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Optimal Susceptibility Testing Conditions for Detection of Azole Resistance in *Aspergillus* spp.: NCCLS Collaborative Evaluation

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The most important role of susceptibility testing is to identify potentially resistant isolates for the agent being evaluated. Standard testing guidelines recently have been proposed for antifungal susceptibility testing of filamentous fungi (molds). This collaborative (eight centers) study evaluated further newly proposed guidelines (NCCLS, proposed standard M38-P, 1998) and other testing conditions for antifungal susceptibility testing of *Aspergillus* spp. to itraconazole and three new triazoles, posaconazole (SCH56592), ravuconazole (BMS-207147), and voriconazole. MICs of itraconazole, posaconazole, ravuconazole, and voriconazole for 15 selected isolates of three species of *Aspergillus* (*A. fumigatus, A. flavus*, and *A. terreus*) with well documented in vitro, clinical, or animal data were determined in each center by using four medium formulations (standard RPMI-1640 [RPMI], RPMI with 2% dextrose, antibiotic medium 3 [M3], and M3 with 2% dextrose) and two criteria of MIC determination (complete [MIC-0s] and prominent [MIC-2s] growth inhibition) at 24, 48, and 72 h. The highest reproducibility (92 to 99%) was seen with the standard RPMI and M3 media. Moreover, the distinction between itraconazole-resistant (MICs of >8 μg/ml for clinically resistant strains) and -susceptible (MICs of 0.03 to 1 μg/ml) isolates, as well as between a voriconazole-resistant laboratory mutant and other isolates (voriconazole MICs of 2 to >8 versus 0.12 to 2 μg/ml), was more consistently evident with the standard RPMI medium and when MIC-0s were determined at 48 h. These results provide further refinement of the testing guidelines for susceptibility testing of *Aspergillus* spp. and warrant consideration for inclusion in the future NCCLS document M38-A.

Among the filamentous fungi (molds), *Aspergillus fumigatus* is responsible for the majority (85 to 90%) of the different clinical manifestations of infections caused by these organisms (2, 4, 17, 18). However, other *Aspergillus* spp. have also been associated with severe infections in the immunocompromised host (4, 14–17, 27–29, 31) and only two antifungal agents are available for the systemic treatment of these infections (6, 12). With the increased incidence of fungal infections and the growing number of new antifungal agents, the laboratory role in the selection and monitoring of antifungal therapy has gained greater attention. The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has proposed reproducible reference testing conditions (NCCLS document M38-P) for the antifungal susceptibility testing of filamentous fungi (21). As a result of two collaborative studies (9, 10), agreement within the subcommittee was achieved regarding the following standard conditions: the use of RPMI-1640 (RPMI) broth and nongerminated conidial inoculum suspensions of approximately 10^4 CFU/ml, with incubation at 35°C for 24 h (*Rhizopus* spp.), 48 h (*Aspergillus* spp., *Fusarium* spp., and other opportunistic filamentous fungi), or 72 h (*Pseudallescheria boydii* [*Scedosporium apiospermum*]), and ≥50% growth inhibition as the criterion for azole MICs. Although an additional study has indicated some degree of correlation between in vitro test results by this method and response to treatment in animal models (25), the results of that study were inconclusive because in vivo experimental conditions could not be established for *Fusarium* spp. and *P. boydii*. Also, the differences of MIC endpoints were narrow for strains infecting animals that either responded or did not respond to treatment (25). Thus, the clinical value of the NCCLS M38-P method for mold testing needs to be established.

Denning et al. (4) have reported high itraconazole MICs (>16 μg/ml) that were determined by non-NCCLS methods for three isolates of *A. fumigatus* from two patients who did not respond to therapy for invasive aspergillosis. In vitro resistance was validated in a neutropenic murine model by these authors. Currently, the concern is whether the M38-P method can clearly distinguish among mold isolates that are potentially resistant or susceptible to itraconazole. The purpose of this third collaborative study was dual: (i) to assess the interlaboratory (eight centers) agreement of MICs obtained for each isolate-drug-testing condition combination and (ii) to describe the correlation between MICs and in vivo data for isolates with recognized susceptible or resistant nature among the 15 *Aspergillus* isolates. The drugs evaluated were the reference agent itraconazole and three investigational agents, posaconazole (SCH56592), ravuconazole (BMS-207147), and voriconazole.

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TABLE 1. Selected isolates of *Aspergillus* spp. used in this study

Species or study isolate no.	Source no.	Provider (reference no.)
A. fumigatus		
1	NCPF 7101 (AF210)[a,b]	D. Denning (4, 5)
2	NCPF 7102 (AF294)[a,b]	D. Denning (4)
3	NCPF 7098 (AF71)[a,b,c]	D. Denning (4, 24)
4	NCPF 7097 (AF65)[a,b]	D. Denning (4, 24)
5	NCPF 7100 (AF91)[c,d]	D. Denning (4, 5, 24)
6	NCPF 7099 (AF72)[d]	D. Denning (4, 5)
7	AZ 606[e]	P. Verweij (NA[f])
8	NIH 4215[d]	T. Walsh (NA)
9	A 1604[a,b]	A. Espinel-Ingroff (10, 25)
A. flavus		
10	A 1340[a]	A. Espinel-Ingroff (7)
11	A 830[a]	A. Espinel-Ingroff (7)
12	A 003[a,b]	A. Espinel-Ingroff (7, 10, 25)
A. terreus		
13	A 122[a]	A. Espinel-Ingroff (7)
14	NIH 961290[a]	T. Walsh (NA)
15	NIH 95644[a]	T. Walsh (NA)

[a] Low itraconazole MICs (≤1.0 μg/ml) by M38-P, Etest, 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 a 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-resistant laboratory mutant.
[f] NA, not available.

MATERIALS AND METHODS

Study design. Each of the eight independent laboratories received the same panel of 15 coded (1 to 15) isolates of *Aspergillus* spp. (Table 1) and two control isolates. Each isolate was tested in each of the participant centers with itraconazole, posaconazole, ravuconazole, and voriconazole by the broth microdilution method following a standard protocol. This protocol included the susceptibility testing guidelines described in the NCCLS M38-P document (21) and a detailed description of the additional testing parameters to be evaluated: (i) four medium formulations, (ii) three incubation times (24, 48, and 72 h), and (iii) two criteria for MIC determination (100 and 50% growth inhibition). The objectives of this study were (i) to determine the reproducibility of MICs obtained by the different testing conditions; (ii) to determine the variability of the broth microdilution test when four medium formulations, three different incubation times, and two criteria of MIC determination were examined; and (iii) to determine which of these testing conditions better correlated with the clinical, animal, and laboratory data available for the isolates tested.

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 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 *Aspergillus flavus* ATCC 204304 (25) and the quality control (QC) strain of *Candida parapsilosis* ATCC 22019 were included as controls. For the *C. parapsilosis* ATCC 22019 strain, there are well-established microdilution MIC ranges of the four 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 a prior study (10), and these values are listed in the M38-P document (21). MIC ranges for the QC and reference isolates were within established values (1, 10, 21).

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

(i) Medium formulations. A single lot of each of the four liquid medium formulations was provided ready to use to all participants by Trek Diagnostic Systems Inc. (Westlake, Ohio). The same lot of each medium was used for the preparation of the drug dilutions. Each batch of standard RPMI and RPMI–2% dextrose (RPMI-2%) was supplemented with 0.3 g of L-glutamine per liter and 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (34.54 g/liter) to pH 7.0 ± 0.1 at 35°C and did not contain sodium bicarbonate. Neither the batch of antibiotic medium 3 (M3) nor of M3–2% dextrose (M3-2%) was buffered and the pH of both media was 7.0 ± 0.1. Each batch of the four media was checked for sterility and pH prior to use in each laboratory.

(ii) Drug dilutions. The four antifungal agents, itraconazole (Janssen Pharmaceutica, Titusville, N.J.), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), ravuconazole (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn.), and voriconazole (Pfizer Pharmaceuticals, New York, N.Y.), were provided by the manufacturers as assay powders. As described in the NCCLS M38-P document (21), additive drug dilutions were prepared at 100 times the final concentrations in 100% dimethyl sulfoxide or 200-molecular-weight polyethylene glycol (posaconazole) followed by further dilutions (1:50) in each of the corresponding media to yield two times the final strength required for the test. Microdilution plates (96 U-shaped wells) containing antifungal dilutions were prepared in a central facility (Trek Diagnostic Systems) and shipped frozen to each participant. Rows 1 to 11 contained the series of drug dilutions in 100-μl volumes; row 12 contained 100 μl of drug-free medium and served as the growth control. QC testing of the prepared microdilution trays was performed prior to the initiation of the study by testing one to two isolates of each species evaluated and both QC and reference isolates *C. parapsilosis* ATCC 22019 and *A. flavus* ATCC 204304 in one of the participant laboratories (Espinel-Ingroff, Medical College of Virginia) and in the central (Trek) facility with *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

(iii) Inoculum preparation. Stock inoculum suspensions were prepared as described in the NCCLS M38-P document (21). Briefly, conidia formation was induced by growing the isolates on potato dextrose agar slants (provided by Remel, Lenexa, Kans.) at 35°C for 7 days. Seven-day-old colonies were covered with approximately 1 ml of sterile saline (provided by Trek Diagnostic Systems) containing 1% Tween 80, and the conidia were harvested by probing the colonies with the tip of a Pasteur pipette. The resulting mixture of mostly nongerminated conidia was transferred to a sterile tube and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (80 to 82% transmittance). These suspensions were diluted 1:50 in each of the four media, which corresponded to two times the density needed for the test of approximately 0.4×10^4 to 5×10^4 CFU/ml.

Inoculum quantification was performed by each laboratory by plating 0.01 μl of a 1:100 dilution of the adjusted inoculum on modified Sabouraud dextrose agar plates (provided by Remel) to determine the viable number of CFU per milliliter. The plates were incubated at 28 to 30°C and were observed for the presence of fungal colonies. The actual stock inoculum suspensions 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 some isolates of *A. flavus, A. fumigatus*, and *Aspergillus terreus*.

(iv) Broth microdilution method (from M38-P document). On the day of the test, each microdilution well containing 100 μl of the diluted (two times) drug concentrations was inoculated with 100 μl of the diluted (two times) conidial inoculum suspensions (final volume in each well was 200 μl). Growth and sterility controls were included for each isolate tested. As described above, *C. parapsilosis* ATCC 22019 and *A. flavus* ATCC 204304 were tested in each laboratory during each time that a set of isolates was evaluated 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. MIC determinations required the visual examination of growth inhibition. The growth in each MIC well was compared with that of the growth control with the aid of a reading mirror. Two MIC endpoints were determined in each laboratory for each isolate and the testing condition was evaluated for the lowest drug concentration that showed prominent reduction of the growth control in the control well (≥50%, or MIC-2) and the lowest drug concentration that showed absence of growth or complete growth inhibition (100% inhibition, or MIC-0).

Analysis of the data. For each species, the overall means and standard errors of the mean for the inoculum sizes were compared. In addition, the percentage of inoculum densities that were within the range of 0.4×10^6 to 5×10^6 CFU/ml was calculated. Both on-scale and off-scale MICs were included in the analysis. As previously analyzed (9, 10), discrepancies between MIC endpoints of no more than 3 dilutions (three wells) from the eight centers were used for the calculation of the percent values of agreement. A measurement of agreement was then determined as the percentage of MIC endpoints within 3 dilutions (i.e., 0.25, 0.5,

TABLE 2. Interlaboratory agreement on broth microdilution antifungal susceptibility for *Aspergillus* spp.[a]

Antifungal agent and MIC criterion	% Agreement [all isolates (itraconazole-susceptible isolates)]			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole				
MIC-0	90 (92)	92 (94)	90 (91)	85 (85)
MIC-2	83 (89)	88 (94)	84 (92)	88 (97)
Posaconazole				
MIC-0	91 (91)	88 (87)	94 (94)	86 (86)
MIC-2	96 (96)	95 (96)	93 (99)	91 (96)
Ravuconazole				
MIC-0	93 (95)	87 (85)	94 (95)	88 (89)
MIC-2	88 (89)	90 (89)	85 (86)	81 (82)
Voriconazole				
MIC-0	97 (97)	98 (99)	99 (100)	93 (92)
MIC-2	88 (96)	94 (95)	90 (92)	96 (96)

[a] Species tested were *A. fumigatus* ($n = 9$), *A. flavus* ($n = 3$), and *A. terreus* ($n = 3$); 48-h MICs are shown.

and 1.0 μg/ml) for each combination of drug, isolate, medium, incubation time, and criterion of MIC determination. The differences in these percentages for each variable and with each drug-isolate pair were calculated. In addition, MICs for each isolate with either clinical or animal data were examined against these data. Comparisons of proportions were performed by chi-square test or Fisher's exact test as appropriate. A two-tailed P value of <0.05 was considered to be significant.

RESULTS

Total MICs evaluated. Each isolate had three incubation time readings with all of the four antifungal agents and by each of the six other testing conditions evaluated (four media and two criteria of MIC determination) in all of the eight participant centers. A total of 96 MICs per isolate in each center, or 768 MICs from the eight centers, were reported; a total of 11,520 MICs were evaluated for the 15 isolates.

Effect of incubation time on MICs. A total of 3,840 MIC pairs for the 15 isolates were compared for the evaluation of the effect of incubation time on the in vitro values. A major increase (3 to 7 dilutions) in MICs was observed between 24 and 72 h with the four agents. Itraconazole MICs increased no more than 3 dilutions between 24 and 48 h for most of the 15 isolates tested; the exceptions were the MICs for the two itraconazole-resistant isolates of *A. fumigatus* (from 0.5 to 2 μg/ml to >8 μg/ml) in four centers. Voriconazole 48-h MICs were either the same as the 24-h results or increased within no more than 3 dilutions, including the values for the two itraconazole-resistant isolates. Similar results were observed with posaconazole; i.e., only MICs for three itraconazole-susceptible *A. fumigatus* were more than 3 dilutions higher at 48 h than at 24 h. For ravuconazole, higher values (3 to 6 dilutions) were obtained at 48 h than at 24 h when testing was performed with both M3 broths. MICs of the four agents were essentially the same after 72 h of incubation as those obtained at 48 h for the four agents. Exceptions to this observation were the values of >8 μg/ml for susceptible isolates.

Interlaboratory agreement. (i) Among the four media. Table 2 summarizes the percentages of interlaboratory agreement for 48-h MICs of the four triazoles obtained with the four media for the 15 isolates by the two criteria of MIC determination. Overall, RPMI demonstrated the best interlaboratory reproducibility. Media containing 2% dextrose had the lowest agreement among the laboratories. All four media supported good growth for MIC determination for all isolates tested after 48 h of incubation. After 24 h, one (both M3 broths) to six (both RPMI broths) readings were scored as 0 (for no growth), especially when testing *A. terreus*. Lower interlaboratory agreement (85 to 92%) was observed among MICs-0s of all four agents with either M3-2% or RPMI-2% than those with the other two formulations (90 to 100%) (Table 2). In general, the differences between the percentages of agreement of MICs obtained with RPMI-2% and M3-2% were drug and growth inhibition criterion dependent as follows: 5 to 9% lower agreement was observed for itraconazole and voriconazole MIC-0s and 4 to 9% lower agreement was observed for posaconazole and ravuconazole MIC-2s with M3-2%. The other percentages of agreement were no more than 3% different. A similar pattern was observed for 24- and 72-h MICs.

(ii) Agreement for the three incubation times. The agreement among the centers was lower (68 to 97%) when 24-h MICs were compared than when those obtained after 48 and 72 h of incubation (81 to 100%) for all the drugs and other testing parameters were evaluated ($P = 0.001$).

(iii) Agreement for the two criteria of MIC determination. Overall, the interlaboratory agreement was higher for MIC-0s than for MIC-2s for three of the four agents with the four media (Table 2). The differences were more evident between MICs with standard RPMI and conventional M3 (5 to 10% difference for 9 of the 12 percentage pairs) than between MICs with the other two formulations (5 to 9% difference for 3 of the 12 percentage pairs). The exceptions were posaconazole MICs, for which a better interlaboratory agreement was seen with MIC-2 endpoints (91 to 99%) than with MIC-0s (86 to 94%).

Interlaboratory agreement for itraconazole MICs. The interlaboratory agreement for itraconazole MICs for the 12 susceptible isolates ranged from 91 to 94% after the three incubation times with both RPMI and conventional M3. The agreement decreased 1 to 2% when MICs for the three resistant isolates were added to the evaluation of the 48-h values (Table 2). The reason was that the interlaboratory agreement was poor for itraconazole MICs for the voriconazole-resistant laboratory mutant.

Interlaboratory agreement for MICs of the investigational triazoles. Higher interlaboratory agreement (90 to 100%) was observed for voriconazole MICs at both 48 and 72 h than for posaconazole (86 to 99%) and ravuconazole (81 to 97%). The exception was the lower agreement (88%) for voriconazole 48-h MIC-2s with standard RPMI (Table 2). The interlaboratory agreement of ravuconazole and posaconazole MICs for the two itraconazole-resistant isolates was good only with the standard RPMI, as demonstrated by the narrower MIC-0 ranges, while the four media provided narrow voriconazole MIC ranges for these two isolates (see Tables 4 to 6).

Optimal testing conditions. Although good to excellent agreement (>90%) was found for the four agents with some of the various combinations of media, criteria of MIC determination, and incubation times, the testing conditions that better allowed the eight centers to identify the two itraconazole-

TABLE 3. Itraconazole MICs for *Aspergillus* spp. in eight laboratories[a]

Isolate(s) (no. tested) and MIC criterion	MIC range (MIC_{90} or modal MIC)[b]			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole susceptible (12)				
MIC-0	0.03–2 (1)	0.03–4 (0.5)	0.06–>8 (1)	0.06–>8 (2)
MIC-2	0.01–1 (0.2)	<0.008–0.5 (0.12)	<0.008–1 (0.2)	0.01–1 (0.2)
Itraconazole resistant (isolate no. 5)				
MIC-0	8–>8 (>8)	8–>8 (>8)	2–>8 (>8)	2–>8 (>8)
MIC-2	0.06–>8 (2)	0.06–>8 (1)	<0.008–>8 (1)	<0.008–>8 (1)
Itraconazole resistant (isolate no. 6)				
MIC-0	1–>8 (>8)	1–>8 (>8)	2–>8 (>8)	1–>8 (>8)
MIC-2	0.12–>8 (1)	0.12–>8 (0.5)	1–>8 (1)	0.2–>8 (1)
Voriconazole-resistant mutant (1)				
MIC-0	0.5–>8 (2)	0.5–>8 (0.5)	1–>8 (>8)	1–>8 (>8)
MIC-2	0.06–0.5 (0.2)	0.03–0.5 (0.12)	0.06–0.5 (0.2)	0.01–0.2 (0.06)

[a] Species tested were *A. fumigatus* ($n = 9$), *A. flavus* ($n = 3$), and *A. terreus* ($n = 3$); see Table 1 for reported data regarding isolates 5 and 6; 48-h MICs are shown.
[b] MICs are reported in micrograms per milliliter; the MIC_{90} is reported for susceptible isolates and the modal MIC is reported for single resistant isolates.

resistant isolates of *A. fumigatus* were (i) the 100% growth inhibition criterion (MIC-0), (ii) the standard RPMI broth, and (iii) 48 to 72 h of incubation. Fifteen of the 16 MIC-0s were >8 μg/ml with each RPMI formulation for these two isolates from the eight laboratories, while by the other parameter combinations 2 to 11 of the 16 MICs were ≤2 μg/ml. The itraconazole MIC-0 of 1 μg/ml with each formulation of RPMI for one of the two resistant isolates (isolate 6; Table 1) was reported from the same laboratory. Furthermore, 8 itraconazole MIC-0s were ≥8 μg/ml with both M3 and RPMI-2% broths for itraconazole-susceptible isolates at 48 h (Table 3) and 21 values were ≥8 μg/ml after 72 h of incubation with all four media. Similar results were observed for the other agents (Tables 4 to 6). The low susceptibility of the voriconazole-resistant laboratory mutant to most of these agents was also better detected with MIC-0 endpoints with the four media after 48 h of incubation.

Interlaboratory agreement for the QC and reference isolates. The MIC ranges of the four triazoles for the QC isolate *C. parapsilosis* ATCC 22019 were within the recently established ranges (1) for each triazole in each of the laboratories. Table 7 provides the MIC data for the reference isolate *A. flavus* ATCC 204304. The itraconazole reference MIC-2 range was 0.12 to 0.5 μg/ml in a previous collaborative study by the NCCLS Subcommittee on Antifungal Susceptibility Tests (10). The MIC-2 range for the present study was 0.06 to 0.2 μg/ml (only 1 dilution lower than the reference range), and the MIC-0 range was 0.2 to 0.5 μg/ml; both ranges are within the 3-dilution limit allowed by the NCCLS for QC ranges.

Antifungal activities of the four triazoles. Tables 3, 4, 5, and 6 depict the 48-h MIC results of the four agents by the other testing parameters for the 12 itraconazole-susceptible strains and the 2 resistant strains as well as for the voriconazole-resistant laboratory mutant. A bimodal itraconazole MIC-0 pattern (0.5 to 2 and 4 to >8 μg/ml) was reported from the participant laboratories after 48 and 72 h of incubation for the voriconazole-resistant mutant; MIC-0s of >8 μg/ml were more frequently reported when the two M3 broths were used.

TABLE 4. Posaconazole (SCH56592) MICs for *Aspergillus* spp. in eight laboratories[a]

Isolate(s) (no. tested) and MIC criterion	MIC range (MIC_{90} or modal MIC)[b]			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole susceptible (12)				
MIC-0	0.01–0.5 (0.2)	0.03–2 (0.2)	0.03–0.5 (0.2)	0.03–>8 (0.5)
MIC-2	0.03–0.12 (0.06)	0.008–0.12 (0.06)	0.008–0.12 (0.06)	0.03–0.06 (0.06)
Itraconazole resistant (isolate no. 5)				
MIC-0	0.2–1 (1)	0.2–2 (0.5)	0.5–2 (0.5)	0.2–2 (0.5)
MIC-2	0.03–0.5 (0.2)	0.03–0.2 (0.12)	<0.008–0.5 (0.5)	<0.008–0.5 (0.2)
Itraconazole resistant (isolate no. 6)				
MIC-0	0.2–1 (0.5)	0.2–1 (0.5)	0.12–2 (1)	0.12–>8 (0.5)
MIC-2	0.06–0.5 (0.12)	0.01–0.2 (0.12)	0.01–1 (0.2)	0.03–0.5 (0.2)
Voriconazole-resistant mutant				
MIC-0	0.12–>8 (0.2)	0.06–2 (0.12)	0.5–8 (0.5)	0.12–2 (0.2)
MIC-2	0.03–0.12 (0.06)	0.03–1 (0.03)	0.03–0.12 (0.03)	0.03–0.06 (0.03)

[a] Species tested were *A. fumigatus* ($n = 9$), *A. flavus* ($n = 3$), and *A. terreus* ($n = 3$); see Table 1 for reported data regarding isolates 5 and 6; 48-h MICs are shown.
[b] MICs are reported in micrograms per milliliter; the MIC_{90} is reported for susceptible isolates and the model MIC is reported for single resistant isolates.

TABLE 5. Ravuconazole (BMS-207147) MICs for *Aspergillus* spp. in eight laboratories[a]

Isolate(s) (no. tested) and MIC criterion	MIC range (MIC_{90} or modal MIC)[b]			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole susceptible (12)				
MIC-0	0.12–4 (2)	0.12–>8 (1)	0.2–>8 (4)	0.2–>8 (4)
MIC-2	0.01–1 (1)	0.01–0.5 (0.5)	0.01–4 (1)	0.06–1 (1)
Itraconazole resistant (isolate no. 5)				
MIC-0	0.2–0.5 (0.2)	0.12–1 (0.2)	0.2–8 (0.5)	0.12–4 (1)
MIC-2	0.01–0.2 (0.12)	0.03–0.12 (0.06)	<0.008–0.5 (0.12)	0.008–0.5 (0.06)
Itraconazole resistant (isolate no. 6)				
MIC-0	2–8 (2)	1–>8 (2)	1–>8 (8)	2–>8 (8)
MIC-2	0.2–4 (0.5)	0.12–1 (1)	0.2–4 (2)	0.062–8 (2)
Voriconazole-resistant mutant (1)				
MIC-0	2–>8 (4)	1–4 (2)	4–>8 (>8)	2–>8 (8)
MIC-2	0.12–4 (1)	0.06–1 (0.5)	0.06–2 (0.5)	0.03–1 (0.2)

[a] Species tested were *A. fumigatus* ($n = 9$), *A. flavus* ($n = 3$), and *A. terreus* ($n = 3$); see Table 1 for reported data regarding isolates 5 and 6; 48-h MICs are shown.
[b] MICs are reported in micrograms per milliliter; the MIC_{90} is reported for susceptible isolates and the modal MIC is reported for single resistant isolates.

Posaconazole MICs were consistently lower than those of the other three compounds: for the susceptible isolates, the posaconazole MIC at which 90% of the isolates were inhibited (MIC_{90}) was 0.2 μg/ml, versus itraconazole, voriconazole, and ravuconazole MIC_{90}s of 1.0 to 2 μg/ml. Voriconazole MICs for the two itraconazole-resistant isolates were similar to those for the susceptible isolates, while posaconazole MICs were higher for both isolates and ravuconazole MICs were higher for only one of the two strains (Tables 4 to 6). Therefore, ravuconazole appeared to have a different activity against each of these two isolates; for one of them the MICs were similar to those of the voriconazole-resistant laboratory mutant, while for the other the MICs were similar to those for the itraconazole-susceptible strains. Overall, the MICs of the other three agents were variable for the voriconazole-resistant mutant strain; i.e., higher MIC-0s (≥2 μg/ml) than those for the susceptible isolates were observed with the four media.

DISCUSSION

The NCCLS M38-P document (21) describes both macro- and microdilution methods for the antifungal susceptibility testing of opportunistic filamentous fungi, which has improved the interlaboratory agreement of MICs (9, 10). The determination of reliable MIC results is one of the two main concerns in the field of antimicrobial susceptibility testing. When the issue of reliability has been addressed for a susceptibility test, the next concern is, do these reliable results have a clinical value as predictors of therapeutic outcome? The identification of strains that are potentially resistant to the agent being evaluated is then the main role of antimicrobial susceptibility testing. With the purpose of addressing these two issues for the antifungal susceptibility testing of *Aspergillus*, we selected strains of *Aspergillus* that had well-documented in vitro as well as in vivo results or consistent in vitro data (Table 1). The reliability was evaluated based on the interlaboratory agree-

TABLE 6. Voriconazole MICs for *Aspergillus* spp. in eight laboratories[a]

Isolate(s) (no. tested) and MIC criterion	MIC range (MIC_{90} or modal MIC)[b]			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole susceptible (12)				
MIC-0	0.12–2 (1)	0.2–2 (1)	0.5–8 (4)	0.2–>8 (4)
MIC-2	0.01–2 (0.5)	<0.008–1 (1)	<0.008–1 (1)	0.12–1 (0.5)
Itraconazole resistant (isolate no. 5)				
MIC-0	0.2–0.5 (0.2)	0.2–0.5 (0.2)	0.2–2 (0.5)	0.2–1 (0.5)
MIC-2 (0.12)	0.03–0.2 (0.12)	0.03–0.2 (0.06)	<0.008–0.2 (0.12)	0.03–0.12 (0.12)
Itraconazole resistant (isolate no. 6)				
MIC-0	1–2 (1)	0.5–2 (1)	2–4 (4)	2–8 (2)
MIC-2	0.12–0.5 (0.5)	0.12–1 (0.2)	0.5–2 (1)	0.2–1 (0.5)
Voriconazole-resistant mutant (1)				
MIC-0	2–>8 (4)	2–8 (4)	4–>8 (8)	4–>8 (8)
MIC-2	0.5–4 (0.5)	0.2–1 (1)	0.2–2 (0.5)	0.2–1 (0.5)

[a] Species tested were *A. fumigatus* ($n = 9$), *A. flavus* ($n = 3$), and *A. terreus* ($n = 3$); 48-h MICs are shown.
[b] MICs are reported in micrograms per milliliter; the MIC_{90} is reported for susceptible isolates and the modal MIC is reported for single resistant isolates.

TABLE 7. Triazole MIC ranges for the reference *A. flavus* ATCC 204304 isolate in eight laboratories

Antifungal agent and MIC criterion	MIC range[a]			
	RPMI	RPMI-2%	M3	M3-2%
Itraconazole				
MIC-0	0.2–0.5	0.12–0.5	0.5–1	0.5–1
MIC-2[b]	0.06–0.2	0.06–0.5	0.06–0.2	0.06–0.5
Posaconazole				
MIC-0	0.06–0.5	0.06–0.5	0.12–0.2	0.12
MIC-2	0.03–0.12	0.03–0.06	0.01–0.12	0.03–0.06
Ravuconazole				
MIC-0	0.5–4	0.5–2	1–4	0.5–4
MIC-2	0.12–1	0.06–1	0.12–1	0.06–2
Voriconazole				
MIC-0	0.5–4	0.5–2	0.5–>8	0.5–4
MIC-2	0.12–0.5	0.06–0.5	0.12–1	0.12–2

[a] Forty-eight-hour MICs are shown; MICs are reported in micrograms per milliliter.
[b] The reference MIC-2 range is 0.12 to 0.5 μg/ml (10).

ment of eight independent laboratories on MICs obtained by different testing parameter combinations. The clinical significance was studied by identifying those parameters that may have potential value as predictors of azole resistance. The selected isolates included nine strains that, according to in vitro and animal data for itraconazole, could be grouped as seven itraconazole-susceptible and two itraconazole-resistant isolates (4, 5). Animal data for posaconazole against two of these isolates characterized one as susceptible and the other as having a degree of cross-resistance with itraconazole (24). Also, we evaluated one voriconazole-resistant laboratory mutant and five other isolates with consistently low MICs (Table 1). The species of *Aspergillus* evaluated are those most frequently associated with severe disease (2, 4, 15, 16, 18, 29).

We examined the combined effect of incubation time and MIC determination criterion on the reliability of MICs as well as their potential clinical use for *Aspergillus* spp. Prior investigations have demonstrated that increases in the MICs of azoles are associated with the combined effect of both the incubation time and inoculum size for certain filamentous fungi, including *A. fumigatus, A. flavus* (13), and *Fusarium* spp. (26). In our study, MIC increases (more than 2 dilutions) occurred more frequently for MIC-0s than for MIC-2s and occurred more frequently between 24 and 72 h than between either 24 and 48 h or 48 and 72 h. Also, more off-scale MICs (>8 μg/ml) of the four triazoles were obtained for the susceptible isolates after 72 h of incubation. Although itraconazole MIC-2s increased between 48 and 72 h (0.2 to >8 μg/ml) for the two itraconazole-resistant isolates in three of the eight centers, the resistant nature of these two isolates was not detected in three others by this criterion, even after 72 h.

The standard RPMI and M3 media consistently yielded ≥90% agreement among the laboratories for MICs of the four triazoles. Although the RPMI-2% medium provided similar or slightly higher (1 to 2%) interlaboratory agreement than that with RPMI for itraconazole and voriconazole, the agreement was 3 to 6% lower with RPMI-2% among posaconazole and ravuconazole MIC-0s. It has been reported that at 24 h, RPMI-2% increases the growth of *Candida albicans* and other yeasts, which facilitates both visual and spectrophotometric evaluation of MICs (28). In contrast, Nguyen and Yu (23) reported that RPMI-2% did not improve significantly the growth density of *Candida* species at 24 h and that this broth may provide falsely high MICs. In our study, all four medium formulations yielded lower percentages of interlaboratory consensus of MICs at 24 h (68 to 97%) than those for the other two incubation times (81 to 100%). The 24-h MICs had little value for differentiating itraconazole-susceptible from -resistant isolates, regardless of the medium formulation. In addition, the augmentation of the dextrose content of RPMI and M3 media lowered the MIC reproducibility. Therefore, increasing the dextrose content of the standard RPMI medium did not appear to improve its performance. Prior studies that have evaluated the RPMI-2% broth for either yeast or mold testing (5, 28) did not assess the reliability of MIC results among different laboratories. Our study differs from those previous reports in this respect.

Because standard RPMI did not appear to be the ideal medium for the determination of amphotericin B MICs for yeasts, the NCCLS M27-A document (22) suggests substitution of the standard broth by M3 medium. Lot-to-lot variation has been demonstrated with M3 broth (19), and Clancy and Nguyen (3) found that the E-test was the good predictor of amphotericin B resistance for *Candida* spp. Data from our study have demonstrated that the use of M3 improved the interlaboratory agreement (1 to 3%) for MIC-0s of the four triazoles for all the isolates tested. However, high MICs of the four agents, like those for the two itraconazole-resistant strains, were obtained for itraconazole-susceptible isolates (Tables 3 to 6). Therefore, when both the reliability and clinical utility of MIC results for these *Aspergillus* spp. were taken into consideration, substitution of the standard RPMI medium with either M3 formulation also did not improve the performance of the M38-P method.

Denning et al. (4, 5) identified two resistant isolates (itraconazole MICs of >16 μg/ml) of *A. fumigatus* among four other isolates (itraconazole MICs of 0.12 to 1.0 μg/ml) by non-NCCLS methods; we included these isolates in our investigation. Their in vitro results correlated with both in vivo data in a neutropenic murine model of invasive aspergillosis (5) and the response to itraconazole therapy for invasive human aspergillosis (4, 5). They reported better growth and clearer MICs with the M3-2% and RPMI-2% media than with the conventional RPMI medium (5). In our study, all four media yielded high itraconazole MICs (Table 3) for these two itraconazole-resistant strains of *A. fumigatus* and low MICs for the four susceptible isolates, despite the different inoculum sizes used in both studies (10^6 versus 10^4 CFU/ml), but the standard RPMI medium gave more consistent results (Tables 2 and 3). Variable MICs (2 to >8 μg/ml) were reported in Denning et al.'s study for the same isolate (isolate 6; Table 1) for which one of the laboratories reported low itraconazole MICs. Different mechanisms of resistance, either an alteration of the target enzyme or a membrane transporter, have been described for the two isolates (4, 5). Recently, Moore et al. (20) have reported that an inoculum size of 5×10^5 CFU/ml, which is their optimal inoculum size to detect itraconazole resistance

in *A. fumigatus*, is not appropriate for some *A. flavus* isolates. The higher inoculum size precluded precise MIC determination due to the presence of trailing growth; a 2.5×10^4-CFU/ml inoculum provided more reliable and relevant results in their study for *A. flavus*.

Data from the present study show a higher interlaboratory agreement (90 to 92%) for itraconazole MIC-0s for the three species of *Aspergillus* evaluated (Table 2) than those (58 to 89%) from the two prior NCCLS multicenter mold studies (9, 10). The agreement was also good to excellent for MIC-0s of the three investigational triazoles (>90% agreement) with standard RPMI. Furthermore, it was the conventional criterion in combination with the use of the standard RPMI medium that allowed the eight laboratories to better distinguish the itraconazole-susceptible isolates from the two resistant isolates after 48 h of incubation. Therefore, this combination of parameters appears to constitute the optimal testing conditions to detect potential azole resistance.

Our study included one voriconazole-resistant laboratory mutant of *A. fumigatus*. Agreement among the centers and recognition of the lower susceptibility of this strain to voriconazole and ravuconazole were more evident with MIC-0s than with MIC-2s (Tables 5 and 6). However, the agreement was lower for itraconazole and posaconazole MICs for this isolate, as evidenced by their wider ranges (Tables 3 and 4). The optimal testing conditions also better detected the different susceptibilities of the two itraconazole-resistant isolates to ravuconazole (the most frequent MIC reported [modal MIC] for one isolate was 10 times higher [2 μg/ml] than for the other isolate [0.2 μg/ml] [Table 5]). Additionally, most ravuconazole MIC-0 ranges for these strains with the other medium formulations and all ravuconazole MIC-2 ranges were wider, indicating a poor agreement among the laboratories. These results suggest that cross-resistance among these four triazoles may be strain dependent. Although posaconazole MICs were mostly higher for the two itraconazole-resistant isolates than for susceptible ones, the cross-reaction with itraconazole was less clear than that previously reported (24).

The MIC data generated by the optimal conditions are similar (Tables 2, 3, 4, and 5) to those obtained in other studies for itraconazole (4, 5, 7, 8), posaconazole (8), ravuconazole (11), and voriconazole (7, 30). It has been demonstrated that in general either MIC-2s and MIC-0s of posaconazole and itraconazole for *Aspergillus* spp. are the same or the differences are within 2 dilutions (8). It appears then that changing the criterion of MIC determination would not alter the MICs of the four triazoles and may facilitate the identification of potential resistance of *Aspergillus* spp. to these agents.

In conclusion, our data indicate that the conventional and more stringent MIC determination criterion (MIC-0s, or complete growth inhibition) can easily and reliably differentiate between susceptible and potentially resistant *Aspergillus* spp. isolates for itraconazole and perhaps for the investigational triazoles. After 48 h of incubation, the standard RPMI medium facilitated more consistent identification of the two itraconazole-resistant isolates among the susceptible isolates by the participant laboratories. Our results warrant consideration for inclusion of this combination of optimal testing conditions for the detection of potential azole resistance among *Aspergillus* isolates in the future NCCLS document M38-A (approved standard).

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