Major Article

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Molecular identification and antifungal susceptibility profiles of *Candida parapsilosis* complex species isolated from culture collection of clinical samples

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ABSTRACT

Introduction: *Candida parapsilosis* is a common yeast species found in cases of onychomycosis and candidemia associated with infected intravascular devices. In this study, we differentiated *Candida parapsilosis sensu stricto*, *Candida orthopsilosis*, and *Candida metapsilosis* from a culture collection containing blood and subungual scraping samples. Furthermore, we assessed the *in vitro* antifungal susceptibility of these species to fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B, and caspofungin. **Methods:** Differentiation of *C. parapsilosis* complex species was performed by amplification of the secondary alcohol dehydrogenase (SADH) gene and digestion by the restriction enzyme *Ban*I. All isolates were evaluated for the determination of minimal inhibitory concentrations using Etest, a method for antifungal susceptibility testing. **Results:** Among the 87 isolates, 78 (89.7%) were identified as *C. parapsilosis sensu stricto*, five (5.7%) were identified as *C. orthopsilosis*, and four (4.6%) were identified as *C. metapsilosis*. Analysis of antifungal susceptibility showed that *C. parapsilosis sensu stricto* isolates were less susceptible to amphotericin B and itraconazole. One *C. parapsilosis sensu stricto* isolate was resistant to amphotericin B and itraconazole. Moreover, 10.2% of *C. parapsilosis sensu stricto* isolates were resistant to caspofungin. Two *C. parapsilosis sensu stricto* isolates and one *C. metapsilosis* isolate were susceptible to fluconazole in a dose-dependent manner. **Conclusions:** We reported the first molecular identification of *C. parapsilosis* complex species in State of Goiás, Brazil. Additionally, we showed that although the three species exhibited differences in antifungal susceptibility profiles, the primary susceptibility of this species was to caspofungin.

Keywords: *Candida parapsilosis. In vitro* antifungal susceptibility. Candidemia. Onychomycosis.

INTRODUCTION

Although *Candida albicans* is the most common isolate from human infections, other *Candida* species have also been observed in the 1990s, supporting the increased frequency of non-*albicans* species[1] [2]. *Candida parapsilosis* is a common yeast species found in cases of onychomycosis and candidemia associated with infected intravascular devices[3] [4] [5] [6]. In State of Goiás, Brazil, *C. parapsilosis* was found in 52.5% of nail samples and was the second most commonly isolated species (24.2%) from blood cultures[4] [7]. Previous work has shown that *C. parapsilosis* isolates may exhibit genotypic differences, allowing the classification of new species by restriction polymorphism analysis of the secondary alcohol dehydrogenase (SADH) gene.

Candida parapsilosis forms a complex composed of three genetically distinct species: *C. parapsilosis sensu stricto*, *Candida orthopsilosis*, and *Candida metapsilosis*[8]. *C. parapsilosis* complex species are usually susceptible to antifungal agents; however, some reports have shown that these isolates may exhibit decreased susceptibility to azoles and echinocandins[9]. This difference in antifungal susceptibility among the three species has led to an increased interest in the study of *C. parapsilosis* complex species[10]. Moreover, antifungal susceptibility surveillance is an important major strategy for the prophylaxis and treatment of candidiasis caused by *C. parapsilosis*.

Because of the medical importance of *C. parapsilosis* in our area and the limited local epidemiological data on this species, we aimed to verify the distribution of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis* species within a culture collection containing blood and subungual scraping samples from patients attending a tertiary hospital

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in Goiás, Brazil. Additionally, we assessed the *in vitro* antifungal susceptibility of these species to fluconazole (FLC), itraconazole (ITC), voriconazole (VOR), posaconazole (POS), amphotericin B (AMB), and caspofungin (CAS).

METHODS

Isolates

A total of 87 *C. parapsilosis* isolates were recovered from different clinical specimens, including blood (737) and nail specimens (136). These isolates were stored at -70°C in yeast extract peptone dextrose (YEPD) broth (Difco) with 10% glycerol and were obtained from the Laboratory of Mycology, Institute of Tropical Pathology and Public Health, Federal University of Goiás from 2007 to 2012.

Candida parapsilosis was obtained from adult patients with candidemia (n = 54) and onychomycosis (n = 33). The study was approved by the Bioethic Committee from *Hospital das Clínicas* in Goiânia-GO (protocol 065/2008). Confirmation of the identification of *C. parapsilosis sensu lato*, after subculture on YEPD agar (Difco) for 48h at 37°C, was based on colony color on CHROMagar *Candida* medium (Difco), micromorphology of colonies on cornmeal agar medium (Difco), and assimilation profiles on ID32C (BioMérieux, France). *C. parapsilosis* ATCC 22019 was included as quality control strain.

Molecular identification of the *Candida parapsilosis* complex species

Genomic deoxyribonucleic acid (DNA) was extracted from each isolate using high-speed cell disruption followed by phenol-chloroform extraction and ethanol precipitation, as described by Tavanti et al.[8].

Polymerase chain reaction (PCR) was carried out to amplify the SADH gene using the primers S1F (5′-GTTGATGCTGTTGGATTGT-3′) and SIR (5′-CAATGCCAAATCTCCCAA-3′) and conditions previously described by Tavanti et al.[8] Amplification was performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA), and the amplified products were loaded onto 2% agarose gels containing ethidium bromide (0.5mg/mL). A 100-bp DNA ladder was used as a molecular size marker (Invitrogen Life Technologies, USA). Amplified products were electrophoresed on 1.5% agarose gels, visualized under ultraviolet light, and analyzed on a photo documenter (Bio-Rad).

The PCR products (fragments of 716bp) were purified with a specialized kit (Qiagen, Valencia, CA, USA), and digestion of the purified PCR product was carried out for 120 min at 37°C using the restriction enzyme *Ban*I (Thermo Scientific, USA). Digestion of the PCR products was subjected to electrophoresis onto 2% agarose gels (Invitrogen Life Technologies). *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96141, and *C. metapsilosis* ATCC 96143 were used as controls.

In vitro susceptibility testing

All isolates were evaluated using Etests (AB Biodisk, Solna, Sweden), as recommended by the manufacturer's guidelines, with Etest strips for ITC, VOR, POS, AMB, and CAS at concentrations ranging from 0.002 to 32μg/mL and for FLC at concentrations ranging from 0.016 to 256μg/mL. *C. parapsilosis* ATCC 22019 was included on each day of testing to verify the reproducibility of the results.

Interpretative criteria for FLC minimum inhibitory concentrations (MICs) were as follows: ≤ 2μg/mL, susceptible; 4μg/mL, susceptible-dose dependent (S-DD), and ≥8μg/mL, resistant. ITC and VOR MICs were interpreted as follows: ≤0.125μg/mL, susceptible; 0.25-0.5μg/mL, S-DD; and ≥ 1μg/mL, resistant. Caspofungin MICs were interpreted as follows: ≤2μg/mL, susceptible; 4μg/mL, intermediate; and ≥8μg/mL, resistant[11][12]. Isolates with MICs of greater than 1μg/mL were considered resistant to AMB and POS, as suggested by Nguyen et al.[13] and Cantón et al.[3].

RESULTS

Among the 87 isolates, 78 (89.7%) were identified as *C. parapsilosis sensu stricto* (two restriction sites), five (5.7%) were identified as *C. orthopsilosis* (no restriction sites), and four (4.6%) were identified as *C. metapsilosis* (four restriction sites). In blood samples, 47 (54.1%), five (5.7%), and two (2.3%) isolates were *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*, respectively. In nail samples, *C. parapsilosis* and *C. metapsilosis* represented 31 (35.6%) and two (2.3%) isolates, respectively. *Ban*I restriction profiles of *SADH* fragments used for distinguishing among the three species of the *C. parapsilosis* complex are shown in **Figure 1**.

According to the interpretative criteria for resistance used for the antifungal drugs described in the Material and Methods, we found that very few isolates were resistant to azoles and AMB. One *C. parapsilosis sensu stricto* isolate was resistant to AMB and ITC. Moreover, 10.2% of *C. parapsilosis sensu stricto* isolates weare resistant to CAS. Two *C. parapsilosis sensu stricto* isolates and one *C. metapsilosis* isolate met the criterion for S-DD to FLC.

Susceptible-dose dependent strains were detected in the three species. *C. orthopsilosis* met the criterion for S-DD to ITC; *C. metapsilosis* and *C. parapsilosis sensu stricto* met the criterion for S-DD to ITC and FLC; and isolates of all three species exhibited intermediate susceptibility to CAS. The profiles of the *in vitro* susceptibility of *C. parapsilosis* complex species to azoles, AMB, and CAS, including MIC ranges, MIC_{50}, MIC_{90}, susceptibility intermediate or S-DD, and resistance, are summarized in **Table 1.**

DISCUSSION

Within the genus *Candida*, *C. parapsilosis* is the second most commonly isolated species from blood cultures in several casuistics[5][14][15]. Moreover, this species represents frequent cause of onychomycosis in several countries, including Brazil[4], Chile[16], México[17], and Iran[18]. Although all 87 isolates of *C. parapsilosis* studied in our work have been identified by

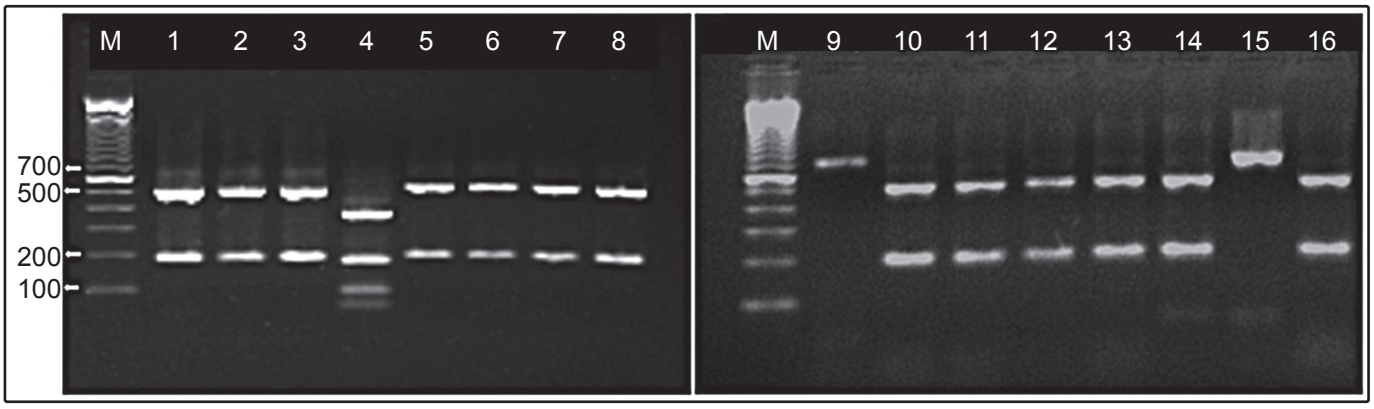

FIGURE 1 - Agarose gel electrophoresis of secondary alcohol dehydrogenase (*SADH*) gene polymerase chain reaction (PCR) products from *Candida parapsilosis sensu lato* after digestion with *Ban*I. *Candida parapsilosis sensu stricto*: lanes 1–3, 5–8, 10–14, 16; *Candida orthopsilosis*: lanes 9 and 15; *Candida metapsilosis:* lane 4. Lane M: molecular size marker 100-bp.

TABLE 1 - *In vitro* susceptibility of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* to azoles (fluconazole, itraconazole, voriconazole, and posaconazole), amphotericin B, and caspofungin.

Isolates (n)	MIC range	MIC50*	MIC90	S-DD		R	
				*n	%	n	%
Candida parapsilosis (78)							
FLU	0.064–4	1	2	2	2.5	-	-
ITRA	0.002–2	0.125	0.125	5	6.4	1	1.3
VOR	0.002–0.25	0.012	0.047	-	-	-	-
POS	0.003–0.094	0.016	0.032	-	-	-	-
AMB	0.064–32	0.5	1	-	-	1	1.3
CAS	0.125–32	0.75	16	3	3.8**	8	10.2
Candida orthopsilosis (5)							
FLU	0.5–2	1	2	-	-	-	-
ITRA	0.125–0.5	0.125	0.5	2	40.0	-	-
VOR	0.008–0.047	0.023	0.047	-	-	-	-
POS	0.008–0.094	0.047	0.094	-	-	-	-
AMB	0.094–0.38	0.25	0.38	-	-	-	-
CAS	2–4	4	4	4	80.0**	-	-
Candida metapsilosis (4)							
FLU	0.5–4	0.75	4	1	25.0	-	-
ITRA	0.064–0.75	0.125	0.75	2	50.0	-	-
VOR	0.008–0.032	0.012	0.032	-	-	-	-
POS	0.008–0.047	0.016	0.047	-	-	-	-
AMB	0.125–0.38	0.125	0.38	-	-	-	-
CAS	0.38–4	0.38	4	2	50.0**	-	-

MIC: minimum inhibitory concentration; **S-DD:** susceptible-dose dependent; **R:** resistant; **FLU:** fluconazole; **ITRA:** itraconazole; **VOR:** voriconazole; **POS:** posaconazole; **AMB**: amphotericin B; **CAS:** caspofungin. *MIC50 and MIC90: MIC at which 50% and 90% of the isolates were inhibited. **Isolates with intermediate sensitivity to caspofungin.

phenotypic methods, we were not able to differentiate among the *C. parapsilosis* complex species, which are phenotypically identical. Amplification of the SADH gene followed by BanI restriction enzyme digestion has been used as a rapid and reliable method with high discriminative power for *C. parapsilosis* complex species[6][19][20][21]. Data have shown that these three species exhibit different prevalence rates, virulence, and *in vitro* antifungal susceptibility[3][10][19][22][23][24]. *In vitro* studies have also shown that *C. parapsilosis sensu stricto* is more pathogenic than *C. orthopsilosis*, which is, in turn, is more pathogenic than *C. metapsilosis*[25].

In our study, *C. parapsilosis sensu stricto* was the dominant species among blood isolates, followed by *C. orthopsilosis* and *C. metapsilosis*. Similar to our results, some researchers have shown that *C. parapsilosis sensu stricto* is the most frequently found species in patients with candidemia[3][15][26][27]. The high frequency of this species in blood samples in critically ill patients may be explained by several factors, including the high affinity of these species for vascular devices and medical instrumentation and their capacity for colonization on the hands of healthcare workers[28][29].

Several studies have found variations in the frequencies of *C. orthopsilosis* and *C. metapsilosis* in candidemia cases. In Spain, among the 171 strains identified as part of the *C. parapsilosis* complex, 2.4% were shown to be *C. orthopsilosis*, and 1.2% were shown to be *C. metapsilosis*[30]. In Brazil, a study by Ruiz et al.[31] showed frequencies of 10.2% for *C. orthopsilosis* and 6.1% for *C. metapsilosis* in strains isolated from blood. Moreover, some studies have shown that *C. metapsilosis* does not cause bloodstream infections[32][33]. A possible explanation for low frequency or absence of *C. metapsilosis* as the cause of candidemia is its poor ability to express virulence factors[34].

Similar to samples obtained from blood, we observed a predominance of *C. parapsilosis sensu stricto* among isolates from nail samples, followed by *C. metapsilosis*. However, *C. orthopsilosis* was not found. Mohammadi et al.[35] have shown that *C. parapsilosis sensu stricto* is the dominant species, followed by *C. orthopsilosis*. In contrast, similar to our results, Ge et al.[19] have found that *C. parapsilosis sensu stricto* and *C. metapsilosis* are the agents responsible for onychomycosis. Among *C. parapsilosis* complex species, the two species newly identified as *C. orthopsilosis* and *C. metapsilosis* have been less frequently isolated from patients with onychomycosis[6][36].

Antifungal resistance or tolerance of *C. parapsilosis* complex species is a growing concern in the context of current and new antifungal agents; this has been a major topic of discussion and concern among infectious disease physicians. According to some researchers, *C. parapsilosis sensu stricto* isolates are less susceptible to some antifungals used for the treatment of candidiasis, such as AMB, FLC, ITC, and CAS, when compared with the susceptibility of *C. orthopsilosis* and *C. metapsilosis* isolates[9][31][37][38]. Among the azole derivatives analyzed in our study, ITC was less active. Consistent with this, Ruiz et al.[31] found resistance in one *C. parapsilosis sensu stricto* isolate and S-DD in 100% of *C. orthopsilosis* isolates and 66.6% of *C. metapsilosis* isolates in response to ITC. Figueiredo-Carvalho et al.[39] verified that *C. orthopsilosis* isolates are less susceptible to ITC than the other species in the *C. parapsilosis* complex.

In this study, *C. parapsilosis* complex isolates were not resistant to FLC; however, S-DD criteria were met for two *C. parapsilosis sensu stricto* isolates and one *C. metapsilosis* isolate. Similarly, FLC resistance in *C. metapsilosis* and *C. parapsilosis sensu stricto* has been observed by Chen et al.[40] Moreover, Gomez-Lopez et al.[41] found an MIC of greater than 4µg/mL FLC for these species, while Silva et al.[6] found an MIC of 16µg/mL FLC for one *C. parapsilosis sensu stricto* isolate. According to Van Asbeck et al.[42], the differences in FLC susceptibility may also reflect the different affinities of azoles for the key ergosterol-synthesizing enzyme 14-α-demethylase or for other enzymes in this pathway.

Interestingly, all isolates of the *C. parapsilosis* complex were susceptible to the new triazole antifungal agents tested in this study (i.e., VOR and POS). These results are consistent with several studies demonstrating the greater efficacy of these new triazoles against *C. parapsilosis* complex isolates[6][9][39][41][43].

In this study, we considered an isolate resistant to AMB when the MIC was greater than 1µg/mL. Thus, in our study, only one *C. parapsilosis sensu stricto* isolate was classified in this category. Although Lockhart et al.[26] reported MICs of more than 1µg/mL for these three species, they found that the proportion of AMB-resistant *C. parapsilosis sensu stricto* is higher than those of *C. orthopsilosis* and *C. metapsilosis.*

Although Trabasso et al.[44] observed some fluctuations in MIC values between the three *C. parapsilosis* complex species, they also showed that these isolates were susceptible to echinocandin, with MIC values within those of the reference strain or the proposed MIC values (CLSI). However, 19.5% (17/87) of *C. parapsilosis* complex isolates studied in our work were less susceptible to CAS, with resistance detected in eight isolates of *C. parapsilosis sensu stricto* **(Table 1)**. Several studies have shown that the MIC of CAS in *C. parapsilosis sensu stricto* is higher than that of the two species within the complex[26][40][41]. However, the reason for the high MIC values for CAS is unclear[45]. Recent works have described a functional point mutation in the glucan synthase (*FKS*) gene of the *C. parapsilosis* complex species, which may explain this observation[46][47]. *FKS* gene mutations have been shown to be associated with resistance to CAS, as demonstrated by increases in MICs in mutant isolates compared with those of wild-type isolates[48][49][50].

In summary, our study reported the first molecular identification of *C. parapsilosis* complex species in State of Goiás, Brazil. We provided evidence showing that although *C. parapsilosis sensu stricto* was the dominant species among the three *C. parapsilosis* complex species, the other two species (*C. metapsilosis* and *C. orthopsilosis*) have clinical importance as pathogens in candidemia and onychomycosis. Moreover, we observed important differences in antifungal susceptibility profiles among the three species, mainly with regard to CAS.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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