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Candida haemulonii complex: species identification and antifungal susceptibility profiles of clinical isolates from Brazil

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Objectives: The emerging fungal pathogens comprising the *Candida haemulonii* complex (*Candida haemulonii*, *Candida haemulonii* var. *vulnera* and *Candida duobushaemulonii*) are notable for their antifungal resistance. Twelve isolates with phenotypic similarity to *C. haemulonii* were recovered from patients in Brazilian hospitals. Here we aimed to identify these isolates by a molecular approach, using the current classification of this fungal complex, and to evaluate their antifungal susceptibility profiles.

Methods: The fungal isolates were rechecked to certify their authentication by mycology methodologies and then characterized by *ITS1-5.8S-ITS2* gene sequencing. A susceptibility assay was performed using the broth microdilution method published by CLSI (M27-A3/M27-S3).

Results: Based on biochemical tests, all Brazilian isolates were identified as *C. haemulonii*. After employing *ITS* sequencing, five isolates were identified as *C. haemulonii*, four as *C. duobushaemulonii* and three as *C. haemulonii* var. *vulnera*. All 12 clinical isolates were resistant to amphotericin B (MICs ranged from 2 to >16 mg/L) and fluconazole (MICs ≥64 mg/L). One isolate of *C. haemulonii* var. *vulnera* and two isolates of *C. duobushaemulonii* were susceptible-dose dependent to itraconazole, while the remaining isolates (75%) were resistant to this antifungal. Eight out of 12 isolates (66.7%) were resistant to voriconazole (MICs ≥16 mg/L), while all isolates were susceptible to caspofungin (MICs ≤0.5 mg/L).

Conclusions: Our results reinforce the importance of molecular identification in differentiating species of the *C. haemulonii* complex. Moreover, the antifungal multiresistant profile of clinical isolates of the *C. haemulonii* complex represents a challenge to the treatment of such infections.

Keywords: *Candida haemulonii* complex, Brazilian hospitals, resistance, antifungal susceptibility

Introduction

Candida haemulonii has emerged as an opportunistic fungal pathogen associated with onychomycosis,[1] vaginal candidiasis,[2] bloodstream infections,[3–5] catheter-related fungemia,[6] osteitis[7] and outbreaks in neonatal intensive care units.[8] Although regarded as a rare *Candida* species, it deserves attention because it is considered a multidrug-resistant yeast,[5,8,9] with clinical failure associated with resistance to amphotericin B and reduced susceptibility to azoles, resulting in difficulty in treating deep infections.[3,10,11]

The first isolation of *C. haemulonii* from humans was reported in 1984, from the blood of a patient who died of renal failure despite therapy with amphotericin B and flucytosine.[12] Since then, several cases of infection due to this yeast have been described in the literature, varying from superficial to deep infections. Cendejas-Bueno *et al.*[9] suggested the reclassification of *C. haemulonii* as a fungal complex formed by three genotypically distinguishable species: *C. haemulonii*, *Candida duobushaemulonii* and *C. haemulonii* var. *vulnera*, based on sequencing of *D1/D2* or *ITS* genes. Moreover, two species related to *C. haemulonii* complex

were described, *Candida pseudohaemulonii*[13] and *Candida auris*,[14] which are responsible for fungaemia and also present antifungal resistance profiles.

Despite the importance of these emergent multiresistant yeasts, little information on the occurrence and distribution of *C. haemulonii* complex in clinical specimens has been available until now. With this in mind, we obtained 12 strains of *C. haemulonii* isolated from Brazilian hospitals and performed molecular identification based on *ITS* gene sequencing to correctly classify them within the current taxonomy of this fungal complex. In parallel, we evaluated the antifungal susceptibility profile of these clinical isolates against amphotericin B, fluconazole, itraconazole, voriconazole and caspofungin.

Materials and methods

Microorganisms, growth conditions and biochemical identification

In this study, we analysed 12 clinical isolates of *C. haemulonii* recovered from 12 patients attending four Brazilian hospitals between 2005 and 2013 (Table 1). Fungal isolates were grown on Sabouraud dextrose medium (37°C/48 h/200 rpm) and then phenotypically identified using CHROMagar Candida® (CHROMagar Company) and VITEK® 2 (bioMérieux) with YST card.

DNA extraction, amplification and nucleotide sequence determination

Yeasts were recovered from Sabouraud dextrose agar and used for DNA extraction with the Gentra® Puregene® Yeast and G+ Bacteria Kit (Qiagen®). All isolates were identified by sequencing the *ITS1-5.8S-ITS2* gene as previously described.[9,10] Amplicons were purified and sequences from both DNA strands were generated and edited with the Sequencher™ version 4.9 (Gene Codes Corporation), followed by alignment using Mega version 4.0.2 software.

Antifungal susceptibility assay

Susceptibility testing was performed according to the standardized broth microdilution technique described by CLSI[15] in document M27-A3 and interpreted according to document M27-S3. Antifungals tested were amphotericin B, fluconazole, itraconazole, voriconazole and caspofungin (Sigma-Aldrich).

Results and discussion

The fungal isolates were initially reconfirmed to certify their authentication by mycology methodologies. All isolates developed a light-to-dark violet colour after 48 h of incubation on CHROMagar Candida®; however, other *Candida* species (e.g. *C. glabrata*) also develop violet pigmentation in this chromogenic medium.[16] The carbohydrate assimilation and metabolic enzymatic profiles evaluated with VITEK® 2 identified all 12 Brazilian clinical isolates as *C. haemulonii* with a probability of identity ranging from 94 to 98%; only two contradictory tests were detected: glycerol assimilation (2/12 isolates, 16.7%) and α-glucosidase (4/12 isolates, 33.3%) (Table 1).

Phenotypic methods are not sufficient to differentiate species of the *C. haemulonii* complex and the closely related species *C. pseudohaemulonii* and *C. auris*;[3,9,10] consequently, molecular

Table 1. Biochemical/molecular identification and antifungal susceptibility profiles of the *C. haemulonii* complex isolates included in this study

				VITEK® 2 YST system			Sequencing of *ITS* gene			Susceptibility profile[a]				
Code	Source of isolate	Collection date	Institution	species	probability (confidence)	atypical biopattern	species	GenBank accession number	AMB	FLC	ITC	VRC	CAS
LIP Ch1	finger nail	2005	IBEx	*C. haemulonii*	99% (EI)	—	*C. duobushaemulonii*	KJ476193	R	R	R	R	S
LIP Ch2	sole of the foot	2009	UFF	*C. haemulonii*	98% (EI)	—	*C. haemulonii*	KJ476194	R	R	R	S	S
LIP Ch3	toe nail	2009	UFF	*C. haemulonii*	98% (EI)	—	*C. haemulonii*	KJ476195	R	R	R	R	S
LIP Ch4	finger nail	2009	UFF	*C. haemulonii*	98% (EI)	—	*C. haemulonii*	KJ476196	R	R	R	R	S
LIP Ch5	toe nail	2009	UFF	*C. haemulonii*	98% (EI)	—	*C. haemulonii* var. *vulnera*	KJ476197	R	R	SDD	S	S
LIP Ch6	toe nail	2009	UFF	*C. haemulonii*	97% (EI)	AGLU	*C. duobushaemulonii*	KJ476198	R	R	SDD	S	S
LIP Ch7	toe nail	2009	UFF	*C. haemulonii*	98% (EI)	—	*C. haemulonii*	KJ476199	R	R	R	R	S
LIP Ch8	blood	2010	USP	*C. haemulonii*	95% (VGI)	AGLU, GLYLa	*C. duobushaemulonii*	KJ476200	R	R	R	R	S
LIP Ch9	urine	2012	INCA	*C. haemulonii*	98% (EI)	—	*C. haemulonii* var. *vulnera*	KJ476201	R	R	R	R	S
LIP Ch10	bronchoalveolar lavage	2013	INCA	*C. haemulonii*	94% (VGI)	AGLU, GLYLa	*C. duobushaemulonii*	KJ476202	R	R	SDD	S	S
LIP Ch11	blood	2013	INCA	*C. haemulonii*	96% (EI)	AGLU	*C. haemulonii* var. *vulnera*	KJ476203	R	R	R	R	S
LIP Ch12	blood	2013	INCA	*C. haemulonii*	98% (EI)	—	*C. haemulonii*	KJ476204	R	R	R	R	S

IBEx, Instituto de Biologia do Exército (Rio de Janeiro, RJ, Brazil); UFF, Universidade Federal Fluminense (Niterói, RJ, Brazil); USP, Universidade de São Paulo (São Paulo, SP, Brazil); INCA, Instituto Nacional do Câncer (Rio de Janeiro, RJ, Brazil); EI, excellent identification; VGI, very good identification; AGLU, α-glucosidase; GLYLa, glycerol assimilation; AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; CAS, caspofungin; R, resistant; SDD, susceptible-dose dependent; S, susceptible.
[a]The MIC values are shown in Table S1.

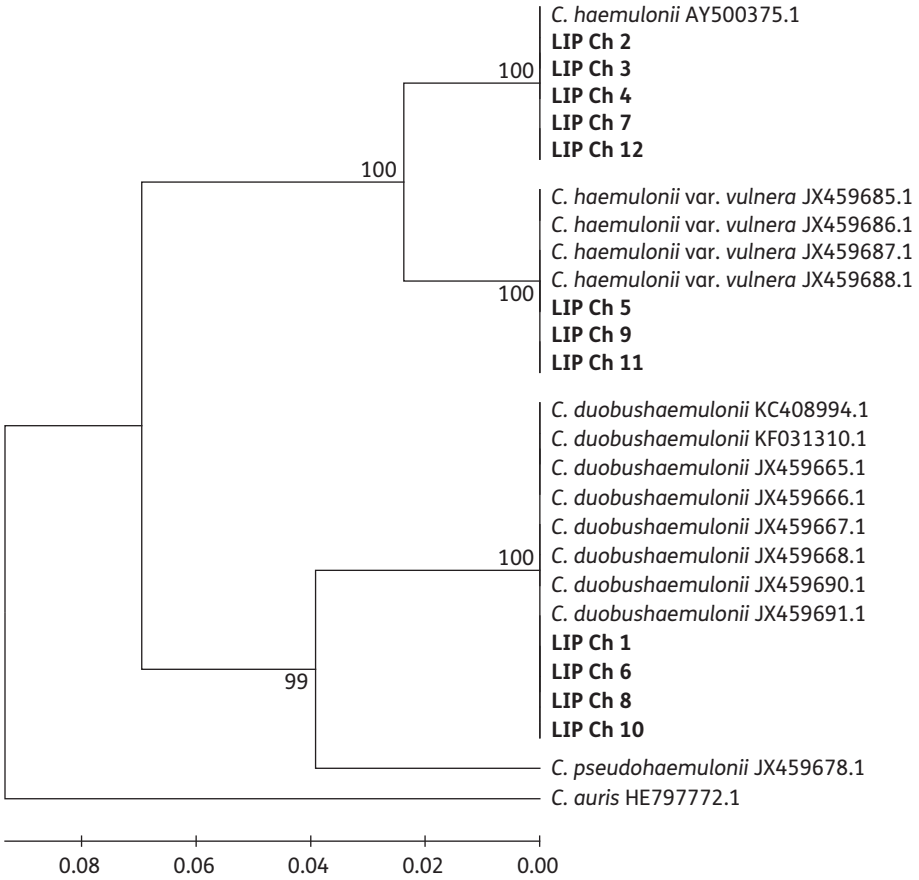

Figure 1. Phylogenetic neighbour-joining dendrogram generated from a genetic similarity matrix based on comparison of the *ITS1-5.8S-ITS2* gene sequence from 12 clinical isolates obtained in the present work (LIP Ch1 to LIP Ch12), as well as 13 sequences of isolates belonging to the *C. haemulonii* complex and two closely related species (*C. auris* and *C. pseudohaemulonii*) obtained from the GenBank database. Data consistency was tested by bootstrapping the alignments 1000 times with corrections for multiple substitutions.

identification was carried out. PCR followed by sequencing of the *ITS* gene was used as the gold standard for identification of this fungal complex.[9] Our results revealed that among the 12 clinical isolates, five were identified as *C. haemulonii* (41.7%), four as *C. duobushaemulonii* (33.3%) and three as *C. haemulonii* var. *vulnera* (25%) (Table 1). The *ITS* sequencing alignment scores of the fungal isolates employed here exhibited 100% identity compared with corresponding *ITS* sequences from reference isolates deposited in GenBank (Figure 1). The *ITS* sequences obtained during this study were deposited in GenBank under the accession numbers listed in Table 1.

To our knowledge, this is the first report of the identification of the three species of *C. haemulonii* complex in clinical isolates from patients attending Brazilian hospitals. It is noteworthy that the isolate LIP Ch8 was previously described as *C. haemulonii*,[4] also based on *ITS* gene sequencing; however, that study was conducted before the aforementioned reclassification of the *C. haemulonii* complex.[9] Our data reallocated the isolate LIP Ch8 as *C. duobushaemulonii*. Additionally, *C. haemulonii*, *C. haemulonii* var. *vulnera* and *C. duobushaemulonii* were identified in both cutaneous and blood samples from Brazilian patients (Table 1). Species of *C. haemulonii* complex had already been isolated from distinct environments, including the gut of *Haemulon sciurus* (fish), *Pyrrhocoris apterus* (insect) and human nails, skin, blood, bone, respiratory specimens and urine.[7,9,11]

Concerning the antifungal susceptibility tests, all 12 isolates of the *C. haemulonii* complex were considered resistant to amphotericin B (MICs ranged from 2 to >16 mg/L) and fluconazole (MICs ≥64 mg/L) (Table 1 and Table S1, available as Supplementary data at *JAC* Online). One isolate of *C. haemulonii* var. *vulnera* and two isolates of *C. duobushaemulonii* were susceptible-dose dependent to itraconazole, while the remaining isolates were resistant to this antifungal agent (Tables 1 and S1). Four fungal isolates (*C. haemulonii* LIP Ch2, *C. haemulonii* var. *vulnera* LIP Ch5 and *C. duobushaemulonii* LIP Ch6 and LIP Ch10) were susceptible to voriconazole with MIC values ≤1 mg/L, while the others were resistant with MIC values ≥16 mg/L. In addition, all the fungal isolates were susceptible to caspofungin (Tables 1 and S1).

Results from the ARTEMIS DISK Global Antifungal Surveillance Study (1997–2007) showed that infections caused by *C. haemulonii* were very infrequent (<0.01%), and at that time 11.1% of these isolates were resistant to fluconazole and itraconazole.[17] A retrospective study[18] showed that, of the species responsible for causing candidaemia, *C. haemulonii* was the sixth most common (1.5%) in an Indian hospital between 2001 and 2005, but no true antifungal resistance was reported. *C. haemulonii*

was the fourth most common species of *Candida* isolated from individuals with cutaneous candidiasis (12% of the total) attending the Human and Veterinary Diagnostic Mycology Sector of UFF (Niterói, RJ, Brazil).[19] The emergence of *C. haemulonii* in five Korean hospitals was reported between 2004 and 2006, with genotyping results suggesting intra- and inter-hospital transmission of a clonal strain.[10] *C. haemulonii* was the third most frequent species responsible for causing candidaemia in Sir Ganga Ram Hospital (New Delhi, India), representing 15.5% of the cases, following *Candida tropicalis* (29.2%) and *C. albicans* (16.8%).[5] Impressively, the authors reported that *C. haemulonii* was first isolated in 2006 and its frequency of isolation increased from 5.45% in 2006 to 18.2% in 2008, showing resistance to amphotericin B and azoles.[5] Interestingly, the authors also analysed the annual usage of antifungal drugs between 2000 and 2008, and observed an increase of 32% in total antifungal use.[5] According to this analysis, fluconazole was the most frequently prescribed antifungal drug in the mentioned period, with an increase of 25% in its use, and a statistically significant correlation was observed between yearly fluconazole usage and the increase in isolation of different non-*albicans Candida* species.[5]

Antifungal resistance is a great concern in the management of patients with invasive candidiasis.[20] *In vitro* resistance to amphotericin B and fluconazole is a common phenotypic characteristic of clinical isolates of the *C. haemulonii* complex already described by several authors, and it has often been associated with clinical treatment failure and fatal outcome.[5,8–11] Concerning the use of itraconazole, some authors have reported variable patterns of susceptibility of the *C. haemulonii* complex to this antifungal.[3,5,8,10] In disagreement with the published literature, the majority (≈67%) of the isolates of the *C. haemulonii* complex studied here were resistant to voriconazole.

Cendejas-Bueno *et al*.[9] observed a subtle difference in azole MICs among the species of the *C. haemulonii* complex, in which *C. haemulonii* showed higher MICs than the others, especially of itraconazole and voriconazole. In this regard, we demonstrated that 100% and 80% of *C. haemulonii* isolates were resistant to itraconazole and voriconazole, respectively, while 50% of *C. duobushaemulonii* and 33.3% of *C. haemulonii* var. *vulnera* were resistant to both antifungal agents. It is remarkable that three out of four isolates (75%) dose-dependently susceptible to itraconazole were also susceptible to voriconazole. Although echinocandins are highly active against *C. haemulonii* complex,[3,10] isolates resistant to this antifungal class have been reported.[6,9]

During recent decades, there has been a change in the epidemiology of *Candida* infections, characterized by a progressive shift from a predominance of *C. albicans* to non-*albicans Candida* species.[20] As a consequence, new *Candida* species have appeared and been recognized as potential pathogens, being described as emerging fungi, some of them innately resistant to commonly used antifungal drugs.[20] Therefore, rapid and correct identification of *Candida* species has become more important in deciding the appropriate starting treatment and providing optimal management of infections.[20] Overall, our results reinforce the importance of molecular identification in differentiating the species comprising the *C. haemulonii* complex. To date, very little is known regarding the clinical characteristics and antifungal susceptibility profiles of clinical isolates of the *C. haemulonii* complex. Clinical experience with *C. haemulonii* complex infection is extremely limited; consequently, no treatment regimen for invasive infections caused by these fungi has been clearly established. Antifungal susceptibility is a particularly relevant problem in managing invasive *C. haemulonii* complex infection due to its multidrug resistance profile, which represents a future therapeutic challenge to clinicians. Finally, continued surveillance regarding non-*albicans Candida* species, such as species of the *C. haemulonii* complex, both locally and on a regional and international basis, is clearly warranted.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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