Development of echinocandins resistance in *Clavispora lusitaniae*

during caspofungin treatment

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Running title

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Abstract

Clavispora lusitaniae is an opportunistic human pathogen responsible for 0.6-2% of candidemia. This species is intrinsically susceptible to echinocandins. Nevertheless, in this study, development of echinocandins resistance in C. lusitaniae isolates was observed during caspofungin treatment. This resistance resulted from missense mutation in the echinocandins 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 (14, 24). Clavispora lusitaniae (anamorph: Candida lusitaniae) is an opportunistic haploid ascomycetous yeast (13, 26), recovered worldwide from plants, animals, and humans (5). This species is able to grow at 37°C and accounts for 0.6 to 2% of the isolates recovered during candidemia (13, 20, 24). 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 (2, 6). Missense mutations in the hotspot (HS)1 and/or HS2 regions, resulting in increased minimal inhibitory concentration (MIC) of echinocandins, had already been described in clinical isolates of C. albicans, C. glabrata, C. tropicalis, and C. krusei from patients treated with caspofungin (3, 4, 8, 12, 15, 16, 21, 22). Clavispora lusitaniae is known for its propensity to develop amphotericin B resistance during therapy (10). It is not intrinsically resistant to echinocandins and modal caspofungin MIC was 0.25µg/mL and 0.06µg/mL (23) (NRCMA unpublished data).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 HS1 region of hypothetic beta 1,3 glucan synthase.

A 77 year-old man was admitted to 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 (16.5x10⁹ cells/L, 90% neutrophils) and increased levels of C-reactive protein (CRP, 184...
mg/L). Culture of both bronchoalveolar lavage (BAL) and urine yielded *C. lusitaniae*. Therapy with cefotaxime (3g/day) and caspofungin (70 mg the first day then 50 mg/day) was started. The patient clinically improved over the next 3 days concurrently to negativation of urine and BAL cultures. Cefotaxime was stopped. On day 16, the patient developed fever, abdominal pain and dyspnea with biological signs of sepsis (leukocytes at 28 X 10^9 cells/L with 85% neutrophils, CRP= 214 mg/L). Clinical and radiologic findings suggested anastomotic stenosis, and the patient underwent surgical revision. Cultures of urines, perianastomotic tissues and fluids samples were positive for *C. lusitaniae*, while blood and BAL cultures were sterile. Fluconazole (800 mg the first day, then 400 mg/d) was added. Urine and abdominal fluids 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 (16g/d) was started. *C. lusitaniae* were recovered from intraabdominal fluid, but blood, urine and BAL cultures were negative. The patient’s clinical condition worsened over the next days. A multidrug-resistant *Acinetobacter baumannii* 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’Etoile, France, code=51573701). Identification was confirmed by sequencing of ITS and D1/D2 regions using universal primers V9D/LS266 (7, 18) and NL1/NL4 (19) respectively. Clinical isolates had 99% and 100% similarity compared to D1/D2 and ITS sequences of the type strain *C. lusitaniae* CBS 4413 (sequence of 323bp, Genbank AJ508571 and sequence of 310 bp, Genbank AF172262). *In vitro* susceptibility was determined for caspofungin, micafungin and anidulafungin by a microdilution technique following the procedure proposed by the Antifungal Susceptibility Testing Subcommittee of EUCAST (AFST-EUCAST,(1)), modified by using AM3 medium for caspofungin and micafungin (8).
Decreased susceptibility to caspofungin was defined by a MIC ≥ 0.5 µg/mL according to previous data showing that clinical isolates of *Candida* spp. exhibiting MICs above these thresholds harboured mutations in target genes (8, 9). Isolates recovered initially from urine (#10BL1-59) and BAL (#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, Pfaffer 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 (0.5µg/mL, 2µg/mL and 0.5µg/mL, respectively) (23).

We then looked for a mutation within the putative FKS1 gene to help explain the high MIC values. In the genome of *C. lusitaniae* (ATCC42720) currently annotated on *Candida* database Broad Institute website (http://www.broadinstitute.org/annotation/genome/candida_lusitaniae/MultiHome.html), one hypothetical beta 1,3 glucan synthase protein of 688 amino-acid (CLUG_01702 Transcript 1, Supercontig2: 965796-967862+), had 83% of similarity with HS2 region of *C. albicans* Fks1p. The DNA sequence localized upstream this sequence (supercontig 2: 964000-967862+) was compared with the nucleotidic sequence of the coding region of *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 (FFLTLSDLRD and WIRRTLSIF) similar to HS1 and HS2 regions of *C. albicans* (FFSTLSDLRD and WIRRTLSIF) 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 compared (BioloMics, v7.2.5, BioAware SA, Hannut, Belgium).
Numbering of the protein sequence was based on *C. albicans* Fks1p. The initial isolates (#10BL1-59, #10BL1-61, Genbank JF304615) showed a protein sequence for HS1 region identical to that of ATCC 42720 and CBS 4413 and were considered as wild-type. The subsequent isolates shared similar nucleotidic 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 (16, 22, 27, 28). Although specific data on caspofungin are lacking, antimicrobial drugs distribution has been shown to be potentially impaired in critically ill patients. This could have resulted in sub inhibitory 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* (11). In the present case, isolates of *C. lusitaniae* with increased echinocandins MIC 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 the more common and lead to the most significant MIC echinocandin increases (25). This is the first time that clinical isolates of *C. lusitaniae* with high echinocandins MIC due to mutation in hypothetic 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 they 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 (17) and the development of echinocandins...
resistance after caspofungin treatment should be taken into account for future therapeutic management.

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

<table>
<thead>
<tr>
<th>Strain/isolate</th>
<th>Time after Caspofungin Initiation (days)</th>
<th>Site of isolation</th>
<th>MIC (μg/mL)</th>
<th>Caspo</th>
<th>Mica</th>
<th>Anidula</th>
<th>HS1 protein sequence</th>
</tr>
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<tbody>
<tr>
<td>CBS4413(^1)</td>
<td>0</td>
<td>Urine</td>
<td>0.06</td>
<td>0.03</td>
<td>0.06</td>
<td>FFLTLSLRD</td>
<td></td>
</tr>
<tr>
<td>10BL1-59</td>
<td>0</td>
<td>BAL</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>FFLTLSLRD</td>
<td></td>
</tr>
<tr>
<td>10BL1-61</td>
<td>2</td>
<td>Urine</td>
<td>0.125</td>
<td>0.06</td>
<td>0.06</td>
<td>FFLTLSLRD</td>
<td></td>
</tr>
<tr>
<td>10BL1-60</td>
<td>11</td>
<td>Urine</td>
<td>4</td>
<td>0.5</td>
<td>1</td>
<td>FFLTLFLRD</td>
<td></td>
</tr>
<tr>
<td>10BL1-62</td>
<td>17</td>
<td>Peritoneal fluid</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>FFLTLFLRD</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Type strain; Caspo: caspofungin, Mica: micafungin, Anidula: anidulafungin

TABLE 2. Primers designed in this study to amplify hypothetical Hot spot (HS) 1 and 2 regions of *Clavispora lusitaniae*

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’- 3’</th>
<th>Locus</th>
<th>Size of amplicon (bp)</th>
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<tr>
<td>MDO002</td>
<td>GCCTTTGGGTGGTTTGTITTA</td>
<td>HS1</td>
<td>696</td>
</tr>
<tr>
<td>MDO003</td>
<td>TCGGAATCTCTTGGGAAGAA</td>
<td>HS1</td>
<td>696</td>
</tr>
<tr>
<td>MDO004</td>
<td>TGCTGTTATGGGTGAACAGA</td>
<td>HS2</td>
<td>425</td>
</tr>
<tr>
<td>MDO005</td>
<td>CGAACACTTCAAGATGAGG</td>
<td>HS2</td>
<td>425</td>
</tr>
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</table>

bp: base pairs
References


