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## Development of Echinocandin Resistance in *Clavispora lusitaniae* during Caspofungin Treatment<sup>∇</sup>

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***Clavispora lusitaniae* is an opportunistic human pathogen responsible for 0.6 to 2% of candidemia. This species is intrinsically susceptible to echinocandins. Nevertheless, in this study, development of echinocandin resistance in *C. lusitaniae* isolates was observed during caspofungin treatment. This resistance resulted from missense mutation in the echinocandin target *Fks1* gene.**

*Candida albicans* remains the most common pathogen responsible for invasive candidiasis. However, increasing rates of candidemia caused by other species, including *Clavispora lusitaniae*, are reported worldwide (13, 23). *Clavispora lusitaniae* (anamorph: *Candida lusitaniae*) is an opportunistic haploid ascomycetous yeast (12, 25), recovered worldwide from plants, animals, and humans (4). This species is able to grow at 37°C and accounts for 0.6 to 2% of the isolates recovered during candidemia (12, 18, 23). Caspofungin, a member of the echinocandin class, demonstrates fungicidal activity against *C. albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida glabrata*, and *C. lusitaniae*. Beta-1,3-glucan synthase encoded by *Fks* genes is the target of the echinocandins (1, 5). Missense mutations in the hot spot 1 (HS1) and/or HS2 regions, resulting in increased MICs of echinocandins, had already been described in clinical isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* from patients treated with caspofungin (2, 3, 7, 11, 14, 15, 20, 21). *Clavispora lusitaniae* is known for its propensity to develop amphotericin B resistance during therapy (9). It is not intrinsically resistant to echinocandins, and modal caspofungin MIC was 0.25 µg/ml and 0.06 µg/ml (22) (NRCMA, unpublished data). Caspofungin can be used as first-line therapy for candidemia due to *C. lusitaniae* and is even recommended for patients preexposed to azoles. Here, we report the first case of clinical isolates of *C. lusitaniae* with high echinocandin MICs recovered from a patient treated with caspofungin associated with a missense mutation localized in the HS1 region of hypothetical beta-1,3-glucan synthase.

A 77-year-old man was admitted to the intensive care unit after coloanal anastomosis and total cystectomy with bilateral nephrostomy for advanced rectal cancer. On day 7 after admission, the patient developed fever, dyspnea, and polypnea associated with hyperleukocytosis ( $16.5 \times 10^9$  cells/liter, 90%

neutrophils) and increased levels of C-reactive protein (CRP; 184 mg/liter). Culture of both bronchoalveolar lavage (BAL) fluid and urine yielded *C. lusitaniae*. Therapy with cefotaxime (3 g/day) and caspofungin (70 mg on the first day and then 50 mg/day) was started. The patient clinically improved over the next 3 days concurrently with urine and BAL fluid cultures turning negative. Cefotaxime was stopped. On day 16, the patient developed fever, abdominal pain, and dyspnea with biological signs of sepsis (leukocytes at  $28 \times 10^9$  cells/liter with 85% neutrophils, CRP = 214 mg/liter). Clinical and radiologic findings suggested anastomotic stenosis, and the patient underwent surgical revision. Cultures of urine, perianastomotic tissues, and fluid samples were positive for *C. lusitaniae*, while blood and BAL fluid cultures were sterile. Fluconazole (800 mg on the first day and then 400 mg/day) was added. Urine and abdominal fluid cultures became sterile 3 days after, and clinical condition improved slightly. On day 24, a second surgical revision was performed because of perianastomotic tissue necrosis. Therapy with piperacillin-tazobactam (16 g/day) was started. *C. lusitaniae* was recovered from intra-abdominal fluid, but blood, urine, and BAL fluid cultures were negative. The patient's clinical condition worsened over the next few days. A multidrug-resistant *Acinetobacter baumannii* strain was isolated from blood cultures 5 days after the third surgery. The patient eventually died 29 days after admission.

All fungal isolates were identified by carbon assimilation patterns (ID32C; bioMérieux, Marcy-l'Étoile, France; code 51573701). Identification was confirmed by sequencing of internal transcribed spacer (ITS) and D1/D2 regions using universal primers V9D/LS266 (6, 17) and NL1/NL4 (19), respectively. Clinical isolates had 99% and 100% similarity compared to D1/D2 and ITS sequences, respectively, of the type strain *C. lusitaniae* CBS 4413 (sequence of 323 bp, GenBank AJ508571, and sequence of 310 bp, GenBank AF172262). *In vitro* susceptibility was determined for caspofungin, micafungin, and anidulafungin by a microdilution technique according to the procedure proposed by the Antifungal Susceptibility Testing Subcommittee of EUCAST (AFST-EUCAST [27]), modified by using AM3 medium for caspofungin and micafungin (7). Decreased susceptibility to caspofungin was defined by a MIC

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TABLE 1. Echinocandin susceptibility and HS1 protein sequence for the 4 clinical isolates of *C. lusitaniae* and type strain CBS 4413

Strain/isolate	Time after caspofungin initiation (days)	Site of isolation	MIC (µg/ml)			HS1 protein sequence <sup>a</sup>
			Caspofungin	Micafungin	Anidulafungin	
CBS4413 <sup>T</sup>			0.06	0.03	0.06	FFLTSLRD
10BL1-59	0	Urine	0.06	0.06	0.06	FFLTSLRD
10BL1-61	2	BAL fluid	0.125	0.06	0.06	FFLTSLRD
10BL1-60	11	Urine	4	0.5	1	FFLTLFLRD
10BL1-62	17	Peritoneal fluid	4	4	2	FFLTLFLRD

<sup>a</sup> Boldface indicates the mutated residue.

of ≥0.5 µg/ml according to previous data showing that clinical isolates of *Candida* spp. exhibiting MICs above these thresholds harbored mutations in target genes (7, 8). Isolates recovered initially from urine (10BL1-59) and BAL fluid (10BL1-61) had low caspofungin MICs, whereas isolates recovered later from urine (10BL1-60) and peritoneal fluid (10BL1-62) had high MICs (Table 1). Of note, Pfaller et al. recently defined epidemiological cutoff values for *C. lusitaniae* for caspofungin, anidulafungin, and micafungin MICs using the CLSI (Clinical and Laboratory Standards Institute) reference method (0.5 µg/ml, 2 µg/ml, and 0.5 µg/ml, respectively) (22).

We then looked for a mutation within the putative *FKSI* gene to help explain the high MIC values. In the genome of *C. lusitaniae* (ATCC 42720) currently annotated in the *Candida* database on the Broad Institute website ([http://www.broadinstitute.org/annotation/genome/candida\\_lusitaniae/MultiHome.html](http://www.broadinstitute.org/annotation/genome/candida_lusitaniae/MultiHome.html)), one hypothetical beta-1,3-glucan synthase protein of 688 amino acids (CLUG\_01702 transcript 1, supercontig 2: 965796-967862+) had 83% similarity with the HS2 region of *C. albicans* Fks1p. The DNA sequence localized upstream from this sequence (supercontig 2: 964000-967862+) was compared with the nucleotide sequence of the coding region of the *C. albicans* *Fks1* gene (orf19-2929, GenBank D88815.1) and had 79% similarity. Resulting protein sequences of *C. lusitaniae* and *C. albicans* (GenBank BAA21535.1) were compared, and 83% similarity was observed for the 867-amino-acid sequence. For *C. lusitaniae*, protein regions (FFLTSLRD and WIRRYTSLIF) similar to HS1 and HS2 regions of *C. albicans* (FFSTLSLRD and WIRRYTSLIF, respectively) were localized. Primers were designed to amplify these hypothetical HS1 and HS2 regions of *C. lusitaniae* (Table 2). The sequences were translated with the standard genetic code (<http://bioinformatics.org/sms/index.html>), and resulting protein sequences were compared (BioMics, v7.2.5; BioAware SA, Hannut, Belgium). Numbering of the protein sequence was based on *C. albicans* Fks1p. The initial isolates (10BL1-59 and 10BL1-61, GenBank JF304615) showed a protein sequence for

the HS1 region identical to that of ATCC 42720 and CBS 4413 and were considered wild type. The subsequent isolates shared similar nucleotide sequences (GenBank JF304613), leading to a missense mutation, S645F, localized in the HS1 region (Table 1). The 4 isolates had a wild-type protein sequence for the HS2 region (GenBank JF304614).

Development of antifungal resistance has been described for yeasts and filamentous fungi after environmental exposure or clinical treatment (15, 21, 26, 28). Although specific data on caspofungin are lacking, antimicrobial drug distribution has been shown to be potentially impaired in critically ill patients. This could have resulted in subinhibitory levels of caspofungin in the patient’s peritoneal fluid and subsequently selection of the resistant mutant. Flucytosine-fluconazole cross-resistance due to nonsense and missense mutations in *FCY2* and *FCY1* genes is also observed for clinical isolates of *C. lusitaniae* (10). In the present case, isolates of *C. lusitaniae* with increased echinocandin MICs were recovered 2 weeks after initiation of caspofungin treatment, and these isolates exhibited missense mutation S645F in the HS1 region. Of note, among *C. albicans* isolates, amino acid changes at Ser 645 are more common and lead to the most significant MIC echinocandin increases (24). This is the first time that clinical isolates of *C. lusitaniae* with high echinocandin MICs due to mutation in hypothetical Fksp after caspofungin treatment are described. There is no available typing method for *C. lusitaniae*, which prevented analysis of the genetic relatedness between the 4 clinical isolates. However, this species is a rare human pathogen and its recovery from multiple anatomical sites and over time in the same patient makes it likely that the isolates are genetically linked. The recent demonstration that exposure to caspofungin influences the epidemiology of candidemia, the potential for *C. lusitaniae* to become an emerging pathogen in this setting (16), and the development of echinocandin resistance after caspofungin treatment should be taken into account for future therapeutic management.

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TABLE 2. Primers designed in this study to amplify hypothetical HS1 and HS2 regions of *Clavispora lusitaniae*

Primer name	Primer sequence, 5'-3'	Locus	Size of amplicon (bp)
MDO002	GCCTTTGGGTGGTTTGTTTA	HS1	696
MDO003	TCGGAATCTCTTGGGAAGAA	HS1	696
MDO004	TGCTGGTATGGGTGAACAGA	HS2	425
MDO005	CGAACACTTCGAAGAATGGAG	HS2	425

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