Journal of Medical Microbiology (2009), **58**, 185–191 DOI 10.1099/jmm.0.004242-0

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

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Received 18 June 2008
Accepted 21 October 2008

The genetic heterogeneity and antifungal susceptibility patterns of *Candida parapsilosis* isolated from blood cultures of patients were investigated in this study. Randomly amplified polymorphic DNA (RAPD) analysis generated 5 unique profiles from 42 isolates. Based on the major DNA fragments of the RAPD profiles, the isolates were identified as RAPD type P1 (29 isolates), P2 (6 isolates), P3 (4 isolates), P4 (2 isolates) and P5 (1 isolate). Sequence analysis of the internal transcribed spacer (ITS) gene of the isolates identified RAPD type P1 as *C. parapsilosis*, P2 and P3 as *Candida orthopsilosis*, P4 as *Candida metapsilosis*, and P5 as *Lodderomyces elongisporus*. Nucleotide variations in ITS gene sequences of *C. orthopsilosis* and *C. metapsilosis* were detected. Antifungal susceptibility testing using Etests showed that all isolates tested in this study were susceptible to amphotericin B, fluconazole, ketoconazole, itraconazole and voriconazole. *C. parapsilosis* isolates exhibited higher MIC_{50} values than those of *C. orthopsilosis* for all of the drugs tested in this study; however, no significant difference in the MICs for these two *Candida* species was observed. The fact that *C. orthopsilosis* and *C. metapsilosis* were responsible for 23.8 and 4.8 % of the cases attributed to *C. parapsilosis* bloodstream infections, respectively, indicates the clinical relevance of these newly described yeasts. Further investigations of the ecological niche, mode of transmission and virulence of these species are thus essential.

INTRODUCTION

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 metapsilopsis* (Tavanti *et al.*, 2005). These two newly described species are phenotypically identical, but genotypically distinct from *C. parapsilosis* (Kocsubé *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; Kocsubé *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. Kocsubé *et al.* (2007) reported the identification of 1 *C. metapsilosis* isolate from a total of 209 *C. parapsilosis* blood isolates (0.5 %). The

Abbreviation: RAPD, randomly amplified polymorphic DNA.

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. parapsilopsis* isolates, Etests were performed to determine the MIC values against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole.

METHODS

Clinical isolates. A total of 42 isolates of *C. parapsilopsis* 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 $MgCl_2$, 0.2 mM dNTPs, 0.15 mM primer, 2.5 U *Taq* DNA polymerase (MBI Fermentas) and 25 ng 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′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-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 604^T (GenBank accession no. AJ635316), *C. orthopsilosis* ATCC 96139^T (AJ698048), *C. metapsilosis* ATCC 96144^T (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.

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 604^T. All six RAPD type P2 isolates demonstrated 100 % sequence similarity with the *C. orthopsilosis* type strain (ATCC 96139^T). 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.

The two RAPD type P4 isolates in this study exhibited 99.3 and 98.3 % (3 and 8 nt differences, respectively) sequence

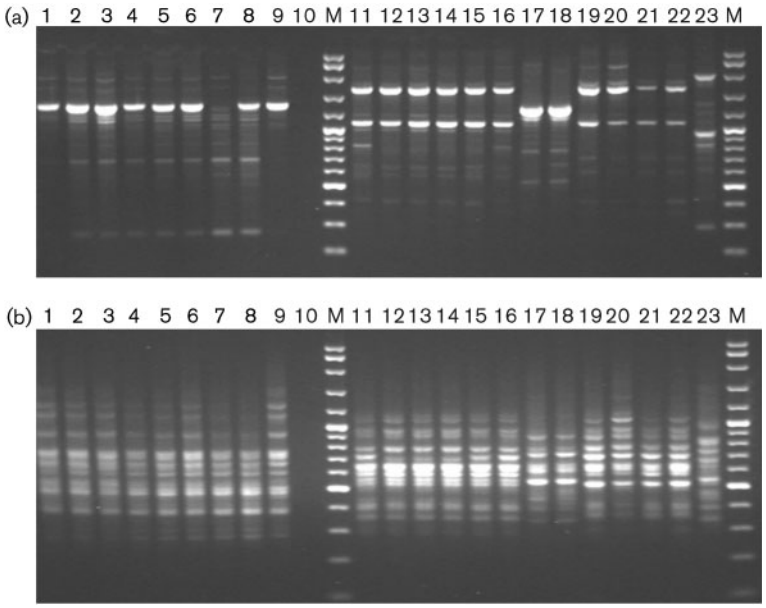

Fig. 1. RAPD profiles generated by primer M13 (a) and $(GTG)_5$ sequence repeats (b) for blood isolates investigated in this study. Lanes: M, 100 bp DNA ladder; 1–9, RAPD type P1 (*C. parapsilosis*); 10, water (negative control); 11–16, RAPD type P2 (*C. orthopsilosis*); 17–18, RAPD type 4 (*C. metapsilosis*); 19–22, RAPD type P3 (*C. orthopsilosis*); 23, RAPD type P5 (*L. elongisporus*).

similarity with *C. metapsilosis* type strain ATCC 96144^T. 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 MIC_{50} values than those of *C. orthopsilosis* for all of the drugs tested in this study, no significant difference in the MIC_{50} 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; Zancopé-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 (Kocsubé *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

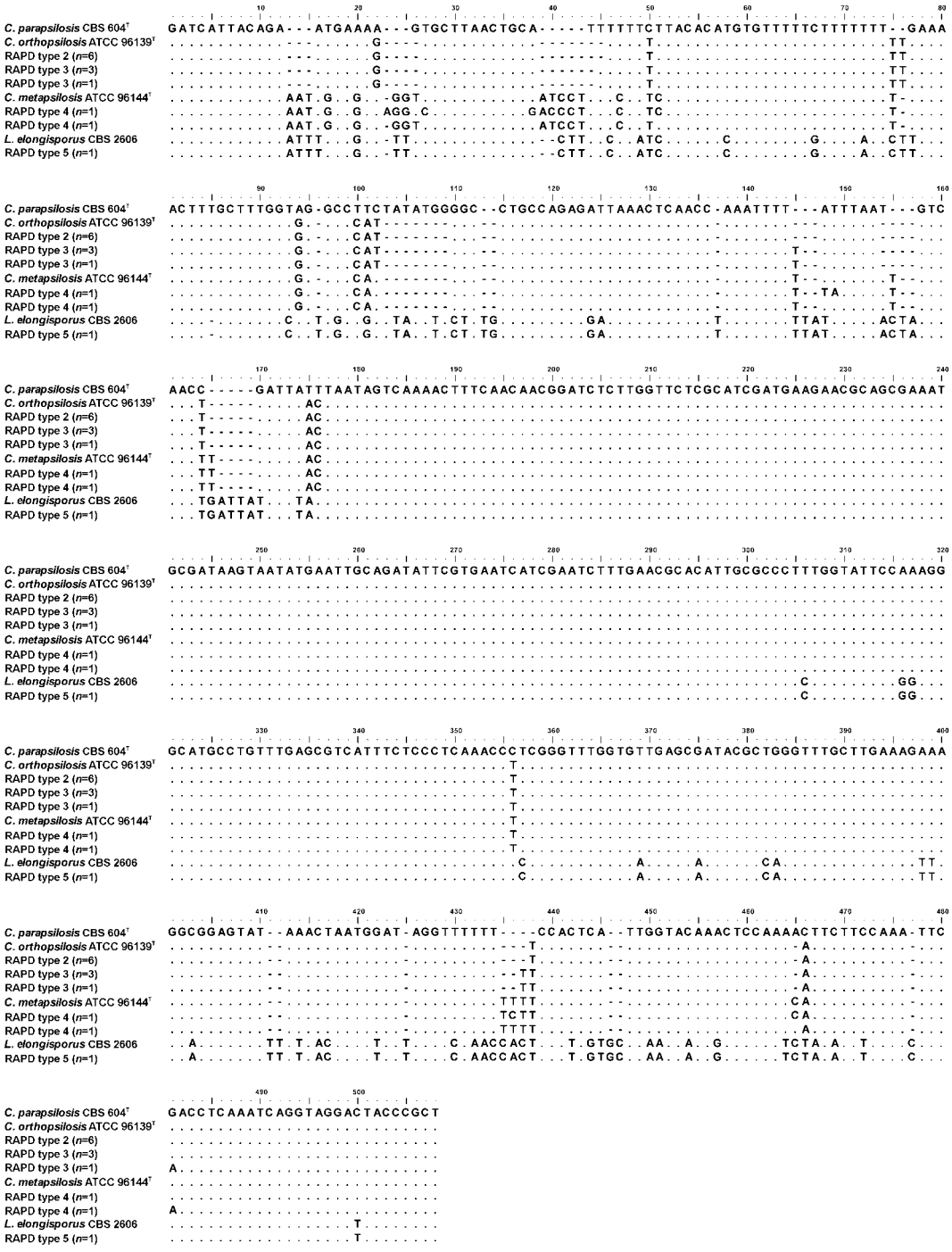

Fig. 2. Multiple alignment of ITS gene sequences of *C. parapsilosis* and related species.

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 geograph-

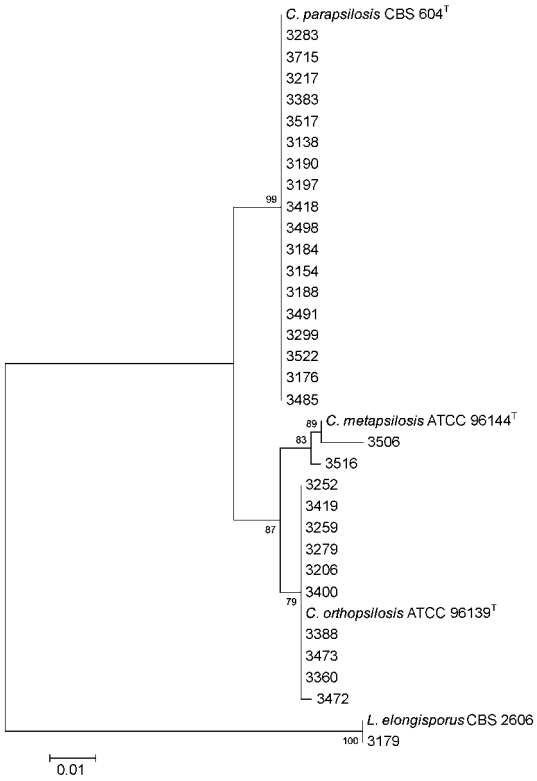

Fig. 3. Dendrogram based on ITS gene sequences of *C. parapsilosis* and related species.

ical areas, with isolates from Mexico showing a significantly lower proportion of VII-1 isolates. The findings in this study also showed that there was a low frequency (69.0 %) of *C. parapsilosis* in the Malaysian isolates. Whether the difference observed in yeast distribution has any association with treatment and management of the infections will require further investigation.

The genetic heterogeneity of *C. orthopsilosis* and *C. metapsilosis*, as reflected by RAPD analysis and detection of nucleotide variations in the ITS gene in this study, has been demonstrated by other investigators (Iida *et al.*, 2005; Lasker *et al.*, 2006; Rycovska *et al.*, 2004; Tavanti *et al.*, 2005, 2007; Van Asbeck *et al.*, 2008). The greater genetic variability of these newly described yeasts compared with *C. parapsilosis* has caused difficulties in the development of molecular techniques for the subtyping of these yeasts (Lasker *et al.*, 2006; Tavanti *et al.*, 2005).

The identification of *C. orthopsilosis* and/or *C. metapsilosis* from clinical isolates has been reported in Europe and the USA (Enger *et al.*, 2001; Gomez-Lopez *et al.*, 2008; Kocsubé *et al.*, 2007; Tavanti *et al.*, 2005, 2007). It is thought that the low frequencies of these newly described species in clinical specimens have prevented further analysis of these yeasts. There is also little information on the frequency of *C. orthopsilosis* and *C. metapsilosis* isolated from cases of *C. parapsilosis* bloodstream infections. The occurrences of *C. orthopsilosis* and *C. metapsilosis* (23.8 and 4.8 % of the cases, respectively) attributed to *C. parapsilosis* bloodstream infections in this study were found to be higher than those reported by others (Gomez-Lopez *et al.*, 2008; Kocsubé *et al.*, 2007; Tavanti *et al.*, 2007). Isolation of both *C. orthopsilosis* and *C. metapsilosis* from the blood cultures of paediatric patients was also documented in this study for what is believed to be the first time. In fact, a higher frequency of *C. orthopsilosis* was noted in the blood cultures of paediatric patients compared with those of

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

Strain	Percentage nucleotide similarity /no. of nucleotide differences		
	***C. parapsilosis* CBS 604^T**	***C. orthopsilosis* ATCC 96139^T**	***C. metapsilosis* ATCC 96144^T**
C. parapsilosis			
CBS 604^T	100/0	94.5/26	91.8/40
RAPD type P1 (n=18)	100/0	94.5/26	91.8/40
C. orthopsilosis			
ATCC 96139^T	94.5/26	100/0	94.1/28
RAPD type P2 (n=6)	94.5/26	100/0	94.1/28
RAPD type P3 (n=3)	94.3/27	99.3/3	94.8/25
RAPD type P3 (n=1)	94.1/28	99.1/4	94.6/26
C. metapsilosis			
ATCC 96144^T	91.8/40	94.1/28	100/0
RAPD type P4 (n=1)	92/39	94.1/28	99.3/3
RAPD type P4 (n=1)	91/44	93.1/33	98.3/8
L. elongisporus			
CBS 2606^T	81.1/95	79.6/103	81.0/96
RAPD type P5 (n=1)	81.1/95	79.6/103	81.0/96

Table 2. *In vitro* antifungal susceptibilities of *C. parapsilopsis* and related species in this study

Clinical isolate	Antifungal drug				
	Amphotericin B	Fluconazole	Ketoconazole	Itraconazole	Voriconazole
***C. parapsilosis* (*n*=21)**					
MIC range (μg ml^{-1})	<0.002–0.75	0.094–6	<0.002–0.25	<0.002–0.38	0.006–0.125
MIC_{50} (μg ml^{-1})	0.047	0.75	0.23	0.047	0.032
MIC_{90} (μg ml^{-1})	0.75	4	0.094	0.19	0.047
***C. orthopsilosis* (*n*=8)**					
MIC range (μg ml^{-1})	0.006–0.125	0.25–1.5	0.008–0.125	0.004–0.047	0.006–0.125
MIC_{50} (μg ml^{-1})	0.023	0.38	0.012	0.016	0.012
MIC_{90} (μg ml^{-1})	0.125	1.5	0.125	0.047	0.125
***C. metapsilosis* (*n*=2)**					
3516	0.023	1.5	0.016	0.023	0.016
3506	0.016	1.5	0.016	0.064	0.032
***L. elongisporus* (*n*=1)**					
3179	0.012	0.125	0.003	0.047	0.004

adult patients in this study. However, as only a small number of isolates (12) from paediatric patients was available for analysis in this study, further investigation is necessary to validate these findings.

The results of antifungal susceptibility testing showed that the *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* isolates tested in this study were susceptible to amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole. According to earlier studies, the MICs of *C. parapsilosis* isolates against amphotericin B, fluconazole, itraconazole and voriconazole ranged from 0.03 to 2, 0.06 to 16, 0.03 to 2 and 0.02 to 1 μg ml^{-1}, respectively (Lin *et al.*, 1995; Pfaller *et al.*, 2001; Tortorano *et al.*, 2006). All of the isolates tested in this study fell within the MIC range reported by others. Although *C. parapsilosis* isolates exhibited higher MIC_{50} values than those of *C. orthopsilosis* for all of the drugs tested, no significant difference in the MIC_{50} values for these two *Candida* species was observed (Table 2). The two *C. metapsilosis* and the single *L. elongisporus* isolates exhibited lower MICs for all of the antifungal drugs tested in this study. Lower MIC values were observed for amphotericin B in *C. metapsilosis* compared with *C. parapsilosis* in an earlier study (Lin *et al.*, 1995).

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.

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