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Recurrent Episodes of Candidemia Due to *Candida glabrata* with a Mutation in Hot Spot 1 of the *FKS2* Gene Developed after Prolonged Therapy with Caspofungin

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We report two episodes of recurrent candidemia caused by echinocandin-resistant *Candida glabrata* in a 69-year-old patient who underwent repeated abdominal surgery. In the first episode of candidemia, an echinocandin-susceptible *Candida glabrata* strain was isolated, and the patient was treated with caspofungin. The isolates from the later episodes showed resistance to echinocandins. Analysis of the HS1 region of the *FKS2* gene showed the amino acid substitution S663P. Microsatellite analysis demonstrated a strong genetic relationship between the isolates.

Candida glabrata represents an important cause of candidemia in Spain (13, 19, 20). Echinocandins, which include anidulafungin (ANF), caspofungin (CSF), and micafungin (MCF), are first-line antifungal therapy for invasive candidiasis by *C. glabrata* (1, 14). They inhibit the 1,3-β-D-glucan synthase (GS) complex, which is encoded by *FKS* genes. Mutations in hot spots 1 and/or 2 (HS1 and HS2) linked with reduced echinocandin susceptibility have been identified in *FKS1* of *Candida albicans*, *Candida krusei*, and *Candida tropicalis* and in *FKS1* and *FKS2* of *C. glabrata* (15). Some reports show a relationship between echinocandin therapy and treatment failure in *C. glabrata* infections (4, 6, 8, 10, 24). Breakthrough mycoses, particularly by non-*C. albicans* species with reduced susceptibility, have been reported for immunocompromised patients after prolonged exposure to echinocandins, with *C. glabrata* and *C. tropicalis* being involved more frequently (22). However, despite the increase in the use of echinocandins, resistance remains uncommon in *Candida* spp. (16).

In 2011, the Clinical and Laboratory Standards Institute (CLSI) revisited the clinical breakpoints (CBP) of anidulafungin, caspofungin, and micafungin for major *Candida* species. These species-specific CBPs can better detect strains with *FKS* mutations and predict the risk of clinical failure (17). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has established CBPs for anidulafungin as follows: for *C. albicans*, susceptible (S), ≤0.03 μg/ml, and resistant (R), >0.03 μg/ml, and for *C. glabrata*, *C. tropicalis*, and *C. krusei*, S, ≤0.06 μg/ml, and R, >0.06 μg/ml (2).

On 16 June 2009, a 69-year-old man developed candidemia caused by *C. glabrata* (CNM-CL-7368) after two successive surgeries, abdominal and biliary. Fluconazole was administered (June 19), but the fever and candidemia persisted after 5 days, so the central venous catheter (CVC) was removed (June 21) and fluconazole was changed (June 26) to caspofungin (70-mg loading dose, followed by 50 mg/day). The anidulafungin and caspofungin MICs determined by Etest were 0.01 and 0.12 μg/ml, respectively. The fever resolved, and blood cultures became negative. Caspofungin was maintained for 30 days, because the patient developed sepsis of abdominal origin and was admitted to the intensive care unit (ICU) on July 13. He underwent two abdominal surgeries due to superior mesenteric vein thrombosis, although no surgical resection was performed because no necrotic bowel segments were found, and broad-spectrum antibiotics were administered. Surveillance cultures showed gastric colonization by *C. glabrata*. On August 28, the patient presented with fever and candidemia. *C. glabrata* (CNM-CL-7369) was isolated from both the blood and the CVC tip which had been removed. The anidulafungin and caspofungin MICs determined by Etest were 2 and 0.5 μg/ml. Since no CBPs for echinocandins and Etest were defined at that time, we used the CBPs suggested by CLSI (S ≤ 2 μg/ml), and the isolate was considered susceptible, although we were well aware that CBPs are method specific and results should be interpreted with caution. Another course of caspofungin was administered for 3 weeks. The fever stopped, and the blood cultures after 4 days of antifungal treatment were negative. On October 21, he presented with fever and sepsis. An abdominal computer tomography revealed chronic ischemia. *C. glabrata* (CNM-CL-7370) was isolated from both the blood and the CVC tip which had been removed. On October 22, he was admitted to the ICU; anidulafungin (200-mg loading dose, followed by 100 mg/day) was started, and the patient underwent a small bowel surgical resection that resolved the ischemic foci. However, fever persisted and blood cultures remained positive for *C. glabrata* after 4 days of treatment with anidulafungin. The MICs of anidulafungin and caspofungin determined by Etest were 2 and 1 μg/ml, respectively, which were significantly higher than those for the first isolate. Anidulafungin was changed to liposomal amphotericin B (5 mg/kg/day), and the episode resolved after 17 days of treatment.

Etest susceptibility profiles (Table 1) were confirmed using the

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TABLE 1 Antifungal MICs for *C. glabrata* isolates by Etest (48 h) and EUCAST

Isolate	Method	MIC (μg/ml)								
		ANF	CSF	MCF[a]	AMB	5FC	FLC	ITC	PSC	VRC
ATCC 90030	Etest	0.01	0.25	0.01	0.12	0.01	8	>32	4	0.25
	EUCAST	0.03	0.5	0.03	0.12	0.12	8	0.5	0.06	0.25
CL7368	Etest	0.01	0.12	0.01	0.12	0.01	8	>32	4	0.5
	EUCAST	0.03	0.25	0.03	0.25	0.12	4	0.12	0.12	0.12
CL7369	Etest	2	0.5	2	0.25	0.01	8	>32	4	0.25
	EUCAST	0.12	1	0.06	0.25	0.12	4	0.12	0.12	0.12
CL7370	Etest	2	1	4	0.25	0.01	8	>32	4	0.25
	EUCAST	0.25	1	0.12	0.25	0.12	4	0.12	0.12	0.12

[a] The MCF Etest was performed later because it was not available at the time when we evaluated the ANF and CSF Etests.

EUCAST microdilution method (21), and strains were classified as S or R according to EUCAST anidulafungin CBPs for *C. glabrata* (2). *C. glabrata* ATCC 90030 was used as the control. The results confirmed that the susceptibility of the strains recovered after the caspofungin treatment was lower than that of the strain recovered before the treatment.

Although *C. glabrata* has three *FKS* genes, the mutations that confer resistance have been found in HS1 of *FKS1* and, in particular, of *FKS2* genes (11), so HS1 from both genes was amplified using the following oligonucleotides: for HS1 from *FKS1*, CGFKS1-HS1-fw (TTCTCAGTCATGCCATTGG) and CGFKS1-HS1-rv (ACAATGTACCACAGATAGG); for HS1 from *FKS2*, CGFKS2-HS1-fw (AAGATATGTTGCTTCTCAGAC) and CGFKS2-HS1-rv (AAGGAGTTAAGATGGAAATACC). Genomic DNA was extracted using the phenol-chloroform protocol (23). PCR conditions for amplifying the HS1 from *FKS1* were 95°C for 10 min, 30 amplification cycles (95°C for 15 s, 50°C for 15 s, and 72°C for 30 s), and 5 min at 72°C. To amplify the HS1 from *FKS2*, PCR cycles were the same, but the annealing temperature was 53°C. The DNA products were sequenced using the Sanger method and an ABI3730XL sequencer. The DNA sequences were analyzed with DNAStar Lasergene 8.0.2 software (DNAStar Inc., Madison, WI). Multiple-sequence alignment was performed using ClustalW2 from the EMBL-EBI (Cambridge, United Kingdom) and Jalview 2.3 (5). In the two resistant strains, there was a mutation (T to C) at nucleotide 1987, which is located in HS1 from *FKS2*, which produced the S663P change in the protein. This mutation causes resistance to echinocandins (8, 10). No changes were found in the region from nucleotides 1516 to 1871 (which contains the HS1) from the *FKS1* gene (data not shown).

To investigate if the mutation appeared during treatment in the strain that was already present or if resistance was due to a different strain acquired during treatment, we performed a microsatellite analysis of the *RPM2*, *MTI*, and *ERG3* genes (9) with the following modifications: the MT1 sense primer was labeled with NED fluorochrome, and the PCR mixture contained 5 mM $MgCl_2$ and 1 U of AmpliTaq polymerase (Applied Biosystems). For fragment size determination, 1 μl of the PCR products was added to 9 μl of Hi-Di formamide (Applied Biosystems) and 1 μl of the internal size standard ROX 500 (Applied Biosystems, Spain). Capillary electrophoresis was performed in an ABI 3730 XL sequencer (Applied Biosystems). Reactions were analyzed in duplicate. Fragment sizes were calculated with Peak Scanner software version 1.0 (Applied Biosystems). The discrimination power of the analysis was calculated according to reference 12. We included the 3 strains described above plus 14 others available from the collection of the Spanish Mycology Reference Laboratory. For each marker, only one peak was observed in each isolate. Fifteen genotypes were identified using a combination of the markers (Table 2). The strains from this study showed the same microsatellite profile for the three markers (discrimination power, 0.98), indicating that these strains are genetically related and suggesting that resistance was acquired by mutation of the *FKS2* gene in the first isolate.

In a recent population-based surveillance study (25), 3.3% of the *C. glabrata* strains isolated from candidemic patients had elevated MICs for echinocandins (CLSI). Most of them had the S663P substitution, which was associated with the highest echinocandin MICs. No mutations were found in the isolates with MICs of ≤0.125 μg/ml.

The previous CLSI CBP of ≤2 μg/ml for echinocandins (7) can classify strains with mutations in the HSs as susceptible (3). Evaluations of CLSI, EUCAST, and Etest methods that apply epidemi-

TABLE 2 Genotypes of the isolates tested

Strain[a]	Fragment size (bp)		
	RPM 2	ERG 3	MTI
CNM-CL 7523*	134	235	241
CNM-CL 7561*	135	0	244
CNM-CL 7580*	209	205	243
CNM-CL 7590*	134	204	234
CNM-CL 6155*	133	204	244
CNM-CL 6221*	133	204	243
CNM-CL 6226*	141	231	243
CNM-CL 6395*	132	233	241
CNM-CL 6449*	141	229	242
CNM-CL 6894*	134	205	244
CNM-CL 7051*	132	204	239
CNM-CL 7083*	134	204	242
CNM-CL 7313*	133	204	241
CNM-CL 7515*	134	204	164
CNM-CL 7368	136	217	244
CNM-CL 7369	136	217	244
CNM-CL 7370	136	217	244

[a] CNM-CL, Yeast Collection of the Spanish National Center for Microbiology. Asterisks indicate the 14 independent clinical isolates used as the control population.

ological cutoff values to a collection of *Candida* isolates characterized for the presence or absence of mutations in HS regions show that all three methods distinguish mutant from wild-type strains, providing evidence that the three methods can be used in resistance surveillance studies (3, 18).

When the EUCAST CBPs for ANF and the revisited CLSI CBPs for ANF and CSF ($S \leq 0.12$ μg/ml, $R \geq 0.5$ μg/ml) and those for MCF ($S \leq 0.06$ μg/ml, $R \geq 0.25$ μg/ml) for *C. glabrata* were used to interpret MICs obtained by Etest, all of them distinguished the two resistant strains.

Etest is easy to perform in the clinical practice and can predict echinocandin therapy failure, but species-specific breakpoints to define *Candida* strains as resistant by this method are lacking.

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