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Wild-Type MIC Distributions and Epidemiological Cutoff Values for Caspofungin and *Aspergillus* spp. for the CLSI Broth Microdilution Method (M38-A2 Document)▽

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Clinical breakpoints have not been established for mold testing. Epidemiologic cutoff values (ECVs) are available for six *Aspergillus* spp. and the triazoles, but not for caspofungin. Wild-type (WT) minimal effective concentration (MEC) distributions (organisms in a species-drug combination with no acquired resistance mechanisms) were defined in order to establish ECVs for six *Aspergillus* spp. and caspofungin. The number of available isolates was as follows: 1,691 *A. fumigatus*, 432 *A. flavus*, 192 *A. nidulans*, 440 *A. niger*, 385 *A. terreus*, and 75 *A. versicolor* isolates. CLSI broth microdilution MEC data gathered in five independent laboratories in Canada, Europe, and the United States were aggregated for the analyses. ECVs expressed in μg/ml that captured 95% and 99% of the modeled wild-type population were for *A. fumigatus* 0.5 and 1, *A. flavus* 0.25 and 0.5, *A. nidulans* 0.5 and 0.5, *A. niger* 0.25 and 0.25, *A. terreus* 0.25 and 0.5, and *A. versicolor* 0.25 and 0.5. Although caspofungin ECVs are not designed to predict the outcome of therapy, they may aid in the detection of strains with reduced antifungal susceptibility to this agent and acquired resistance mechanisms.

Invasive fungal infections caused by *Aspergillus fumigatus* are associated with high morbidity and mortality, especially in the immunocompromised host. In addition, the incidence of other species, such as *A. flavus*, *A. niger*, and *A. terreus* has increased as the cause of severe opportunistic infections (4, 9, 11, 32). Echinocandins have a broad spectrum of *in vitro* activity against *Aspergillus* spp. and other molds; caspofungin is an important therapeutic agent for the systemic treatment of refractory invasive aspergillosis as well as for empirical or prophylactic therapy (32). The target of echinocandin antifungal activity is the protein Fksp (glucan synthase) encoded by three *FKS* genes. Drug binding with this target leads to fungal cell glucan depletion. Elevated echinocandin MICs for a variety of *Candida* sp. clinical strains have been associated with genetic mutations conferring amino acid substitutions in the *FKS1* and *FKS2* gene products, therapeutic failure, and/or breakthrough infection (5, 10, 17, 18, 26, 28). However, resistance mechanisms are not as clearly determined for *Aspergillus* spp. as those for *Candida* spp. (19, 31). In a laboratory mutant of *A. fumigatus*, an S678P amino acid change (equivalent to a mutation in resistant *Candida* spp.) conferred *in vitro* resistance to caspofungin (minimal effective concentration [MEC] of >16 μg/ml) (27).

The Clinical and Laboratory Standards Institute (CLSI) has developed a reference broth microdilution method for antifungal susceptibility testing of molds (CLSI M38-A2 document) (6). The most recent edition of the CLSI document describes guidelines for testing the echinocandins, including the determination of caspofungin and other echinocandin MECs for *Aspergillus* and other mold species instead of the traditional MIC. The availability of reference methodologies has enabled the development of CLSI epidemiologic cutoff values (ECVs) for six species of *Aspergillus* and the triazoles (15, 24). An ECV (or the highest wild-type [WT] susceptibility endpoint) is the critical drug concentration that may aid in the evaluation of clinical isolates by identifying those strains with decreased susceptibility or serve as an early warning of emerging subtle changes in organisms' patterns of susceptibility to the agent being evaluated (8). Although caspofungin MECs for *Aspergillus* spp. are usually below 1 μg/ml for most isolates (13, 14, 16), neither MEC distributions nor ECVs are available for any echinocandin-mold combination. In the absence of clinical breakpoints and using the CLSI guidelines to test echinocandins (6), ECVs could help to characterize the susceptibility of *Aspergillus* isolates to caspofungin and to monitor the emergence of strains with mutations or reduced susceptibility to caspofungin. The more frequent use of echinocandins has increased selection pressure, and the monitoring of echinocandin resistance in *Aspergillus* spp. has therefore become important.

We are proposing ECVs for six species of *Aspergillus* (*A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, *A. terreus*, and *A. versicolor*). The purpose of the study was to define the WT distributions (see "Definitions") of each *Aspergillus* sp. and caspofungin by using aggregated MEC data gathered in five laboratories in Canada, Europe, and the United States (75 to 1,691 MECs according to species) and to use these data to propose caspofungin ECVs for each species.

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MATERIALS AND METHODS

Isolates. Each isolate was recovered from unique clinical specimens at five medical centers: the University of Texas Health Science Center, San Antonio, TX; the HPA Mycology Reference Laboratory, Kingsdown, Bristol, United Kingdom; the Hospital General Universitario Gregorio Marañón, Madrid, Spain; the University of Alberta Hospital, Edmonton, Canada; and VCU Medical Center, Richmond, VA. Isolates were identified and stored at each medical center using standardized methodologies; isolates were not genetically characterized for mutations. The total number of aggregated available MEC data from the five laboratories per species was 1,691 isolates of *A. fumigatus*, 432 isolates of *A. flavus*, 192 isolates of *A. nidulans*, 440 isolates of *A. niger*, 385 isolates of *A. terreus*, and 75 isolates of *A. versicolor*.

Two quality control (QC) isolates, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258, and three reference isolates, *Paecilomyces variotii* ATCC MYA-3630 (6) and *Aspergillus fumigatus* NCPF 7100 and NCPF 7097, were used by the participant laboratories; Table 1 depicts the MIC (QC *Candida* strains) and MEC data (reference mold strains) for these isolates.

Antifungal susceptibility testing. In order to include MEC results in the total set (Table 2) of available caspofungin MECs, MECs were obtained at each center by following the CLSI M38-A2 broth microdilution method (standard RPMI-1640 broth [0.2% dextrose], final inoculum concentrations that ranged from 0.4×10^4 to 5×10^4 CFU/ml, and 24 to 48 h of incubation at 35°C). The MEC was the lowest caspofungin concentration that led to the growth of small, rounded, compact microcolonies compared to hyphal growth in the growth control (caspofungin-free RPMI-1640) (6). At least one of the QC and/or one of the reference strains was utilized during the years of testing in each center.

Definitions. WT is the subpopulation of isolates/MECs in a species-drug combination with no detectable acquired resistance mechanisms (8, 29, 30). The high WT MEC has been defined as either the WT cutoff value (COwt) or the ECV; the latter term has been used previously in similar fungal reports (15, 23).

Data analysis. The MEC distribution of each species from each laboratory was reviewed for outlier results, and modal MECs were determined for species from each laboratory. MEC distributions of the aggregated data from the five laboratories for each species were obtained, and ECVs were calculated by the previously reported statistical technique (29). Briefly, the modeled population is based on fitting a normal distribution at the lower end of the MEC range, working out the mean and standard deviation of that normal distribution, and using those numbers to calculate the MEC that captured both 95% and 99% of the modeled WT population. MEC values that captured greater than or equal to 95% of the observed population were also calculated, as this method has been used previously in an attempt to define ECVs for azoles and echinocandins (15, 23, 24).

RESULTS AND DISCUSSION

The utility of susceptibility testing is achieved when the test result can define the likely response to treatment of infections caused by the organism being tested against a specific agent. The drug concentration that classifies the organisms as treatable or nontreatable or predicts the likelihood of clinical outcome is now defined as the clinical breakpoint (8, 30). Clinical breakpoints are based on clinical trial data, global susceptibility surveillance, resistance mechanisms, and pharmacokinetic/pharmacodynamic (PK/PD) parameters from model systems (30). Most of these data are not currently available for molds, owing to the low volume of mold infections and the scarcity of isolates with high MICs or MECs that might predict failure. This is particularly the case for caspofungin and other echinocandin MECs obtained during clinical trials. Instead, the CLSI Antifungal Subcommittee has recently elected to publish ECVs for nondermatophyte filamentous fungi in the CLSI M51-A document (disk diffusion testing of molds) (7). Published CLSI ECVs for *Aspergillus* spp. are available for testing the triazoles (15, 24), and preliminary ECVs from a single laboratory for testing the echinocandins (25). In the present study, caspofungin ECVs for six species of *Aspergillus* were defined. These ECVs may aid in the evaluation of clinical

TABLE 1. MICs/MECs of caspofungin for QC and reference strains as determined at 24 h/48 h[a]

QC or laboratory control isolate	QC MIC range (mode)	% MICs within CLSI range	MIC/MEC range (mode)[c]				
			Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Paecilomyces variotii ATCC MYA-3630[b]	NA	NA	ND	0.06–0.12 (0.06)	0.06–0.25 (0.25)	ND	ND
Aspergillus fumigatus NCPF 7097	NA	NA	ND	ND	ND	0.25–0.25 (0.25)	ND
Aspergillus fumigatus NCPF 7100	NA	NA	ND	ND		0.25–0.25 (0.25)	0.25–0.25 (0.25)
Candida parapsilosis ATCC 22019	0.25–1 (0.5)/0.5–4 (1)	97.1/92.9	0.25–1 (0.5)/0.5–4 (0.5)	ND	0.25–1 (0.5)/0.5–1 (2)	0.5–2 (1)/1–2 (2)[d]	0.25–1 (0.25)/0.5–2 (1)
Candida krusei ATCC 6258	0.12–1 (0.5)/0.25–1 (0.5)	98.8/97.5	0.12–2 (1)/0.25–2 (1)[d]	0.12–0.25 (0.25)/0.25–0.5 (0.5)	0.12–1 (0.5)/0.25–1 (1)	0.5–1 (0.5)/0.5–1 (1)	ND

[a] MICs for QC *Candida* isolates and MECs for *P. variotii* and *Aspergillus* spp. as recommended by the CLSI M38-A2 document.
[b] Caspofungin QC MEC limits are not available for this or any other mold isolate.
[c] ND, not done.
[d] MICs were outside the range for QC isolates of *C. parapsilosis* at 24 h 2% of 64 times and of *C. krusei* 3% of 30 times.

TABLE 2. MEC distributions and epidemiologic cutoff values of caspofungin for six *Aspergillus* spp. from five laboratories, using CLSI M38-A2 microdilution method

Species	No. of isolates	MEC (μg/ml) of:											
		≤0.016	0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
A. fumigatus	1,691	8	81	166	394	866[a]	143[b,d]	19[c]	9	2	0	1	2
A. flavus	432	4	36	158[a]	137	76[b]	12[c,d]	4	3	1	0	0	1
A. nidulans	192	0	6	17	77[a]	57	8[b,c]	3	11	2	4[d]	7	0
A. niger	440	1	45	180[a]	137	55[b,c,d]	18	3	1	0	0	0	0
A. terreus	385	1	31	168[a]	105	48[b]	20[c,d]	10	2	0	0	0	0
A. versicolor	75	4	19	20	21[a]	6[b]	2[c,d]	0	3	0	0	0	0

[a] Most frequent minimal effective concentration (MEC).
[b] Statistically calculated ECV value includes at least 95% of the statistically modeled population.
[c] Statistically calculated ECV value includes at least 99% of the statistically modeled population.
[d] Observed ECV value includes at least 95% of the observed overall distribution.

isolates by identifying those strains with reduced caspofungin susceptibility and may serve as an early warning of emerging subtle changes in the susceptibility patterns of these organisms.

Variability is expected when MECs or MICs from different laboratories are compared despite standardization efforts, including the interlaboratory variation that has been observed under controlled conditions with single strains in QC or in collaborative studies designed to identify optimal testing conditions for *Aspergillus* (triazoles and caspofungin) (12, 22). The CLSI has not selected a mold as a QC isolate nor has it established MEC QC limits for testing echinocandins against molds. Instead MIC limits for both QC isolates *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 are listed in the latest edition of the M38-A2 document as controls for mold testing (6). Because of that, each of the five laboratories provided MIC data for one or both QC *Candida* isolates, and four laboratories generated additional MEC data for their respective internal mold controls when clinical isolates were tested (Table 1). Although most MIC ranges were within the CLSI established limits (97 to 100%) for the QC isolates (6), the modes were variable (mostly ± one 2-fold dilution). The same applied to the two laboratories that used the *P. variotii* ATCC MYA-3630 strain as their control isolate (different modes), but the MEC range was within the accepted three-dilution range (35 and 120 replications); the intralaboratory reproducibility was also excellent in the laboratory that used the two strains of *A. fumigatus* (NCPF 7100 and 7097). This modal MIC/MEC variability among the five laboratories for QC isolates reflected the differences among their modal MECs for the clinical *Aspergillus* isolates. Similar modal variability was also observed for the aggregated data used to establish ECVs for the same *Aspergillus* spp. and the triazoles (15). Although these sources of variability have not been explored formally, they appear to reflect individual interpretations of susceptibility endpoints.

Table 2 shows the caspofungin aggregated MEC distributions for the six *Aspergillus* spp. The trimodal distribution of *A. nidulans* was a noteworthy feature. This distribution, caused by the presence of a small but noticeable number of strains with elevated MECs, also requires explanation. It was explained largely but not completely by the data from one laboratory, which had a significant proportion of strains with MECs above 1 μg/ml. However, all participating laboratories had some strains with MECs above 1 μg/ml for this species. Whether this phenomenon represents identification problems, acquired *FKS* mutations, or natural *FKS* polymorphisms remains to be determined. However, this highlighted the challenge of using the MEC that captures 95% or more of the observed population for determining ECVs; *A. nidulans* showed an implausible value that was much higher than that of the statistical method.

Table 2 also depicts the proposed caspofungin ECVs (using both ≥95% and ≥99% of the modeled population) and modal MECs for each of the six *Aspergillus* spp. The lowest modal MECs (0.06 μg/ml) were for *A. flavus*, *A. niger*, and *A. terreus*, and the highest modal MEC was for *A. fumigatus* (0.25 μg/ml). Modal MEC values were similar (equal to or within one 2-fold dilution) for individual contributing laboratories with all the species tested. Each caspofungin modal MEC ± one 2-fold dilution comprised similar percentages of the populations: 76.8% of *A. flavus*, 78.6% of *A. nidulans*, 79% of *A. terreus*, 80% of *A. versicolor*, 82.3% of *A. niger*, and 83.6% of *A. fumigatus* isolates. In the previous single-laboratory study of caspofungin WT distributions for *Aspergillus* spp. (25), caspofungin modal MECs were two to four 2-fold dilutions lower (0.015 and 0.03 μg/ml) than those for the modes that we observed (*A. nidulans* was not examined in that study). The reason for this major difference is unclear. However, none of our participant laboratories had modal MECs as low as those observed in that previously published study. Also, laboratories did not differ significantly in the observed modal MECs for any of the *Aspergillus* species we tested; all were within one 2-fold dilution for each species. Therefore, given this and the wide geographical range over which MECs were collected for our study, we are confident of the validity of our MEC data.

ECVs encompassing ≥95% of the modeled MEC population were either 0.25 μg/ml (*A. flavus*, *A. niger*, *A. terrus*, and *A. versicolor*) or 0.5 μg/ml (*A. fumigatus* and *A. nidulans*); ECVs were mostly the same or one dilution higher when these values were encompassing ≥99% of each population (Tables 2 and 3). The frequency of caspofungin MECs above the ECV varied according to the species, and it was observed in at least two of the five laboratories for each species as follows: lower for *A. fumigatus* (2% or 33 MECs and 1% or 14 MECs, encompassing ≥95 and ≥99% of the population, respectively) than for the other species (5% or 22 MECs for *A. niger* to 14.1% or 22 MECs for *A. nidulans*, both ≥95 and ≥99% of the MEC population) (Table 3). Caspofungin MECs above ECVs as defined in the present study have recently been reported for isolates of the *Aspergillus* section *Nigri* (caspofungin MECs of

TABLE 3. Caspofungin MEC distributions, ECVs, and percentages of isolates of six *Aspergillus* spp. above the wild-type (WT) distribution from five laboratories as determined by the CLSI M38-A2 broth microdilution method

Species	No. isolates tested	MEC (μg/ml)		Cutoff value (% above the WT distribution)		
		Range	Mode[a]	Statistical ECV ≥95%[b]	Statistical ECV ≥99%[b]	Observed MEC ≥95%[c]
A. fumigatus	1,691	0.016–32	0.25	0.5 (2)	1 (1)	0.5
A. flavus	432	0.016–≥32	0.06	0.25 (5.1)	0.5 (2.1)	0.5
A. nidulans	192	0.032–16	0.12	0.5 (14.1)	0.5 (14.1)	8
A. niger	440	0.016–2	0.06	0.25 (5)	0.25 (5)	0.25
A. terreus	385	0.016–2	0.06	0.25 (8.3)	0.5 (8.3)	0.5
A. versicolor	75	0.032–2	0.12	0.25 (6.7)	0.5 (6.7)	0.5

[a] Most frequent minimum effective concentration (MEC).
[b] Calculated ECVs comprising ≥95 or ≥99% of the statistically modeled population.
[c] MEC value that includes at least 95% of the observed (rather than modeled) overall distribution.

0.5 to 1 μg/ml) as well as for *A. terreus* (caspofungin MEC_{50} and MEC_{90} of 1 and 2 μg/ml, respectively) (1, 20). These regional differences underscore the utility of susceptibility testing and WT cutoffs as a practical means to detect reduced susceptibility to caspofungin. Among the triazoles, the number of non-WT isolates was also *Aspergillus* species dependent, with the highest values being for *A. niger* and *A. nidulans* with itraconazole (8.8% of 427 and 6.3% of 141 MICs, respectively) and *A. versicolor* with posaconazole and voriconazole (13.3% and 6% of 41 and 80 MICs, respectively) (15). This and the previous study with the triazoles (15) highlight the fact that ECVs are species specific and cannot generally be merged if they are to achieve their aim of assisting in the early detection of acquired resistance.

In contrast to *Candida* spp., the relationship between *FKS1* gene mutations and high caspofungin MECs for *Aspergillu*s spp. has not been determined. A breakthrough *A. fumigatus* infection during caspofungin treatment was reported in the absence of characteristic *FKS1* resistance mutations, but the MEC result was not available (2). Overexpression but no mutations of the *FKS* gene in an *A. fumigatus* isolate from another patient failing caspofungin therapy were reported; the MEC was below our proposed ECV of 0.5 μg/ml (3). High MECs (caspofungin MEC of ≥1 μg/ml for 3 of 4 isolates) for *A. fumigatus* were obtained from breakthrough infections in patients receiving either empirical or prophylactic caspofungin therapy (21), but genetic studies were not performed. The latter results are more in agreement with those for the laboratory mutant of *A. fumigatus* (caspofungin MEC of ≥16 μg/ml, with an S678P amino acid change) (27).

In conclusion, we propose caspofungin species-specific ECVs of 0.25 to 1 μg/ml for six *Aspergillus* spp. Further studies are needed to determine the relationship between resistant molecular mechanisms and our proposed caspofungin non-WT values. Although ECVs do not predict clinical outcome to therapy, they should be considered for inclusion in future revised versions of the CLSI M38-A2 document. In the absence of clinical breakpoints, caspofungin ECVs may aid in detecting isolates with reduced caspofungin susceptibility or non-WT strains.

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