

Quality Control and Reference Guidelines for CLSI Broth Microdilution Method (M38-A Document) for Susceptibility Testing of Anidulafungin against Molds[∇]

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The CLSI (formerly NCCLS) M38-A document for antifungal susceptibility testing of filamentous fungi does not describe guidelines for echinocandins. A multicenter study (eight centers) evaluated inter- and intralaboratory reproducibilities of two reading times (24 and 48 h or 48 and 72 h) and two end points (MICs and minimum effective concentrations [MECs]) for evaluating anidulafungin against molds. Anidulafungin MICs ($\geq 50\%$ inhibition) and MECs (morphological hyphal changes) were determined for seven *Aspergillus* isolates (four species) and one isolate each of *Fusarium moniliforme*, *Fusarium solani*, and *Paecilomyces variotii* and for two *Scedosporium apiospermum* isolates. The inter- and intralaboratory reproducibilities of 10 replicate tests performed in each laboratory on 10 different days for each isolate was 100% at 24 h (MECs, ≤ 0.015 $\mu\text{g/ml}$) for six *Aspergillus* and *P. variotii* isolates. The reproducibility was 94 to 96.7% at 72 h (MECs, 1 to 8 $\mu\text{g/ml}$) for *S. apiospermum* and 96.7 to 97.5% at 48 h (MICs, ≥ 32 $\mu\text{g/ml}$) for both *Fusarium* isolates. Introduction of these identified optimum testing conditions for anidulafungin into future versions of the M38 document is warranted.

The Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS) has developed a reference method for broth microdilution antifungal susceptibility testing of filamentous fungi (CLSI M38-A document) (5, 6, 8). This document describes guidelines for testing the susceptibility of filamentous fungi (molds) that are associated with invasive fungal infections to established agents and newer triazoles (posaconazole, ravuconazole, and voriconazole). However, the document does not describe testing conditions for the echinocandins. More recently, quality control (QC) MIC data were established for three triazoles and amphotericin B (7). Anidulafungin also was evaluated during that multicenter study (eight centers) with 12 mold isolates and following the requirements described in the CLSI M23-A2 document for establishing QC ranges for antibacterial agents (10). The purpose of the evaluation was dual: (i) to identify optimum conditions for testing anidulafungin against mold isolates and (ii) to select QC or reference isolates among the 12 mold isolates tested and to establish anidulafungin MIC or minimum effective concentration (MEC) QC limits for broth microdilution testing of anidulafungin versus molds.

MATERIALS AND METHODS

Study design. The experimental design followed the requirements described in the CLSI M23-A2 document for establishing QC limits for antibacterial agents (10) but with the appropriate modification for mold testing (8). The inter- and

intralaboratory reproducibilities of 10 replicate tests performed for each isolate were determined by a modified CLSI M38-A broth microdilution method in each laboratory on 10 different days. The same coded set of 12 molds and the QC *Candida krusei* ATCC 6258 isolate were sent to each laboratory. Each isolate was tested in each laboratory with anidulafungin following a standard protocol that included the testing guidelines of both the CLSI M23-A2 and M38-A documents (8, 10), as well as a detailed description of the two reading times and two susceptibility end points to be evaluated: (i) for isolates of *Aspergillus* spp., *Fusarium* spp., and *Paecilomyces variotii* (MECs and MICs [$\geq 50\%$ growth inhibition] at both 24 and 48 h) and (ii) for *Scedosporium apiospermum* (MECs and MICs [$\geq 50\%$ growth inhibition] at both 48 and 72 h). The objective of this 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 medium for each incubation time and end point combination (10).

Isolates. The set of isolates evaluated included one isolate each of *Aspergillus flavus*, *Aspergillus nidulans*, *Fusarium moniliforme*, *Fusarium solani*, and *P. variotii*; three isolates of *Aspergillus fumigatus* (one isolate is not included in Table 1); and two isolates each of *Aspergillus terreus* and *S. apiospermum* obtained from the culture collections of the University of Texas Health Science Center, San Antonio, TX, and the National Cancer Institute, Bethesda, MD. The QC *C. krusei* ATCC 6258 isolate was included each time that 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 (10). Therefore, a total of 240 MICs were determined for each isolate and testing condition evaluated. 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 0.11 (78 to 82% transmittance) and were diluted (1:25 [*S. apiospermum*] and 1:50 [other isolates]) in each of the three lots of RPMI 1640 broth. The three lots of standard RPMI 1640 medium were Sigma (Saint Louis, MO) lots 062K83121 and 062K83122 and Irvine Scientific (Santa Ana, CA) lot 951321042A. The density of the inoculum suspension of the QC yeast isolate was adjusted by a spectrophotometer to the density of a 0.5 McFarland standard and diluted 1:1,000 in the three lots of medium (9). The participant laboratories were provided with sam-

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TABLE 1. Summary of anidulafungin MIC and MEC ranges and interlaboratory agreement at two incubation times in seven of eight laboratories

Species and isolate	MIC or MEC, $\mu\text{g/ml}$ (% interlaboratory agreement)			
	24 h		48 h	
	MIC ^b	MEC	MIC ^b	MEC
<i>A. fumigatus</i>				
ATCC MYA-3626	<0.015 (75)	<0.015 (100)	<0.015 (63)	<0.015 (100)
ATCC MYA-3627	<0.015 (75)	<0.015 (100)	<0.015 (62)	<0.015 (100)
<i>A. flavus</i> ATCC MYA-3631	<0.015 (75)	<0.015 (100)	<0.015 (54)	<0.015 (91.7)
<i>A. terreus</i>				
ATCC MYA-3628	<0.015 (63)	<0.015 (100)	<0.015 (49)	<0.015 (75)
ATCC MYA-3633	<0.015 (63)	<0.015 (99.6)	<0.015 (54)	<0.015 (99.6)
<i>A. nidulans</i> ATCC MYA-3632	<0.015 (75)	<0.015 (100)	<0.015 (67)	<0.015 (96.7)
<i>F. solani</i> ATCC MYA-3636	>32 (87)	>32 (59)	>32 (96.7)	>32 (87)
<i>F. moniliforme</i> ATCC MYA-3629	>32 (94.2)	>32 (82.5)	>32 (97.5)	>32 (87.5)
<i>S. apiospermum</i> ^a				
ATCC MYA-3634	1–4 (59)	1–4 (90)	1–4 (50)	1–4 (96.7)
ATCC MYA-3635	1–4 (47)	1–4 (79)	1–4 (46)	1–4 (94)
<i>P. variotii</i> ATCC MYA-3630	<0.015 (75)	<0.015 (100)	<0.015 (75)	<0.015 (100)

^a For *S. apiospermum*, incubation was for 48 and 72 h.

^b $\geq 50\%$ growth inhibition MICs.

ples of the three lots of the medium for the dilution of all stock inocula (Trek Diagnostic Systems Inc., Westlake, OH).

Anidulafungin was provided by the manufacturer (Pfizer Central Research, New York, NY) as a standard assay powder. Microdilution trays (96-well, U-bottom shaped) containing anidulafungin 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 32 $\mu\text{g/ml}$. 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 the three lots of medium for each of the 12 molds and QC isolates on each of the 10 testing days. MICs were determined by visual examination at 48 and 72 h (*S. apiospermum*) and at 24 and 48 h (remaining isolates) and corresponded to $\geq 50\%$ growth inhibition. MECs were also determined at the same incubation times and were defined by reference photographs as the lowest drug dilution that produced small, rounded, compact microcolonies compared to the filamentous growth seen in the growth control wells. MICs for the QC yeast isolate (*C. krusei*) were determined at both 24 and 48 h and corresponded to 50% or more growth inhibition (9).

RESULTS AND DISCUSSION

For the 12 mold isolates 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*) (8, 9). 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; control limits were not proposed for these isolates. Our reproducibility results for inoculum suspensions were similar to those obtained in previous collaborative studies (92 to 95%) (5, 6).

The reference criterion of MIC determination for amphotericin B and triazoles is 100% growth inhibition (8). Anidulafungin, like other echinocandins, does not generally yield 100% inhibition susceptibility end points because trailing growth similar to that observed when testing triazoles against yeast isolates is usually seen, especially when testing molds.

The determination of the MEC (measurement of morphological hyphal changes) has been shown to assess better the in vitro antifungal activity of caspofungin to mold isolates (2, 11). The MEC of caspofungin also has generated more reproducible results than the conventional MIC end point. However, the reliability of either the anidulafungin MEC or MIC end point for mold testing has not been evaluated in collaborative studies.

As the activity of anidulafungin and other echinocandins has been reported using either MIC or MEC end points (3, 4), we evaluated the reliability of both MIC and MEC end points at two incubation times for each mold isolate. Table 1 lists the summary and interlaboratory agreements of MIC and MEC end points for 11 of the 12 isolates tested at corresponding reading times. The reproducibility (within three dilutions) with the MEC end point was higher (75 to 100%) than that with the MIC end point (46 to 75%) for six of the seven *Aspergillus* isolates, the isolate of *P. variotii*, and the two isolates of *S. apiospermum*. While the MEC appears to be more reliable than the MIC for testing anidulafungin against *Aspergillus* spp., *S. apiospermum*, and *P. variotii*, the MIC was more reliable (96.7 to 97.5% versus 87 to 87.5% at 48 h) when testing anidulafungin against the two isolates of *Fusarium* spp.

The standard incubation time for testing amphotericin B and triazoles against molds is 48 h (8). The suitable incubation time for testing the echinocandins has been controversial. For yeast testing, the 24-h incubation time is the most reliable and clinically relevant MIC end point for caspofungin (11). In the present study, the reproducibility of MEC results was lower at 48 h than at 24 h for four of the seven *Aspergillus* isolates (75 to 100% and 99.6% to 100%, respectively), but higher reproducibility was observed at 72 h (94 to 96.7%) than at 48 h (79

to 90%) for both isolates of *S. apiospermum* (Table 1). Therefore, the reading parameters 48 h (8) and $\geq 50\%$ growth inhibition had the best performance for testing anidulafungin against *Fusarium* spp., but the shorter incubation time (24 h) and the MEC end point provided superior results for testing *Aspergillus* isolates. Agreement of MEC results for *P. variotii* and three *Aspergillus* isolates was not dependent on the incubation time.

MICs for the QC isolate *C. krusei* ATCC 6258 were within the established limit (0.03 to 0.12 $\mu\text{g/ml}$) (1) in seven of the eight laboratories; 15 of the 30 MICs determined for this isolate were lower (0.015 $\mu\text{g/ml}$) in one laboratory. Because of that, results from that laboratory were excluded from the analysis. However, according to the M23-A2 document, the MIC limit is determined when at least 95% of the 210 or more end points from at least seven independent laboratories are included in the proposed QC limit. This requirement was fulfilled for various isolates. As expected, there were no major differences (>2 dilutions) between lots of RPMI 1640 broths; intralaboratory discrepancies (>3 dilutions) were also low ($>95\%$ agreement). MEC and MIC data for the following isolates met M23-A2 requirements (10): *A. fumigatus* ATCC MYA-3626 and ATCC MYA-3627, *A. terreus* ATCC MYA-3633, *F. solani* ATCC MYA-3636, *P. variotii* ATCC MYA-3630, and *S. apiospermum* ATCC-3634. These isolates could be selected as either QC or at least reference isolates for testing anidulafungin against mold isolates. The *P. variotii* ATCC MYA-3630 isolate has been selected as the QC isolate for testing molds with triazoles and amphotericin B (7). MEC or MIC ranges for most of these isolates are off scale. However, an isolate with an off-scale itraconazole MIC (≥ 8 $\mu\text{g/ml}$) has been previously proposed (7), as we do in the present study (*F. solani* isolate [Table 1]), because these isolates could aid in the identification of potential azole or echinocandin resistance. Isolates with anidulafungin MECs of ≤ 0.015 $\mu\text{g/ml}$ are intended to monitor the performance of daily testing as well as to aid in the determination of this novel end point that is to be introduced as the echinocandin end point in the future version of the M38-A document. A more suitable mold isolate(s) with on-scale MEC ranges for the echinocandins needs to be identified in future collaborative studies according to M23-A guidelines.

In conclusion, the following optimum testing conditions for evaluating the in vitro activity of anidulafungin against molds were identified: the determination of MECs at 24 h (*Aspergillus* spp. and *P. variotii*) or 72 h (*S. apiospermum*) and the determination of MICs at 48 h (*Fusarium* spp.). To our knowledge, this is the first study that has proposed QC or reference MIC limits for six mold isolates (*A. fumigatus* ATCC MYA-3626 and ATCC MYA-3627, *A. terreus* ATCC MYA-3633, *F. solani*

ATCC MYA-3636, *P. variotii* ATCC MYA-3630, and *S. apiospermum* ATCC MYA-3634) for testing anidulafungin against molds. These MIC or MEC limits would aid in monitoring the performance of in vitro antifungal susceptibility testing by the M38-A method and alternative methods. The resistant isolate (*F. solani*) could aid in the identification of potential anidulafungin-resistant isolates in the clinical laboratory.

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