum suspensions of yeast cells (11). This recommendation has been substantiated by ensuing collaborative studies of the subcommittee and other studies (2, 3, 5, 6, 12).

The spectrophotometric method has been evaluated further in a single laboratory for the preparation of conidial suspensions of selected medically important filamentous fungi (1). In the study described here the investigators used different percent transmissions (*T*) for each species tested. It was reported that inoculum suspensions of 1×10^6 to 5×10^6 CFU/ml can be reliably prepared (*P* value for between-day variability values, ≥ 0.05) for isolates of *Aspergillus* spp., *Pseudallescheria boydii*, and *Sporothrix schenckii*. Although cell suspensions of isolates of *Mucor* spp. and *Rhizopus* spp. resulted in lower densities, the range in CFU of these suspensions per milliliter also was reliable and was sufficient to be used for the antifungal susceptibility testing of these filamentous fungi (1). Comparative studies between laboratories were needed to assess the utility of this method of inoculum preparation (4).

The present collaborative (six-center) study had a dual objective: the determination of interlaboratory variability of the spectrophotometric method for inoculum preparations of five species of conidium-forming (size range, 2 to 7 μm) filamentous fungi and the adaptation of the proposed NCCLS reference method for yeasts (m27-P), with certain modifications, for the broth antifungal susceptibility testing of these filamentous fungi. The intralaboratory comparison of broth macro- and microdilution tests as well as the interlaboratory agreement of each test were evaluated. Amphotericin B, fluconazole, itraconazole, ketoconazole, and miconazole were tested against five strains each of *Aspergillus flavus*, *Aspergillus fumigatus*, *P. boydii*, *Rhizopus arrhizus*, and *S. schenckii.*

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MATERIALS AND METHODS

Study design. Six laboratories, coded as laboratories 1, 2, 3, 4, 5, and 6, were able to participate in the study, and each laboratory received the same panel of 25 filamentous fungi and two quality control (QC) isolates from the Medical College of Virginia/Virginia Commonwealth University (Richmond, Va.) and the University of Texas (San Antonio, Tex.). Each isolate was tested in each of

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TABLE 1. *T* range and mean inoculum sizes for filamentous fungi in six laboratories

Fungus (no. tested)[a]	Conidium size range (μm)	*T* range (%)[b]	Inoculum (CFU/ml [10^6])	
			Range	Mean (SD)
A. flavus (5)	3–6	78–82	0.2–4.6	1.2 (0.7)
A. fumigatus (5)	2–3.5	80–82	0.4–3.2	1.5 (1.1)
S. apiospermum (*P. boydii*) (5)	5–7	68–71	0.2–2.9	1.1 (0.7)
R. arrhizus (*R. orizae*) (5)	5–7	68–71	0.6–4	1.1 (0.7)
S. schenckii (5)	2–3	80–82	0.9–5	2.4 (1.2)
QC isolates[c]				
C. parapsilosis (1)	2–4	80–82	1.1–4	2.2
P. variotii (1)		74–76	0.5–1.7	1.1

[a] Each isolate was tested at least once in each laboratory.
[b] Percent *T* at 530 nm.
[c] QC isolates were tested three to five times in each laboratory.

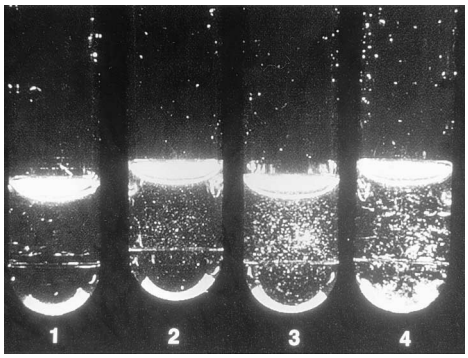

FIG. 1. Photograph "standards" of *A. fumigatus* growth controls. Tube 1, no growth; tube 2, suggested growth for scores of 1 (25% of growth control); tube 3, suggested growth for scores of 2 (50% of growth); tube 4, equal to drug-free growth control.

the participating centers with four of the five antifungal drugs by both broth macro- and microdilution antifungal susceptibility tests by following a standard protocol. Inoculum suspensions were prepared on at least one occasion in each laboratory. The standard protocol provided to each laboratory included the percent *T* ranges, from an earlier study (1), which were used to adjust the conidial suspensions of the isolates of each species and the two QC isolates (Table 1). Isolates of *P. boydii* were evaluated with miconazole in addition to the other drugs tested in each laboratory. The standard protocol was identical to the NCCLS reference method for yeasts (m27-P), with a slight modifications that included a higher inoculum size density and slightly different MIC endpoint criteria. The objectives of the study were (i) to determine the intralaboratory agreement between broth macro- and microdilution tests, (ii) to determine the interlaboratory reproducibility of both broth macro- and microdilution tests, and (iii) to determine the variability of broth macro- and microdilution tests when two different MIC endpoint scoring criteria were evaluated.

Antifungal drugs. The MICs of amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), fluconazole (Pfizer Pharmaceuticals, New York, N.Y.), and itraconazole, miconazole, and ketoconazole (Janssen Research Foundation, Beerse, Belgium) were determined by broth macro- and microdilution techniques. Itraconazole, miconazole, and ketoconazole were supplied to each participating laboratory by the manufacturer as standard powders of the same lot. Amphotericin B and fluconazole were distributed by the University of Texas as liquid suspensions and were received in each laboratory as 1,000- and 2,000-μg/ml drug concentrations, respectively. Itraconazole powder was dissolved in heated (75°C) polyethylene glycol for about 1 h to obtain stock drug suspensions of 5,000 μg/ml, with the weight adjusted according to the potency of the drug. Miconazole was received as a 10-mg/ml suspension.

Cultures. A set of 25 well-characterized clinical isolates of selected conidium-forming (size range, 2 to 7 μm) filamentous fungi was sent to each laboratory as coded isolates. The identification of each isolate was confirmed, prior to shipment, by the New York State Health Department (Albany, N.Y.). The 25 isolates included five isolates each of *A. flavus*, *A. fumigatus*, *Scedosporium apiospermum* (anamorph of *P. boydii*), *R. arrhizus* (synonym, *Rhizopus orizae*), and *S. schenckii*. The isolates of *R. arrhizus* belonged to the culture collection of the University of Texas, and the other isolates were from the collection at the Medical College of Virginia. Each isolate originated from a different patient and was maintained as a suspension in water at room temperature in each laboratory until testing was initiated. The isolate of *Candida parapsilosis* (ATCC 90018) used in previous NCCLS studies (3, 5) and an isolate of *Paecilomyces variotii* (ATCC 22319) also were provided to each laboratory and were included as QC isolates each time that any testing was performed by both methods and with each drug. Previously determined MICs of the different drugs tested for the two QC isolates ranged from 0.06 to 2 μg/ml for *C. parapsilosis* and from ≤0.25 to 2.5 μg/ml for *P. variotii*. These MIC endpoints were supplied to all of the laboratories before the study began.

Procedure. The standard protocol supplied to each laboratory included detailed instructions concerning the preparation of twofold drug dilutions and inoculum suspensions and the scoring of MIC tubes and wells. A set of photograph "standards" for each one of the species tested also was provided to aid in the reading and scoring of MIC tubes or wells. Figure 1 depicts the set used for the scoring of MICs for *A. fumigatus.*

(i) Medium. The same lot of liquid RPMI 1640 medium (RPMI medium; Whittaker Bioproducts, Inc., Walkersville, Md.) supplemented with both L-glutamine and 0.165 M MOPS (morpholinepropanesulfonic acid) buffer (34.54 g/liter) and without sodium bicarbonate and ready for use was provided to all participants. The pH of the medium was 7.0 ± 0.1 at 35°C. Sterility control of each bottle was performed prior to use.

(ii) Drug dilutions. Both broth macro- and broth microdilution tests were performed by following the standard additive twofold drug dilution schema described in the NCCLS reference method for yeasts (10). The drug dilutions for the broth macrodilution tests were prepared at 10 times the strength of the final test concentration, with medium used as the diluent (e.g., 640 to 1.25 μg/ml for fluconazole and 160 to 0.3125 μg/ml for amphotericin B, itraconazole, ketoconazole, and miconazole). For the microdilution test, each 10× drug dilution was further diluted 1:5 in medium to obtain the 2× strength used for the test.

(iii) Inoculum preparation. The isolates were grown from the stock water suspensions on potato dextrose agar (PDA) slants (Remel, Lenexa, Kans.) at 35°C. Each isolate was subcultured twice to ensure its viability. Inoculum suspensions of each filamentous fungal isolate were prepared for each experiment from fresh, mature (7-day-old) cultures grown on PDA slants (Remel). The isolate of *C. parapsilosis* was grown on the same agar for 24 h at 35°C. The fungal colonies were covered with approximately 1 ml of sterile 0.85% saline, and the suspensions were made by gently probing the colony with the tip of a Pasteur pipette. The resulting mixture (conidia and hyphal particles) was withdrawn and transferred to a sterile tube. Heavy particles of the suspensions (when they were present) were allowed to settle for 3 to 5 min, and the upper homogeneous suspensions were used for further testing.

For the spectrophotometric procedure, the turbidities of the conidial suspensions were measured with a spectrophotometer as described previously (1). The suspensions were mixed for 15 s with a vortex mixer, and their densities were read at 530 nm and were adjusted to the percent *T* ranges listed in Table 1 for isolates of each species tested.

Inoculum quantitation (each isolate) was performed in each laboratory by quantitative plating on modified Sabouraud glucose agar (SAB; Difco Laboratories, Detroit, Mich.) to determine the viable number of CFU per milliliter (1, 7). The adjusted suspensions were mixed for 15 s with a vortex mixer and were diluted 1:100, and 0.01-ml aliquots were spread onto SAB plates with a glass hockey stick. The plates were incubated at room temperature and were observed daily for the presence of fungal colonies. Colonies were counted as soon as possible after growth became visible, especially with isolates of *R. arrhizus*. The range of incubation times was 24 h or less (*R. arrhizus*) to 5 days (*P. boydii*) in the different laboratories.

For the preparation of the inoculum for the macrodilution test, the adjusted stock conidial suspensions were diluted 1:100 with RPMI medium to obtain the final desired inoculum size of approximately 0.5×10^4 to 5×10^4 CFU/ml. The conidial suspensions were mixed with a vortex mixer for 15 s prior to the dilution step. The test inoculum was made in a sufficient volume to directly inoculate each MIC tube with 0.9 ml.

For the preparation of the inoculum for the microdilution test, the adjusted stock conidial suspension was diluted 1:50 with RPMI medium to obtain approximately 0.2×10^5 to 1×10^5 CFU/ml or two times the final test inoculum. The cell suspension was mixed with a vortex mixer for 15 s before the dilution step.

(iv) Broth macrodilution test. The 10× drug dilutions were pipetted in 0.1-ml volumes into round-bottom, polystyrene, snap-cap, sterile tubes (12 by 75 mm; Falcon 2054; Becton Dickinson Labware, Lincoln Park, N.J.). Each tube was inoculated by adding 0.9-ml volumes of the corresponding well-mixed, diluted conidial suspension. This step diluted each drug to the final test concentrations (16 to 0.03125 μg/ml for amphotericin B, itraconazole, ketoconazole, and miconazole and 64 to 0.125 μg/ml for fluconazole). The growth control tube contained a 0.9-ml volume of inoculum suspension and a 0.1-ml volume of drug-free medium. Both QC isolates were tested in the same manner as that for the other isolates and were included each time that a set of isolates was tested in each laboratory with each drug. Sterility control was performed by including 1 ml of uninoculated, drug-free medium.

(v) Broth microdilution test. The broth microdilution tests were performed as described previously (2, 3) by using sterile, disposable, multiwell microdilution

plates (96 U-shaped wells; Dynatech Laboratories, Inc., Alexandria, Va.). The 2× drug concentrations were dispensed into the wells of rows 1 to 10 of the microdilution plates in 100-μl volumes with a multichannel pipette. Row 1 contained the highest drug concentration, and row 10 contained the lowest drug concentration. 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 and inoculum densities to the final desired test concentrations mentioned above. The growth control wells contained 100 μl of sterile drug-free medium and were inoculated with 100 μl of the corresponding diluted inoculum suspension. Both QC isolates also were included each time that a set of isolates was tested in each laboratory with each drug, as described above. The wells of row 11 contained 100 μl of uninoculated, drug-free medium and served as sterility controls.

(vi) Incubation and scoring of MIC tubes or wells. All tubes and plates were incubated at 35°C. Growth control tubes and wells were observed for the presence or absence of visible growth. When growth was visible, each tube was vortexed for 10 s immediately prior to being scored, which allowed the detection of small amounts of growth. The growth in each tube was compared with that of the growth control (drug-free) tube and the set of photograph "standards" (for each species tested) provided to each laboratory (Fig. 1). These photograph "standards" depicted the suggested growth corresponding to scores of 1 (25% of growth control; tube 2 of Fig. 1), 2, 3, and 4 (drug-free control; tube 4 of Fig. 1). The broth microdilution wells were scored with the aid of a reading mirror (Cooke Engineering Co., Alexandria, Va.); the growth in each well was also compared with that in the growth control (drug-free) well and that of the photograph "standards" mentioned above. Each tube or well was given a numerical score as follows: 0, optically clear or the absence of growth; 1, approximately 75% reduction in growth; 2, approximately 50% reduction in growth; 3, approximately 25% reduction in growth, and 4, no reduction in growth. Each laboratory recorded the scores for the MIC tubes or wells on standardized working sheets, which were mailed to the Medical College of Virginia, where the results were entered into a computer database.

Analysis of the data. The mean inoculum sizes were compared by species and site by an analysis of variance (ANOVA) procedure. In addition, each inoculum value was compared with the range of 0.4×10^6 to 3.3×10^6 CFU/ml. If the value was in this range it was categorized as in agreement; otherwise, it was considered not in agreement. Logistic regression techniques were then used to determine if differences in the agreement rate existed by site or species. *P* values of 0.05 were considered statistically significant.

Each of the 25 isolates was tested by both methods with four of the five drugs, and isolates of *P. boydii* were also tested with miconazole. For each isolate there was only one reading per test run, which was performed on the day that the growth control showed clearly detectable growth. Each MIC endpoint was determined in two ways by using a computer program (14), as follows: the lowest drug concentration (tube or well) which had a score of 1 (approximately 75% growth reduction or less) and that which had a score of 2 (approximately 50% growth reduction or less). For each *P. boydii* isolate 20 MIC endpoints per laboratory (including miconazole MICs) were determined; for the other isolates 16 MICs were determined by each laboratory.

Two analyses of the MIC data were performed: Both on-scale and off-scale MICs were included in the first analysis, and off-scale MICs were excluded for the second analysis. The high off-scale MICs (>64 and >16 μg/ml) were converted to the next highest concentration (either 128 or 32 μg/ml), and the low off-scale MICs (≤0.12 and ≤0.03 μg/ml) were converted to the next lowest concentration (either 0.06 or 0.001 μg/ml). When skips (uneven patterns) were present, the MIC endpoint was the higher drug concentration. Discrepancies between MIC endpoints of no more than 2 dilutions (two tubes or wells) were used to obtain the percent values of agreement. A measure of agreement was then determined as follows. For each combination of drug, isolate, and testing method the percentage of MIC endpoints within 2 dilutions was determined. The differences in these percentages for the macro- and microdilution methods for each drug-isolate combination were then calculated. If these two methods are equivalent, the differences in the percent agreement would be zero. A sign test was used to analyze if the median differences in the percent agreement were statistically different from zero for each drug-isolate combination.

RESULTS

Spectrophotometric procedure. One hundred forty-eight inoculum preparations were analyzed. The CFU-per-milliliter range for 90% of the inoculum size values was 0.4×10^6 to 3.3×10^6. Higher inoculum sizes (>3.3×10^6 CFU/ml) were obtained in laboratories 1, 2, and 4 with four isolates of *S. schenckii* and in laboratory 3 with one isolate of *A. fumigatus*. Lower values were found in laboratory 5 with two isolates of *A. flavus* (0.2×10^6 CFU/ml) and in laboratory 6 with one isolate of *P. boydii* (0.2×10^6 CFU/ml). The ranges, means, and standard deviations for inoculum size counts of each of the five species tested and the two QC isolates in the six centers combined are summarized in Table 1. The range of the mean values in the six centers was 1.1×10^6 CFU/ml for the isolates of *R. arrhizus* and *P. boydii* to 2.4×10^6 CFU/ml for *S. schenckii*, with an overall mean value of 1.4×10^6 CFU/ml (standard deviation, 1.0). The mean values for the multiple sets (three to five sets per laboratory) of inoculum sizes of the QC isolates were 2.2×10^6 CFU/ml for *C. parapsilosis* and 1.1×10^6 CFU/ml for *P. variotii.*

The intralaboratory (each center) and interlaboratory (among the centers) percent agreement of inoculum values that were within the range of 0.4×10^6 to 3.3×10^6 CFU/ml were obtained for each species tested. Overall, intralaboratory agreement was 90% or greater in each center with most of the species tested. Less than 80% agreement was seen in one of the six laboratories with isolates of *A. flavus* and *P. boydii* and with isolates of *S. schenckii* in two other centers. Interlaboratory agreement of 94 to 97% (within 0.4×10^6 to 3.3×10^6 CFU/ml) was observed with isolates of three (*A. fumigatus*, *P. boydii*, and *R. arrhizus*) of the five species tested. Lower percent agreement (80 to 83%) was observed with isolates of *A. flavus* and *S. schenckii*. The overall interlaboratory agreement for all the species combined was 90%.

In order to test for differences in the mean inoculum values by site and species, an ANOVA procedure was used. In addition to the main effects of site and species, an interaction was considered and was found to be significant ($P = 0.0001$). Therefore, the mean inoculum sizes differed by species, but the directions and magnitudes of the differences were dependent on the site. For each site separate ANOVA tests were run on each species. Differences in the means were found at each site. A Tukey post hoc test was then used to determine what differences were found. In general, at four of the six sites *S. schenckii* was found to have a mean inoculum size greater than that of at least one of the other species. At one other site, *P. boydii* had a mean inoculum size significantly greater than those of three of the other species. By using a logistic regression procedure no differences in the percent agreement between sites or species could be found at the 0.05 level.

Antifungal susceptibility. *Aspergillus* spp. and *R. arrhizus* produced clearly detectable growth at 24 h, and *P. boydii* and *S. schenckii* produced clearly detectable growth at between 3 and 4 days in RPMI broth. Both macro- and microdilution MIC endpoints were determined after those periods of incubation in the six laboratories. Macrodilution MIC results obtained by the MIC endpoint criterion for a score of 1 (75% growth reduction; MIC-1) are summarized in Table 2. Table 2 provides the modal MIC-1 (±1 dilution) as well as the MIC-1 range that encompassed 95% of the values for each species in the six centers. Itraconazole MICs were so spread out that it was not possible to obtain the modal MIC of this drug for any of the species tested. Macrodilution MIC-2 endpoints and microdilution MICs gave similar results (data not shown in Table 2).

Interlaboratory agreement of microdilution MIC endpoints. Table 3 represents the summary of interlaboratory agreement for broth microdilution MIC endpoints across the species for the 25 filamentous isolates tested. The results are stratified by antifungal agents and MIC scoring criteria. The values are the mean percentages of total MICs in agreement of the total microdilution MICs with off-scale MICs included; the mean percent values when off-scale MICs were excluded are listed in parentheses. For each set of MICs from the six centers, MICs were considered in agreement when they belonged in the largest MIC subset with a range of no greater than 2 (±1 dilution) dilutions. The interlaboratory comparison of MIC endpoints demonstrated 90 to 95% agreement among the six laboratories with four of the five drugs MIC-1 endpoints (all MICs includ-

TABLE 2. Macrodilution antifungal susceptibilities of five drugs against 25 filamentous fungi at six centers

Fungus (no. tested)	Broth macrodilution MIC-1 range (MIC-1 mode) (μg/ml)[a]				
	A	Fl	I	K	M
A. fumigatus (5)	0.25–1 (0.5)	64 (64)	0.03–8	2–4 (4)	ND
A. flavus (5)	0.5–16 (1)	64 (64)	0.03–1	0.5–16 (2)	ND
R. arrhizus (5)	0.25–2 (0.5)	64 (64)	0.12–16	1–16 (8)	ND
P. boydii (5)	0.5–16 (1)	2–32 (8)	0.03–1	0.03–2 (1)	0.06–1 (0.25)
S. schenckii (5)	0.5–16 (1)	64 (64)	0.03–16	0.25–4 (2)	ND

[a] A, amphotericin B; Fl, fluconazole; I, itraconazole; K, ketoconazole; M, miconazole. The MIC-1 range, encompasses 95% of the MICs; modal MIC-1, most frequent MIC; ND, not determined.

ed). The exceptions were itraconazole MIC-1 and MIC-2, which showed lower levels of agreement (80 and 64%, respectively) than the MICs of the other four drugs. Exclusion of off-scale MICs increased percentage (91 to 97% scored as MIC-1) of agreement values with four of the five drugs; the exception was fluconazole. In general, MICs scored as MIC-1 provided higher levels of agreement than MICs scored as MIC-2, with the exception of miconazole MICs, which had higher levels of agreement (93 to 100%) with MICs scored as MIC-2 than with MICs scored as MIC-1 (90 to 93%). However, the percentages of interlaboratory agreement by the two scoring criteria (scores of 1 and 2) were similar, and the differences were not statistically significant, as demonstrated by *P* values of 0.15 or greater (four of five drugs).

Interlaboratory agreement of macrodilution MIC endpoints. Table 3 also summarizes the interlaboratory reproducibilities for the broth macrodilution MICs. The mean percentages of agreement were obtained in the same manner described above for the microdilution MICs. The pattern of reproducibility for the macrodilution MICs was similar to that for the microdilution MICs, but the differences between the results of the two scoring criteria were smaller by the macrodilution test. The percentage of agreement (within 2 dilutions) was the same or very similar for broth macrodilution MICs scored as MIC-1 and MIC-2 with four of the five antifungal agents tested (itraconazole was the exception). Fluconazole, ketoconazole, and miconazole MICs showed the highest percentage of interlaboratory agreement (90 to 100%) by the macrodilution test among the six laboratories, and itraconazole showed the lowest percentage of interlaboratory agreement (58 to 74%). The percentage of interlaboratory reproducibility was slightly higher by the macrodilution test than by the microdilution test with fluconazole (MIC-1 and MIC-2) and was slightly higher by the microdilution test with amphotericin B, itraconazole, and ketoconazole with MIC-1. An analysis of the differences in the percent agreement, however, found no significant differences between the macrodilution and microdilution methods. This result is consistent when the off-scale values are included and when they are excluded.

Comparison of micro- and macrodilution methods. Two thousand four hundred eighty-eight micro- and macrodilution MICs were used for the intralaboratory comparison of pairs of MICs for the 25 filamentous isolates tested. MIC pairs were in agreement when the differences between the two MIC results were within 2 dilutions. The comparison of macro- and microdilution MIC pairs (all MICs included), when all species and centers were combined, demonstrated good intralaboratory agreement (96 to 97%) with amphotericin B and fluconazole (Table 4). The agreement was lower (67 to 84%) with itraconazole, miconazole, and ketoconazole (all MICs included). As with other comparisons, the agreement between macro- and microdilution MICs was optimized by using the MIC-1 endpoint criterion and excluding off-scale MICs.

Interlaboratory agreement for the QC isolates. Each time that a set of MICs (three to five in each laboratory) was determined by either test, both QC isolates were included as controls of drug activity. A total of 240 MIC endpoints were analyzed for the two QC isolates with the five drugs by both tests. MICs were within previously demonstrated ranges with the five drugs for *C. parapsilosis* (3, 5) in each laboratory and for *P. variotii* (13) in most laboratories. In general, ≥95% of the MICs for each drug-organism combination could be encompassed by a 3- to 4-dilution range (Table 5). Two of the six centers reported higher (up to 3 dilutions) fluconazole and itraconazole microdilution MICs than the other four centers for *P. variotii* (8 to 64 and 8 μg/ml, respectively) (Table 5).

DISCUSSION

The purpose of the present study was to evaluate the adaptation of the proposed NCCLS reference method for yeasts (m27-P) for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. It was conducted as a collaborative multicenter (six-center) study to initiate the development of a reference method for these fungi. The reference method testing conditions that were adopted included buffered (MOPS) RPMI medium, the drug dilution schema, and the 35°C incubation temperature (10). The following steps of the reference method were modified and tailored for the filamentous fungi: (i) the inoculum suspensions were adjusted by the spectrophotometric method, but different percents *T* (Table 1) and inoculum densities were used, and (ii) the tubes or wells were scored in the same manner but photograph "standards" (Fig. 1) were introduced to aid in their scoring. The study evaluated the antifungal agents that are considered important as treatments for infections with the filamentous fungi tested, that is, amphotericin B, itraconazole, and ketoconazole. In addition, miconazole was evaluated against iso-

TABLE 3. Interlaboratory agreement for broth macro- and microdilution antifungal tests for 25 filamentous isolates

Drug	Mean % agreement with all MICs included (with off-scale MICs excluded)[a]			
	Micro		Macro	
	1	2	1	2
Amphotericin B	91 (97)	87 (94)	87 (93)	91 (96)
Fluconazole	95 (92)	95 (83)	100 (100)	97 (98)
Itraconazole	80 (91)	64 (71)	74 (83)	58 (71)
Ketoconazole	90 (96)	84 (91)	89 (93)	87 (90)
Miconazole	90 (93)	93 (100)	90 (100)	90 (100)

[a] Agreement is defined as the largest MIC subset within 2 dilutions and is based on 2,488 MIC endpoints in six laboratories. Micro and macro, micro- and macrodilution MICs, respectively. Scores were as follows: 1, approximately 75% reduction in growth; 2, approximately 50% reduction in growth.

TABLE 4. Intralaboratory comparison of broth micro- and macrodilution antifungal tests

Drug	Mean % agreement with all MICs included (with off-scale MICs excluded)[a]	
	1	2
Amphotericin B	97 (97)	90 (93)
Fluconazole	96 (97)	92 (86)
Itraconazole	67 (79)	64 (59)
Ketoconazole	84 (90)	77 (83)
Miconazole	77 (79)	80 (94)

[a] MIC endpoints were scored as 1 (approximately 75% reduction in growth) and 2 (approximately 50% reduction in growth). Agreement between macro- and microdilution MIC endpoint pairs in six laboratories was within 2 dilutions.

TABLE 5. Tentative QC limits for five antifungal drugs in six laboratories

Drug	Species[a]	MIC QC range (μg/ml)[b]	
		Micro MIC-1	Macro MIC-1
Amphotericin B	c.p.	0.25–0.5	0.25–1
	p.v.	0.5–1	0.25–1
Fluconazole	c.p.	0.25–4	0.25–1
	p.v.	4–64	0.5–4
Itraconazole	c.p.	0.03–1	0.03–0.5
	p.v.	0.03–4	0.03–1
Miconazole	c.p.	0.06–0.12	0.12–2
	p.v.	0.03–0.25	0.03–0.25
Ketoconazole	c.p.	0.03–0.12	0.06–0.5
	p.v.	0.06–0.5	0.06–0.5

[a] The Q.C. isolates (c.p., *C. parapsilosis* ATCC 90018; p.v., *P. variotii* University of Texas) were tested with each drug and by both tests 12 times in the six laboratories.

[b] The MICs encompass 95% of the values. MIC-1, approximately 75% reduction in growth by the broth microdilution (micro) and broth macrodilution (macro) methods.

lates of *P. boydii* and fluconazole was also tested against the 25 isolates.

The inoculum sizes of the stock conidial suspensions for the 25 isolates ranged between 0.4×10^6 and 3.3×10^6 CFU/ml for 90% of the cell suspensions in the six laboratories (Table 1). This range of inoculum densities was only slightly lower than the expected range (0.8×10^6 to 5×10^6 CFU/ml) reported before for similar conidium-forming filamentous species determined by the spectrophotometric method (1). The activity being measured by using these inoculum suspensions (predominantly dormant conidial cells) is the antifungal effect on conidial germination rather than on cell growth. However, previous antifungal susceptibility testing performed with inoculum suspensions of both the conidial and the hyphal invasive phases of some filamentous fungi has demonstrated a good correlation between the two phases (7). In addition, inoculum suspensions of *S. schenckii* isolates may need a higher dilution factor for antifungal susceptibility testing than those required by cell suspensions of the other four species.

Buffered RPMI medium supported adequate growth of the filamentous fungi tested and produced detectable growth at 24 h for isolates of *Aspergillus* spp. and *R. arrhizus* and between 3 and 4 days for isolates of *P. boydii* and *S. schenckii*. Previous evaluations of this medium for the yeasts indicated that visible growth was obtained at 24 h for *Candida* spp. and *T. glabrata* and at 48 to 72 h for *C. neoformans* (3, 5). Therefore, RPMI seems to be an adequate medium for the susceptibility testing of both yeasts and filamentous fungi.

The persistent partial inhibition that is observed with the azoles was accounted for by using endpoint criteria that approximated 75% inhibition of growth relative to that of the growth control. The evaluation of these less stringent criteria (versus the complete absence of growth) has been addressed in previous studies of the NCCLS subcommittee for the determination of MIC endpoints (3, 5). In those studies an 80% inhibition endpoint was used. This less stringent criterion provided higher interlaboratory reproducibility and a shift in the MIC distribution toward lower drug concentrations for *Candida albicans* and *Candida tropicalis*, especially with the azoles (3, 5). Our evaluation of the two scoring criteria for filamentous fungi indicated higher reproducibilities with MICs scored as MIC-1 by both methods (Table 3 and 4). The MIC-1 endpoint in the present study corresponded to approximately ≥75% inhibition. Therefore, our findings with the filamentous fungi corroborated the results obtained with the yeasts.

The percent reproducibility shown with amphotericin B and fluconazole among the laboratories was similar to that observed previously in testing yeasts-fungi (3, 5). Ketoconazole and miconazole MICs demonstrated ≥90% interlaboratory agreement by both macro- and microdilution tests for the isolates tested with these two drugs. The data for itraconazole were more variable by both tests. The reason for the higher variability of itraconazole MIC data is unknown at present but may be due in part to the poor solubility of this drug in the medium used for susceptibility testing.

Prior NCCLS comparisons of macro- and microdilution pairs for the yeasts demonstrated good intralaboratory agreement for amphotericin B and fluconazole (2, 3). In our evaluation with molds-fungi, agreements between macro- and microdilution tests were 96 and 97% (higher than for the yeasts) with fluconazole and amphotericin B, respectively. The agreements with ketoconazole (84%), miconazole (77%), and itraconazole (67%) were somewhat lower but nevertheless offered the promise that filamentous fungi could be tested by the microdilution test. Further evaluations should be conducted in order to achieve higher intra- and interlaboratory agreement; this should be followed by the needed in vitro versus in vivo correlations of drug efficacy.

To summarize, our study suggests that it may be possible to develop a reference method for antifungal susceptibility testing of the filamentous fungi. The optimal testing conditions seem to be comparable to the testing parameters for yeasts. Similar to yeast testing, less stringent MIC endpoint criteria for molds (≥75% inhibition relative to growth control) resulted in improved interlaboratory agreement. The less stringent (75%) inhibition criterion was necessary to allow for the trailing frequently seen with the azole antifungal agents. Our study also indicates that antifungal susceptibility testing of the filamentous fungi may be performed by either the macro- or the microdilution test, as was demonstrated for yeast cells. It is hoped that further collaborative evaluations will refine the steps of antifungal susceptibility tests of the filamentous fungi in order to improve their reproducibilities among the laboratories, especially with itraconazole. In vivo and in vitro correlations of drug efficacy also are needed.

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