Molecular differentiation and antifungal susceptibilities of *Candida parapsilosis* isolated from patients with bloodstream infections

Sun Tee Tay, Shiang Ling Na and Jennifer Chong

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

The incidence of candidiasis among hospitalized patients has increased generally in recent years, with a larger proportion of bloodstream infections being caused by non-*albicans* *Candida* spp. (Pfaller & Diekema, 2007). Among the *Candida* spp., *Candida parapsilosis* has been described as the second or third most common yeast species isolated from patients with bloodstream infections in Europe, Canada, Asia and Latin America (Almirante et al., 2006; Messer et al., 2006; Pfaller et al., 2005). This yeast is believed to be of low virulence, but is well adapted to the human commensal environment, and occurrence of infection among hospitalized patients is particularly associated with carriage by the hands of health-care workers (Almirante et al., 2006; Bonassoli et al., 2005; Lin et al., 1995; Lupetti et al., 2002; Messer et al., 2006). *C. parapsilosis* bloodstream infections have been reported in association with catheter colonization and intravenous hyperalimentation in neonates (Sarvikivi et al., 2005), due to the capability of the yeast to form a biofilm on plastic intravascular devices (Branchini et al., 1994; Pfaller et al., 1995). Catheter removal has been shown to be effective in clearance of fungaemia (Levy et al., 1998; Rex, 1996).

*C. parapsilosis* has been considered a clonal organism in the past. However, this yeast is now differentiated into three species using molecular typing techniques (Lin et al., 1995; Roy & Meyer, 1998; Tavanti et al., 2005). Whilst the species *C. parapsilosis* is retained for genotype I isolates, genotypes II and III have been proposed as the separate species *Candida orthopsilosis* and *Candida metapsilosis* (Tavanti et al., 2005). These two newly described species are phenotypically identical, but genotypically distinct from *C. parapsilosis* (Kocsbè et al., 2007; Lin et al., 1995; Tavanti et al., 2005).

Both *C. orthopsilosis* and *C. metapsilosis* have been reported from bloodstream infections and infections at other anatomical sites (Gomez-Lopez et al., 2008; Kocsbè et al., 2007; Tavanti et al., 2007). *C. orthopsilosis* was found to be responsible for 4.5% of infections/colonization attributed to *C. parapsilosis* (Tavanti et al., 2007), and the organism was isolated from the blood samples of 2 out of 13 patients examined in their study. Kocsbè et al. (2007) reported the identification of 1 *C. metapsilosis* isolate from a total of 209 *C. parapsilosis* blood isolates (0.5%).
prevalence of *C. orthopsilosis* and *C. metapsilosis* was 1.4 and 1.7 %, respectively, in a study conducted in Spain (Gomez-Lopez et al., 2008).

*Lodderomyces elongisporus* has been recognized as a third yeast species that is found to be closely related to *C. parapsilosis*. Data on small-subunit rRNA gene sequencing show that it is a distinct species that is closely related to *C. parapsilosis* (James et al., 1994). This species has been reported in bloodstream infections of patients from Asia and Mexico (Lockhart et al., 2008).

In this study, *C. parapsilosis* isolates from patients with fungaemia in our hospital were differentiated by randomly amplified polymorphic DNA (RAPD) analysis. The identities of isolates with unique RAPD profiles were determined by sequence analysis of the ITS1–5.8S rRNA gene–ITS2 region of the yeasts. As there were no data on the antifungal susceptibility of our *C. parapsilosis* isolates, Etests were performed to determine the MIC values against *C. parapsilosis* type strain, whilst the remaining one exhibited 99.1 % sequence similarity (4 nt differences) with the *C. orthopsilosis* type strain, whilst the remaining one exhibited 99.1 % sequence similarity (4 nt differences) with the type strain.

The two RAPD type P4 isolates in this study exhibited 99.3 and 98.3 % (3 and 8 nt differences, respectively) sequence similarity with the *C. orthopsilosis* type strain, whilst the remaining one exhibited 99.1 % sequence similarity (4 nt differences) with the type strain.

**RESULTS**

**RAPD analysis.**

Based on the major DNA fragments (ranging from 200 bp to approximately 2 kb) generated by primer M13 (Fig. 1a), five RAPD profiles (designated P1–P5) were obtained. RAPD types P1, P4 and P5 could easily be recognized based on their unique profiles. RAPD types P2 and P3 were differentiated based on several faintly stained DNA fragments of <1 kb generated in the profiles. Similar typing results were obtained using (GTG)₅ sequence repeats (Fig. 1b). A total of 29 (69.0 %) of the isolates were identified as RAPD type P1, 6 (14.3 %) as P2, 4 (9.5 %) as P3, 2 (4.8 %) as P4 and 1 (2.4 %) as P5.

**ITS sequence analysis.**

A total of 461–504 nt of the ITS1–5.8S rRNA gene–ITS2 regions of 18 RAPD type P1 isolates and all RAPD type P2–P5 isolates were determined. Fig. 2 shows the multiple alignment of the ITS sequences of *C. parapsilosis* and its closely related species. Fig. 3 is a dendrogram illustrating the genetic relationships of *C. parapsilosis* and its related species based on their ITS sequences. *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* isolates were grouped in the same cluster as their respective type strains. *L. elongisporus* was distinctly differentiated from the *Candida* spp.

Table 1 shows the percentages of sequence similarity of the yeasts when compared with their respective type strains. The ITS gene sequences of RAPD type P1 isolates showed 100 % sequence similarity with *C. parapsilosis* type strain CBS 604ᵀ. All six RAPD type P2 isolates demonstrated 100 % sequence similarity with the *C. orthopsilosis* type strain (ATCC 96139ᵀ). Three RAPD type P3 isolates exhibited 99.3 % sequence similarities (3 nt differences) with the *C. orthopsilosis* type strain, whilst the remaining one exhibited 99.1 % sequence similarity (4 nt differences) with the type strain.

**METHODS**

**Clinical isolates.** A total of 42 isolates of *C. parapsilosis* obtained from different patients with bloodstream infections, randomly collected from 2004 to 2007, were used in this study. The ages of the patients ranged from 1 month to 83 years, with a mean age of 36.7 years. Twelve individuals (28.6 %) were paediatric patients with ages ranging from 1 month to 13 years. All isolates were identified as *C. parapsilosis* using the API 20C AUX system (bioMérieux).

**DNA extraction.** Yeast genomic DNA was extracted as described by Makimura et al. (1994) with slight modifications. Briefly, yeast cells were boiled for 15 min in lysis buffer containing 100 mM Tris/HCl (pH 8), 0.5 % SDS and 30 mM EDTA. The lysate was then added to 2.5 M potassium acetate solution and incubated on ice for 1 h before centrifuging at 13 226 g for 5 min. The yeast DNA in the supernatant was precipitated with isopropanol, washed twice with ethanol, air dried and resuspended in 50 μl distilled water prior to use in the PCR.

**RAPD analysis.** Amplification was performed using the single primer M13 (5′-GAGGGTGGCGGTTCT-3′) or (GTG)₅ sequence repeats, as described by Meyer et al. (1997). The PCR master mix (25 μl) contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.15 mM primer, 2.5 U Taq DNA polymerase (MBI Fermentas) and 25 μg yeast DNA. PCR conditions were 35 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 1 min and extension at 72 °C for 20 s, followed by a final extension at 72 °C for 6 min. A 10 μl aliquot of the amplification products was separated by electrophoresis on a 2.0% agarose gel. The RAPD profiles were visualized under UV light and photographed. Isolates were assigned to their respective RAPD types based on the presence or absence of DNA fragments generated from the PCR.

**ITS sequence analysis.** Amplification of the ITS1–5.8S rRNA gene–ITS2 fragment was performed using primer ITS1 (5′-TCCGTA-GGTAACCCGCGG-3′) and ITS4 (5′-TCCTCCGCTATTGATATGTCG-3′) (White et al., 1990). The amplicon was purified using a GeneAll PCR SV kit (General Biosystem) and the subsequent sequencing reaction was performed with a BigDye terminator cycle sequencing kit (Applied Biosystems) on an ABI-3730 Genetic Analyzer (Applied Biosystems) using ITS1 and ITS4 as primers. Sequences were imported into the BioEdit sequence alignment program and inspected manually (Hall, 1999). The neighbour-joining method of MEGA software (version 4.0) was employed to determine the phylogenetic status of the isolates (Kumar et al., 2004). The reliability of different phylogenetic groupings was evaluated using bootstrap tests (1000 bootstrap replicates). Type strains included for analysis were: *C. parapsilosis* CBS 604ᵀ (GenBank accession no. AJ635316), *C. orthopsilosis* ATCC 96139ᵀ (AJ698048), *C. metapsilosis* ATCC 96144ᵀ (AJ698049) and *L. elongisporus* CBS 2606 (AY391845).

**Antifungal susceptibility tests.** Etests were performed according to the manufacturer’s instructions (AB Biodisk). The antifungal agents used were amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole (in strips provided in the Etest kit). Comparisons of proportions were performed using the Mann–Whitney test of the SPSS statistical package (version 13.0). A P value of <0.05 was considered statistically significant.
similarity with *C. metapsilosis* type strain ATCC 96144T. The only RAPD type P5 isolate in this study showed 100% sequence similarity with the *L. elongisporus* type strain CBS 2606 (Table 1).

*C. parapsilosis* accounted for 69.0% of the isolates in this study. This was followed by *C. orthopsilosis* (23.8%) and *C. metapsilosis* (4.8%). The distribution of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* among the paediatric patients were seven (58.3%), three (25.0%) and two (16.7%) isolates. The distribution of *C. parapsilosis* and *C. orthopsilosis* in the adult patients were 22 (73.3%) and 7 (23.3%) isolates, respectively. No. *C. metapsilosis* was isolated from adult patients in this study.

**Antifungal susceptibility tests**

The isolates were susceptible to all of the antifungal drugs tested, with MICs <1 μg ml⁻¹ for amphotericin B, ketoconazole, itraconazole, and voriconazole and <6 μg ml⁻¹ for fluconazole (Table 2). The lowest MIC values were obtained for ketoconazole. Although *C. parapsilosis* isolates exhibited higher MIC₅₀ values than those of *C. orthopsilosis* for all of the drugs tested in this study, no significant difference in the MIC₅₀ values for these two *Candida* species was observed (Table 2). The MIC range of *C. metapsilosis* and *L. elongisporus* isolates fell within the MIC range of *C. parapsilosis* (Table 2). Overall, the MICs of our isolates were well below the plasma levels normally achieved for these drugs.

**DISCUSSION**

Identification of *Candida* spp. from clinical specimens, particularly blood culture, is important to facilitate optimal antifungal therapy and patient management (Edwards et al., 1997; Rex et al., 2000). Molecular techniques are excellent tools for identification and strain typing of yeasts. Analysis of new species that are closely related to *C. parapsilosis* has been performed using various molecular techniques including RAPD analysis (Kocsubé et al., 2007; Lehmann et al., 1992; Lin et al., 1995; Tavanti et al., 2007; Zancope-Oliveira et al., 2000), nucleotide sequence analysis (Iida et al., 2005; Kato et al., 2001; Lin et al., 1995; Lockhart et al., 2008; Nosek et al., 2002; Pryce et al., 2006), DNA–DNA hybridization (Roy & Meyer, 1998), probe hybridization (Enger et al., 2001), analysis of mitochondrial DNA (Rycovska et al., 2004), multilocus sequence typing (Tavanti et al., 2005), microsatellite analysis (Lasker et al., 2006), amplification fragment length polymorphism analysis (Tavanti et al., 2007) and RFLP analysis of genomic DNA (Van Asbeck et al., 2008). In this study, the occurrence and susceptibility profiles of our isolates were investigated by RAPD and sequence analysis of ITS genes.

RAPD analysis using a single primer, M13, was used initially in this study to provide a quick screening of the genetic heterogeneity of the yeasts. The RAPD profiles generated were stable, especially with the major DNA fragments. The typing results were reproducible using another primer, (GTG)₅ sequence repeats, in the RAPD analysis (Fig. 1). The genomic homogeneity among *C. parapsilosis* isolates as observed by RAPD analysis and sequence analysis of ITS genes in this study has been demonstrated previously by various molecular techniques (Kocsué et al., 2007; Lehmann et al., 1992; Tavanti et al., 2005, 2007). However, a recent investigation of the size polymorphisms in loci harbouring microsatellite repeat sequences of *C. parapsilosis* group I isolates (Lasker et al., 2006) demonstrated 30 different microsatellite genotypes
from 42 isolates. Due to the high discriminatory power of microsatellite analysis, this technique has been proposed for *C. parapsilosis* outbreak and epidemiological investigations (Lasker et al., 2006).

In a recent study to analyse the distribution of the subtypes of *C. parapsilosis* from various geographical localities, Van Asbeck et al. (2008) divided their isolates into two groups: VII-1 (dominant subtype showing RAPD profiles consistent with *C. parapsilosis sensu stricto*) and non-VII-1 (all remaining subtypes). Approximately 82% VII-1 isolates were reported for the USA, Europe, Brazil and Israel strains; however, there was diversity in the genotypic groups of *C. parapsilosis* isolated from different geographi-

**Fig. 2.** Multiple alignment of ITS gene sequences of *C. parapsilosis* and related species.
的各项区域，其中来自墨西哥的分离株显示VII-1分离株比例显著较低。该研究的发现还显示，在马来西亚分离株中，C. parapsilosis的低频率（69.0%）。

C. parapsilosis是否存在与治疗和管理这些感染相关的关联将需要进一步调查。

C. orthopsilosis和C. metapsilosis的遗传异质性，如由RAPD分析和检测ITS基因中的核苷酸变异所反映的，已由其他研究者（Iida et al., 2005; Lasker et al., 2006; Rycovska et al., 2004; Tavanti et al., 2005, 2007; Van Asbeck et al., 2008）所证实。这些新描述的酵母的更大遗传变异性使得开发用于这些酵母分型的分子技术变得困难（Lasker et al., 2006; Tavanti et al., 2005, 2007）。

分离的C. orthopsilosis和/or C. metapsilosis从临床标本报告在欧洲和美国（Enger et al., 2001; Gomez-Lopez et al., 2008; Kocsobé et al., 2007; Tavanti et al., 2005, 2007）。人们认为这些新描述的酵母的低频率导致了临床标本的进一步分析。

C. orthopsilosis和C. metapsilosis的分离株频率（C. parapsilosis血流感染的23.8和4.8%）在该研究中比其他研究者（Gomez-Lopez et al., 2008; Kocsobé et al., 2007; Tavanti et al., 2007）报告的要高。在该研究中，C. orthopsilosis和C. metapsilosis从血液培养的儿童患者的分离株被首次报道，事实上，C. orthopsilosis的分离株频率在儿童血液培养中被观察到比其他更为频繁。

### Table 1. Percentage sequence similarity and nucleotide differences of blood isolates compared with the type strains of C. parapsilosis, C. orthopsilosis, C. metapsilosis and L. elongisporus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage nucleotide similarity /no. of nucleotide differences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. parapsilosis CBS 604T</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>CBS 604T (n=18)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P1 (n=18)</td>
</tr>
<tr>
<td>C. orthopsilosis</td>
<td>ATCC 96139T (n=6)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P2 (n=3)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P3 (n=1)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P3 (n=1)</td>
</tr>
<tr>
<td>C. metapsilosis</td>
<td>ATCC 96144T (n=1)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P4 (n=1)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P4 (n=1)</td>
</tr>
<tr>
<td>L. elongisporus</td>
<td>CBS 2606T (n=1)</td>
</tr>
<tr>
<td></td>
<td>RAPD type P5 (n=1)</td>
</tr>
</tbody>
</table>
Table 2. In vitro antifungal susceptibilities of C. parapsilosis and related species in this study

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>Antifungal drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>C. parapsilosis (n=21)</td>
<td>0.02–0.75</td>
</tr>
<tr>
<td></td>
<td>MIC50 (µg ml⁻¹)</td>
</tr>
<tr>
<td></td>
<td>MIC90 (µg ml⁻¹)</td>
</tr>
<tr>
<td>C. orthopsilosis (n=8)</td>
<td>0.006–0.125</td>
</tr>
<tr>
<td></td>
<td>MIC50 (µg ml⁻¹)</td>
</tr>
<tr>
<td></td>
<td>MIC90 (µg ml⁻¹)</td>
</tr>
<tr>
<td>C. metapsilosis (n=2)</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>MIC50 (µg ml⁻¹)</td>
</tr>
<tr>
<td></td>
<td>MIC90 (µg ml⁻¹)</td>
</tr>
<tr>
<td>L. elongisporus (n=1)</td>
<td>0.012</td>
</tr>
</tbody>
</table>

In conclusion, this study presented the identification of newly described yeast species that are closely related to C. parapsilosis from the blood cultures of our patients. Although the blood isolates in this study were identified initially by conventional biochemical tests as C. parapsilosis, these organisms could be differentiated further into C. parapsilosis, C. orthopsilosis, C. metapsilosis and L. elongisporus using RAPD and sequence analysis of ITS genes. The fact that C. orthopsilosis and C. metapsilosis were responsible for 23.8 and 4.8% of these cases attributed to C. parapsilosis bloodstream infections, respectively, indicates the clinical relevance of these yeast species. Further investigations of the ecological niche, mode of transmission and virulence of these newly described species are thus essential.

ACKNOWLEDGEMENTS

This study was supported by a research grant (SF062-2007A) provided by the University of Malaya, Kuala Lumpur, Malaysia. We thank Professor K. P. Ng for his support in this study.

REFERENCES


of the internal transcribed spacers (ITS) and 5.8S rRNA genes among clinical isolates of Candida parapsilosis in Brazil and Japan. Nippon Ishinkin Gakkai Zasshi 46, 133–137.


