

## Comparative Evaluation of Disk Diffusion with Microdilution Assay in Susceptibility Testing of Caspofungin against *Aspergillus* and *Fusarium* Isolates

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**We compared the disk diffusion and broth microdilution methods for susceptibility testing of caspofungin against *Aspergillus* ( $n = 78$ ) and *Fusarium* ( $n = 22$ ) isolates. Microdilution testing followed the NCCLS M-38P guidelines but was performed in antibiotic medium 3 supplemented to 2% glucose (AM3). Disk diffusion assays were performed on AM3 agar plates with a 2- $\mu$ g caspofungin disk. By both methods, caspofungin showed favorable activity against *Aspergillus* isolates and no activity against *Fusarium* isolates. In the disk-based format, intrazonal growth that was not influenced by the drug concentration gradient was consistently observed for all of the *Aspergillus* isolates tested.**

The development of novel, water-soluble echinocandins is an exciting development in systemic antifungal therapy. These agents exert antifungal activity via inhibition of (1,3)- $\beta$ -D-glucan synthesis. Because of the lack of this target in mammalian tissue and the dissimilarity between the mechanism of action of echinocandins and other systemic antifungal drugs (particularly polyenes and azoles), echinocandins appear to be advantageous and promising for the treatment of invasive mycoses (2, 4, 5, 7, 8, 10, 12, 13, 19, 22). Caspofungin (Cancidas; Merck Research Laboratories) is a novel echinocandin that was licensed in January 2001 in the United States.

While caspofungin has proven to be efficacious in the treatment of candidiasis, aspergillosis, and possibly other mycoses (9; J. Hiemenz, I. Raad, M. Boogaerts, J. Maertens, A. Saah, C. A. Sable, J. A. Chodakewitz, M. Severino, P. Sadder, R. Berman, M. J. DiNubile, T. F. Patterson, D. W. Denning, and T. J. Walsh, Focus on Fungal Infections 11, abstr. 21, 2001; J. Maertens, I. Raad, C. A. Sable, A. Ngai, R. Berman, T. F. Patterson, D. Denning, and T. Walsh, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1103, 2000; M. A. Powles, J. Anderson, P. Liberator, and D. M. Schmatz, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-42, 1996; C. A. Sable, A. Villanueva, E. Arathon, E. Gotuzzo, G. Turcato, D. Uip, L. Nriega, C. Rivera, E. Rojas, V. Taylor, R. Berman, G. B. Calandra, and J. Chodakewitz, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. LB-33, 1997), in vitro susceptibility testing method and interpretive test parameters have not been fully established for this novel drug. In addition, there are few data correlating its in vitro activity and the in vivo response to it. Previous studies have focused on broth dilution methods for susceptibility testing of caspofungin against yeasts and molds (6, 17, 20, 21). When testing caspofungin and other echinocandins against molds, a distinctive microscopic interpretive parameter called

the minimum effective concentration (MEC) has been found to be more consistent and also appears to be better correlated with clinical outcome than the conventional macroscopic MIC-2 (1, 9, 11, 14; C. M. Douglas, J. C. Bowman, G. K. Abruzzo, A. M. Flattery, C. J. Gill, L. Kong, C. Leighton, J. G. Smith, V. B. Pikounis, K. Bartizal, M. B. Kurtz, and H. Rosen, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1683, 2000). Agar-based testing methods are the other obvious avenue of investigation. Some investigators have explored the use of disk diffusion susceptibility testing for caspofungin against yeasts (specifically, *Candida* isolates) (16), but data on caspofungin testing against molds by the disk diffusion method are lacking. In this study, we investigated the applicability of a disk diffusion assay in susceptibility testing of caspofungin against *Aspergillus* and *Fusarium* isolates and compared the results of this disk diffusion assay with those of the broth microdilution method for these isolates.

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*Aspergillus* strains ( $n = 78$ ; 27 *Aspergillus flavus*, 26 *Aspergillus fumigatus*, 16 *Aspergillus niger*, and 9 *Aspergillus terreus*) and *Fusarium* strains ( $n = 22$ ; 18 *Fusarium solani* and 4 *Fusarium oxysporum*) isolated from clinical samples were included in this study. One of the *Aspergillus fumigatus* isolates (strain 2-160) was included in each run of susceptibility tests as a reference strain to validate the reproducibility and quality of the test results. The isolates were defined to the species level by standard methods (15) and stored on Sabouraud dextrose agar slants at  $-70^{\circ}\text{C}$  until tested. Standard caspofungin powder was provided by Merck Research Laboratories for use in susceptibility tests.

Caspofungin susceptibility tests were done by using the broth microdilution and disk diffusion methods. Except as noted otherwise, broth microdilution tests were done in accordance with the NCCLS guidelines for conidium-forming filamentous fungi (18). Antibiotic medium 3 (lot JD4ZSG; BBL, Becton Dickinson) buffered by addition of 1 g of  $\text{Na}_2\text{HPO}_4$  and

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TABLE 1. Microdilution and disk diffusion test results at 24, 48, and 72 h of incubation

Species (no. of isolates)	Incubation period (h) <sup>a</sup>	IZ diam (mm)		MIC-2 (μg/ml)		MEC (μg/ml)	
		AM <sup>b</sup>	Range	GM <sup>c</sup>	Range	GM	Range
<i>Aspergillus</i> spp. (78)							
<i>A. flavus</i> (27)	24			2.27	0.125->16	0.26	0.125-0.5
	48	21.2	16-25	3.7	0.125->16	0.26	0.125-0.5
	72	20.1	15-23	>16	>16	0.26	0.125-0.5
<i>A. fumigatus</i> (26)	24			0.43	0.25->16	0.31	0.25-0.5
	48	16.2	12-23	2.83	0.25->16	0.31	0.25-0.5
	72	15.3	11-19	17.4	0.25->16	0.31	0.25-0.5
<i>A. niger</i> (16)	24			0.23	0.125-1	0.2	0.125-0.25
	48	18.1	15-24	0.26	0.125-0.5	0.2	0.125-0.25
	72	17.4	15-24	0.32	0.25-1	0.27	0.25-0.5
<i>A. terreus</i> (9)	24			0.25	0.25	0.25	0.25
	48	19.6	12-26	0.25	0.25	0.25	0.25
	72	17.4	10-23	1.26	0.25->16	0.25	0.25
<i>Fusarium</i> spp. (22)							
<i>F. solani</i> (18)	24			19.4	16->16	16.63	4->16
	48		NZ <sup>d</sup>	26.4	16->16	26.4	16->16
	72		NZ	>16	>16	>16	>16
<i>F. oxysporum</i> (4)	24			16	16	16	16
	48		NZ	26.91	16->16	26.91	16->16
	72		NZ	>16	>16	>16	>16

<sup>a</sup> Disk diffusion test results could not be evaluated at 24 h because of the poor growth of the isolates.

<sup>b</sup> AM, arithmetic mean.

<sup>c</sup> GM, geometric mean.

<sup>d</sup> NZ, no IZ detected.

1g of NaH<sub>2</sub>PO<sub>4</sub> to each liter and supplemented to 2% glucose (AM3) was used as the test medium. Serial twofold dilutions of caspofungin over a range of 16 to 0.03 μg/ml were prepared in microdilution plates. The results were read after 24, 48, and 72 h of incubation by using two different parameters: the visual MIC-2 (the minimum concentration [micrograms per milliliter] of caspofungin that provides an ~50% reduction in growth compared to the growth in the control well) and the microscopic MEC (the minimum concentration [micrograms per milliliter] of caspofungin that results in the formation of abnormal hyphal growth with short, abundant branchings [14]). The broth microdilution assay results for the isolates included in this study were published in one of our previous reports (1).

Disk diffusion tests were done by using empirically defined parameters. Caspofungin disks were prepared in house. Blank paper disks (6.3 mm in diameter; Becton Dickinson Microbiology Systems, Cockeysville, Md.) were impregnated with 20 μl of a caspofungin suspension (concentration, 100 μg/ml), resulting in a final concentration of 2 μg/disk. The disks were allowed to dry at room temperature. (This disk concentration was chosen on the basis of the results of preliminary experiments in which blank disks were impregnated with 20 μl from suspensions containing caspofungin at concentrations of 200, 100, 50, and 25 μg/ml. The concentration of 100 μg/ml was chosen because it yielded inhibition zones [IZs] that are wide enough to be measured and sufficiently narrow to be accurately determined.) The strain to be tested was initially suspended in saline, adjusted spectrophotometrically to ~81 or ~70% transmittance (for *Aspergillus* and *Fusarium*, respectively), and then

diluted 1/100 in distilled water to achieve a final concentration of ~10<sup>4</sup> CFU/ml. The prepared inoculum was swabbed onto an AM3 agar plate, and the plate was left to dry at room temperature for 20 min. A caspofungin disk was then placed onto the center of the inoculated agar plate. The plates were incubated for 72 h at 35°C, and the IZ diameters (millimeters) were measured at 24, 48, and 72 h of incubation. The edges of the IZs were taken as the points of a marked decrease in fungal density.

For comparative evaluation of the broth microdilution and disk diffusion methods, the geometric mean (GM) and range of the MICs and MECs and the arithmetic mean and range of the IZ diameters were calculated for each genus-species combination. For computation of GM values, high off-scale MICs and MECs were converted to the twofold concentration just above the highest drug concentration tested.

The results obtained by the microdilution and disk diffusion methods at 24, 48, and 72 h of incubation of the test isolates and reference strain 2-160 are shown in Tables 1 and 2, respectively. While broth microdilution test results could be determined at all of the reading time points (24, 48, and 72 h), disk diffusion test results could not be interpreted at 24 h because of poor growth. At 48 and 72 h, IZs with sharply defined edges were observed on agar plates.

All of the *Aspergillus* strains generated measurable IZs, and the diameters of these IZs were distributed over a relatively narrow range. Interestingly and in addition, *Aspergillus* microcolonies were visualized inside the IZs and these colonies were found uniformly distributed right up to and even under the

TABLE 2. Microdilution and disk diffusion test results obtained for reference strain 2-160<sup>a</sup>

Incubation period (h)	IZ diam range (mm)	MIC-2 range ( $\mu\text{g/ml}$ )	MEC range ( $\mu\text{g/ml}$ )
24		0.125–0.25	0.125–0.25
48	15–17	0.25–>16	0.25
72	13–17	0.25–>16	0.25

<sup>a</sup> The results shown were obtained in four runs and by testing the isolate in duplicate in each run. Disk diffusion test results could not be evaluated at 24 h because of poor growth.

disks without any evident effect of the drug concentration gradient (Fig. 1). These colonies were observed for all of the *Aspergillus* species and strains tested. It is noteworthy that the number of intrazonal colonies observed for *A. niger* was lower than the number observed for other *Aspergillus* species. When examined under a microscope, these intrazonal colonies produced short, stubby hyphal branchings and a star-like morphology. This appearance was similar to that observed at the MEC. The colonies outside the zone, on the other hand, showed a normal, elongated, branching hyphal morphology. When the intrazonal colonies were subcultured and retested, they yielded a pattern identical to that seen with the original isolate. On the other hand, confluent growth and absence of an IZ were consistently observed for all of the *Fusarium* isolates. Comparison of the results obtained by the broth microdilution and disk diffusion assays showed that, as for the *Fusarium* isolates, the high MECs obtained by the microdilution method correlated very well with absence of IZs on disk diffusion agar plates. Relatedly and as for *Aspergillus* isolates, relatively lower MECs correlated with the production of measurable IZs around caspofungin disks.

In the present study, our major goal was to investigate the relevance of the disk diffusion assay for caspofungin susceptibility testing against *Aspergillus* and *Fusarium* strains. We thus compared the disk diffusion assay results with our MECs pre-

viously determined by the broth microdilution method. Since MICs tended to increase inconsistently, particularly with extended incubation (1), we focused more on the correlation of MECs with IZ diameters. The comparative evaluation of the two methods showed that while lower MECs corresponded to the generation of IZs, higher MECs were in absolute correlation with the absence of IZs. Relatively lower MECs and measurable IZs were obtained with *Aspergillus* isolates, while *Fusarium* spp. consistently generated very high MECs and no IZs. Being less time-consuming and less labor-intensive, the disk diffusion method is preferable to the microdilution method. However, the inability to determine the susceptibility test result at 24 h for an individual isolate appears to be a notable limitation of the disk diffusion assay. Further investigation is required to determine whether higher inoculum concentrations yield satisfactory growth on disk diffusion agar plates at 24 h.

Our observation of the growth of *Aspergillus* microcolonies inside the IZs was noteworthy. Since the intrazonal colonies produced the same growth pattern when retested, the possibility of heterogeneous resistance was ruled out. Thus, this finding appeared to be a special growth pattern. This might originate from the partial inhibitory nature of caspofungin and other echinocandins and correspond to the lack of complete inhibition of growth observed both in microdilution plate wells with high caspofungin concentrations and by examination of the intrazonal colonies under a microscope. The mechanism, meaning, and clinical significance of this observation remain unclear and merit investigation.

In this study, we used the disk diffusion assay for susceptibility testing of caspofungin against *Aspergillus* and *Fusarium* isolates and compared the results with those obtained by the microdilution method. A preliminary report has been previously published on the use of the disk diffusion assay for candidin derivatives L-733560, L-705589, and L-731373 against *Aspergillus* spp. In that study, potato dextrose agar was seeded with  $10^6$

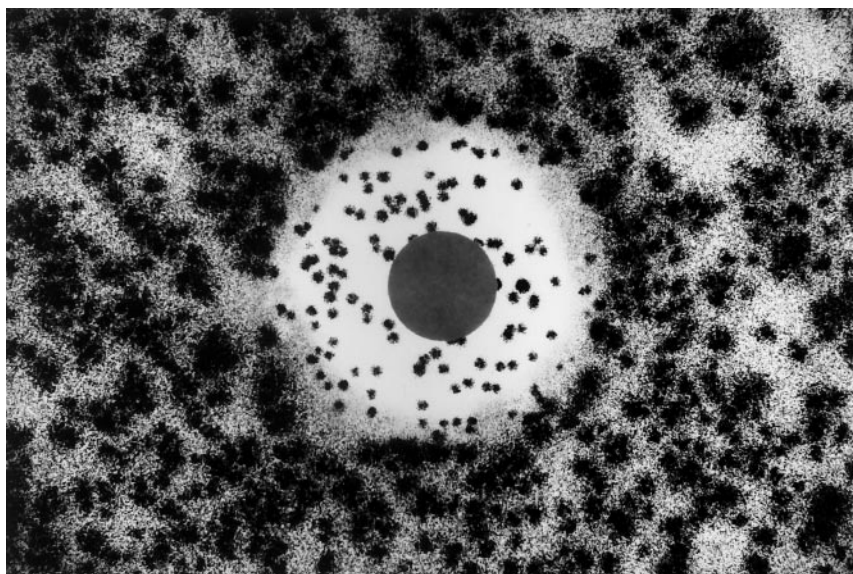


FIG. 1. *Aspergillus* growth pattern obtained by the disk diffusion test method. Note the clear zone edges and the microcolonies inside the IZ. The disk contained 2  $\mu\text{g}$  of caspofungin.

CFU of *A. fumigatus* spores and poured into petri dishes. Disks impregnated with each of the candins (at concentrations of 128 to 0.06 µg/ml) were placed on the agar plates. IZs were observed after 24 h of incubation, demonstrating the favorable in vitro activity of the compounds against *Aspergillus* strains. No observation was noted regarding intrazonal growth (3).

We conclude that the disk diffusion and microdilution methods appear to be correlated in the susceptibility testing of caspofungin against *Aspergillus* and *Fusarium* isolates. The intrazonal growth of colonies of *Aspergillus* spp. is of uncertain relevance.

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