

Caspofungin Susceptibility Testing of *Candida inconspicua*: Correlation of Different Methods with the Minimal Fungicidal Concentration

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Minimal inhibitory and minimal fungicidal concentrations of caspofungin were determined for 48 *Candida inconspicua* isolates. By using CLSI (formerly NCCLS) methodology with the partial inhibition endpoint criterion, caspofungin exhibited a good fungicidal effect against *C. inconspicua* (the MIC₉₀ was 0.25 µg/ml and the minimum fungicidal concentration [MFC] was 0.5 µg/ml after 24 h). Total inhibition yielded falsely elevated MICs, exceeding even the respective MFCs.

Caspofungin is an echinocandin antifungal exhibiting good in vitro activity against the majority of *Candida* spp., including *C. krusei* (4). Caspofungin MICs for *Candida* spp. determined by broth microdilution (BMD) strongly depend on the applied test conditions, such as incubation time, culture medium, and endpoint criterion (1, 11). According to Bartizal and Odds (1), lower caspofungin MICs could be obtained by using antibiotic medium 3 than with RPMI medium. Pfaller et al. (11) demonstrated that discrepant MIC results obtained with antibiotic medium 3 and RPMI 1640 disappear when 24 h of incubation and the partial inhibition endpoint criterion are used. A higher concentration of starting inoculum seems to be less important; Chrysanthou and Cuenca-Estrella (3) used a 100-times-higher concentration (10⁵ CFU/ml) of starting inoculum in their modified BMD together with a 24-h incubation time and total or nearly total (95%) inhibition as the endpoint criterion. They obtained 96 to 100% agreement with the standard BMD method (9).

Caspofungin exhibits a rapid fungicidal effect against *Candida* species, which has been proven by flow cytometry (4). A standard method for the determination of the minimum fungicidal concentration (MFC) has not yet been proposed (10), although Cantón et al. (2) recently described a method which seems reliable to detect the ≥99.9% killing rate caused by amphotericin B in the case of *Candida* species. Similar experiments determining caspofungin MFCs have not yet been performed (10).

C. inconspicua was the sixth most frequent *Candida* species in our laboratory in 2002 (L. Majoros, G. Kardos, C. Misztai, J. Szabó, and B. Szabó, Abstr. 23rd Int. Spec. Symp. Yeast, abstr. O-6-05, 2003), and due to its decreased susceptibility to fluconazole, it represents a therapeutic problem. The aim of this study was to examine the in vitro efficacy of caspofungin against fluconazole-resistant clinical isolates of *C. inconspicua* by using the standard BMD method (9) and Etest. In the BMD test, MICs were determined by using partial inhibition and total inhibition endpoint criteria after either 24 or 48 h incu-

bation. We also determined MFCs to *C. inconspicua* clinical isolates by using the method described by Cantón et al. (2) with modifications (see below).

We used the same 48 clinical isolates of *C. inconspicua* as in our previous study (8). The identification of isolates was performed as described earlier (7). The testing of isolates was performed in duplicate, and all tests were repeated at least twice with each method. For interpretation, we used the breakpoint proposed by Stone et al. (12): isolates with MICs of >1 µg/ml were considered resistant, and isolates with lower MICs were regarded as susceptible.

BMD. The reference BMD method was performed according to the guidelines of CLSI (formerly NCCLS) (9). Caspofungin (Merck Research Laboratories) was dissolved in sterile distilled water. Stock solutions were diluted with RPMI 1640 medium (with l-glutamine but without bicarbonate) (Sigma), supplemented with glucose (2%), and buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) (Sigma). The final concentration range of caspofungin was 0.03 to 8 µg/ml.

Test plates were incubated at 35°C and read visually after 24 and 48 h. We used two endpoint criteria for MIC determination: (i) total inhibition (MIC_{TI}), the lowest concentration of caspofungin that yielded no visible growth (a clear well); and (ii) partial inhibition (MIC_{PI}), the lowest concentration that produced a prominent decrease in turbidity compared to that of the drug-free control. MIC_{PI}s read after 24 h were used as reference MICs. Quality control strains of *Candida parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were included in each test.

Etest. Caspofungin Etest strips (Merck Research Laboratories) were applied to RPMI 1640 agar with 2% glucose and buffered to pH 7.0 with MOPS. The plates were incubated at 35°C and read after 24 and 48 h.

MFC. For MFC determination, we used the method described by Cantón et al. (2) with the following modifications. BMD was performed using approximately 10⁵ CFU/ml yeast inoculum (3); otherwise, the method was according to the CLSI guidelines (9). The content of each well containing drug concentrations corresponding to and higher than the MIC_{PI} read after 24 h was transferred onto drug-free Sabouraud dextrose agar plates. Plates were incubated at 35°C for 48 h. The

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TABLE 1. Distribution of caspofungin MICs and MFCs for *C. inconspicua* isolates ($n = 48$) obtained by different methods

Method or measurement (time in h) ^a	No. of isolates with indicated MIC (μg/ml)						MIC ₅₀ / MIC ₉₀	MFC ₅₀ / MFC ₉₀	% Overall agreement ^b
	0.06	0.12	0.25	0.5	1	2			
BMD _{PI}									
24	4	27	17	0	0	0	0.12/0.25		
48	0	13	31	4	0	0	0.25/0.25		81.1
BMD _{TI}									
24	0	0	2	19	27	0	1/1		16.7
48	0	0	1	19	26	2	1/1		14.6
Etest									
24	0	8	35	5	0	0	0.25/0.25		87.5
48	0	3	24	21	0	0	0.25/0.5		62.5
MFC									
24	0	10	28	8	2	0		0.25/0.5	
48	0	31	17	0	0	0		0.12/0.25	

^a BMD_{PI}, broth microdilution method with partial inhibition endpoint criterion; BMD_{TI}, broth microdilution method with total inhibition endpoint criterion.

^b Percent agreement represents the percentage of MICs within ± 1 dilution of those obtained by the reference method (9, 11).

MFC was defined as the lowest caspofungin concentration that killed $\geq 99.9\%$ of the final inoculum (≥ 3 -log reduction). In a repeated experiment, MIC_{PI} determination and transfer to antifungal-free medium were performed after 48 h of incubation.

We summarize our results in Table 1. Caspofungin demonstrated excellent activity (48/48 susceptible) against clinical isolates of *C. inconspicua* when we used the partial inhibition criterion read at 24 and 48 h (MIC range, 0.06 to 0.25 and 0.12 to 0.5 $\mu\text{g/ml}$, respectively). MIC_{TI}s obtained after both 24- and 48-h incubation tended to be higher (generally with two dilutions) than the MIC_{PI} read at 24 h. Our findings are concordant with the results published by Pfaller et al. (11), who demonstrated one- to two-dilutions-higher caspofungin MICs by using the total inhibition endpoint criterion than MICs obtained using partial inhibition in the case of *Candida albicans*.

The Etest provided clearly readable sharp interception zones; growth of microcolonies was never observed. MICs obtained by Etest read at 24 h were similar to the MICs found with the BMD method that were read at 24 h (MIC range, 0.12 to 0.5 $\mu\text{g/ml}$).

Comparing the MICs obtained with the methods mentioned above to our reference MICs (partial inhibition and evaluation at 24 h), good agreement was observed only with the MIC_{PI}s read at 48 h and with the Etest results read at 24 h (Table 1). Comparing MICs obtained with total inhibition endpoint criterion read at 48 h, Etest MICs read at 24 and 48 h showed only 39.6 and 60.4% agreement, respectively. Similarly, Laverdiere et al. (6) and Chryssanthou and Cuenca-Estrella (3) obtained good agreement between the standard BMD method (total inhibition and 48 h incubation) and Etest only when they defined agreement less stringently at ± 2 dilutions.

After 24- and 48-h incubations using high inoculum size (10^5 CFU/ml) and partial inhibition, MICs were the same or one

dilution higher than the MIC_{PI}s read at 24 h, in accordance with previously described findings (3).

The MFC range of caspofungin after 24 h incubation was 0.12 to 1 $\mu\text{g/ml}$ (90% MFC [MFC₉₀], 0.5 $\mu\text{g/ml}$). The MFC was equal to $1\times$ or $2\times$ MIC_{PI} in the cases of 83.3% (40/48) of the isolates and never exceeded $4\times$ MIC.

After a 48-h incubation, the obtained MFCs were lower (0.12 to 0.25 $\mu\text{g/ml}$; MFC₉₀, 0.25 $\mu\text{g/ml}$), apparently due to the longer exposure. MFCs were equal to the MIC_{PI}s read at 24 h, with the exception of five isolates, for which MFCs were $2\times$ MIC_{PI}.

Agreement between the MIC_{TI} at 48 h and MFC at 24 h within ± 1 dilution was only 45.8%. MFCs, with the exception of two isolates, did not reach the MIC_{TI} read at 48 h. The correlation between the MIC_{TI} at 48 h and the MFC at 48 h was similarly poor. These findings suggest that after 24-h incubation, virtually all cells are unviable in the wells showing partial inhibition.

This assumption is supported by the findings of Klepser et al. (5), who demonstrated by using scanning electron microscopy that yeast cells exposed to a concentration greater than the MIC₈₀ of another echinocandin, LY303366, exhibited substantial ultrastructure abnormalities and lack of viability signs. Similarly, we proved by using transmission electron microscopy that in the case of the total inhibition endpoint criterion, the falsely elevated MICs (MIC₉₀, 1 $\mu\text{g/ml}$) were caused by unviable cells and cell debris (data not shown). Our results, together with the findings mentioned above (5), indicate that differences between the reference MICs and MIC_{TI}s read at 48 h are most probably due to dead yeast cells and cell debris rather than living cells.

The good agreement between MIC_{PI} read at 24 h and MFCs after 24 and 48 h incubation time and the inconsistency of MFCs and MIC_{TI}s suggest that the MIC_{TI} read at either 24 h or 48 h overestimates the MIC considerably and can frequently lead to falsely elevated MICs, at least in the case of *C. inconspicua*. However, Pfaller et al. (11) and Bartizal and Odds (1) similarly reported lower MIC_{PI}s than MIC_{TI}s in cases of other species, including *C. albicans*, *Candida glabrata*, and *C. krusei*, suggesting that our findings are likely to be applicable to other *Candida* species as well. To confirm this assumption, further studies are currently going on in our laboratory.

Summarizing our results, we demonstrated good in vitro efficacy of caspofungin against the fluconazole-resistant *C. inconspicua* strains by using MIC_{PI} after a 24-h incubation. Our results also showed that the caspofungin Etest read at 24 h may be a reliable substitute for the BMD, at least in the case of *C. inconspicua*. We found that MFCs of caspofungin correspond well to MIC_{PI}s read after 24-h incubation but not to MIC_{TI}s read at either 24 or 48 h. This difference was caused by trailing wells containing mainly unviable cells.

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