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Wild-Type MIC Distributions and Epidemiological Cutoff Values for the Echinocandins and *Candida* spp.▽

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We tested a global collection of *Candida* sp. strains against anidulafungin, caspofungin, and micafungin, using CLSI M27-A3 broth microdilution (BMD) methods, in order to define wild-type (WT) populations and epidemiological cutoff values (ECVs). From 2003 to 2007, 8,271 isolates of *Candida* spp. (4,283 *C. albicans*, 1,236 *C. glabrata*, 1,238 *C. parapsilosis*, 996 *C. tropicalis*, 270 *C. krusei*, 99 *C. lusitaniae*, 88 *C. guilliermondii*, and 61 *C. kefyr* isolates) were obtained from over 100 centers worldwide. The modal MICs (in μg/ml) for anidulafungin, caspofungin, and micafungin, respectively, for each species were as follows: *C. albicans*, 0.03, 0.03, 0.015; *C. glabrata*, 0.06, 0.03, 0.015; *C. tropicalis*, 0.03, 0.03, 0.015; *C. kefyr*, 0.06, 0.015, 0.06; *C. krusei*, 0.03, 0.06, 0.06; *C. lusitaniae*, 0.05, 0.25, 0.12; *C. parapsilosis*, 2, 0.25, 1; and *C. guilliermondii*, 2, 0.5. 05. The ECVs, expressed in μg/ml (percentage of isolates that had MICs that were less than or equal to the ECV is shown in parentheses) for anidulafungin, caspofungin, and micafungin, respectively, were as follows: 0.12 (99.7%), 0.12 (99.8%), and 0.03 (97.7%) for *C. albicans*; 0.25 (99.4%), 0.12 (98.5%), and 0.03 (98.2%) for *C. glabrata*; 0.12 (98.9%), 0.12 (99.4%), and 0.12 (99.1%) for *C. tropicalis*; 0.25(100%), 0.03 (100%), and 0.12 (100%) for *C. kefyr*; 0.12 (99.3%), 0.25 (96.3%), and 0.12 (97.8%) for *C. krusei*; 2 (100%), 0.5 (98.0%), and 0.5 (99.0%) for *C. lusitaniae*; 4 (100%), 1 (98.6%), and 4 (100%) for *C. parapsilosis*; 16 (100%), 4 (95.5%), and 4 (98.9%) for *C. guilliermondii*. These WT MIC distributions and ECVs will be useful in surveillance for emerging reduced echinocandin susceptibility among *Candida* spp. and for determining the importance of various *FKS1* or other mutations.

The members of the echinocandin class of antifungal agents (anidulafungin, caspofungin, and micafungin) are now well recognized as the preferred, systemically active antifungal agents for the treatment of invasive candidiasis (IC), including candidemia (19). The *in vitro* activity of these agents against *Candida* spp. is also well-known (17, 24), and the Clinical and Laboratory Standards Institute (CLSI) Antifungal Subcommittee has established a clinical breakpoint (CBP) for susceptibility of ≤2 μg/ml for all three agents and all species of *Candida* (3, 4, 25). Recently, however, it has become evident that *Candida* infections involving strains with mutations in *FKS1* (encodes the echinocandin target) do not necessarily have MICs above this CBP (2, 5–8, 14, 28). Likewise, kinetic studies of the glucan synthesis enzyme complex suggest that a lower MIC cutoff of 0.5 μg/ml may be more sensitive in detecting those strains with *FKS1* mutations (7, 8). Given these considerations, we have conducted global surveillance of *Candida* spp. by using CLSI broth microdilution (BMD) methods to ascertain the wild-type (WT) MIC distribution for the three echinocandins and the eight most common species of *Candida* causing bloodstream infections (BSI). This information allows us to establish epidemiological cutoff values (ECVs) that may be used to assess the emergence of strains with *FKS1* mutations and the decreased susceptibility to these agents (10, 27, 30).

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MATERIALS AND METHODS

Organisms. A total of 8,271 clinical isolates obtained from more than 100 medical centers worldwide from 2003 through 2007 were tested. The collection included 4,283 strains of *Candida albicans*, 1,236 of *Candida glabrata*, 1,238 of *Candida parapsilosis*, 996 of *Candida tropicalis*, 270 of *Candida krusei*, 99 of *Candida lusitaniae*, 88 of *Candida guilliermondii*, and 61 of *Candida kefyr*. All isolates were obtained from blood or other normally sterile sites and represented the incident isolate from individual infectious episodes. The isolates were collected at individual study sites and were sent to the University of Iowa (Iowa City) for identification and susceptibility testing as described previously (20–23). The isolates were identified by standard methods (9) and stored as water suspensions until used in the study. Prior to testing, each isolate was passaged at least twice onto potato dextrose agar (Remel) and Chromagar *Candida* medium (Becton Dickinson and Company, Sparks, MD) to ensure purity and viability.

Antifungal agents. Reference powders of anidulafungin, caspofungin, and micafungin were obtained from their respective manufacturers. Stock solutions were prepared in water (caspofungin and micafungin) or dimethyl sulfoxide (anidulafungin), and serial 2-fold dilutions in RPMI 1640 medium (Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) buffer (Sigma) were made.

Antifungal susceptibility testing. BMD testing was performed in accordance with the guidelines in CLSI document M27-A3 (3) by using RPMI 1640 medium, an inoculum of 0.5×10^3 to 2.5×10^3 cells/ml, and incubation at 35°C. MICs were determined visually, after 24 h of incubation, as the lowest concentration of drug that caused a significant diminution (≥50% inhibition) of growth below control levels (16, 20, 25).

Quality control. Quality control was performed by testing CLSI-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (3, 4).

Definitions. The definitions of WT and ECVs were those outlined previously (10, 26, 29, 30). A WT organism is defined as a strain which does not harbor any acquired resistance to the particular antimicrobial agent being examined (29, 30). The typical MIC distribution for WT organisms covers three to four 2-fold dilution steps surrounding the modal MIC (1, 11). Inclusion of WT strains in the present study was ensured by testing only the incident isolate for each infectious episode.

The ECV for each echinocandin and each species of *Candida* was obtained as

TABLE 1. WT MIC distributions of anidulafungin, caspofungin and micafungin for eight species of *Candida*, using CLSI BMD methods

Species	Antifungal agent	No. of isolates tested	No. of isolates with MIC (μg/ml) of:										
			0.007	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	>8
C. albicans	Anidulafungin	4,283	338	1,278	1,542	896	216	12		1			
	Caspofungin	4,283	92	1,181	2,037	898	68	6	1				
	Micafungin	4,283	608	2,952	625	90	5	1	1				
C. glabrata	Anidulafungin	1,236		7	161	715	320	26	2	2	2	1	
	Caspofungin	1,236		132	731	329	26	8	7	1			2
	Micafungin	1,236	208	935	71	12	4	2	1	2	1		
C. tropicalis	Anidulafungin	996	41	254	493	173	24	7	1		3		
	Caspofungin	996	17	318	482	161	12	4		1			1
	Micafungin	996	46	400	375	149	17	6	1	2			
C. krusei	Anidulafungin	270		4	159	91	14	1	1				
	Caspofungin	270		1		140	79	40	8	2			
	Micafungin	270		4	28	211	21	6					
C. kefyr	Anidulafungin	61		1	6	31	23						
	Caspofungin	61	8	47	6								
	Micafungin	61		4	27	30							
C. lusitaniae	Anidulafungin	99				5	14	33	43	4			
	Caspofungin	99			3	2	42	46	4	2			
	Micafungin	99	1		4	9	52	31	1	1			
C. parapsilosis	Anidulafungin	1,238		1	2	1	1	14	49	319	765	86	
	Caspofungin	1,238		2	5	31	126	545	399	113	16	1	
	Micafungin	1,238		2	2	1	10	66	261	676	220		
C. guilliermondii	Anidulafungin	88				1	5	7	5	31	32	7	
	Caspofungin	88			1	10	7	21	32	12	1		4
	Micafungin	88		2		5	8	16	31	23	2		1

described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (10), by considering the WT MIC distribution, the modal MIC for each distribution, and the inherent variability of the test (usually within 1 $\log_2$ dilution). In general, the ECV should encompass at least 95% of isolates in the WT distribution (29, 30). Statistical determination of ECVs for each species and antifungal agent was performed as described previously (29). Organisms with acquired resistance mechanisms may be included among those for which the MICs are higher than the ECV (1, 10, 11, 26).

The CBPs for susceptibility (MIC, ≤2 μg/ml) for all three echinocandins used in this study were those defined by Pfaller et al. (25) and CLSI (4).

RESULTS AND DISCUSSION

The WT MIC distributions for the three echinocandins and each of the eight species of *Candida* are shown in Table 1. These distributions clearly show the very low MICs typical of WT strains of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. kefyr* and the higher MICs typical of *C. parapsilosis*, *C. guilliermondii*, and *C. lusitaniae* for all three echinocandins.

The modal MICs (percentage of isolates with MICs equal to the mode is shown in parentheses) for anidulafungin, caspofungin, and micafungin, respectively, and each species are as follows (Table 2): *C. albicans*, 0.03 μg/ml (36.0%), 0.03 μg/ml (47.6%), 0.015 μg/ml (68.9%); *C. glabrata*, 0.06 μg/ml (57.8%), 0.03 μg/ml (59.1%), 0.015 μg/ml (75.6%); *C. tropicalis*, 0.03 μg/ml (49.5%), 0.03 μg/ml (48.4%), 0.015 μg/ml (40.2%); *C. krusei*, 0.03 μg/ml (58.9%), 0.06 μg/ml (51.9%), 0.06 μg/ml (78.1%); *C. kefyr*, 0.06 μg/ml (50.8%), 0.015 μg/ml (77.0%), 0.06 μg/ml (49.2%); *C. lusitaniae*, 0.5 μg/ml (43.4%), 0.25 μg/ml (46.5%), 0.12 μg/ml (52.5%); *C. parapsilosis*, 2 μg/ml (61.8%), 0.25 μg/ml (44.0%), 1 (54.6%); *C. guilliermondii*, 2 μg/ml (36.4%), 0.5 μg/ml (36.4%), 0.5 μg/ml (35.2%).

The ECVs (percentage of isolates with MICs that were less than or equal to the ECVs is shown in parentheses) were calculated as described by Turnidge et al. (29), taking into consideration the WT MIC distributions and the inherent variability of the BMD test method, and were as follows for each species and anidulafungin, caspofungin, and micafungin, respectively (Table 2): 0.12 μg/ml (99.7%), 0.12 μg/ml (99.8%), and 0.03 μg/ml (97.7%) for *C. albicans*; 0.25 μg/ml (99.4%), 0.12 μg/ml (98.5%), and 0.03 μg/ml (98.2%) for *C. glabrata*; 0.12 μg/ml (98.9%), 0.12 μg/ml (99.4%), and 0.12 μg/ml (99.1%) for *C. tropicalis*; 0.25 μg/ml (100%), 0.03 μg/ml (100%), and 0.12 μg/ml (100%) for *C. kefyr*; 0.12 μg/ml (99.3%), 0.25 μg/ml (96.3%), and 0.12 μg/ml (97.8%) for *C. krusei*; 2 μg/ml (100%), 0.5 μg/ml (98.0%), and 0.5 μg/ml (99.0%) for *C. lusitaniae*; 4 μg/ml (100%), 1 μg/ml (98.6%), and 4 μg/ml (100%) for *C. parapsilosis*; and 16 μg/ml (100%), 4 μg/ml (95.5%), and 4 μg/ml (98.9%) for *C. guilliermondii*.

Compared to the CBP value of ≤2 μg/ml, the ECVs are between 8- and 66-fold lower for the three echinocandins and *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. kefyr* (Table 2). Whereas the CBP encompasses 99.9% to 100% of the isolates of these five species, the ECVs of each agent encompass 96% to 100% of the isolates, highlighting the small number of isolates of each species that fall outside of the WT distribution yet remain susceptible to each agent

TABLE 2. ECVs for anidulafungin, caspofungin, and micafungin and eight species of *Candida*

Species	Antifungal agent	No. of isolates tested	MIC (μg/ml)			% Isolates with MIC of ≤2 μg/ml
			Range	Mode	ECV (%[a])	
C. albicans	Anidulafungin	4,283	0.007–1	0.03	0.12 (99.7)	100.0
	Caspofungin	4,283	0.007–0.5	0.03	0.12 (99.8)	100.0
	Micafungin	4,283	0.007–0.5	0.015	0.03 (97.7)	100.0
C. glabrata	Anidulafungin	1,236	0.015–4	0.06	0.25 (99.4)	99.9
	Caspofungin	1,236	0.015–8	0.03	0.12 (98.5)	99.8
	Micafungin	1,236	0.007–2	0.015	0.03 (98.2)	100.0
C. tropicalis	Anidulafungin	996	0.007–2	0.03	0.12 (98.9)	100.0
	Caspofungin	996	0.007–>8	0.03	0.12 (99.4)	99.9
	Micafungin	996	0.007–1	0.015	0.12 (99.1)	100.0
C. kefyr	Anidulafungin	61	0.015–0.12	0.06	0.25 (100.0)	100.0
	Caspofungin	61	0.007–0.03	0.015	0.03 (100.0)	100.0
	Micafungin	61	0.015–0.06	0.06	0.12 (100.0)	100.0
C. krusei	Anidulafungin	270	0.015–0.5	0.03	0.12 (99.3)	100.0
	Caspofungin	270	0.015–1	0.06	0.25 (96.3)	100.0
	Micafungin	270	0.015–0.25	0.06	0.12 (97.8)	100.0
C. lusitaniae	Anidulafungin	99	0.06–1	0.5	2 (100)	100.0
	Caspofungin	99	0.03–1	0.25	0.5 (98.0)	100.0
	Micafungin	99	0.007–1	0.12	0.5 (99.0)	100.0
C. parapsilosis	Anidulafungin	1,238	0.015–4	2	4 (100.0)	93.1
	Caspofungin	1,238	0.015–4	0.25	1 (98.6)	99.9
	Micafungin	1,238	0.015–2	1	4 (100)	100.0
C. guilliermondii	Anidulafungin	88	0.06–4	2	16 (100.0)	92.0
	Caspofungin	88	0.03–>8	0.5	4 (95.5)	95.5
	Micafungin	88	0.015–>8	0.5	4 (98.9)	98.9

[a] Percentage of isolates for which MIC is less than or equal to the ECV.

according to the CBP. In contrast, the ECVs for the three less susceptible species, *C. lusitaniae*, *C. parapsilosis*, and *C. guilliermondii*, are similar to the CBPs for all three of the echinocandins.

Generally speaking, CBPs are used to indicate those isolates that are likely to respond to treatment with a given antimicrobial agent administered at the approved dosing regimen for that agent, whereas the ECV can be used as the most sensitive measure of the emergence of strains with reduced susceptibility to a given agent (10, 11, 27). Although organisms whose MICs exceed the ECV show reduced susceptibility compared with the WT population and may exhibit one or more acquired resistance mechanisms, they may yet respond to clinical treatment, as their MIC may lie below the CBP (27).

Although the various clinical trials have shown that each of the three echinocandins can be used to treat candidemia and IC due to isolates of *Candida* spp. for which MICs are as high as 2 μg/ml (12, 13, 15, 18, 25), several recent reports of clinical resistance to caspofungin therapy (Table 3), as well as studies of glucan synthase (GS) enzyme kinetics (6–8), suggest that the CBP of ≤2 μg/ml may need to be adjusted to predict both clinical resistance as well as the emergence of strains with *FKS1* mutations. In each of the cases shown in Table 3, clinical failure of caspofungin therapy was associated with *FKS1* mu-

TABLE 3. Clinical and *in vitro* resistance: caspofungin in candidiasis patients[b]

Species (reference)	Infection type	Antifungal treatment[a]	Agents (MICs in μg/ml)	Comment(s)
C. glabrata (28)	Candidemia	CSF	CSF (2), ANF (0.5), MCF (0.25)	Mutation in *FKS2*, F659V
C. albicans (2)	Esophagitis	FLC, VRC, CSF, AMB	CSF (2), MCF (1)	Mutation in *FKS1*, F641S
C. tropicalis (6)	Candidemia	CSF, VRC	CSF (4), ANF (2), MCF (2)	Mutation, 50× increase in IC_{50}
C. tropicalis (6)	Candidemia	CSF, AMB	CSF (4), ANF (1), MCF (2)	Mutation, 50× increase in IC_{50}
C. tropicalis (6)	Candidemia	CSF, FLC	CSF (1), ANF (0.5), MCF (0.5)	Mutation, 38× increase in IC_{50}
C. albicans (14)	Esophagitis	CSF, AMB, FLC, VRC, ITZ, MCF	CSF (2), ANF (1), MCF (2)	Mutations, S645F and R1361H

[a] Antifungal agents administered to patient.
[b] AMB, amphotericin B; ANF, anidulafungin; CSF, caspofungin; FLC, fluconazole; ITZ, itraconazole; MCF, micafungin; VRC, voriconazole; IC_{50}, concentration that inhibits 50% of enzyme activity.

tations and MICs for all three echinocandins that were elevated compared to the WT but not necessarily higher than the CBP of ≤2 μg/ml. Application of the ECVs in Table 2 would have recognized these strains as non-WT and thus likely to contain an acquired resistance mutation.

It is evident that only a small number (<4%) of isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* fall outside of the respective ECVs for each of the three echinocandins (Tables 1 and 2). Almost all would be classified as susceptible using the CBP criteria despite the possibility that they may have an acquired *FKS1* mutation. The questions that must be answered are (i) what proportion of these isolates do in fact contain a target enzyme mutation and (ii) is the presence of a mutation that does not result in an MIC that is greater than the CBP meaningful or necessary to detect?

Garcia-Effron et al. (7, 8) demonstrated that clinically resistant isolates of *C. albicans* and *C. glabrata* with mutations in *FKS1* and/or *FKS2* showed elevated MICs and altered GS enzyme kinetics for all three echinocandins. Importantly, an MIC of >0.5 μg/ml identified those strains with resistant GS for anidulafungin, caspofungin, and micafungin.

Likewise, Wiederhold et al. (31) examined 12 strains of *C. albicans* for which the MICs of anidulafungin (MIC range, 0.12 to 1 μg/ml), caspofungin (MIC range, 2 to 8 μg/ml), and micafungin (MIC range, 0.5 to 4 μg/ml) were elevated relative to the control (WT) MIC for each agent (0.03 μg/ml, 0.125 μg/ml, and 0.06 μg/ml, respectively). All 12 isolates were found to contain mutations in *FKS1*; however, the MICs exceeded the CBPs for 0 of 12 strains with anidulafungin, 9 of 12 with caspofungin, and 2 of 12 with micafungin. In contrast, all 12 would have been considered to have reduced susceptibility to caspofungin and micafungin, and nine would have been considered to have reduced susceptibility to anidulafungin, using the ECVs shown in Table 2. Unfortunately, no clinical data concerning these strains were presented by the authors.

Thus, the ECVs determined for *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. kefyr* will be important in detecting the emergence of decreased susceptibility to the echinocandins in ongoing surveillance efforts. The CBPs for these agents may serve the same purpose for *C. parapsilosis* and *C. guilliermondii* but appear to be too insensitive to be of epidemiological value in monitoring the more susceptible species. Future studies must include molecular analysis of *FKS1* and *FKS2* for the mutant strains with values that fall between the ECV and CBP to better understand the frequency and clinical importance of such mutations. The establishment of the WT MIC distributions and ECVs for each echinocandin and species of *Candida* will be useful in resistance surveillance and may prove to be an important step in the development of species-specific CBPs for this important class of antifungal agents.

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