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Quality Control and Reference Guidelines for CLSI Broth Microdilution Susceptibility Method (M38-A Document) for Amphotericin B, Itraconazole, Posaconazole, and Voriconazole

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Although standard conditions are available for testing the susceptibilities of filamentous fungi to antifungal agents by the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) broth microdilution assay, quality control (QC) MIC limits have not been established for any mold-agent combination. This multicenter (eight-center) study documented the reproducibility of tests for one isolate of *Paecilomyces variotii* ATCC MYA-3630 and 11 other mold isolates (three isolates of *Aspergillus fumigatus*; two isolates of *A. terreus*; one isolate each of *A. flavus*, *A. nidulans*, *Fusarium moniliforme*, and *F. solani*; and two isolates of *Scedosporium apiospermum*) by the CLSI reference broth microdilution method (M38-A document). Control limits (amphotericin B, 1 to 4 μg/ml; itraconazole, 0.06 to 0.5 μg/ml; posaconazole, 0.03 to 0.25 μg/ml; voriconazole, 0.015 to 0.12 μg/ml) for the selected QC *P. variotii* ATCC MYA-3630 were established by the analysis of replicate MIC results. Reference isolates and corresponding MIC ranges were also established for 6 of the 12 molds evaluated. MIC limits were not proposed for the other five molds tested due to low testing reproducibility for these isolates.

The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) has developed a reference method for broth microdilution antifungal susceptibility testing of filamentous fungi (CLSI/NCCLS M38-A document) (4). This document describes guidelines for testing the susceptibility of filamentous fungi (molds) associated with invasive fungal infections to established and new antifungal agents, including *Aspergillus* spp., *Fusarium* spp., *Rhizopus arrhizus*, *Pseudallescheria boydii* (*Scedosporium apiospermum*), *Sporothrix schenckii*, and other less-common opportunistic fungi. Testing conditions for the new triazoles, posaconazole, ravuconazole, and voriconazole, in addition to itraconazole and amphotericin B, are described in the CLSI document. However, the document does not describe quality control (QC) parameters for any mold-drug combination. For QC purposes, the document lists the available QC isolates and established QC MIC ranges for yeast testing (*Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) (1, 5) and two reference isolates (*A. fumigatus* ATCC 204305 and *A. flavus* ATCC 204304); data for these two isolates were collected in two previous collaborative studies (2, 3) but not under M23-A2 guidelines for selection of QC isolates (6).

The purpose of this multicenter (eight-center) study was to select QC isolates among 12 mold strains and to establish MIC limits for broth microdilution tests of amphotericin B, itraconazole, posaconazole, and voriconazole.

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MATERIALS AND METHODS

Study design. The experimental design followed the requirements described in the CLSI M23-A2 document for establishing QC ranges for antibacterial agents (6) but with the appropriate modification for mold testing (4). The inter- and intralaboratory reproducibility of ten replicate tests performed for each drug and strain combination were determined by the CLSI M38-A broth microdilution method in each laboratory on 10 different days. The same coded set of 12 molds and the QC *C. krusei* ATCC 6258 isolate were sent to each laboratory. Each isolate was tested in each laboratory with amphotericin B, itraconazole, posaconazole, and voriconazole according to a standard protocol that included the testing guidelines of both CLSI M23-A2 and M38-A documents (4, 6) and the detailed description of the additional reading conditions to be evaluated for isolates of *Fusarium* spp. (50 and 100% growth inhibition azole MICs at both 24 and 48 h) and *Paecilomyces variotii* (50 and 100% growth inhibition azole MICs and 100% amphotericin B MICs at both 24 and 48 h). The objective of the present study was to determine the reproducibility of the method within each laboratory, as well as among the laboratories and between three lots of standard RPMI 1640 broth (4, 6).

Isolates. The set of isolates included one isolate each of *A. flavus*, *A. nidulans*, *Fusarium moniliforme*, *F. solani*, and *P. variotii*; three isolates of *A. fumigatus*; and two isolates each of *A. terreus* and *S. apiospermum* from the culture collections of the University of Texas Health Science Center, San Antonio, and the National Cancer Institute, Bethesda, Md. The QC *C. krusei* ATCC 6258 strain was included each time isolates were tested in each laboratory with each medium.

Antifungal susceptibility testing. The 12 molds were tested by the M38-A microdilution method in each center with the three lots of medium (RPMI 1640) on 10 different working days as recommended in the CLSI M23-A document (6).

TABLE 1. Distribution of MICs reported by eight laboratories on 10 separate days for seven of the twelve filamentous fungi tested

Antifungal agent and strain[a]	No. of times the indicated MICs (μg/ml) were reported (n = 240)[b]												%
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16	≥32	
Amphotericin B													
P. variotii ATCC MYA-3630							[**90**	**146**	**4**]				100
A. flavus ATCC MYA-3631						3	[**27**	**121**	**83**	**6**]			98.8
A. fumigatus∗ ATCC MYA3626						[**29**	**70**	**109**	**23**]	3			98.7
A. fumigatus ATCC MYA-3627					2	[**42**	**69**	**105**	**22**]				99.2
A. terreus ATCC MYA-3633							4	[**71**	**136**	**29**]			98.3
F. moniliforme∗ ATCC MYA-3629							1	[**47**	**152**	**38**]			99.6
S. apiospermum ATCC MYA-3635								2	[**73**	**157**	**7**]	1	98.8
Itraconazole													
P. variotii∗ ATCC MYA-3630			[**12**	**104**	**84**	**10**]							100
A. flavus ATCC MYA-3631†													84.2
A. fumigatus∗ ATCC MYA3626				10	**51**	[**79**	**94**	**0**]					95.7
A. fumigatus ATCC MYA-3627									4	8	[**228**]		95
A. terreus ATCC MYA-3633†													62
F. moniliforme∗ ATCC MYA-3629								1		4	[**234**]		97.9
S. apiospermum ATCC MYA-3635†													89
Posaconazole													
P. variotii∗ ATCC MYA-3630	1	[**16**	**109**	**64**	**20**]								99.5
A. flavus ATCC MYA-3631		7	[**53**	**56**	**119**	**5**]							97.1
A. fumigatus ATCC MYA-3626†													74.8
A. fumigatus ATCC MYA-3627			1		[**79**	**44**	**112**	**2**]	1			1	98.8
A. terreus ATCC MYA-3633†													43.7
F. moniliforme∗ ATCC MYA-3629						[**34**	**151**	**18**]	4				98.1
S. apiospermum ATCC MYA-3635							[**71**	**159**	**6**]	4			98.3
Voriconazole													
P. variotii ATCC MYA-3630	[**2**	**92**	**126**	**20**]									100
A. flavus ATCC MYA-3631					3	[**11**	**205**	**20**]			1		98.3
A. fumigatus ATCC MYA-3626				[**15**	**186**	**33**]							100
A. fumigatus ATCC MYA-3627					[**9**	**175**	**54**]	2					99.2
A. terreus ATCC MYA-3633				2	[**39**	**175**	**24**]						99.2
F. moniliforme∗ ATCC MYA-3629							[**7**	**186**	**19**]				100
S. apiospermum ATCC MYA-3635							[**76**	**155**	**9**]				100

[a] ∗, data from one laboratory were excluded because MICs for the QC *C. krusei* isolate were outside the established limits and/or another laboratory did not report all values. †, no range recommended, i.e., agreement was <95%, and MIC ranges were within more than four dilutions.
[b] Proposed QC and reference MIC limits are indicated by ranges of values in boldface.

Therefore, a total of 240 MICs were determined for each isolate-drug combination. Briefly, stock inoculum suspensions were prepared from 7-day cultures grown on potato dextrose agar (Remel, Lenexa, KS) and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.17 (68 to 82% transmittance) and diluted (1:50) in each of the three lots of RPMI 1640 broth. The three lots of standard RPMI 1640 medium were Sigma lots 062K83121 and 062K83122 and the Irvine Scientific lot 951321042A; the participant laboratories were provided with samples of all three lots of broth for inoculum preparation (Trek Diagnostic Systems, Inc., Westlake, Ohio). The density of the inoculum suspension of the QC yeast isolate was adjusted by the spectrophotometer to the density of a 0.5 McFarland standard and diluted 1:1,000 in the three lots of medium (5).

Amphotericin B (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn.), itraconazole (Janssen, Beerse, Belgium), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), and voriconazole (Pfizer Central Research, New York, N.Y.) were provided by the manufacturers as standard assay powders. Microdilution trays (96 U-bottom shaped) containing antifungal dilutions diluted with each lot of medium were prepared in a central facility (Trek Diagnostic Systems) and shipped frozen to each participant. Drug concentration ranges were 0.015 to 16 μg/ml for itraconazole and voriconazole, 0.008 to 8 μg/ml for posaconazole, and 0.015 to 32 μg/ml for amphotericin B. After inoculation of the trays, all microdilution trays were incubated at 35°C in ambient air. Colony counts were performed in each center for at least one of the inoculum suspensions prepared with one of three lots of medium for each of the 12 molds and the QC isolate on each of the 10 testing days.

As described in the M38-A document (4), MICs were determined by visual examination at 72 h (*S. apiospermum*) and 48 h (remaining isolates); MICs were defined as the lowest drug concentration that showed absence of growth or complete growth inhibition (100%). Because isolates of *P. variotii* had not been evaluated by the M38-A method in a collaborative study and triazole MICs for *Fusarium* spp. may increase with extended incubation (7), MICs for these two species were also determined at 24 and 48 h (50 and 100% growth inhibition values) (50% growth inhibition values). MICs for the QC yeast isolate (*C. krusei*) were determined at both 24 and 48 h and corresponded to 100% with amphotericin B and 50% or more growth inhibition with the triazoles (5).

TABLE 2. Proposed QC and/or reference MIC ranges of four antifungal agents for seven filamentous fungus isolates tested by the CLSI M38-A broth microdilution method

Isolate	Purpose[a]	Agent	MIC range (μg/ml [% within range])	Mode (μg/ml)
P. variotii ATCC MYA-3630	QC	Amphotericin B	1–4 (100)	2
		Itraconazole	0.06–0.5 (100)	0.12
		Posaconazole	0.03–0.25 (99.5)	0.06
		Voriconazole	0.015–0.12 (100)	0.06
A. flavus ATCC MYA-3631	R	Amphotericin B	1–8 (98.8)	2
		Posaconazole	0.12–1 (97.1)	0.5
		Voriconazole	0.5–2 (98.3)	1
A. fumigatus ATCC MYA-3626	R	Amphotercin B	0.5–4 (98.7)	2
		Itraconazole	0.25–2 (95.7)	1
		Voriconazole	0.25–1 (100)	0.5
A. fumigatus ATCC MYA-3627	R	Amphotericin B	0.5–4 (99.2)	2
		Itraconazole	≥16 (95)	>16
		Posaconazole	0.25–2	NA[b]
		Voriconazole	0.25–1 (99.2)	0.5
A. terreus ATCC MYA-3633	R	Amphotericin B	2–8 (98.3)	4
		Voriconazole	0.25–1 (99.2)	0.5
F. moniliforme ATCC MYA-3629	R	Amphotericin B	2–8 (99.6)	4
		Itraconazole	>16 (97.9)	>16
		Posaconazole	0.5–2 (98.1)	1
		Voriconazole	1–4 (100)	2
S. apiospermum ATCC MYA-3635	R	Amphotericin B	4–16 (98.8)	8
		Posaconazole	1–4 (98.3)	2
		Voriconazole	0.5–2 (100)	1

[a] QC, quality control isolate for testing the antifungal agents listed; R, reference isolate for testing the antifungal agents listed.
[b] NA, bimodal.

RESULTS AND DISCUSSION

The performance of antimicrobial susceptibility testing is monitored by the introduction of at least one QC strain for which MIC limits of acceptable reproducibility have been established (6). In order to establish QC limits, MIC data from at least seven independent laboratories from distinct institutions should be included in the final analysis. The MIC range is determined when at least 95% of 210 or more MIC endpoints are included in the proposed range. The design of the present study followed these guidelines (1, 6); MICs from one of the eight laboratories were excluded (itraconazole and posaconazole for *P. variotii* isolate ATCC MYA-3630) due to either MICs for the QC *C. krusei* isolate being out of range or due to poor intralaboratory agreement for the drug-isolate combination (Table 1).

For the 12 molds and the QC yeast isolate, 97% of the inocula were within the target ranges of 0.4×10^4 to 5×10^4 CFU/ml (molds) and 0.5×10^3 to 2.5×10^3 CFU/ml (*C. krusei*) (4, 5). Lower inoculum densities (0.1×10^4 to 0.8×10^3 CFU/ml) were reported for *A. nidulans* ATCC MY 3632 and one of the *S. apiospermum* isolates from three centers, which perhaps accounted for the lower reproducibility of MIC results observed for these two isolates (data not shown in Table 1); control limits were not proposed for these two isolates. Our reproducibility results for inoculum suspensions were similar to those obtained in previous collaborative studies (92 to 95%) (2, 3).

It has been reported that triazole MICs for *Fusarium* spp. substantially increase between 24 and 48 h and that the 24 h results appear to have more clinical relevance (7); however, the standard incubation time is 48 h (4). Our MIC data for both *Fusarium* isolates indicated that the best reproducibility was at 48 h by the 100% inhibition criterion, especially for itraconazole (*F. moniliforme* ATCC MY 3629) and posaconazole (*F. solani* ATCC MY 3636). Therefore, we have proposed the isolate of *F. moniliforme* ATCC MYA-3629 as one of the reference strains by the most appropriate testing conditions. However, for *F. moniliforme* ATCC MYA-3629 the 24 h MIC ranges for 99% of the values were very similar (0.25 to 2 and 0.5 to 4 μg/ml with posaconazole and voriconazole, respectively) to the 48 h results (Table 2); this suggests that perhaps these data can be obtained at 24 h. The same reading conditions were evaluated for the *P. variotii* ATCC MYA-3630, because this species was not included during the development of the M-38-A document (4); again, the M38-A reading parameters (48 h and 100% growth inhibition) had the best performance.

Table 1 lists the MIC distribution with the three triazoles and amphotericin B for 7 of the 12 isolates tested by the conditions described in the M38-A document (4). As indicated in the table, no MIC range was recommended for some isolate-drug combinations because MIC results did not meet the requirements described in the M23-A2 document for selection of QC strains. That is, the interlaboratory agreement was <95%.

We also did not include the results of 5 of the 12 isolates because, again, no MIC range was recommended for those strains with any of the antifungal agents for *F. solani* ATCC MY 3636 and *S. apiospermum* ATCC MY 3634 and only with voriconazole for *A. fumigatus* ATCC MY 3626, *A. nidulans* ATCC MY 3632, and *A. terreus* ATCC MY 3628 (MIC range, 0.12 to 1 μg/ml; interlaboratory agreement, 99.2 to 100%). Therefore, Table 2 presents a summary of selected isolates and corresponding QC and reference MIC limits and provides the percentages of agreement, as well as the mode for each isolate-drug combination. As expected, there were no major differences (>2 dilutions) between lots of RPMI 1640 broths or consistent discrepancies between the laboratories included in the study. Therefore, results were pooled for the analysis. QC and reference limits were defined as ranges that included one doubling concentration on either side of the mode (1, 6), as was observed for 10 of the 12 isolates with voriconazole. However, when two contiguous concentrations had similar frequencies, the mode was assumed to be between the even $\log_2$ concentrations that were tested, as was the case for the *P. variotii* isolate ATCC MY 3630 with the three triazoles (Table 1). For this reason, we proposed a four-dilution range for *P. variotii* ATCC MY 3630 with the triazoles (Table 2) as it was established for QC yeast isolates (1, 5); the establishment of four-dilution ranges is described in the M23-A2 document (6). We are also proposing four and three dilution MIC ranges for the *F. moniliforme* isolate ATCC MY 3629 with the four antifungal agents and for another five isolates with the corresponding agents listed in Table 2. MIC ranges that were within the parameters described in the M23-A document for the selection of QC isolates were not observed for any other isolate with itraconazole, but they were observed with two more isolates with posaconazole and amphotericin B (data not shown in Table 1).

In conclusion, this is the first study that has proposed QC and reference MIC limits for seven mold isolates and selected one of these seven strains (*P. variotii* ATCC MY 3630) as the QC isolate for testing the most clinically relevant antifungal agents (voriconazole, itraconazole, amphotericin B, and posaconazole) against molds. Because these MIC limits have been proposed according to the available guidelines for the selection of QC isolates and the establishment of MIC limits, these ranges would assist in monitoring the performance of in vitro antifungal susceptibility testing by the M38-A and alternative methods. Our selection of reference strains includes isolates that are resistant to amphotericin B (MICs of ≥2 μg/ml) and itraconazole (≥8 μg/ml), which could aid in the identification of potential azole- and amphotericin B-resistant isolates among susceptible isolates in the clinical laboratory.

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