CASE REPORT

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. lambica*, *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 reinitiated. 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℃. 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 $MgCl_2$, 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

No potential conflict of interest was reported.

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

VITEK 2 (%ID*)	API 20AUX (%ID*)	ITS sequencing alignment score (%ID*)	
		ITS 1 Forward	ITS 4 Reverse
	52.2%	100%	100%
	C. parapsilosis	*C. haemulonii***	*C. haemulonii***
97%	34.2 %	94%	94%
C. haemulonii	*C. guilliermondii*	*C. pseudohaemulonii*#	*C. pseudohaemulonii*#
	13.1%		
	C. famata		

*:Percentual identification given; **:CBS6915 – GenBank access number AB118790;
#: GenBank access number EU881976.1

initial denaturation step at 95℃ for 5 minutes; 30 cycles of 1 minute at 95℃, 1 minute at 55℃ and 1 minute at 72℃; and a final extension at 72℃ 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), voriconazole (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. parapsilosis* 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.

DISCUSSION

Sunyong Kim *et al*. (11) identified reports of *C. haemulonii* bloodstream infections in the literature and summarized the clinical characteristics of the patients who developed these infections. These authors found that among ten cases, nine were related to CVCs. Most isolates had elevated MICs for amphotericin, fluconazole and itraconazole, and treatment failure occurred when one of these drugs was prescribed (11). Among four patients treated with an echinocandin, only one experienced treatment failure (11).

Mi-Na Kim *et al*. (9) described eight cases of fungemia caused by *C. haemulonii* and *C. pseudohaemulonii* in five hospitals in Korea. Four of these patients had received previous antifungal therapy, and all of the patients had a severe underlying disease and a CVC (9).

Oh *et al*. (21) investigated the *in vitro* production of biofilms by *C. haemulonii* and C. *pseudohaemulonii*. The formation of biofilms reflects the potential of these two species to cause catheter-related bloodstream infections. In accordance with these previous reports, our patient had a severe underlying disease and a CVC when candidemia was diagnosed.

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 VITEK 2™ and confirmed by ITS1 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 2 - Antifungal susceptibility profile of *Candida haemulonii* determined using a Sensititre YeastOne™ panel.

Drug	MIC (mg/L)	CLSI Interpretation (M23-A3)
Amphotericin	4	*
Fluconazole	8	S
Itraconazole	0.25	SDD
Voriconazole	0.064	S
Caspofungin	0.25	S
Posaconazole	0.125	**
Flucytosine	64	R

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.

Author	Year of Report	Country	Isolate	MIC (mg/L)/Antifungal Susceptibility Profile*				
				AMB**	FLU	VOR	CAS	ITRA
Rodero *et al*. (7).	2002	Argentina	1	4	32/S-DD	NT	NT	0.12/S
Giusiano *et al*. (8).	2006	Argentina	1	1	32/S-DD	NT	NT	MD/R
Khan *et al*. (12).	2007	Kuwait	1	4	96/R	0.047/S	0.5/S	2/R
			2	6	>256/R	0.125/S	0.023/S	3/R
			3	4	>256/R	0.125/S	0.125/S	2/R
			4	8	>256/R	0.125/S	0.125/S	4/R
Mi-Na Kim *et al*. (9).	2009	Korea	1	1	64/R	1/S	0.125/S	4/R
Ruan *et al*. (10).	2010	China	1	2	16/S-DD	0.25/S	1/S	0.25/S-DD
			2	2	16/S-DD	0.25/S	1/S	0.25/S-DD
Sunyong Kim *et al*. (11).	2010	Korea	1	0.5	8/S	0.5/S	0.125/S	0.25/S-DD
Almeida Junior *et al*. (our case).	2012	Brazil	1	4	8/S	0.064/S	0.25/S	0.25/S-DD

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 Diz Mdel P provided clinical data. Almeida Junior JN, Bernard G and Del Negro GM wrote the paper.

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