First report of a clinical isolate of *Candida haemulonii* in Brazil

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INTRODUCTION

The spectrum of *Candida* species associated with invasive fungal infections is evolving. New microbiology diagnostic tools, the increasing number of immunosuppressed patients with invasive devices and the use of prophylaxis with fluconazole could contribute to this phenomenon (1). In recent years, an increasing number of rare species with reduced susceptibility to antifungal molecules have been described, including *C. ciferrii*, *C. inconspicua*, *C. guilliermondii*, *C. humicola*, *C. lambia*, *C. lipolytica*, *C. norvegensis*, *C. palmioleophila*, *C. rugosa*, *C. valida*, *C. fermentati*, and *C. lusitaniae* (2-5). Data from the SENTRY Antimicrobial Surveillance Program-Fungal Objective (5) concerning bloodstream infections from 2008 and 2009 show that 4.5% of 348 episodes from 10 centers in Latin America were caused by species other than *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*.

In 1984, Lavarde et al. (6) reported the first clinical isolate of *C. haemulonii* from a blood culture. Since then, rare cases of human infections with *C. haemulonii* have been reported worldwide, including central venous catheter (CVC)-related bloodstream infections in patients from Argentina, Korea, and China (7-11); in preterm neonates receiving parenteral nutrition in Kuwait (12); and in a 37-year-old French patient with osteomyelitis of the left hallux (13). This pathogen has not been identified in previous reports of candidemia from Brazil (14-17).

The present paper reports for the first time a case of fungemia caused by *C. haemulonii* in a tertiary hospital in the city of São Paulo. Clinical features and laboratory analyses, including phenotypic and molecular identification and antifungal susceptibility testing (AST), are described.

CASE DESCRIPTION

A 26-year-old woman diagnosed with ovarian carcinoma with a low degree of differentiation two months before hospitalization was admitted to the Institute of Cancer in May 2010 for abdominal pain. The patient was submitted to laparotomy and tumor resection. Four days later, the patient developed fever and low back pain, and a diagnosis of pyelonephritis was established. Piperacillin/tazobactam 4.5 g three times per day was prescribed. Because of persistent clinical deterioration, the antimicrobial therapy was changed to imipenem 0.5 g four times per day and vancomycin 1 g two times per day. After stabilization of the patient's clinical condition and the cessation of antibiotics, chemotherapy comprising carboplatin and paclitaxel was started through a CVC. In June 2010, after the second cycle of chemotherapy, the patient was diagnosed with febrile neutropenia, and two sets of Bactec™ aerobic bottles (Becton Dickinson Diagnostics, USA) were collected for blood cultures. Imipenem and vancomycin were reintroduced. After two days, a provisional analysis of the blood culture showed the presence of budding yeast cells. Due to the poor prognosis and the lack of response to chemotherapy, all antibiotics were stopped, and no antifungal agent was prescribed. The patient died six days after the positive blood culture.

Microbial growth was detected after 24 hours in Bactec 9249 incubator (Becton Dickinson Diagnostics, USA). Gram staining of the positive blood cultures showed the presence of yeast. The samples were seeded on CHROMagar *Candida*™ medium (Becton Dickinson Diagnostics, USA). Pink colonies grew after 24 hours of incubation at 37°C. After 72 hours, the colonies developed darker violet central pigmentation. Further phenotypic identification was conducted using API 20C™ AUX panels (BioMérieux, Marcy-L’Etoile, France) but was inconclusive. A VITEK 2™ system (BioMérieux, Marcy-L’Etoile, France) identified the isolate as *C. haemulonii* with 97% certainty.

The DNA from the isolate was extracted as described by Loffler et al. (18) using 250 U/mL of recombinant lyticase (L-4276, Sigma-Aldrich, St. Louis, MO, USA) and the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. Amplification targeted the *Candida* ITS sequence and was performed in a total volume of 25 μL containing 1 × enzyme buffer, 200 mM dNTPs, 0.4 mM each of the ITS1 and ITS4 primers (19), 2 mM MgCl₂, 2.5 U of Taq DNA polymerase (Invitrogen, São Paulo, Brazil) and 5 ng of the template DNA from the *Candida* isolate. The PCR was conducted in a thermocycler (Veriti, Applied Biosystems, Carlsbad, CA, USA) with an

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No potential conflict of interest was reported.
initial denaturation step at 95°C for 5 minutes; 30 cycles of 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C and a final extension at 72°C for 5 minutes. The amplification product was purified using the PureLink™ PCR purification kit (Invitrogen, Carlsbad, CA, USA) and sequenced in both directions in a MegaBace-1000 analyzer (GE Healthcare, Buckinghamshire, UK). The sequences were compared with the reference sequences available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov).

The results of the phenotypic and molecular analyses are summarized in Table 1. Molecular methods confirmed the identity of C. haemulonii.

Antifungal susceptibility testing (AST) was performed using Sensititre YeastOne™ colorimetric plates (Trek Diagnostic Systems, Cleveland, USA) according to the manufacturer's instructions, and interpretation was performed according to the breakpoints established by the Clinical Laboratory Standards Institute (CLSI) (20). The drugs included in these panels and their concentrations were as follows: posaconazole (0.004-8 mg/L), fluconazole (0.125-256 mg/L), itraconazole (0.008-16 mg/L), ketoconazole (0.008-16 mg/L), 5-flucytosine (0.03-64 mg/L), caspofungin (0.008-16 mg/L) and amphotericin (0.008-16 mg/L). Reference strains (C. para- psilosis ATCC 22019 and C. krusei ATCC 6258) were used for quality control purposes. The AST results are summarized in Table 2. The isolate had elevated minimal inhibitory concentrations (MICs) for amphotericin and flucytosine.

The darker violet central pigmentation of the colonies after 72 hours of incubation in chromogenic medium is not a characteristic specific to C. haemulonii. Hospenthal et al. (22) studied the appearance of 83 isolates of different Candida species on CHROMagar™ Candida medium (CHROMagar Microbiology, Paris, France) and found that C. glabrata also developed darker violet pigmentation after three or four days of incubation.

Ruan et al. (10) described three C. haemulonii infections that were identified by VITEK2™ and confirmed by ITS and 18S rRNA sequencing. However, Mi-Na Kim et al. (9) showed that most isolates obtained from blood cultures that are identified as C. haemulonii by VITEK 2™ were determined to be C. pseudohaemulonii based on the sequences of D1/D2 regions of the rRNA gene. These authors also reported that these two species are closely related according to phylogenetic analysis (9). Genotypic identification of the present isolate by ITS sequencing showed 100% identity with C. haemulonii but only 94% identity with C. pseudohaemulonii, thus confirming the identification of our isolate as the former species according to the CLSI MM-18A guidelines (23). Fluconazole, caspofungin, voriconazole, and posaconazole showed good in vitro activity. According to other reports, most isolates of C. haemulonii related to candidemia are resistant to amphotericin and fluconazole (10). Table 3 summarizes the antifungal susceptibility profile of C. haemulonii isolates from Argentina, Korea, Kuwait, China, and Brazil.

To our knowledge, this is the first report of a clinical isolate of C. haemulonii in Brazil. VITEK2™ correctly identified the etiologic agent, but DNA sequencing was necessary for final identification. Echinocandins and voriconazole should be empirical treatment options when C. haemulonii infection is suspected and an antifungigram is not available.

Table 1 - Identification of Candida haemulonii by two commercial biochemical systems and by sequencing analysis.

<table>
<thead>
<tr>
<th>VITEK 2 (%ID*)</th>
<th>API 20AUX (%ID*)</th>
<th>ITS sequencing alignment score (%ID*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. haemulonii</td>
<td>97%</td>
<td>52.2% C. parapsilosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.2% C. guilliermondii</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.1% C. famata</td>
</tr>
</tbody>
</table>

*:Percentual identification given; **: CBS6915 – GenBank access number AB8118790; #: GenBank access number EU881976.1

Table 2 - Antifungal susceptibility profile of Candida haemulonii determined using a Sensititre YeastOne™ panel.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (mg/L)</th>
<th>CLSI Interpretation (M23-A3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin</td>
<td>4</td>
<td>*</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.25</td>
<td>SDD</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.064</td>
<td>S</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>0.125</td>
<td>**</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>64</td>
<td>R</td>
</tr>
</tbody>
</table>

S: susceptible; SDD: susceptible dose dependent; R: resistant; *: likely resistant when MIC>1 mg/L; **: breakpoints not established by CLSI.
Table 3 - Comparison of the antifungal susceptibility of Candida haemulonii bloodstream isolates according to author and the year of publication.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year of Report</th>
<th>Country</th>
<th>Isolate</th>
<th>AMB***</th>
<th>FLU</th>
<th>VOR</th>
<th>CAS</th>
<th>ITRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruan et al. (7)</td>
<td>2002</td>
<td>Argentina</td>
<td>1 4</td>
<td>325/DD</td>
<td>NT</td>
<td>NT</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Giusiano et al. (8)</td>
<td>2006</td>
<td>Argentina</td>
<td>1 1</td>
<td>325/DD</td>
<td>NT</td>
<td>NT</td>
<td>MD/R</td>
<td></td>
</tr>
<tr>
<td>Khan et al. (12)</td>
<td>2007</td>
<td>Kuwait</td>
<td>2 6</td>
<td>256/R</td>
<td>0.0475</td>
<td>0.5/5</td>
<td>2/R</td>
<td></td>
</tr>
<tr>
<td>Mi-Na Kim et al. (9)</td>
<td>2009</td>
<td>Korea</td>
<td>1 1</td>
<td>64/R</td>
<td>1/5</td>
<td>0.125</td>
<td>4/R</td>
<td></td>
</tr>
<tr>
<td>Ruan et al. (10)</td>
<td>2010</td>
<td>China</td>
<td>2 2</td>
<td>16/S-DD</td>
<td>0.25/5</td>
<td>1/5</td>
<td>0.25/DD</td>
<td></td>
</tr>
<tr>
<td>Sunyong Kim et al. (11)</td>
<td>2010</td>
<td>Korea</td>
<td>1 0.5</td>
<td>8/S</td>
<td>0.5/5</td>
<td>0.125</td>
<td>0.25/DD</td>
<td></td>
</tr>
</tbody>
</table>

MIC: minimal inhibitory concentration; AMB: amphotericin B; FLU: fluconazole; VOR: voriconazole; CAS: caspofungin; ITRA: itraconazole; *interpretation according to CLSI breakpoints document M27; **likely resistant if MIC $>$ 1 mg/L; S: susceptible; S-DD: susceptible dose dependent; R: resistant; NT: not tested; MD: missing data.

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AUTHOR CONTRIBUTIONS
Almeida Junior JN, Motta AL, and Del Negro GM provided experimental data. Rossi F, Abdala E, Bernard G and Del Negro GM designed the study. Pierroti LC, Kono ASG and Díaz Mdel P provided clinical data. Almeida Junior JN, Bernard G and Del Negro GM wrote the paper.

REFERENCES