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Evaluation of Etest Method for Determining Caspofungin (MK-0991) Susceptibilities of 726 Clinical Isolates of *Candida* Species

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The performance of the Etest for testing the susceptibilities to caspofungin (MK-0991) of 726 isolates of *Candida* spp. was assessed against the National Committee for Clinical Laboratory Standards (NCCLS) microdilution broth method. The NCCLS method employed RPMI 1640 broth medium, and MICs were read after incubation for 48 h at 35°C. MICs were determined by Etest for all 726 isolates with RPMI agar containing 2% glucose (RPG) and were read after incubation for 48 h at 35°C. The *Candida* isolates included *Candida albicans* ($n = 486$), *Candida glabrata* ($n = 96$), *Candida tropicalis* ($n = 51$), *Candida parapsilosis* ($n = 47$), *Candida krusei* ($n = 11$), *Candida lusitaniae* ($n = 2$), and *Candida guilliermondii* ($n = 33$). In addition, a subset of 314 isolates were also tested by Etest using Casitone agar (CAS) and antibiotic medium 3 agar (AM3). The Etest results obtained using RPG correlated well with reference MICs. Overall agreement was 94% with RPG, 82% with CAS, and 79% with AM3. When RPG was used, agreement ranged from 79% for *C. parapsilosis* to 100% for *C. krusei*, *C. lusitaniae*, and *C. guilliermondii*. When CAS was used, agreement ranged from 0% for *C. lusitaniae* to 100% for *C. glabrata*. With AM3, agreement ranged from 0% for *C. lusitaniae* to 100% for *C. guilliermondii*. All three media supported growth of each of the *Candida* species. Etest results were easy to read, with sharp zones of inhibition. In most instances (75%) where a discrepancy was observed between the Etest and the reference method, the Etest MIC was lower. The Etest method using RPG appears to be useful for determining caspofungin susceptibilities of *Candida* species.

The standardization of broth dilution methods for performing antifungal susceptibility testing of yeasts has set the stage for the development of alternative testing methods that may be easier to perform in the clinical laboratory (1, 4–7, 10, 12, 14, 15). These include both broth- and agar-based methods. Agar-based methods for antimicrobial susceptibility testing are widely used in microbiology laboratories and include disk diffusion testing, agar dilution, and the Etest stable-gradient method (8). In many instances agar-based methods may allow for enhanced detection of antimicrobial resistance (7, 9, 14, 15, 23). Although agar-based methods are not widely used for performing antifungal susceptibility testing, recent studies have shown that both disk diffusion testing and the Etest may be useful for testing yeasts and moulds (1, 4–7, 9, 10, 14–17, 19–21).

Among the newer antifungal agents, the water-soluble glucan synthesis inhibitor caspofungin (MK-0991; Merck Research Laboratories, Rahway, N.J.), has potent fungicidal activity against pathogenic yeasts including most species of *Candida* (3, 10, 11, 13, 22). This agent has been tested extensively in broth but has not been widely evaluated using an agar-based testing method. Recently, Lozano-Chiu et al. (10) reported a simple disk diffusion method for determining the in vitro susceptibility testing of caspofungin against *Candida* spp. They demonstrated a good correlation between zone diameter and MICs determined using the National Committee for Clinical Laboratory Standards (NCCLS) recommended broth microdilution method. Although simple to perform, the disk test provides qualitative data only. Thus, the development of a quantitative agar-based test such as the Etest may be desirable.

The Etest has proven useful for testing amphotericin B and the azoles against a variety of fungal pathogens (4–7, 9, 16, 17, 19–21, 23). The choice of agar medium may be important in optimizing the performance of Etest for some antifungal agents (6, 9, 16, 17, 19, 20, 23). Thus far, the Etest has not been applied to the testing of caspofungin or other glucan synthesis inhibitors against *Candida*. In the present study, we evaluated the Etest for caspofungin, using RPMI, Casitone, and antibiotic medium 3 agars, in comparison to the NCCLS reference microdilution method for testing 726 clinical isolates of *Candida* spp.

MATERIALS AND METHODS

Test organisms. A total of 726 clinical isolates of *Candida* species were selected for testing. The collection included 486 *Candida albicans*, 96 *Candida glabrata*, 51 *Candida tropicalis*, 47 *Candida parapsilosis*, 33 *Candida guilliermondii*, 11 *Candida krusei*, and 2 *Candida lusitaniae* isolates. The members of this collection were all recent clinical isolates from geographically diverse medical centers in North and Latin America. The majority were isolated from blood or normally sterile body fluids (18). The isolates were identified by standard methods (24) and were stored as suspensions in water at ambient temperature until used in the study. Prior to testing, each isolate was subcultured at least twice to Sabouraud dextrose agar (Remel, Lenexa, Kars.) to ensure optimal growth characteristics.

Antifungal agents. Etest strips containing caspofungin (MK-0991) were supplied by AB BIODISK (Solna, Sweden). Caspofungin was obtained as a reagent-grade powder from Merck Research Laboratories. Stock solutions were prepared in water and further diluted in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma, St. Louis, Mo.) and dispensed into 96-well microdilution trays. Trays containing a 0.1-ml aliquot

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TABLE 1. In vitro activity of caspofungin against 726 *Candida* clinical isolates as determined by the reference broth microdilution method[a]

Organism	No. of isolates tested	MIC (μg/ml)[b]		
		Range	50%	90%
C. albicans	486	0.03–1.0	0.12	0.25
C. glabrata	96	0.03–1.0	0.12	0.25
C. tropicalis	51	0.06–1.0	0.25	0.5
C. parapsilosis	47	0.25–8.0	1.0	2.0
C. krusei	11	0.5–1.0	0.5	1.0
C. lusitaniae	2	1.0–2.0	1.0	2.0
C. guilliermondii	33	>8.0	>8.0	>8.0
All	726	0.03–>8.0	0.12	1.0

[a] Performed as described by NCCLS documents M27-A (12).
[b] 50 and 90%, MICs at which 50 and 90% of isolates tested, respectively, are inhibited.

TABLE 2. Agreement between Etest and reference caspofungin MICs for *Candida* clinical isolates tested on RPG, Casitone agar, and (AM3) agar

Organism	% agreement when tested on[a]		
	RPG	CAS	AM3
C. albicans	95 (486)	85 (173)	88 (173)
C. glabrata	99 (96)	100 (57)	100 (57)
C. tropicalis	86 (51)	40 (30)	23 (30)
C. parapsilosis	79 (47)	77 (39)	49 (39)
C. krusei	100 (11)	80 (5)	100 (5)
C. lusitaniae	100 (2)	0 (2)	0 (2)
C. guilliermondii	100 (33)	83 (6)	100 (6)
All	94 (726)	82 (312)	79 (312)

[a] Percentage of Etest MICs (read at 48 h) determined on RPG, Casitone, and AM3 agar medium that are within two $\log_2$ dilutions of the reference microdilution MICs (RPMI broth at 48 h). Numbers of isolates tested are given in parentheses.

of appropriate drug solution (twice the final concentration) in each well were subjected to quality control (QC) testing and then sealed and stored at −70°C until used in the study. The final concentrations of caspofungin were 0.007 to 8 μg/ml.

Media. Agar formulations used for the Etest were RPMI 1640 (American Biorganics, Buffalo, N.Y.) supplemented with 1.5% agar and 2% glucose (RPG) and buffered with MOPS, Casitone agar (CAS; Difco) and antibiotic medium 3 agar (AM3; BBL). The RPMI 1640 broth medium used for the microdilution testing was buffered with MOPS in accordance with the NCCLS M27-A method (12).

Antifungal susceptibility testing methods. Broth microdilution tests were performed as specified by NCCLS document M27-A (12). A stock inoculum suspension of 1×10^6 to 5×10^6 cells per ml was standardized spectrophotometrically at 530 nm to match the turbidity of a 0.5 McFarland standard, diluted 1:1,000 with medium, and validated by quantitative plate counts, to provide a test inoculum of 0.5×10^3 to 2.5×10^3 cells per ml. The microdilution trays were incubated at 35°C and read after 48 h of incubation. For caspofungin (MK-0991), the MIC end point was defined as the lowest concentration of antifungal agent that completely inhibited growth.

For the Etest, 90-mm-diameter plates containing agar at a depth of 4.0 mm were used. The agar surface was inoculated by using a nontoxic swab dipped in a cell suspension adjusted spectrophotometrically at 530 nm to the turbidity of a 0.5 McFarland standard. After excess moisture was absorbed into the agar and the surface was completely dry (15 min at room temperature), an Etest strip was applied to each plate. The plates were incubated at 35°C and read at 48 h. The MIC was taken as the lowest concentration at which the zone of complete inhibition intersected the strip.

QC. QC testing was performed as described by NCCLS document M27-A using *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (12). QC determinations made on each day of testing were within the control limits for caspofungin as established by Barry et al. (2): *C. krusei* ATCC 6258, 0.25 to 1.0 μg/ml; *C. parapsilosis* ATCC 22019, 0.5 to 4.0 μg/ml.

Analysis of results. Etest MICs read at 48 h on the three media were compared to reference microdilution MICs read at 48 h. All 726 isolates were tested on RPG agar. A subset of 314 isolates were also tested by Etest on CAS and AM3 agar. Since the Etest scale has a continuous gradient of concentrations, the MICs in between twofold dilutions were raised to the next twofold level of the reference method for comparison (16, 17, 19, 20). Off-scale MICs at the upper limit were converted to the next higher concentration, and off-scale results at the lower limit were left unchanged. Discrepancies between MICs of no more than two dilutions were used to calculate the percent agreement.

RESULTS AND DISCUSSION

Table 1 summarizes the in vitro susceptibilities of 726 *Candida* spp. isolates to caspofungin as determined by the reference broth microdilution method. The caspofungin MICs obtained were consistent with values reported previously for the individual *Candida* species tested in RPMI 1640 medium (3, 11, 13, 22). Lower MICs of caspofungin have been reported when isolates of *Candida* spp. were tested in A3 broth (11). Thus, the MIC data shown in Table 1 may underestimate the activity of caspofungin.

Table 2 summarizes the percentage of 48-h caspofungin MICs obtained for all 726 isolates by the Etest on RPG agar and for the 312 isolates tested on Casitone and AM3 agar that were within two dilutions of the reference method result. Overall, the percent agreement was 94% with results obtained for RPG agar. The agreement between the Etest using RPG agar and microdilution MICs was >90% for all species except *C. tropicalis* and *C. parapsilosis*. Of the 10 discrepancies observed with *C. parapsilosis*, 4 were corrected when Etest results obtained after a 24-h incubation were used. In general, however, the 24-h Etest results yielded a lower percent agreement with the reference method than that obtained with 48 h readings. The agreement between Etest and microdilution MICs was poorer when either Casitone (82% agreement) or AM3 (79% agreement) was used (Table 2); however, ≥80% agreement was achieved on both media with *C. albicans*, *C. glabrata*, *C. krusei*, and *C. guilliermondii*. In most instances (75%) when a discrepancy was observed between Etest and the reference method, the Etest provided a lower MIC.

The results of this study provide the first documentation of the applicability of the Etest stable-agar-gradient method for determining the in vitro susceptibilities of *Candida* species to the glucan synthesis inhibitor caspofungin. Recently, Lozano-Chiu et al. (10) demonstrated the feasibility of agar-based methods for testing this agent when they reported a disk diffusion method using RPG agar for determining the susceptibilities of *Candida* spp. to caspofungin. These authors tested 94 isolates by both disk and reference broth microdilution methods and found that the results obtained in the disk test correlated well with those obtained by the broth microdilution method (10). Similar to our results, they found that when discrepancies between agar- and broth-based methods were observed, the agar-based disk method result was always more susceptible.

In the present study, we found that RPG provided optimal growth of all species tested and excellent agreement with the MICs obtained in the broth microdilution method (Table 2). As noted by Lozano-Chiu with the disk test (10), the inhibition

ellipses were sharply defined and the MICs were easily determined with the Etest.

Neither Casitone agar nor AM3 agar performed particularly well in this study. In most instances, the MIC of caspofungin was underestimated by more than two dilutions when determined on these media relative to the broth MIC. Both media supported adequate growth of *Candida* spp., as reported previously (16, 17, 20, 23). It is notable that the antifungal activity of caspofungin (MK-0991) has been shown to be enhanced in AM3 broth (11). Thus, it is conceivable that better agreement between broth dilution and Etest MICs could be achieved if organisms were tested in AM3 broth as well as agar, but this was not done in the present study.

In summary, we have provided the first documentation of the ability of the Etest to generate caspofungin MIC data that are comparable to those obtained by the NCCLS broth microdilution method. It is now apparent that Etest using RPG may be used to test *Candida*, *Cryptococcus*, and other yeasts against polyenes, triazoles, and inhibitors of glucan synthesis (9, 16, 17, 20, 23). This provides great flexibility to laboratories that may want to test only one or two agents yet provide quantitative MIC data that are comparable to that generated by the NCCLS reference broth dilution method. The flexibility of the Etest technology will stimulate additional unique applications that may add to our understanding of the in vitro interactions between fungi and antifungal agents.

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