Frequency of Decreased Susceptibility and Resistance to Echinocandins among Fluconazole-Resistant Bloodstream Isolates of *Candida glabrata*

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The echinocandin class of antifungal agents is considered to be the first-line treatment of bloodstream infections (BSI) due to *Candida glabrata*. Recent reports of BSI due to strains of *C. glabrata* resistant to both fluconazole and the echinocandins are of concern and prompted us to review the experience of two large surveillance programs, the SENTRY Antimicrobial Surveillance Program for the years 2006 through 2010 and the Centers for Disease Control and Prevention population-based surveillance conducted in 2008 to 2010. The *in vitro* susceptibilities of 1,669 BSI isolates of *C. glabrata* to fluconazole, voriconazole, anidulafungin, caspofungin, and micafungin were determined by CLSI broth microdilution methods. Fluconazole MICs of $\geq 64 \mu\text{g/ml}$ were considered resistant. Strains for which anidulafungin and caspofungin MICs were $\geq 0.5 \mu\text{g/ml}$ and for which micafungin MICs were $\geq 0.25 \mu\text{g/ml}$ were considered resistant. A total of 162 isolates (9.7%) were resistant to fluconazole, of which 98.8% were nonsusceptible to voriconazole (MIC $> 0.5 \mu\text{g/ml}$) and 9.3%, 9.3%, and 8.0% were resistant to anidulafungin, caspofungin, and micafungin, respectively. There were 18 fluconazole-resistant isolates that were resistant to one or more of the echinocandins (11.1% of all fluconazole-resistant isolates), all of which contained an acquired mutation in *fkp1* or *fkp2*. By comparison, there were no echinocandin-resistant strains detected among 110 fluconazole-resistant isolates of *C. glabrata* tested in 2001 to 2004. These data document the broad emergence of coresistance over time to both azoles and echinocandins in clinical isolates of *C. glabrata*.

Resistance to the echinocandin antifungal agents, anidulafungin, caspofungin, and micafungin, among the broad population of *Candida* spp. causing invasive candidiasis (IC), is distinctly uncommon (1, 18, 39, 46, 48, 55–58, 62). Among 15,269 clinical isolates of *Candida* spp. tested at the University of Iowa (Iowa City), only 25 (0.2%) showed resistance to one or more echinocandins using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) method and interpretive criteria (39, 44). As such, the echinocandin class of antifungal agents is recommended as first-line therapy for candidemia and IC, especially in patients with severe sepsis, those previously exposed to azoles, and/or those infected with *Candida glabrata* (35).

Ever since the introduction of fluconazole in 1990 for the treatment of candidiasis, including IC, empirical antifungal therapy has been driven by fear of *C. glabrata* (56, 57). Decreased susceptibility of *C. glabrata* to fluconazole and cross-resistance to other azoles are well known (21, 22, 32, 35, 41, 43, 45, 56, 57). In the United States, *C. glabrata* has increased as a cause of IC from 18% of all bloodstream infection (BSI) isolates in the time period of 1992 to 2001 to 25% in 2001 to 2007, with a concomitant increase in fluconazole resistance from 9% to 14% (45). Given the distinct differences between the mechanisms of action and resistance for the azoles and the echinocandins (37, 38), the documented lack of cross-resistance between the two classes (27, 31, 42) is not surprising, and thus the recommendation that the echinocandins be used for treatment of IC in patients with prior exposure to azoles and/or infection with *C. glabrata* is well founded (24, 35, 56, 57). Global surveys have documented the exquisite *in vitro* susceptibility of *C. glabrata* to each of the echinocandins (MIC₉₀, 0.015 $\mu\text{g/ml}$ [micafungin], 0.06 $\mu\text{g/ml}$ [caspofungin], or 0.12 $\mu\text{g/ml}$ [anidulafungin]) (39). Furthermore, the clinical experience with

the use of echinocandins to treat IC due to *C. glabrata* is quite favorable (2, 20, 28, 36, 51, 55).

Although documentation of acquired resistance to the echinocandins remains sporadic (3, 18, 37, 44, 48, 58, 62), several recent reports of acquired resistance in clinical isolates of *C. glabrata* focus concern on this species (5, 7, 11–15, 18, 19, 48, 58, 59, 62). Data from global surveys demonstrate that the frequency of echinocandin resistance among clinical isolates of *C. glabrata* ranges from 1 to 3% and is higher among isolates from North America (3%) than among those from Europe (1%), Latin America (0.0%), or the Asia-Pacific region (0.0%) (39, 47). It is now clear that clinical isolates of *C. glabrata* with decreased susceptibility to one or more echinocandins harbor mutations in *fkp1* and/or *fkp2* and are also resistant clinically (5, 15, 37, 48, 59, 62). Chapeland-Leclerc et al. (5) reported a case of IC due to *C. glabrata* in which the infecting strain acquired resistance to flucytosine, fluconazole, voriconazole, and caspofungin through successive independent events following prolonged exposure to each class of antifungal agent. The recovery of different isolates exhibiting clonality for microsatellite markers but genetic diversity for antifungal resistance markers (three unique resistance mechanisms) demonstrates the high propensity of *C. glabrata* to readily mutate *in vivo*

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in a single patient (5). Additional reports from medical centers in the United States and Denmark provide further documentation of multidrug-resistant (MDR) (resistant to two or more classes of antifungal agents) strains of *C. glabrata* (13, 18, 19, 48, 62). One potential explanation for the emergence of MDR in *C. glabrata* is that the haploid nature of the organism makes it particularly adept at acquiring and expressing resistance mutations in response to drug pressure (5, 37, 48, 62).

The generally excellent wild-type (WT) susceptibility of *C. glabrata* to the echinocandins coupled with broadening azole resistance has driven the use of echinocandins for treatment of infections due to *C. glabrata* and at the same time has generated selection pressure for resistant organisms (4, 5, 11, 18, 24, 33–35, 48, 56, 57, 62). The fact that echinocandins are recommended for use in the setting of prior azole exposure and specifically for the treatment of IC due to *C. glabrata* raises the concern that acquired resistance to the echinocandins may emerge independently in fluconazole-resistant strains due to mutations in *fkf* (5, 24, 48, 62). In an effort to further examine this issue, we have addressed the frequency of decreased susceptibility to the echinocandin class of antifungal agents among fluconazole-resistant strains of *C. glabrata* BSI isolates from two large antifungal surveys, the SENTRY Global Surveillance Program for the years 2006 through 2010 and the Centers for Disease Control and Prevention (CDC) population-based surveillance conducted in the Atlanta, GA, and Baltimore, MD, metropolitan areas between 2008 and 2010. Those isolates with decreased susceptibility (either intermediate [I] or resistant [R]) to one or more of the echinocandins were further examined for mutations in *fkf1* and *fkf2*.

MATERIALS AND METHODS

Organisms. A total of 1,669 isolates of *C. glabrata* from blood or other normally sterile sites were obtained from diverse medical centers worldwide (SENTRY; 847 isolates) or from those in the Atlanta and Baltimore metropolitan areas (CDC; 822 isolates). All isolates represented incident episodes of IC (first positive blood culture). Isolates were identified by standard methods and stored in water at ambient room temperature (SENTRY) or in glycerol at -70°C (CDC) until used in the study. Before testing, each isolate was passaged on Sabouraud dextrose agar (Remel, Lenexa, KS) and CHROMagar (Becton, Dickinson, Sparks, MD) to ensure purity and viability.

A total of 21 fluconazole-resistant isolates of *C. glabrata* for which MICs for one or more echinocandin were I or R were further characterized for the presence or absence of mutations in the hot spot (HS) regions of *fkf1* and *fkf2* as described previously (3, 62).

Antifungal susceptibility testing. All isolates were tested for *in vitro* susceptibilities to anidulafungin, caspofungin, micafungin, fluconazole, and voriconazole using CLSI BMD methods (8, 9). MIC results for all agents were read visually following 24 h of incubation as the lowest concentration of drug that caused a significant diminution ($\geq 50\%$ inhibition) of growth compared with control levels (8, 9). The recently revised CLSI clinical breakpoints were used to identify strains of *C. glabrata* that were either I or R to the echinocandins and R or nonsusceptible (NS) to the azoles (40, 41, 44): anidulafungin and caspofungin MIC values of 0.25 $\mu\text{g/ml}$ were considered I, and MIC values of $\geq 0.5 \mu\text{g/ml}$ were considered R; micafungin MIC values of 0.12 $\mu\text{g/ml}$ were considered I, and MIC values of $\geq 0.25 \mu\text{g/ml}$ were considered R; fluconazole MIC values of $\geq 64 \mu\text{g/ml}$ were considered R, and voriconazole MIC values of $>0.5 \mu\text{g/ml}$ were considered NS. Quality control was performed by testing the CLSI-recommended strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (8, 9).

RESULTS AND DISCUSSION

Frequency of resistance to fluconazole and voriconazole.

Among 1,669 BSI isolates of *C. glabrata* collected during the course of the two surveillance programs, 162 (9.7%) were resistant to fluconazole, including 62 of 847 (7.3%) isolates from the SENTRY Program (years 2006 to 2010) and 100 of 822 (12.2%) isolates from the CDC population-based surveillance (years 2008 to 2010). Among the 162 fluconazole-resistant isolates, 160 (98.8%) were NS to voriconazole (MIC $> 0.5 \mu\text{g/ml}$) (40).

Decreased susceptibility to echinocandins among fluconazole-resistant *C. glabrata* isolates. Among the 162 fluconazole-resistant isolates of *C. glabrata*, there were 21 isolates (13.0%) that were either I or R to one or more of the echinocandins (Table 1): 3.7% and 9.3% were I or R, respectively, to anidulafungin, 3.7% and 9.3% to caspofungin, and 4.9% and 8.0% to micafungin. Mutations in either *fkf1* or *fkf2* were detected in 19 of the 21 (90.5%) echinocandin I or R isolates (Table 1). There were 18 fluconazole-resistant isolates (11.1% of all fluconazole-resistant isolates) that were R to one or more of the echinocandins (MIC $\geq 0.25 \mu\text{g/ml}$ [micafungin] or $\geq 0.5 \mu\text{g/ml}$ [anidulafungin and caspofungin]), all (100.0%) of which demonstrated an acquired mutation in *fkf1* or *fkf2*.

Table 2 provides a comparison of the frequency of I or R among 110 fluconazole-resistant strains of *C. glabrata* collected from diverse medical centers worldwide between 2001 and 2004 (27, 42) and that of the present collection, representing the time period 2006 to 2010. Results from the two time periods were obtained with CLSI reference methods in different laboratories; however, quality control procedures were rigorously performed during all studies, suggesting that testing conditions did not influence the differences noted.

Whereas there were no echinocandin-resistant strains among 110 fluconazole-resistant isolates of *C. glabrata* tested between 2001 and 2004, a period where only caspofungin was available, there were 18 echinocandin-resistant strains (11.1%) among the 162 fluconazole-resistant isolates representing the latter time period. All of these had mutations in *fkf* (Tables 1 and 2).

These data document for the first time the broad emergence of coreistance over time to both azoles and echinocandins in clinical isolates of *C. glabrata*. Whereas resistance to azoles in isolates of *C. glabrata* has been documented to be due to the overexpression of CDR efflux pumps (52, 53), there is little or no evidence that efflux pumps are involved in resistance to the echinocandins (31, 50). The documentation of *fkf* mutations in isolates of *C. glabrata* showing *in vitro* resistance to both azoles and echinocandins suggests the sequential accumulation of acquired resistance mechanisms as demonstrated by Chapeland-Leclerc (5). The lack of coreistance in *C. glabrata* in the 2001-to-2004 time period is not surprising given that among the echinocandins only caspofungin was available, having been just approved in the United States and Europe in 2001. Since that time, the overall use of echinocandins in the United States has increased significantly, from 7.7 ± 5.3 days of therapy (DOT) per 1,000 patient-days in 2004 to 13.1 ± 8.6 DOT per 1,000 patient-days in 2008 (mean $\pm$ standard deviation [SD]) ($P < 0.001$) (33). During the same time period (2004 to 2008), the use of azoles increased as well, from 67.6 ± 29 to 72 ± 33 DOT per 1,000 patient-days, but this change was not significant ($P = 0.1570$) (33).

Whereas a shift to non-*albicans* species of *Candida*, particularly

TABLE 1 Fluconazole-resistant bloodstream isolates of *C. glabrata* with decreased susceptibility to one or more echinocandin agent as determined by CLSI broth microdilution methods^a

Isolate no.	Yr	Location	Gender/age (yr) of patient	MIC (μ g/ml)			FKS mutation ^b
				ANF	CSF	MCF	
CAS08-0016	2008	Georgia	M/47	1	0.5	1	S629P(1)
127F	2008	NA	F/49	2	16	2	S629P(1)
CAS11-3112	2010	Georgia	M/47	0.5	0.25	0.12	S629P(1)
CAS11-2978	2010	Maryland	F/70	0.5	0.5	0.12	S629P(1)
CAS11-3129	2010	Maryland	F/70	4	>16	2	S629P(1)
CAS09-1437	2009	Georgia	F/66	0.25	0.25	0.5	R631G(1)
CAS09-1680	2009	Georgia	F/65	0.25	0.5	1	R631G(1)
748F	2008	Washington	M/58	0.25	0.25	0.12	D632Y(1)
30011F	2010	NA	NA	0.25	0.5	0.03	D648E(1)
10956A	2006	Virginia	F/69	1	2	0.12	F659V(2)
CAS09-0869	2009	Maryland	M/39	1	2	0.25	F659Y(2)
CAS08-0425	2008	Maryland	F/23	2	16	4	S663P(2)
CAS08-0094	2008	Georgia	NA	4	>16	4	S663P(2)
CAS09-1786	2009	Georgia	NA	2	>16	2	S663P(2)
CAS09-1225	2009	Georgia	M/25	4	>16	4	S663P(2)
CAS09-1083	2009	Georgia	F/51	0.5	0.25	0.12	S663F(2)
36670F	2010	Washington	NA	0.25	0.25	0.06	NM
53301F	2010	NA	NA	0.25	0.12	0.12	NM
CAS10-2732	2010	Maryland	M/38	0.12	0.25	0.015	NM

^a M, male; F, female; ANF, anidulafungin; CSF, caspofungin; MCF, micafungin; NA, data not available.^b Value in parentheses indicates FKS1 (1) or FKS2 (2); NM, no mutation.

C. glabrata, has been a cause for alarm among clinicians caring for profoundly ill patients in the hospital (23, 26, 61), others have suggested that this is not a concern in the current “echinocandin era” (16, 56, 57). Prior to the availability of the echinocandins, concern over the reduced azole susceptibility of *C. glabrata* was a major issue for clinicians (10, 22, 29, 32, 54). Both *in vitro* and clinical studies have documented excellent activities of the echinocandins against *C. glabrata* and other potentially azole-resistant species, and the echinocandins have been rapidly replacing the azoles as first-line therapy for IC (4, 17, 18, 24, 30, 33, 35, 55, 58). Thus, in addition to the continued high use of azoles, the significant increase in the use of echinocandins places considerable selection pressure on *C. glabrata*, resulting in the emergence of MDR strains. It must be noted that thus far, *Candida* isolates demonstrating reduced echinocandin susceptibility appear following prolonged exposure to the drug, and these infections are manifested as recurrent episodes of candidemia in severely immunocompromised patients (48, 58).

Exposure to fluconazole is recognized by some (6, 22, 25, 49, 60) but not all (10, 54) investigators as a risk factor for fluconazole-resistant *C. glabrata*. Aside from reports of breakthrough fungemias, some involving *fks* mutations, there is little comparable data for the echinocandins and their influence on species distribution or resistance patterns in IC (58). Recently Lortholary et al. (24) examined the effect of exposure to fluconazole ($n = 159$) or caspofungin ($n = 61$) on the proportions of the five major species of *Candida*. For both drugs, preexposure was associated with a decreased prevalence of *C. albicans* in favor of less drug-susceptible species (*C. glabrata* and *C. krusei* for the former; *C. parapsilosis*, *C. glabrata*, and *C. krusei* for the latter; $P = 0.001$). Not only did the species distribution change in patients with recent exposure to fluconazole and caspofungin, but the overall susceptibilities of the isolates to these drugs decreased. Notably, two *C. glabrata* isolates recovered from patients previously exposed to caspofungin during incident candidemia had high caspofungin MICs and harbored mutations in *fks1* (24). The authors caution

TABLE 2 Comparison of the activities of anidulafungin, caspofungin, and micafungin against fluconazole-resistant isolates of *C. glabrata* from two time periods, 2001 to 2004 and 2006 to 2010^a

Time period (yr)	Antifungal agent	No. of isolates tested	No. (%) of isolates ^b		Reference(s)
			I	R	
2001–2004	Anidulafungin	110	2 (1.8)	0 (0.0)	27, 42
	Caspofungin	110	4 (3.6)	0 (0.0)	
	Micafungin	110	0 (0.0)	0 (0.0)	
2006–2010	Anidulafungin	162	6 (3.7)	15 (9.3)	Present study
	Caspofungin	162	6 (3.7)	15 (9.3)	
	Micafungin	162	8 (4.9)	13 (8.0)	

^a All isolates were tested in accordance with CLSI document M27-A3 (8). Fluconazole resistance was defined as an MIC of $\geq 64 \mu$ g/ml.^b Number of isolates for which the echinocandin MICs were intermediate (I) (anidulafungin and caspofungin MIC of 0.25μ g/ml; micafungin MIC of 0.12μ g/ml) or resistant (R) (anidulafungin and caspofungin MIC of $\geq 0.5 \mu$ g/ml; micafungin MIC of $\geq 0.25 \mu$ g/ml).

that a new episode of sepsis after a recent prescription of antifungals may be due to isolates with decreased susceptibility to the prescribed drugs, including caspofungin (24). In light of this, it should be noted that among the CDC isolates of *C. glabrata* with *fks* mutations for which epidemiological data were available, all came from patients with prior exposure to an echinocandin (62).

In summary, we have documented the emergence of coresistance to the echinocandins among BSI isolates of fluconazole-resistant *C. glabrata*. Among the 18 fluconazole-resistant isolates with coresistance to the echinocandins, all were from the United States, consistent with our previous observations concerning the geographic distribution of echinocandin resistance in *C. glabrata*, where the highest frequency of resistance was seen in North America (3%) versus Europe (1%), Latin America (0.0%), and the Asia-Pacific region (0.0%) (47). The increased use of both azoles and echinocandins will most certainly bring selection pressure to bear against *C. glabrata*, a species that appears to be unique in its ability to sequentially acquire and express resistance mutations. Specifically, the targeted use of echinocandins in severely immunocompromised individuals with prior exposure to azoles and/or infection with *C. glabrata* will ensure that this dual drug pressure is maintained. Although the vast majority of *C. glabrata* isolates remain highly susceptible to the echinocandin class of antifungal agents, the increase in MDR *C. glabrata* strains is a serious concern and argues for continued close resistance surveillance and the increased application of standardized antifungal susceptibility testing. Heightened awareness, rather than complacency, concerning the importance of *C. glabrata* should be the watchword for those caring for patients at risk for IC.

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