Prevalence, distribution and antifungal susceptibility profiles of Candida parapsilosis, Candida orthopsilosis and Candida metapsilosis bloodstream isolates

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INTRODUCTION

For the last three decades, systemic fungal infections have become increasingly important because of their high rates of incidence and mortality (Wey et al., 1988; Shin et al., 2001; Gudlaugsson et al., 2003; Morgan, 2005; Colombo et al., 2006; Horn et al., 2009; Leroy et al., 2009). In Brazil, studies by Colombo et al. (2006) and Pereira et al. (2010) recorded 1.66 and 2.4 infections per 100,000 cases, respectively, highlighting the relevance of this life-threatening disease.

Candida species are the most common fungal agent of bloodstream infections, particularly in intensive care units. Candida parapsilosis is the second or even the most prevalent species in many studies on candidaemia around the world (Krcmery & Barnes, 2002; Pereira et al., 2010; Pfaller et al., 2011). Both new cryptic members of the C. parapsilosis group, Candida orthopsilosis and Candida metapsilosis, have been associated with bloodstream infections or other anatomical sites (Kocsübe et al., 2007; Tavanti et al., 2007; Gomez-Lopez et al., 2008). Rapid diagnosis and the early use of specific antifungal therapy is key for the proper management of candidaemia. Fluconazole, caspofungin and amphotericin B are used as first-line therapy in the treatment of candidaemia, as recommended by the Infectious Diseases Society of America. Voriconazole anditraconazole may also be employed as alternative therapies to treat cases of candidaemia (Pappas et al., 2009).

Antifungal resistance of the infective strain is becoming recognized as a main factor in clinical failure, and the occurrence of clinical resistance and the appearance of in vitro resistant strains have been described systematically around the world. Decreased susceptibility to azoles depends on the species, and, for echinocandins, C. parapsilosis
demonstrates less in vitro susceptibility than other Candida members, although the clinical relevance is still uncertain (Gleason et al., 1997; Rocco et al., 2000; Lopez et al., 2001; Rex et al., 2001; da Matta et al., 2007; Pappas et al. 2009; Arendrup, 2010). Some species that are generally considered fully susceptible to fluconazole may include subpopulations with reduced susceptibility (Oxman et al., 2010). Additionally, newly described cryptic species, as seen in the C. parapsilosis group, have presented distinct drug susceptibility patterns (Lockhart et al., 2008). Given these considerations, local routine monitoring of the susceptibility profiles of C. parapsilosis group members may be highly advisable. Here, we conducted the largest Brazilian study to date to investigate the susceptibility of C. parapsilosis bloodstream isolates to caspofungin, voriconazole, fluconazole, itraconazole and amphotericin B.

METHODS

Isolates. Between 2006 and 2010, 340 isolates of C. parapsilosis were banked at the Instituto Adolfo Lutz Reference Laboratory of São Paulo, Brazil, as part of the Ibero-American Network for Surveillance of Invasive Fungal Infections Study (Cuenca-Estrella et al., 2008). Of these isolates, 152 came from Brazilian hospitals and were selected for species identification and susceptibility testing. The bloodstream isolates were obtained from only a single candidaemia episode for each patient attending at 17 hospital centres located in two different states.

Identification. All strains were identified using morphological characteristics on cornmeal agar and assimilation profiles using API 20 C AUX (bioMérieux), and were stored in 15% glycerol at –20 °C. For this study, the isolates were thawed and plated on CHROMagar Candida (CHROMagar Microbiology) to confirm the purity and viability of the previous phenotype identification. The C. parapsilosis group identity was confirmed by amplification of the gene encoding secondary alcohol dehydrogenase (SADH) by PCR using primers S1F and S1R (MyGene Series Gradient Thermal Cycler, LongGene Scientific Instruments; Tavanti et al., 2005). Amplification conditions consisted of a 5 min denaturation at 94 °C, 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 1 min, and a final extension step of 10 min at 72 °C. The PCR product was digested with the restriction enzyme BanII (Fermentas) and separated on a 2% agarose gel and stained with ethidium bromide (0.05 μg ml−1). TBE was used as the running buffer, and a 100 bp DNA ladder was used as a molecular size marker (Invitrogen). DNA bands were visualized using a UV transilluminator. The presence of one BanII restriction site was indicative of C. parapsilosis sensu stricto, three restriction sites indicated C. metapsilosis and none indicated C. orthopsilosis (Tavanti et al., 2005). Strains of C. parapsilosis ATCC 22019, C. orthopsilosis ATCC 96139 and C. metapsilosis ATCC 96144 were used as quality controls.

Susceptibility testing. Antifungal susceptibility testing was performed using the broth microdilution assay method of the Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST) 2008. The following antifungal drugs, supplied by the manufacturers as pure standard compounds, were tested: amphotericin B (Sigma Chemical), fluconazole and voriconazole (Pfizer), itraconazole (Janssen Pharmaceutica) and caspofungin (Merck). Candida krusei ATCC 6258 and C. parapsilosis ATCC 22019 strains were used in all tests for quality control. The end point in tests with amphotericin B was the lowest concentration able to prevent 90% of growth. The MICs for each azole were defined as the lowest concentration resulting in 50% inhibition or more of growth compared with growth of the control. The breakpoints for fluconazole and voriconazole were applied following AFST-EUCAST recommendations (Rodriguez-Tudela et al., 2010). Fluconazole MICs >4 mg l−1 or voriconazole MICs >0.12 mg l−1 were defined as resistance. According to the AFST-EUCAST methodology, there is no interpretative breakpoint for itraconazole or caspofungin, and MICs >0.5 mg l−1 and >2 mg l−1, respectively, were considered high values. For amphotericin B, MIC values >1 mg l−1 were used to classify strains as resistant (http://www.euast.org). The MIC90 and MIC90 values, defined as the concentration inhibitory to 50 and 90% of the isolates, respectively, were measured for each drug.

RESULTS

According to the BanII restriction pattern of the PCR fragment, isolates were identified molecularly as C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis. In addition, C. parapsilosis accounted for 138 (90.8%) of the isolates, whilst 13 (8.5%) were C. orthopsilosis and one (0.7%) was C. metapsilosis.

The antifungal susceptibilities of the 152 bloodstream isolates to the azoles, caspofungin and amphotericin B are summarized in Table 1. All isolates were susceptible to caspofungin, amphotericin B and itraconazole, five isolates (3.3%) were intermediate to fluconazole and a single (0.7%) isolate was resistant (MIC 16 mg l−1). No fluconazole intermediate or resistant strains were detected among the C. orthopsilosis and C. metapsilosis isolates. Three (2.0%) isolates of C. parapsilosis sensu stricto were resistant to voriconazole. All isolates of C. orthopsilosis and C. metapsilosis were susceptible to voriconazole. Similar findings were recorded for caspofungin: all C. orthopsilosis and C. metapsilosis isolates were susceptible to the drug, but higher MICs (2 mg l−1) were observed for C. orthopsilosis (Table 2).

DISCUSSION

Although Candida albicans represents the most frequent aetiological agent of candidaemia globally, the expanding role of C. parapsilosis reinforces the necessity of monitoring the incidence and susceptibility profile of this agent (Almirante et al., 2006; Trofa et al., 2008). Genetic heterogeneity of C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis has been demonstrated using RAPD analysis, detection of nucleotide variations in the internal transcribed spacer gene and other techniques (Lida et al., 2005; Tavanti et al., 2005, 2007; Lasker et al., 2006; van Asbeck et al., 2008; Tay et al., 2009). These findings add new questions concerning the C. parapsilosis group, and investigations are needed to address the mode of transmission and virulence of these agents.

In the present study, Candida isolates identified as C. parapsilosis during a continuing surveillance of candidaemia were screened genotypically for C. orthopsilosis and C.
metapsilosis identification. A total of 152 isolates were screened by PCR amplification, followed by digestion with the restriction enzyme BanI. C. parapsilosis was responsible for 90.8% of the cases attributed to C. parapsilosis bloodstream infections. In accordance with previous data, we found that C. orthopsilosis (8.5%) and C. metapsilosis (0.7%) accounted for <10% of the total number of formerly identified C. parapsilosis isolates (Gomez-Lopez et al., 2008; Kocsübe et al., 2007; Tavanti et al., 2007; Lockhart et al., 2008; Silva et al., 2009; Gonçalves et al., 2010). Interestingly, the percentage of presumed C. parapsilosis isolates we found to be C. orthopsilosis varied greatly between the two Brazilian geographical regions studied, with the highest percentage from hospitals located in the state of Mato Grosso do Sul (13.5%) compared with those from the state of São Paulo (5%) (data not shown). Furthermore, a single C. metapsilosis isolate was found in a case from São Paulo. Although rare, C. metapsilosis isolates have been described in many countries from all six continents (Tavanti et al., 2005, 2007; Pryce et al., 2006; Kocsübe et al., 2007; Gomez-Lopez et al., 2008; Lockhart et al., 2008; Asadzadeh et al., 2009; Silva et al., 2009; Tay et al., 2009; Gonçalves et al., 2010). In the largest study, with almost 2000 clinical isolates, Lockhart et al. (2008) reported that Europe has almost as many C. metapsilosis isolates as C. orthopsilosis. In contrast, C. metapsilosis occurrence is less frequent (0.3%) in Brazil (Gonçalves et al., 2010). Gomez-Lopez et al. (2008) described similar rates of 1.4 and 1.7% of C. orthopsilosis and C. metapsilosis, respectively, in Spanish isolates. In Portugal and Denmark, analogous proportions of C. orthopsilosis and C. metapsilosis isolates were also detected (Silva et al., 2009; Mirhendi et al., 2010). Chen et al. (2010) described one of the highest rates of C. metapsilosis occurrence (4.4%) among 45 nonduplicating bloodstream isolates from Taiwan. In Malaysia, higher percentages of C. orthopsilosis (23.8%) and C. metapsilosis (4.8%) have been described (Tay et al., 2009). It is unclear whether these differences are influenced by the methodology used in each study. Mirhendi et al. (2010) considered that methodologies using SADH gene RFLP with BanI were unreliable for identifying C. metapsilosis, and PCR sequencing of the D1/D2 region of the 26S rRNA gene is not a suitable target for separation of the three C. parapsilosis group species. Furthermore, the authors concluded that use of SADH gene PCR followed by NlaIII digestion was a more reliable method for differentiating these species.

We observed that the MIC distributions for the C. orthopsilosis isolates were lower than those for C. parapsilosis for all drugs except itraconazole. The lowest MIC values were obtained for itraconazole and voriconazole. These data contrast with those from de Toro et al. (2011), who found a lower susceptibility to voriconazole for C. orthopsilosis than for C. parapsilosis sensu stricto. The MICs for all isolates analysed fell within previously published MIC ranges: amphotericin B, 0.03–16 mg l⁻¹; itraconazole, 0.03–2 mg l⁻¹; and voriconazole, 0.02–1 mg l⁻¹ (Lin et al., 1995; Pfaller et al., 2001; Tortorano et al., 2006; Tay et al., 2009).

No C. orthopsilosis isolates were fluconazole-resistant, whereas one isolate of C. parapsilosis possessed a high MIC for both fluconazole (16 mg l⁻¹) and voriconazole (0.25 mg l⁻¹). Another isolate of C. parapsilosis resistant to voriconazole (MIC 0.25 mg l⁻¹) exhibited an intermediate MIC value for fluconazole (4 mg l⁻¹). We detected a multi-azole-resistant phenotype for C. parapsilosis sensu stricto, as reported previously by Moudgal et al. (2005), Ghannoun et al. (2009) and Silva et al. (2009). The expanding azole-resistant phenotypes of C. parapsilosis sensu stricto should represent clinical complications and raise concerns regarding

<table>
<thead>
<tr>
<th>Specie (no. tested)</th>
<th>Antifungal agent</th>
<th>No of isolates at each MIC (mg l⁻¹)</th>
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<tr>
<td></td>
<td></td>
<td>0.015</td>
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<tr>
<td>C. parapsilosis (138)</td>
<td>Fluconazole</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>0</td>
</tr>
<tr>
<td>C. orthopsilosis (13)</td>
<td>Fluconazole</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>8</td>
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<tr>
<td></td>
<td>Amphotericin B</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>0</td>
</tr>
<tr>
<td>C. metapsilosis (1)</td>
<td>Fluconazole</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>0</td>
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<tr>
<td></td>
<td>Voriconazole</td>
<td>1</td>
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<tr>
<td></td>
<td>Amphotericin B</td>
<td>0</td>
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<td></td>
<td>Caspofungin</td>
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</table>
the potential for cross-resistance among the newer generation of azoles, such as posaconazole and ravuconazole.

Although *C. parapsilosis* sensu stricto is not considered to be a resistant species (Gomez-Lopez et al., 2008; Lockhart et al., 2008; van Asbeck et al., 2008; Tay et al., 2009; Chen et al., 2010), previous reports have suggested that both *C. orthopsilosis* and *C. metapsilosis* possess amphotericin B and caspofungin MICs that are lower than those for *C. parapsilosis* but have slightly higher fluconazole MICs overall than *C. parapsilosis* (Lockhart et al., 2008; Silva et al., 2009; Pfaller et al., 2011). As described by van Asbeck et al. (2008), the distinct affinity of azoles for the key ergosterol-synthesizing enzyme 14-demethylase, or other enzymes involved in this metabolic pathway, could explain the differences in susceptibility to voriconazole and fluconazole among the three species.

The single *C. metapsilosis* isolate exhibited low MICs for all the antifungal drugs tested in this study. A previous study conducted in Brazil also demonstrated a high susceptibility of four *C. metapsilosis* isolates (Gonçalves et al., 2010). Lin et al. (1995) observed lower MIC values for amphotericin B in *C. metapsilosis* compared with *C. parapsilosis* in non-bloodstream isolates from the USA. Further investigation with a larger number of *C. metapsilosis* isolates is necessary to achieve a better understanding of the susceptibility pattern of *C. metapsilosis* bloodstream isolates.

There is little information on the occurrence of *C. orthopsilosis* and *C. metapsilosis* isolated from cases of *C. parapsilosis* bloodstream infections. Here, we have conducted the largest study to date analysing *C. parapsilosis* group bloodstream isolates in Brazil, confirming a higher number of *C. orthopsilosis* isolates than *C. metapsilosis* isolates. We found that both *C. orthopsilosis* and *C. metapsilosis* together accounted for <10% of the *C. parapsilosis* group infections. Moreover, we confirmed that there were differences among the antifungal susceptibility patterns of the species. *C. parapsilosis* sensu stricto demonstrated less susceptibility than *C. orthopsilosis* to the majority of the antifungal agents tested: caspofungin, fluconazole, amphotericin B and voriconazole. This tendency was not significant, and additional clinical trials should be carried out to evaluate the implications of these data for therapeutic use. Moreover, these data improve our knowledge of the national and global distribution of *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*. These data also contribute to information on the susceptibility profiles of these clinically relevant species to azoles, amphotericin B and caspofungin, particularly the occurrence of multiazole-resistant phenotypes.

**ACKNOWLEDGEMENTS**

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**Table 2. In vitro susceptibility of 152 *C. parapsilosis* complex bloodstream isolates tested against the antifungal agents fluconazole, itraconazole, voriconazole, amphotericin B and caspofungin.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Fluconazole</th>
<th>Itraconazole*</th>
<th>Voriconazole</th>
<th>Amphotericin B</th>
<th>Caspofungin*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. parapsilosis</em></td>
<td>0.12–16</td>
<td>0.25–1</td>
<td>0.5–1</td>
<td>0.25–1</td>
<td>0.25–1</td>
</tr>
<tr>
<td><em>C. orthopsilosis</em></td>
<td>0.06–0.015</td>
<td>0.06–0.015</td>
<td>0.06–0.015</td>
<td>0.06–0.015</td>
<td>0.06–0.015</td>
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<tr>
<td><em>C. metapsilosis</em></td>
<td>1–100</td>
<td>1–100</td>
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</table>

*S* Susceptible; *I* Intermediate; *R* Resistant.

*S* | **Species** | **MIC (mg l⁻¹)** | **MIC (mg l⁻¹)** | **MIC (mg l⁻¹)** | **MIC (mg l⁻¹)** |
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<td></td>
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<td>Range</td>
<td>MIC₉₀/MIC₉₀</td>
<td>MIC₉₀/MIC₉₀</td>
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<td></td>
<td><em>C. parapsilosis</em></td>
<td>0.12–16</td>
<td>0.25–1</td>
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<td></td>
<td><em>C. orthopsilosis</em></td>
<td>0.06–0.015</td>
<td>0.06–0.015</td>
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<tr>
<td></td>
<td><em>C. metapsilosis</em></td>
<td>1–100</td>
<td>1–100</td>
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<td>1–100</td>
</tr>
</tbody>
</table>

*There are no specific breakpoints for these antifungal agents.*
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REFERENCES


