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Use of Epidemiological Cutoff Values To Examine 9-Year Trends in Susceptibility of *Candida* Species to Anidulafungin, Caspofungin, and Micafungin[▽]

M. Pfaller,* L. Boyken, R. Hollis, J. Kroeger, S. Messer, S. Tendolkar, and D. Diekema

University of Iowa, Iowa City, Iowa

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The CLSI clinical breakpoint (CBP) for echinocandin susceptibility (S; MICs of ≤2 μg/ml) may classify isolates with acquired resistance (R) mutations as susceptible. Epidemiological cutoff values (ECVs) have been established to distinguish wild-type (WT) *Candida* strains from those that may exhibit R mutations. The CLSI-developed ECVs for anidulafungin, caspofungin, and micafungin were applied to 15,269 isolates of *Candida* spp. collected from over 100 centers worldwide between 2001 and 2009 to determine the frequency of non-WT strains of each species. The collection included 8,378 isolates of *Candida albicans*, 2,352 isolates of *C. glabrata*, 2,195 isolates of *C. parapsilosis*, 1,841 isolates of *C. tropicalis*, and 503 isolates of *C. krusei*. The mean percentages of non-WT isolates per year for anidulafungin, caspofungin, and micafungin, respectively, were as follows: for *C. albicans*, 0.3, 0.1, and 2.1; for *C. glabrata*, 0.8, 1.3, and 1.6; for *C. parapsilosis*, 0.0, 1.5, and 0.5; for *C. tropicalis*, 0.9, 0.7, and 0.9; and for *C. krusei*, 0.5, 6.4, and 3.5. We noted increases in the percentage of non-WT isolates, from 0.5% (2001) to 3.1% (2009) for caspofungin and *C. parapsilosis*, from 0.4% (2004) to 1.8% (2009) for anidulafungin and *C. glabrata*, from 2.4% (2004) to 5.7% (2009) for micafungin and *C. krusei*, and from 0.0% (2004) to 3.1% (2009) for micafungin and *C. parapsilosis*. No trends were noted for any species and drug when we used the CBP. Echinocandin CBPs are insensitive for detecting emerging R. Although uncommon, decreased S among *Candida* isolates was observed for each of the echinocandins and varied by species. Using ECVs is important in determining R trends among echinocandins and *Candida*.

The echinocandins anidulafungin, caspofungin, and micafungin are important antifungal agents in the prevention and treatment of invasive candidiasis (IC) (22). Although resistance (R) to these agents is uncommon, reports of acquired R have increased in recent years (2–7, 10, 11, 17–21, 23, 24, 32–34). The mechanism of R to the echinocandins in *Candida* spp. is now well defined and involves mutations in the *fks* genes, encoding the target enzyme (11–13, 23, 24). It is now recognized that the Clinical and Laboratory Standards Institute (CLSI) MIC clinical breakpoint (CBP) for susceptibility (S) of ≤2 μg/ml (27) may misclassify isolates with acquired R mutations as being susceptible (3, 11–13, 28, 29, 37). For this reason, epidemiological cutoff values (ECVs) have been established as a means of distinguishing wild-type (WT) isolates of *Candida* from those non-WT strains that may exhibit acquired R mechanisms (29). Although we previously used the ARTEMIS global antifungal surveillance database to examine temporal trends in the *in vitro* susceptibility of *Candida* spp. to the echinocandins (25, 26), we employed the CBP of 2 μg/ml as a threshold MIC to detect the emergence of decreased susceptibility to this class of antifungal agents over time. Subsequently, it was shown that this CBP lacks the sensitivity of the ECVs for detecting the emergence of strains with *fks* mutations that may account for clinical resistance (2, 21, 25–27). In the present study, we applied the ECV for each echinocandin and species of *Candida* to a global collection of 15,269 clinical isolates of *Candida* spp. collected from more than 100 medical centers and tested using CLSI broth microdilution (BMD) methods (8) to determine the frequency of reduced susceptibility (emergence of non-WT strains) of each species over the period 2001–2009.

MATERIALS AND METHODS

Organisms. A total of 15,269 clinical isolates obtained internationally from more than 100 medical centers from 2001 through 2009 were tested against caspofungin. A subset of these isolates representing the years 2004 to 2009 were tested against anidulafungin (9,238 isolates) and micafungin (8,922 isolates). The collection included 8,378 isolates of *C. albicans*, 2,352 isolates of *C. glabrata*, 2,195 isolates of *C. parapsilosis*, 1,841 isolates of *C. tropicalis*, and 503 isolates of *C. krusei*. All isolates were obtained from blood or other normally sterile sites and represented individual infectious episodes. The isolates were collected at individual study sites and were sent to the University of Iowa (Iowa City, IA) for identification and susceptibility testing as described previously (26, 29). The isolates were identified by standard methods (14) and stored as water suspensions until used in the study. Prior to being tested, 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 were made in RPMI 1640 medium (Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) buffer (Sigma).

Antifungal susceptibility testing. BMD testing was performed in accordance with the guidelines in CLSI document M27-A3 (8), 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 concentrations

* Corresponding author. Mailing address: Medical Microbiology Division, C606 GH, Department of Pathology, University of Iowa College of Medicine, Iowa City, IA 52242-1009. Phone: (319) 356-8615. Fax: (319) 356-4916. E-mail: michael-pfaller@uiowa.edu.

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TABLE 1. Trends in distribution of the top five species of *Candida* from bloodstream infections over a 9-year period (2001 to 2009)

Species	Species distribution (%) by year (*n*)								
	2001	2002	2003	2004	2005	2006	2007	2008	2009
C. albicans	58 (1,000)	56 (1,208)	54 (1,178)	58 (1,119)	56 (1,045)	56 (869)	49 (749)	48 (650)	53 (560)
C. glabrata	17 (287)	15 (332)	14 (291)	13 (252)	16 (291)	15 (238)	16 (239)	19 (247)	17 (175)
C. parapsilosis	12 (206)	13 (275)	17 (360)	15 (284)	13 (243)	14 (209)	15 (220)	18 (236)	15 (162)
C. tropicalis	10 (174)	12 (265)	12 (268)	11 (211)	12 (227)	13 (203)	14 (208)	12 (161)	12 (124)
C. krusei	3 (50)	4 (73)	3 (65)	3 (53)	3 (59)	2 (34)	6 (94)	3 (40)	3 (35)
Total	100 (1,717)	100 (2,153)	100 (2,162)	100 (1,919)	100 (1,865)	100 (1,553)	100 (1,510)	100 (1,339)	100 (1,056)

of drug that caused a significant diminution (≥50% inhibition) of growth below control levels (8).

Quality control. Quality control was performed by testing CLSI-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (8, 9).

Definitions. The definitions of WT organisms and ECVs were those outlined previously (28, 29). A WT organism is defined as a strain which does not harbor any acquired resistance to the particular antimicrobial agent being examined (35, 36). The typical MIC distribution for a WT organism covers three to five 2-fold dilution steps surrounding the modal MIC (1, 15, 16).

The ECV for each echinocandin and each species of *Candida* was obtained as described previously (29), by considering the WT MIC distribution, the modal MIC for each distribution, and the inherent variability of the test. In general, the ECV should encompass at least 95% of isolates in the WT distribution (35, 36). Statistical determination of the ECV for each species and antifungal agent was performed as described by Turnidge et al. (35). Organisms with acquired resistance mechanisms may be included among those for which the MIC is higher than the ECV (3, 15, 28–30, 35, 36). The ECV can be used as the most sensitive measure of the emergence of strains with reduced susceptibility to a given agent (3, 28, 29).

The CBPs for S (MICs of ≤2 μg/ml) and nonsusceptibility (NS; MICs of >2 μg/ml) for all three echinocandins used in this study were those defined by Pfaller et al. (27) and the CLSI (9). We used the chi-square test to examine temporal trends in the proportion of isolates with MICs higher than the ECV by year.

RESULTS AND DISCUSSION

Table 1 demonstrates the species distribution of *Candida* isolates from the bloodstream and normally sterile sites according to each year of the survey, 2001 to 2009. In general, the rank order remained the same from year to year: *C. albicans* > *C. glabrata* > *C. parapsilosis* > *C. tropicalis* > *C. krusei*. The only exceptions to this rank order came in the years 2003 and 2004, when the frequency of *C. parapsilosis* exceeded that of *C. glabrata*. Overall, there was no consistent trend indicating an increase or decrease in the frequency of any given species over time.

The ECVs for each echinocandin and the five *Candida* species studied are shown in Table 2. The ECVs for these five species were determined in a previous study of 8,023 isolates of *Candida* spp. representing the years 2003 to 2007 and were tested against all three echinocandins by use of the CLSI BMD method (29). We have demonstrated the ability of these echinocandin ECVs to discriminate WT strains (with MICs less than or equal to the ECV) from those with acquired resistance mechanisms (5, 28, 30). In an earlier study of 136 isolates of *Candida* (105 WT strains and 31 non-WT strains with *fks* mutations), the ECVs for anidulafungin, caspofungin, and micafungin identified 90% (anidulafungin) to 100% (caspofungin and micafungin) of the 31 mutant strains as non-WT strains (28). Furthermore, the ECVs correctly classified 74% (micafungin) to 96% (anidulafungin) of the 105 WT strains. In a subsequent study of *C. glabrata* (30), we also demonstrated that 6 of 12 non-WT strains for each echinocandin were *fks* mutants.

TABLE 2. ECVs for anidulafungin, caspofungin, and micafungin and five species of *Candida*[a]

Species	No. of isolates tested	ECV (μg/ml [% of isolates that were at or below the ECV])		
		Anidulafungin	Caspofungin	Micafungin
C. albicans	4,283	0.12 (99.7)	0.12 (99.8)	0.03 (97.7)
C. glabrata	1,236	0.25 (99.4)	0.12 (98.5)	0.03 (98.2)
C. tropicalis	996	0.12 (98.9)	0.12 (99.4)	0.12 (99.1)
C. krusei	270	0.12 (99.3)	0.25 (96.3)	0.12 (97.8)
C. parapsilosis	1,238	4 (100.0)	1 (98.6)	4 (100.0)

[a] The data are compiled from the work of Pfaller et al. (29).

Although ECVs may be considered an early step in the development of CBPs (15, 16), the most important role for these cutoffs is to detect the emergence of reduced susceptibility to the agent of interest in the context of a resistance surveillance program. This is especially noteworthy in the case of the echinocandins and *Candida*, where the existing CBP for NS of >2 μg/ml is clearly not sensitive enough to detect the emergence of non-WT strains (Tables 3 and 4). The insensitivity of the CBP to detect the emergence of potential R to caspofungin is shown in Table 3, where both the CBP for NS and the ECV for each species are applied to the collection of *Candida* isolates spanning the 9-year period from 2001 through 2009. Application of the ECVs shows that the mean proportion of non-WT isolates per year was 0.1% for *C. albicans*, 1.3% for *C. glabrata*, 1.5% for *C. parapsilosis*, 0.7% for *C. tropicalis*, and 6.5% for *C. krusei*, whereas the CBP criteria for NS show only 0.0% to 0.3% of each species to be NS (Table 3). Notably, the application of the ECV for *C. parapsilosis* of 1 μg/ml documents a steady emergence of strains with decreased susceptibility to caspofungin from 2001 (0.5%) through 2009 (3.1%) ($P = 0.04$). No such trend was detected for any species by use of the CBP to detect the emergence of NS isolates.

A similar analysis is seen in Table 4, where the ECVs for anidulafungin and micafungin are applied to isolates from the years 2004 through 2009. Using the ECV for each echinocandin and species of *Candida*, the mean proportions of non-WT isolates per year for anidulafungin and micafungin, respectively, were as follows: 0.3% and 2.1% for *C. albicans*, 0.8% and 1.6% for *C. glabrata*, 0.0% and 0.5% for *C. parapsilosis*, 0.9% and 0.9% for *C. tropicalis*, and 0.5% and 3.5% for *C. krusei* (Table 4). Conversely, most years showed a complete

TABLE 3. Trends in susceptibility of *Candida* bloodstream infection isolates to caspofungin: comparison of ECVs with the CLSI clinical breakpoint MICs

Species (ECV [μg/ml])	Yr	No. of isolates tested	MIC (μg/ml)		% of MICs	
			Range	Mode	>ECV	>2 μg/ml
C. albicans (0.12)	2001	1,000	0.007–0.25	0.03	0.1	0.0
	2002	1,208	0.007–0.12	0.007	0.0	0.0
	2003	1,178	0.007–0.25	0.03	0.1	0.0
	2004	1,119	0.007–0.25	0.03	0.4	0.0
	2005	1,045	0.007–0.12	0.03	0.0	0.0
	2006	869	0.007–0.5	0.03	0.2	0.0
	2007	749	0.007–0.12	0.03	0.0	0.0
	2008	650	0.007–0.12	0.03	0.0	0.0
	2009	560	0.007–0.25	0.03	0.4	0.0
C. glabrata (0.12)	2001	287	0.015–4	0.03	1.4	0.3
	2002	332	0.007–0.5	0.03	1.5	0.0
	2003	291	0.015–0.5	0.03	0.3	0.0
	2004	252	0.015–0.5	0.03	1.2	0.0
	2005	291	0.015–8	0.03	2.1	0.3
	2006	238	0.015–0.5	0.03	1.3	0.0
	2007	239	0.015–0.5	0.03	0.4	0.0
	2008	247	0.015–8	0.03	2.0	0.4
	2009	175	0.015–8	0.03	1.7	0.6
C. tropicalis (0.12)	2001	174	0.007–0.25	0.03	0.6	0.0
	2002	265	0.007–1	0.03	1.9	0.0
	2003	268	0.007–0.12	0.03	0.0	0.0
	2004	211	0.007–>8	0.03	1.9	0.5
	2005	227	0.007–0.25	0.015	0.9	0.0
	2006	203	0.007–0.12	0.03	0.0	0.0
	2007	208	0.007–0.12	0.03	0.0	0.0
	2008	161	0.007–0.06	0.03	0.0	0.0
	2009	124	0.007–0.5	0.015	0.8	0.0
C. krusei (0.25)	2001	50	0.06–1	0.25	12.0	0.0
	2002	73	0.06–1	0.12	8.2	0.0
	2003	65	0.03–2	0.06	18.5	0.0
	2004	53	0.015–0.5	0.06	7.5	0.0
	2005	59	0.06–1	0.12	5.1	0.0
	2006	34	0.06–0.25	0.12	0.0	0.0
	2007	94	0.06–0.5	0.06	1.1	0.0
	2008	40	0.06–1	0.06	5.0	0.0
	2009	35	0.03–0.25	0.12	0.0	0.0
C. parapsilosis (1)	2001	206	0.06–2	0.25	0.5	0.0
	2002	275	0.06–4	0.5	0.7	0.4
	2003	360	0.015–2	0.5	0.8	0.0
	2004	284	0.06–4	0.5	2.1	1.8
	2005	243	0.06–2	0.25	2.5	0.0
	2006	209	0.06–2	0.25	1.4	0.0
	2007	220	0.03–1	0.25	0.0	0.0
	2008	236	0.015–2	0.25	2.5	0.0
	2009	162	0.015–4	0.5	3.1	0.6

lack of NS isolates detected by the CBP. Temporal trends of interest uncovered by the ECVs included increases in the proportion of non-WT isolates, from 0.4% (2004) to 1.8% (2009) for anidulafungin and *C. glabrata* ($P = 0.09$), from 2.4% (2004) to 5.7% (2009) for micafungin and *C. krusei* ($P = 0.01$), and from 0.0% (2004 to 2008) to 3.1% (2009) for micafungin and *C. parapsilosis* ($P = 0.001$). Although the CBPs for anidulafungin and micafungin identified more isolates of *C. parapsilosis* as NS than those detected by the ECVs, the CBPs for this species may need to be adjusted to avoid bisecting the WT population (1).

In light of the data presented in Tables 3 and 4, one may ask (i) what is the importance of detecting non-WT strains of *Candida* species for each echinocandin and (ii) given the slight differences in the percentage of non-WT strains for each species and echinocandin, what is the level of cross-resistance among the three echinocandins? Regarding the first question, application of the ECV criteria for each species and each echinocandin is simply a way to identify those strains that are phenotypically different from WT strains in their *in vitro* response to a given agent. The ECVs were generated earlier from a subset of the total collection described in the present study and were determined using the statistical methods of Turnidge et al. (35), which take into account the variation

TABLE 4. Trends in susceptibility of *Candida* bloodstream infection isolates to anidulafungin and micafungin: comparison of ECVs with the CLSI clinical breakpoint MICs

Antifungal agent and species (ECV [μg/ml])	Yr	No. of isolates tested	MIC (μg/ml)		% of MICs	
			Range	Mode	>ECV	>2 μg/ml
Anidulafungin						
C. albicans (0.12)	2004	1,120	0.007–0.25	0.03	0.9	0.0
	2005	1,045	0.007–0.25	0.03	0.1	0.0
	2006	869	0.007–1	0.03	0.2	0.0
	2007	749	0.007–0.12	0.03	0.0	0.0
	2008	650	0.007–0.12	0.03	0.0	0.0
	2009	560	0.007–2	0.015	0.4	0.0
C. glabrata (0.25)	2004	252	0.03–1	0.06	0.4	0.0
	2005	291	0.03–4	0.12	0.7	0.3
	2006	238	0.015–0.5	0.06	0.4	0.0
	2007	239	0.015–0.25	0.06	0.0	0.0
	2008	247	0.015–2	0.06	1.2	0.0
	2009	175	0.015–2	0.06	1.8	0.0
C. tropicalis (0.12)	2004	211	0.007–2	0.03	2.8	0.0
	2005	227	0.007–2	0.03	1.8	0.0
	2006	203	0.007–0.12	0.03	0.0	0.0
	2007	208	0.007–0.12	0.03	0.0	0.0
	2008	161	0.007–0.25	0.03	0.6	0.0
	2009	124	0.007–0.12	0.015	0.0	0.0
C. krusei (0.12)	2004	53	0.015–0.12	0.03	0.0	0.0
	2005	59	0.015–0.5	0.06	1.7	0.0
	2006	34	0.03–0.12	0.03	0.0	0.0
	2007	94	0.03–0.25	0.03	1.1	0.0
	2008	40	0.03–0.12	0.03	0.0	0.0
	2009	35	0.015–0.06	0.03	0.0	0.0
C. parapsilosis (4)	2004	284	0.5–4	2	0.0	3.9
	2005	243	0.5–4	2	0.0	12.1
	2006	209	0.25–4	2	0.0	7.2
	2007	220	0.25–4	2	0.0	6.8
	2008	236	0.015–4	2	0.0	5.9
	2009	162	0.015–4	1	0.0	3.7
Micafungin						
C. albicans (0.03)	2004	943	0.007–0.12	0.015	7.1	0.0
	2005	1,045	0.007–0.06	0.015	1.3	0.0
	2006	869	0.007–0.5	0.015	0.7	0.0
	2007	749	0.007–0.25	0.015	0.9	0.0
	2008	650	0.007–0.06	0.015	0.2	0.0
	2009	560	0.007–1	0.015	2.2	0.0
C. glabrata (0.03)	2004	200	0.007–0.25	0.015	3.0	0.0
	2005	291	0.007–1	0.015	3.1	0.0
	2006	238	0.007–0.03	0.015	0.0	0.0
	2007	239	0.007–0.06	0.015	0.8	0.0
	2008	247	0.007–2	0.015	1.6	0.0
	2009	175	0.007–2	0.015	1.2	0.0
C. tropicalis (0.12)	2004	176	0.007–1	0.03	2.3	0.0
	2005	227	0.007–1	0.03	0.4	0.0
	2006	203	0.007–0.25	0.015	2.0	0.0
	2007	208	0.007–0.06	0.015	0.0	0.0
	2008	161	0.007–0.12	0.03	0.0	0.0
	2009	124	0.007–0.25	0.015	0.8	0.0
C. krusei (0.12)	2004	42	0.015–0.25	0.06	2.4	0.0
	2005	59	0.015–0.12	0.06	0.0	0.0
	2006	34	0.03–0.25	0.06	11.8	0.0
	2007	94	0.015–0.25	0.06	1.1	0.0
	2008	40	0.03–0.12	0.06	0.0	0.0
	2009	35	0.03–0.25	0.06	5.7	0.0
C. parapsilosis (4)	2004	243	0.25–2	1	0.0	0.0
	2005	243	0.25–2	1	0.0	0.0
	2006	209	0.25–2	1	0.0	0.0
	2007	220	0.06–2	1	0.0	0.0
	2008	236	0.015–2	1	0.0	0.0
	2009	162	0.015–8	1	3.1	6.2

inherent in determining MICs due to the time of testing and reader variation. The non-WT strains detected by application of the ECVs to a population of MIC values represent strains that may have an acquired resistance mechanism and which may require additional testing to confirm the species identification, MIC value, and resistance mechanism. One of the main goals of the present work is to demonstrate that the use of ECVs provides data that may be "hidden" by CBPs. Such an approach thus highlights strains that should be characterized further. Although the non-WT strains detected in this study were not characterized further for the presence of *fks* mutations, we have demonstrated the sensitivity and specificity of these ECVs in distinguishing those isolates with a WT MIC for each echinocandin from non-WT strains containing *fks* mutations in previous studies (28, 30). Thus, the data herein should represent a reasonable approximation of the frequency of strains with decreased susceptibility to each of the echinocandins.

The issue of cross-resistance among the echinocandins has certainly been examined with respect to the molecular and biochemical characteristics of strains containing different *fks* mutations (11–13, 23, 24, 31, 34, 37). Whereas a given mutation may have a class effect in reducing the sensitivity of the modified enzyme to inhibition by drug, it does not necessarily manifest itself phenotypically with the same magnitude of change in various MIC values. Despite such differences, we demonstrated previously that ECVs do provide a phenotypic marker that indicates those strains most likely to contain an *fks* mutation and that they do so for each echinocandin, despite differences in the absolute values of the MICs (28, 30, 31). In assessing cross-resistance in the present study, we examined a subset of 3,400 MIC results where the isolates were tested against all three echinocandins (data not shown). We compared the levels of categorical agreement (CA) of the different agents by using the respective ECVs to separate the populations tested into WT and non-WT MIC groups. The overall CA between anidulafungin and caspofungin was 98.6% (0.8% very major discrepancies [VMD]), that between micafungin and caspofungin was 96.6% (0.7% VMD), and that between anidulafungin and micafungin was 98.0% (1% VMD) (data not shown). Thus, although there were some differences in the percentages of non-WT strains detected by the ECVs for the three echinocandins, the overall concordance was excellent and demonstrated a high degree of cross-resistance and -susceptibility across the three agents.

In summary, we have demonstrated the importance of ECVs in detecting the emergence of decreased susceptibility to the echinocandins in surveys of antifungal resistance. Whereas CBPs may serve the same purpose for *C. parapsilosis*, they appear to be too insensitive to be of epidemiological value in monitoring the more susceptible *Candida* species. Although uncommon, decreased S among *Candida* spp. was observed for each of the echinocandins and varied according to species over the 9-year study period. Application of *in vitro* susceptibility testing and the use of ECVs are important in determining R trends among the echinocandins and *Candida* spp. and will also serve as important steps in developing improved species-specific CBPs.

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