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Further Standardization of Broth Microdilution Methodology for In Vitro Susceptibility Testing of Caspofungin against *Candida* Species by Use of an International Collection of More than 3,000 Clinical Isolates

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The influence of test variables on in vitro susceptibility testing of caspofungin was examined with 694 isolates of *Candida albicans* including seven laboratory-derived glucan synthesis mutants. The conditions providing the greatest separation between the mutant strains and the clinical isolates were RPMI medium, MIC end point criterion of partial inhibition, and incubation for 24 h. These testing conditions were then applied to 3,322 isolates of *Candida* spp. (3,314 clinical isolates and eight glucan synthesis mutants). Among the 11 isolates for which caspofungin MICs were ≥2 μg/ml, eight were accounted for by the glucan synthesis mutants. The MICs for >99% of isolates were ≤1 μg/ml, and thus these isolates were differentiated from strains with reduced in vitro and in vivo susceptibilities to caspofungin.

Caspofungin is an echinocandin-class antifungal agent with potent in vitro and in vivo activities against *Candida* spp (1, 4, 7, 10, 11). Caspofungin has recently been approved for primary treatment of candidemia and other forms of invasive candidiasis (4, 11).

In vitro susceptibility studies using National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution (BMD) methods have documented the excellent spectrum and potency of caspofungin versus a wide range of *Candida* spp. (3, 13, 16, 17). For the most part these studies have employed 48-h incubation in RPMI 1640 medium and a conservative MIC end point criterion of complete inhibition of growth (MIC-0) relative to control tubes or wells (2). By the use of these criteria, *C. albicans*, *C. glabrata*, and *C. tropicalis* have been shown to be the most susceptible species (MIC at which 90% of the isolates tested are inhibited [MIC_{90}], ≤0.5 μg/ml; 99% of MICs were ≤1 μg/ml) and *C. parapsilosis* (MIC_{90}, 4 μg/ml) and *C. guilliermondii* (MIC_{90}, >8 μg/ml) have been shown to be the species least susceptible to caspofungin (16).

It is well known that lower MICs of caspofungin may be obtained with *Candida* spp. when testing is performed in antibiotic medium 3 (AM3) rather than RPMI medium (2, 13). To some extent this medium effect may be due to falsely elevated MICs caused by trailing growth patterns in RPMI that are not observed in AM3 (2, 8, 13). Indeed, earlier studies using scanning electron microscopy to study the interaction between *Candida* spp. and the echinocandin LY303366 demonstrated that apparent trailing in RPMI broth beyond the concentration at which a prominent decrease in growth was observed was due to nonviable cellular debris rather than intact yeast cells (8). Thus, the MIC end point criterion for echinocandins should probably be less stringent than MIC_0 to avoid falsely high MICs due to dead organisms and cellular debris (2, 8).

A recent multicenter (17 laboratories) study by Odds and colleagues indicated that highly reproducible MIC results (>80% of MICs within ±1 dilution of the modal MIC) were obtained when *Candida* spp. were tested against caspofungin by the NCCLS BMD method with RPMI 1640 broth, incubation for no longer than 24 h, and a MIC end point criterion that specified the concentration at which the first prominent reduction in growth (MIC-2 or ≥50% inhibition relative to control growth) was observed (14). Furthermore, these test conditions were sufficient to differentiate isolates with "normal" susceptibilities from glucan synthesis mutant strains with "low" susceptibilities to caspofungin. Although similar results were obtained when AM3 was used in place of RPMI medium, concerns regarding batch-to-batch variability with AM3 make this a less acceptable choice of medium (2, 14).

The present study was designed to address several of the findings and concerns raised by the studies noted above. First (phase 1), the issue of medium choice, duration of incubation, and MIC end point criterion was examined using a large collection of *C. albicans* clinical isolates supplemented with seven laboratory-derived glucan synthesis mutants of *C. albicans* as markers of reduced in vitro and in vivo susceptibilities to caspofungin (5, 9). Second (phase 2), the optimal testing conditions, as described by Odds et al. (14) and confirmed by our initial (phase 1) studies, were used to test an international collection of more than 3,000 clinical isolates of *Candida* spp. encompassing nine different species. Again, the seven glucan synthesis mutants of *C. albicans*, plus an additional strain of *C. krusei* with reduced in vitro and in vivo susceptibilities to

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TABLE 1. Caspofungin MIC distribution for 694 isolates of *C. albicans:* influence of test medium, reading time, and end point criterion

Test medium	End point inhibition criterion	Incubation time (h)	No. of isolates at MIC (μg/ml):											
			0.007	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	>8
RPMI	Complete	24	0	2	24	164	399	90	8	0	0	2[a]	0	5[a]
		48	0	2	12	74	303	228	63	5	0	0	1[a]	6[a]
	Partial	24	54	127	338	156	12	0	0	0	4[a]	3[a]		
		48	9	133	348	180	13	2	1	1	4[a]	3[a]		
AM3	Complete	24	35	216	381	51	4	0	2[a]	1[a]	3[a]	1[a]	1[a]	
		48	9	70	457	138	9	4	2[b]	1[a]	1[a]	2[a]	0	2[a]
	Partial	24	109	422	148	8	0	1[a]	2[a]	2[a]	2[a]			
		48	74	406	192	12	2	1	2[a]	1[a]	4[a]			

[a] Glucan synthesis mutant.
[b] One of two isolates is a glucan synthesis mutant.

caspofungin (14), were used to identify a potentially resistant category.

MATERIALS AND METHODS

Organisms. (i) Phase 1. A total of 687 clinical isolates of *C. albicans* representing a broad geographical distribution were used to assess the effect of test medium, MIC end point criterion, and duration of incubation. These isolates were obtained from various surveillance studies conducted by the University of Iowa, and each isolate represented an individual infectious episode (15). In addition, seven laboratory-derived glucan synthesis mutants (mutation in the *FKS1* gene) of *C. albicans* were included to represent strains with reduced in vitro and in vivo susceptibilities to caspofungin (2, 5, 9). The isolates were identified by standard methods (6) and stored as water suspensions until they were used in the study. Prior to testing, each isolate was passaged at least twice on potato dextrose agar (Remel, Lenexa, Kans.) and CHROMagar Candida (Hardy Laboratories, Santa Monica, Calif.) to ensure purity and viability.

(ii) Phase 2. A total of 3,314 clinical isolates obtained from more than 100 different medical centers internationally were tested (17). The collection included 1,993 strains of *C. albicans*, 481 of *C. glabrata*, 310 of *C. tropicalis*, 361 of *C. parapsilosis*, 100 of *C. krusei*, 27 of *C. guilliermondii*, 24 of *C. lusitaniae*, 9 of *C. kefyr*, and 9 of *C. pelliculosa*. The isolates were all recent clinical isolates, and each represented an individual infectious episode (17). In addition, the seven glucan synthesis mutants of *C. albicans* described above (2) and one strain of *C. krusei* with reduced susceptibility (14) were included as markers of decreased susceptibility to caspofungin. The isolates were identified by standard methods (6) and stored as water suspensions until used in the study. Prior to testing, each isolate was passaged at least twice on potato dextrose agar (Remel) and CHROMagar Candida to ensure purity and viability.

Antifungal agents. Caspofungin reference powder was obtained from the manufacturer (Merck Co., Whitehouse Station, Pa.). A stock solution was prepared in water, and serial twofold dilutions were prepared exactly as outlined in NCCLS document M27-A2 (12). Final dilutions were made in RPMI 1640 medium (Sigma, St. Louis, Mo.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) (phases 1 and 2) and in AM3 (Becton Dickinson, Sparks, Md.) (phase 1).

Antifungal susceptibility studies. BMD testing was performed in accordance with the guidelines in NCCLS document M27-A2 (12). In phase 1, both RPMI and AM3 broth were used, whereas in phase 2 only RPMI broth was used. The trays were incubated at 35°C, and MIC end points were read visually. Drug-free and yeast-free controls were included.

In phase 1, the 694 isolates of *C. albicans* (687 clinical isolates and seven glucan synthesis mutants) were tested in both RPMI and AM3 broth and MICs were determined after 24 and 48 h of incubation. MICs were determined at each time point with two visual criteria: complete inhibition, or MIC-0, the lowest caspofungin concentration that supported no visible growth (a clear well), and partial inhibition, or MIC-2, the lowest caspofungin concentration that caused a significant diminution (≥50%) of growth below control growth levels (12).

In phase 2, the 3,322 isolates of *Candida* spp. (3,314 clinical isolates plus eight glucan synthesis mutants [seven of *C. albicans* and one of *C. krusei*]) were tested in RPMI broth and the partial inhibition (MIC-2) end point was determined after 24 h of incubation.

Quality control. Quality control was performed by testing the NCCLS-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (12).

RESULTS AND DISCUSSION

Phase 1. Table 1 summarizes the caspofungin MIC distribution for 694 isolates of *C. albicans* (clinical isolates plus glucan synthesis mutants) tested in both RPMI and AM3 broth. Little difference was observed in the MIC distributions between 24- and 48-h readings irrespective of the testing conditions, although the 48-h complete inhibition MICs in RPMI tended to be higher than those read at 24 h. As expected, the partial inhibition end point criterion resulted in lower MICs with both media. The MICs determined in AM3 were also lower than those determined in RPMI. Notably, all of the testing conditions differentiated the mutant strains with decreased susceptibilities to caspofungin from the clinical isolates tested. The conditions producing the greatest separation between the mutant strains and the clinical isolates were RPMI medium, the partial inhibition end point criterion, and 24-h incubation. These findings are consistent with those reported by Odds et al. (14) and suggest that this approach may be useful in testing other species of *Candida* versus caspofungin.

Phase 2. Table 2 summarizes the in vitro susceptibilities of 3,322 isolates of *Candida* spp. (3,314 clinical isolates and eight glucan synthesis mutants) to caspofungin when tested in RPMI 1640 medium with 24-h incubation and the partial inhibition end point criterion. Among the 11 isolates for which caspofungin MICs were ≥2 μg/ml, eight were accounted for by the glucan synthesis mutants.

These data indicate that the use of RPMI medium, 24-h incubation, and the partial inhibition end point criterion provides an in vitro test method that reliably differentiates strains of *Candida* spp. with known *FKS1* mutations and reduced in vitro and in vivo susceptibilities to caspofungin from the vast majority of clinical isolates of *Candida* spp.

The MIC distributions generated for 3,314 clinical isolates of *Candida* spp. (Table 2) reveal two important findings. First, isolates for which caspofungin MICs exceed 1 μg/ml rarely occur in clinical infections. Only three (two of *C. parapsilosis* and one of *C. guilliermondii*) out of 3,314 (0.09%) clinical isolates exhibited decreased susceptibilities to caspofungin with MICs (≥2 μg/ml) comparable to those observed with the laboratory-derived glucan synthesis mutants (Table 2). Second, among the nine species tested in phase 2 of the study there appear to be two groups that can be differentiated by the degree of susceptibility to caspofungin. Group I includes three

TABLE 2. Caspofungin MIC distribution for 3,322 isolates of *Candida* spp. tested in RPMI 1640 broth with 24-h incubation and a partial inhibition end point criterion

Species	No. of isolates tested	No. of isolates at MIC (μg/ml):											
		0.007	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	>8
C. albicans	2,000	677	381	646	272	17				4[a]	3[a]		
C. glabrata	481	1	22	262	164	25	5	2					
C. tropicalis	310	80	74	97	42	12	3	1	1				
C. kefyr	9	2	4	2	1								
C. pelliculosa	9		7	2									
C. parapsilosis	361	1		2	8	30	138	146	34	1	1		
C. krusei	101			2	28	36	26	7	1		1[a]		
C. guilliermondii	27				2	6	2	11	5				1
C. lusitaniae	24				11	8	4	1					
All species	3,322	761	488	1,013	528	134	178	168	41	5[b]	5[b]		1

[a] Glucan synthesis mutant.
[b] Eight of 10 isolates are glucan synthesis mutants.

common species, *C. albicans*, *C. glabrata*, and *C. tropicalis* (as well as the less common *C. kefyr* and *C. pelliculosa*), and exhibits exquisite susceptibility to caspofungin (MIC_{90}, 0.06 μg/ml), whereas group II includes *C. parapsilosis* and less common species such as *C. krusei*, *C. lusitaniae*, and *C. guilliermondii* and is approximately 10-fold less susceptible (MIC_{90}, 0.5 μg/ml) to caspofungin. All of these species appear to respond equally well clinically to caspofungin treatment (11), and MICs for >99% of isolates in both groups are ≤1 μg/ml; thus, these isolates are differentiated from strains with documented *FKS1* gene mutations (Table 2). However, the differences in the MIC distributions of the two groups suggest possible biological differences in the way that they respond to caspofungin that warrant further investigation.

In summary, we have provided further validation of in vitro methods for determining the susceptibilities of *Candida* spp. to caspofungin. Using a large international collection of clinical isolates of *Candida* spp., we have shown that the use of RPMI medium, 24-h incubation, and the partial inhibition end point criterion provides an in vitro test method that reliably differentiates strains of *Candida* spp. with known glucan synthesis mutations and reduced in vivo susceptibilities to caspofungin from the vast majority (99.9%) of clinical isolates of *Candida* spp. We have also defined potentially important differences in the degree of susceptibility of two broad groups of *Candida* spp. headed by *C. albicans* and *C. parapsilosis*, respectively. The clinical usefulness of this testing method must ultimately be validated by clinical outcomes; however, for now the use of this standardized means of testing caspofungin will be important in monitoring susceptibility trends of this new agent over time.

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