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Species-Specific Differences in the Susceptibilities of Biofilms Formed by *Candida* Bloodstream Isolates to Echinocandin Antifungals[▽]

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The echinocandin susceptibilities of bloodstream *Candida* isolates growing in a biofilm was investigated. Within the therapeutic range of concentrations of each drug, caspofungin and micafungin were active against biofilms formed by *Candida albicans* or *C. glabrata* but not those formed by *C. tropicalis* or *C. parapsilosis*.

Biofilm-mediated antifungal resistance is a well-documented phenomenon for *Candida* species and probably contributes to *Candida* pathogenicity in catheter-related bloodstream infections (BSIs) (6, 10, 16). Although fungal biofilm-associated infections are frequently refractory to conventional antifungal therapy, the echinocandins, which constitute a new class of antifungals that inhibit 1,3-β-D-glucan synthase, have recently been demonstrated to be active against *Candida albicans* biofilms (3, 14). While *C. albicans* is the most commonly isolated *Candida* species, other non-*C. albicans* species have been increasingly recognized as catheter-related BSI pathogens (4, 16). However, there have been few comparisons of the activities of echinocandins against biofilms formed by different *Candida* species. We compared the in vitro activities of caspofungin, micafungin, fluconazole, and amphotericin B against biofilms formed by BSI isolates of four different *Candida* species.

We examined 43 *Candida* species isolates, including 12 *C. albicans*, 12 *Candida parapsilosis*, 10 *Candida tropicalis*, and 9 *Candida glabrata* isolates. All of the isolates were from blood cultures acquired at Chonnam National University Hospital, Gwangju, South Korea, between January 1999 and December 2003. The MICs for planktonic cells were determined by the standard CLSI (Clinical and Laboratory Standards Institute) M27-A2 broth microdilution method (5). The MICs of the two echinocandins for planktonic cells were defined as the lowest concentrations resulting in the prominent inhibition of growth as determined after 24 h of incubation (11).

The MICs for sessile cells (biofilms) were determined using a microtiter-based assay (3, 15). In this system, mature biofilms were allowed to form in 96-well microtiter plates for 48 h and the cell densities of the biofilms were estimated using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrozolium-5-carboxanilide (XTT) absorbance assay. The drugs were prepared in a series of twofold dilutions as follows: fluconazole, 1,024 to 2 μg/ml; amphotericin B, 32 to 0.06 μg/ml; and the two echinocandins, 16 to 0.03 μg/ml. The inhibitory effects of the antifungals were measured as the optical densities (ODs) of the antifungal-treated wells relative to those of the control (antifungal-free) wells (considered to be 100%) as determined

TABLE 1. Distributions of fluconazole MICs for different *Candida* species strains under planktonic or biofilm (sessile-cell) growth conditions

Species	No. of isolates tested	Type of MIC[a]	No. of isolates for which indicated MIC (μg/ml) was:											
			0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>1,024
C. albicans	12	MIC for planktonic cells			3	4	1	3			1				
		MIC_{50} for sessile cells									1	1	2	1	7
		MIC_{80} for sessile cells													12
C. parapsilosis	12	MIC for planktonic cells				1	4	4	3						
		MIC_{50} for sessile cells											2		10
		MIC_{80} for sessile cells													12
C. tropicalis	10	MIC for planktonic cells			1	6	1	2							
		MIC_{50} for sessile cells									2				8
		MIC_{80} for sessile cells													10
C. glabrata	9	MIC for planktonic cells								1	2	5	1		
		MIC_{50} for sessile cells									1				8
		MIC_{80} for sessile cells													9

[a] MIC_{50}s and MIC_{80}s were determined by measuring XTT activity reduction.

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TABLE 2. Distributions of amphotericin B MICs for different *Candida* species strains under planktonic or biofilm growth conditions

Species	No. of isolates tested	Type of MIC[a]	No. of isolates for which indicated MIC (μg/ml) was:										
			0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32
C. albicans	12	MIC for planktonic cells				3	9							
		MIC_{50} for sessile cells				3	3	6						
		MIC_{80} for sessile cells						4	4					4
C. parapsilosis	12	MIC for planktonic cells			2	2	8							
		MIC_{50} for sessile cells					2	5	5					
		MIC_{80} for sessile cells							2	2	1			7
C. tropicalis	10	MIC for planktonic cells				1	9							
		MIC_{50} for sessile cells				4	2	4						
		MIC_{80} for sessile cells						1	1	2	1	1		4
C. glabrata	9	MIC for planktonic cells			1	1	6	1						
		MIC_{50} for sessile cells			1	2	4	1	1					
		MIC_{80} for sessile cells					1	1	2	1	1	1		2

[a] MIC_{50}s and MIC_{80}s were determined by measuring XTT activity reduction.

in the XTT assays and are expressed as percentages of the values for control wells. The MIC_{50} and MIC_{80} of each drug for sessile cells were determined and compared to the controls (15). All isolates were tested at least twice.

The ODs of the different *Candida* species were compared by the Mann-Whitney U test by using the SPSS Win 10.0 program. Differences between the species were considered to be significant for P of <0.05. Correlations between the MICs for planktonic cells and those for sessile cells and between the MICs of caspofungin and micafungin for sessile cells were examined by the least-squares method (13). Alpha was set at 0.05, and all the P values were two tailed.

The distributions of antifungal MICs for planktonic and sessile cells of the different *Candida* species are shown in Tables 1, 2, and 3. The median MIC_{50}s and MIC_{80}s of fluconazole for sessile cells of all *Candida* species were >1,024 μg/ml. The median MIC_{50} of amphotericin B for sessile cells of each of the four *Candida* species ranged from 0.5 to 1 μg/ml, which was similar to the median MIC for planktonic cells (0.5 μg/ml), while the median MIC_{80} for sessile cells ranged from 2 to >32 μg/ml. These data show that amphotericin B is moderately effective against the biofilms of all four species, whereas fluconazole is ineffective.

The caspofungin MIC_{50}s for *C. albicans* sessile cells ranged from 0.06 to 0.5 μg/ml, similar to the values reported previously (3). The median caspofungin MIC_{80}s for sessile cells of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* were 0.5, >16, >16, and 1 μg/ml, respectively. The median mica-

TABLE 3. Distribution of caspofungin and micafungin MICs for different *Candida* species strains under planktonic or biofilm growth conditions

Drug	Species	No. of isolates tested	Type of MIC[a]	No. of isolates for which indicated MIC (μg/ml) was:									
				0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>16
Caspofungin	*C. albicans*	12	MIC for planktonic cells	6	4	2								
			MIC_{50} for sessile cells		1	1	1	9						
			MIC_{80} for sessile cells					9	3					
	C. parapsilosis	12	MIC for planktonic cells				6	5	1					
			MIC_{50} for sessile cells						1	2	2	2		5
			MIC_{80} for sessile cells											12
	C. tropicalis	10	MIC for planktonic cells	4	5	1								
			MIC_{50} for sessile cells						6	2				2
			MIC_{80} for sessile cells											10
	C. glabrata	9	MIC for planktonic cells	2	5	2								
			MIC_{50} for sessile cells			1	1	6	1					
			MIC_{80} for sessile cells					3	6					
Micafungin	*C. albicans*	12	MIC for planktonic cells	12										
			MIC_{50} for sessile cells		2	6	3	1						
			MIC_{80} for sessile cells			1	2	6	3					
	C. parapsilosis	12	MIC for planktonic cells				3	7	2					
			MIC_{50} for sessile cells							2	5	1		4
			MIC_{80} for sessile cells								1			11
	C. tropicalis	10	MIC for planktonic cells	10										
			MIC_{50} for sessile cells			1	1	1	1	1	1			4
			MIC_{80} for sessile cells								1			9
	C. glabrata	9	MIC for planktonic cells	9										
			MIC_{50} for sessile cells		2	6		1						
			MIC_{80} for sessile cells			3	2	2	1	1				

[a] MIC_{50}s and MIC_{80}s were determined by measuring XTT activity reduction.

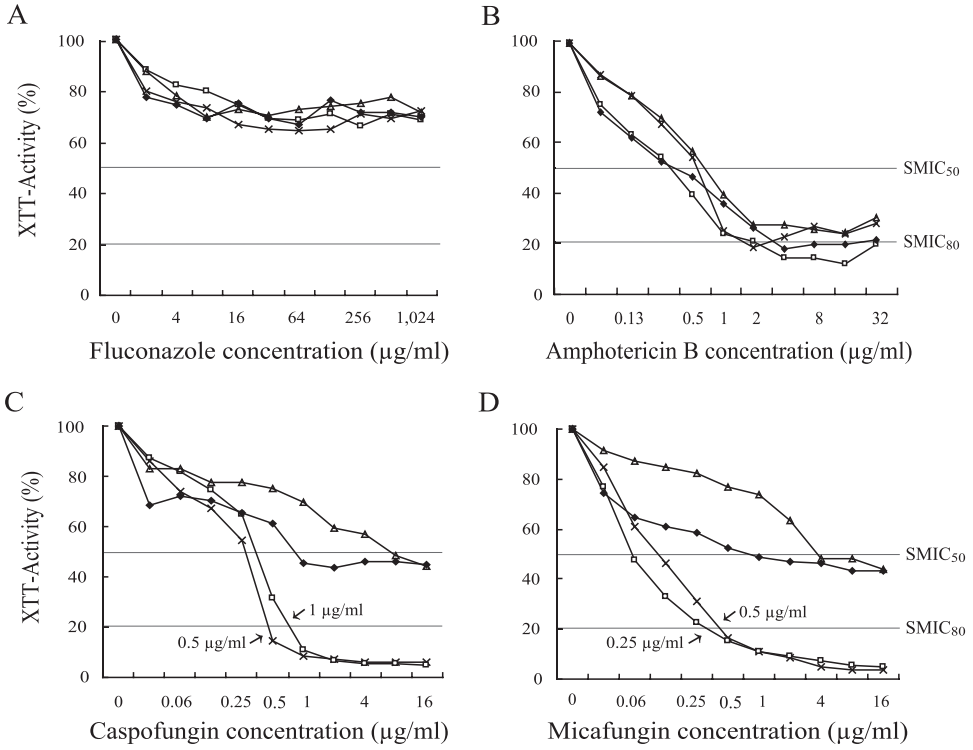

FIG. 1. In vitro activities of different concentrations of drugs against biofilms formed by four individual bloodstream isolates representing four different *Candida* species. Symbols: ×, *C. albicans*; △, *C. parapsilosis*; ◆, *C. tropicalis*; □, *C. glabrata*. The inhibitory effect of each concentration of antifungal was measured as the average OD of all antifungal-treated wells and expressed as a percentage of the OD of control (antifungal-free) wells (considered to be 100%) as determined in XTT reduction assays. Species-specific differences are evident for the activities of caspofungin and micafungin against *Candida* biofilms ($P < 0.05$), in contrast to the activities of fluconazole and amphotericin B. $SMIC_{50}$, MIC_{50} for sessile cells; $SMIC_{80}$, MIC_{80} for sessile cells.

fungin MIC_{80}s for sessile cells were 0.5 μg/ml and 0.25 μg/ml for *C. albicans* and *C. glabrata*, respectively, and >16 μg/ml for both *C. parapsilosis* and *C. tropicalis* ($P < 0.01$). There was no correlation between the MICs for planktonic cells and those for sessile cells of a given strain, but a positive correlation between the caspofungin and micafungin MICs for sessile cells was noted (R, 0.4 for MIC_{50} for sessile cells; $P = 0.014$; R, 0.9 for MIC_{80} for sessile cells; $P < 0.01$).

Figure 1 shows the inhibition curves for biofilm-grown *Candida* species in the presence of different concentrations of the four antifungal agents. The inhibitory effects of fluconazole and amphotericin B showed no significant differences among the *Candida* species. However, there were significant species-specific differences in the echinocandin activities against the *Candida* biofilms ($P < 0.05$). In contrast to that in the *C. parapsilosis* and *C. tropicalis* biofilms, a reduction in XTT activity of up to 80% in the *C. albicans* and *C. glabrata* biofilms was observed following exposure to relatively low concentrations (0.25 to 1 μg/ml) of caspofungin and micafungin.

Kuhn et al. (9) have shown that both echinocandins inhibit one of two *C. parapsilosis* strains while exhibiting high MICs for the other strain. In the present study, the biofilms formed by *C. parapsilosis* (12 strains) were less susceptible to both echinocandins in vitro. These interstudy differences may be due to the differences in the *Candida* biofilm models used or to the biofilm-forming abilities of the *Candida* isolates tested (8, 16, 17).

We selected 43 out of the 95 isolates from preliminary experiments in which the biofilms had high turbidities at 48 h (OD > 0.3), since the biofilms with lower turbidities gave nonreproducible MIC results for sessile cells, mainly due to the low ODs of the control wells. Therefore, the criterion that we used for isolate selection may have introduced a bias. Further studies are needed on the potential associations between the densities of biofilms (particularly those formed by *C. parapsilosis* and *C. tropicalis* isolates) and echinocandin susceptibilities.

The mechanisms of echinocandin activity against biofilms formed by different *Candida* species are unknown (7). However, differences related to the composition of the *Candida* biofilm matrix (1, 8), metabolic activity (12), and the rate of drug diffusion through the biofilm (2) have been reported for different *Candida* species. These data suggest different drug resistance mechanisms for different biofilm-forming *Candida* species. Clinical significance remains to be verified, given the variations in testing methods and isolate selection. The observed differences in echinocandin susceptibilities among different *Candida* species suggest the involvement of novel biochemical and genetic mechanisms in biofilm formation.

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