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

M. A. Pfaller,1,2* S. A. Messer,1 L. Boyken,1 C. Rice,1 S. Tendolkar,1 R. J. Hollis,1 and D. J. Dickema1,3

Departments of Pathology,1 Epidemiology,2 and Medicine,3 University of Iowa College of Medicine and College of Public Health, University of Iowa, Iowa City, Iowa 52242

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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 [MIC90] ≤0.5 μg/ml; 99% of MICs were ≤1 μg/ml) and C. parapsilosis (MIC90 4 μg/ml) and C. guilliermondii (MIC90 >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 MIC0 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
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.

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 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...
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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**REFERENCES**


### 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

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates tested</th>
<th>No. of isolates at MIC (µg/ml):</th>
<th>0.007</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.12</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>&gt;8</th>
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<tr>
<td><em>C. albicans</em></td>
<td>2,000</td>
<td></td>
<td>677</td>
<td>381</td>
<td>646</td>
<td>272</td>
<td>17</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
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<tr>
<td><em>C. glabrata</em></td>
<td>481</td>
<td></td>
<td>1</td>
<td>22</td>
<td>262</td>
<td>164</td>
<td>25</td>
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<tr>
<td><em>C. tropicalis</em></td>
<td>310</td>
<td></td>
<td>80</td>
<td>74</td>
<td>97</td>
<td>42</td>
<td>12</td>
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<td>1</td>
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<tr>
<td><em>C. kefyr</em></td>
<td>9</td>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
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<td><em>C. pelliculosa</em></td>
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<td>7</td>
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<td>30</td>
<td>138</td>
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<tr>
<td><em>C. krusei</em></td>
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<td>2</td>
<td>28</td>
<td>36</td>
<td>26</td>
<td>7</td>
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<tr>
<td><em>C. guilliermondii</em></td>
<td>27</td>
<td></td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>11</td>
<td>5</td>
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<tr>
<td><em>C. lusitaniae</em></td>
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<td>528</td>
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<td>168</td>
<td>41</td>
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<td>5^b</td>
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</table>

*Glucan synthesis mutant.

^b Eight of 10 isolates are glucan synthesis mutants.