Occurrence and genetic variability of *Candida parapsilosis sensu lato* in Hungary

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The occurrence and genetic variability of *Candida parapsilosis* isolates in two Hungarian hospitals, located in Debrecen and Pécs, were examined. Among the 209 *Candida* isolates examined, 20 were found to belong to *C. parapsilosis sensu lato*, based on morphological, physiological and molecular data. The frequency of occurrence of *C. parapsilosis* isolates (9.6 %) was lower than that observed in Europe but higher than that observed previously in Hungary. The genetic variability of *C. parapsilosis sensu lato* isolates was also examined using random amplified polymorphic DNA (RAPD) analysis and sequence analysis of the intergenic transcribed spacer (ITS) region of the rRNA gene cluster. The genetic variability of the isolates was relatively high, as revealed by RAPD analysis. Two isolates were found to belong to the recently described *Candida metapsilosis* species (*C. parapsilosis* group III), based on ITS sequence data, RAPD analysis and phenotypic data. These two isolates could also be distinguished from *C. parapsilosis sensu stricto* isolates using a primer pair developed for the detection of *C. parapsilosis* group I isolates. To the best of the authors' knowledge, this is the first report on the identification of *C. metapsilosis* from bloodstream infection.

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INTRODUCTION

Infections caused by *Candida* species are widespread throughout the world. Although *Candida albicans* is the most common *Candida* species encountered as a cause of human infection, other *Candida* species have been increasingly associated with disseminated disease since the 1990s. Among them, *C. parapsilosis* has been the second most common yeast species isolated from bloodstream infections

in several surveys (Safdar *et al.*, 2002; Messer *et al.*, 2006; Bassetti *et al.*, 2006). This species has emerged as an important nosocomial pathogen, with clinical manifestations that include fungaemia, endocarditis, endophthalmitis, septic arthritis and peritonitis, and infection usually occurs in association with invasive procedures or prosthetic devices (Weems, 1992). In some children's hospitals, *C. parapsilosis* has become the predominant species causing candidaemia (Levy *et al.*, 1998). This species is more frequent in bloodstream infections of neonates, in transplant recipients and in patients who have received parenteral nutrition or previous antifungal therapy (Almirante *et al.*, 2006). This species is also frequently associated with catheter-associated candidaemia and intravenous hyperalimentation (Kremery & Barnes, 2002).

Previous studies have clarified that *C. parapsilosis sensu lato* isolates can be divided into three groups that can be

Abbreviations: ITS, intergenic transcribed spacer; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the ITS sequences of *C. metapsilosis* 12821 and Bp57 are DQ786952 and DQ786953, respectively.

Neighbour-joining tree-based ITS sequence data of the examined *Candida* isolates are available as supplementary data with the online version of this paper.

distinguished based on several criteria, including randomly amplified polymorphic DNA (RAPD) analysis (Lehmann *et al.*, 1992), isoenzyme electrophoresis (Lin *et al.*, 1995), sequences of the internal transcribed spacer (ITS) region of the rRNA gene cluster (Lin *et al.*, 1995; Pryce *et al.*, 2006), hybridization to a fingerprinting probe (Enger *et al.*, 2001), DNA relatedness (Roy & Meyer, 1998), morphotyping (Cassone *et al.*, 1995), electrophoretic karyotypes (Lott *et al.*, 1993), single nucleotide polymorphisms (Fundyga *et al.*, 2004), mitochondrial DNA sequence differences (Nosek *et al.*, 2002; Kosa *et al.*, 2006) and biofilm-producing abilities (Song *et al.*, 2005). Recently, Tavanti *et al.* (2005) have recognized *C. parapsilosis* groups II and III as separate species, *Candida orthopsilosis* and *Candida metapsilosis*, respectively, based on multilocus sequence typing studies. Accordingly, *C. parapsilosis sensu lato* includes *C. parapsilosis sensu stricto* and the two newly described species. The two latter species are recovered relatively rarely in clinical samples (Tavanti *et al.*, 2005). In addition, *C. parapsilosis* group IV has also been recognized recently among Brazilian clinical *Candida* isolates by Iida *et al.* (2005).

In this study, we examined the occurrence of *C. parapsilosis* isolates among *Candida* isolates collected in Hungarian hospitals, and examined the genetic variability of these isolates using sequence analysis of the ITS region and the RAPD technique.

METHODS

Clinical samples. Altogether, 209 *Candida* isolates from blood samples from two Hungarian hospitals, located in Debrecen and Pécs, were examined. All examined isolates came from different patients. *C. parapsilosis* isolates were identified by standard morphological and physiological methods within the hospitals (Barnett *et al.*, 2000).

Phenotyping. The API 20C AUX version 3.0 kit (bioMérieux) was used to type the species. Isolates from stored slants were streaked onto nutrient agar and incubated at 30 °C for 24 and 48 h. Samples were then analysed according to the manufacturer's prescribed methods. The antifungal susceptibilities of isolates were determined by the Etest method (AB Biodisk). The Etest was performed in accordance with the manufacturer's instructions, with the use of Casitone agar plates (AB Biodisk, 2003). A 0.5 McFarland turbidity suspension was used as a standard in the test. The drug concentrations of Etest strips were 0.016–256 mg l⁻¹ for fluconazole, and 0.002–32 mg l⁻¹ for itraconazole, voriconazole and amphotericin B. The incubation time was 24–48 h at 30 °C, depending on the growth characteristics. The MICs for amphotericin B were taken as the drug concentrations causing 100 % inhibition. MICs for azoles were read at the visually selected end point of 80 % inhibition of growth. Interpretative susceptibility criteria for these antifungal agents were used as published by the Clinical and Laboratory Standards Institute (CLSI) and in the literature (National Committee for Clinical Laboratory Standards, 2002; de Hoog *et al.*, 2000).

Genotyping. Isolates were cultivated in YPD medium (0.5 % Bacto yeast extract, 1 % glucose, 0.5 % Bacto peptone) and centrifuged, and DNA was extracted from the cells using the Masterpure yeast DNA purification kit (Epicentre Biotechnologies) according to the instructions of the manufacturer. For confirmation of the species assignment of the isolates, the species-specific primer pair developed for *C. parapsilosis* by Luo & Mitchell (2002) was used.

For the identification of *C. parapsilosis sensu stricto* (group I) isolates, the primer pair developed by Pontieri *et al.* (2001) was applied. The ITS region of the isolates was amplified using primers ITS1 and ITS4 (White *et al.*, 1990). DNA fragments were purified from the excised agarose blocks using Genelute spin columns (Sigma-Aldrich). Direct sequencing of the fragments was performed on an ABI 373A DNA sequencer (Applied Biosystems) using dideoxy terminator reaction chemistry. Sequences were determined from both strands using ITS1 and ITS4 as primers. The GenBank accession numbers for the ITS sequences of *C. metapsilosis* 12821 and Bp57 are DQ786952 and DQ786953, respectively.

RAPD analysis of the isolates was carried out according to the literature (Williams *et al.*, 1990). Fungal DNA sequences were amplified by using the following decamer primers of the Operon random primer kit (Operon Technology): OPC-02 (5'-GTGAGGCGTC-3'), OPC-05 (5'-GATGACCGCC-3'), OPC-07 (5'-GTCCCGACGA-3'), OPC-09 (5'-CTCACCCTCC-3'), OPC-12 (5'-TGTCATCCCC-3'), OPC-14 (5'-TGCGTGCTTG-3'), OPC-16 (5'-CACACTCCAG-3'), OPC-18 (5'-TGAGTGGGTG-3'), OPC-20 (5'-ACTTCGCCAC-3'), OPD-12 (5'-CACCCTATCC-3'), OPE-17 (5'-CTACTGCCGT-3'), OPG-07 (5'-GAACCTGCGG-3'), OPG-19 (5'-GTCAGGGCAA-3'), OPH-18 (5'-GAATCGGCCA-3'), OPK-04 (5'-CCGCCCAAAC-3'), OPM-18 (5'-CACCATCCGT-3'), OPR-15 (5'-GGACAACGAG-3') and OPW-17 (5'-ACCGGCTTGT-3'), and UBC (University of British Columbia) primers UBC 8 (5'-CCTGGCGGTA-3'), UBC 18 (5'-GGGCCGTTTA-3') and UBC 66 (5'-GAGGGCGTGA-3').

Data analysis. Sequence alignments were performed by using CLUSTAL_X (Thompson *et al.*, 1997) and improved manually. Evolutionary distances between the sequences were calculated by Kimura's formula (Kimura, 1980) using the program DNADIST. Phylogenetic trees were prepared by the neighbour-joining method (Saitou & Nei, 1987) using the program NEIGHBOR of the PHYLIP package. Bootstrap values were calculated from 1000 replications of the bootstrap procedure using programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the package (Felsenstein, 1995).

RAPD bands were scored visually. Only those bands were taken into account which were amplified in all three separate RAPD reactions. The binary matrices of RAPD data were converted to distance matrices using PhylTools (Buntjer, 1997). RAPD data were analysed by the neighbour-joining method using the program NEIGHBOR of the PHYLIP package (Saitou & Nei, 1987; Felsenstein, 1995).

RESULTS AND DISCUSSION

Occurrence of *C. parapsilosis* in Hungarian hospitals

Altogether, 103 and 106 *Candida* isolates were collected in two Hungarian hospitals located in Debrecen and Pécs, respectively, during 2004–2005. Among these, 10 and 16 isolates, respectively, were found to belong to *C. parapsilosis sensu lato*, based on morphological and physiological criteria (Table 1). The *C. parapsilosis* species-specific primer pair (Luo & Mitchell, 2002) was successfully used to confirm the identity of eight and 12 isolates as *C. parapsilosis sensu lato*. API 20C AUX tests also confirmed that these isolates belonged to *C. parapsilosis sensu lato*. Overall, 9.6 % of the collected isolates were found to belong to *C. parapsilosis sensu lato*, while the remaining isolates were found to belong to *C. albicans*, *Candida lusitanae* (teleomorph *Clavispora lusitanae*; Kurtzman & Fell, 1998) or *Candida krusei*.

Table 1. Origin and species assignment of the *Candida* isolates examined in this study

Strain number	Origin	Species assignment based on ITS sequences
12821	Blood, Debrecen	<i>C. metapsilosis</i>
3929	Blood, Debrecen	<i>C. parapsilosis</i>
1188/1	Blood, Debrecen	<i>C. parapsilosis</i>
6761	Blood, Debrecen	<i>C. parapsilosis</i>
11811	Blood, Debrecen	<i>C. parapsilosis</i>
5312	Blood, Debrecen	<i>C. parapsilosis</i>
5308	Blood, Debrecen	<i>C. parapsilosis</i>
25329	Blood, Debrecen	<i>C. parapsilosis</i>
Bp57	Throat, Pécs	<i>C. metapsilosis</i>
Bp73	Stomach, Pécs	<i>C. parapsilosis</i>
Bp42	Blood, Pécs	<i>C. parapsilosis</i>
Bp101	Blood, Pécs	<i>C. parapsilosis</i>
Bp113	Blood, Pécs	<i>C. parapsilosis</i>
Bp59	Nail, Pécs	<i>C. parapsilosis</i>
Bp75	Nail, Pécs	<i>C. parapsilosis</i>
Bp46	Blood, Pécs	<i>C. parapsilosis</i>
Bp54	Fingernail, Pécs	<i>C. parapsilosis</i>
Bp3	Nail, Pécs	<i>C. parapsilosis</i>
Bp90	Sputum, Pécs	<i>C. parapsilosis</i>
Bp94	Blood, Pécs	<i>C. parapsilosis</i>
5311	Blood, Debrecen	<i>C. lusitaniae</i>
5068/1	Blood, Debrecen	<i>C. lusitaniae</i>
Bp47	Blood, Pécs	<i>C. krusei</i>
Bp39	Clinical sample, Pécs	<i>C. albicans</i>
Bp63	Blood, Pécs	<i>C. krusei</i>
Bp86	Blood, Pécs	<i>C. krusei</i>

(teleomorph *Issatchenkia orientalis*; Kurtzman & Fell, 1998) (Table 1). This observation could have been caused by misidentifications or, more probably, by contamination of some of the cultures during storage of the isolates after morphological identification.

Previously, *C. parapsilosis* has been found to be responsible for about 19 % of bloodstream *Candida* infections in Europe (Pfaller *et al.*, 2001). More recent surveys have revealed that *C. parapsilosis* is responsible for more than 20 % of candidaemias in Italy (Bassetti *et al.*, 2006; Bedini *et al.*, 2006), Spain (Almirante *et al.*, 2006; Rodriguez *et al.*, 2006; San Miguel *et al.*, 2006) and South American countries (Brito *et al.*, 2006; Rodero *et al.*, 2005), while this species is encountered less frequently in Turkey (12.5 %; Yapar *et al.*, 2006) and Norway (6 %; Sandven *et al.*, 2006). In a worldwide survey, Messer *et al.* (2006) have found *C. parapsilosis* to be responsible for candidaemias in 16.36 % of the 336 cases examined from Europe. In another survey, Tortorano *et al.* (2004, 2006) have found that *C. parapsilosis* is responsible for 6.9–30 % (mean 13.54 %) of candidaemias in different European countries, with the highest incidence found in Spain. In a previous study, Dóczy *et al.* (2002) found that *C. parapsilosis* was responsible for 4.9 % of bloodstream infections caused by *Candida* species in a university hospital in Hungary. These data indicate that the frequency of *C. parapsilosis* infections varies widely among

different institutions and countries (Pfaller *et al.*, 2001). The 9.6 % incidence of this species found in this survey is higher than that observed previously in Hungary, but lower than the mean observed in European countries.

Genetic variability of *C. parapsilosis* isolates

RAPD analysis of the isolates was carried out by using 21 random decamer primers. The genetic variability observed among the isolates was low: most isolates exhibited very similar or the same RAPD patterns with most primers tested (Fig. 1). Similarly low genetic variability has been observed among *C. parapsilosis sensu stricto* isolates by Zeng *et al.* (1996), Lehmann *et al.* (1992) and Enger *et al.* (2001). For preparation of the similarity matrix, the presence or absence of 72 DNA bands was taken into account. The dendrogram based on RAPD data indicates that the *C. parapsilosis* isolates form two main clusters which are supported by high bootstrap values (Fig. 2). One of these includes two isolates, 12821 and Bp57, which could also be distinguished from other *C. parapsilosis* isolates using the *C. parapsilosis* group I-specific primer pair developed by Pontieri *et al.* (2001). This primer pair did not amplify the expected product from isolates 12821 and Bp57 (data not shown).

Sequences of the ITS region of the isolates were also determined. Phylogenetic analysis of sequence data indicated

Fig. 1. RAPD profiles of the examined *C. parapsilosis sensu lato* isolates generated by primer OPR-15. Lanes: 1, 100 bp DNA ladder; 2, *C. parapsilosis* 5312; 3, *C. parapsilosis* 5308; 4, *C. parapsilosis* Bp73; 5, *C. parapsilosis* Bp46; 6, *C. parapsilosis* 25329; 7, *C. parapsilosis* Bp42; 8, *C. metapsilosis* 12821; 9, *C. metapsilosis* Bp57; 10, *C. krusei* Bp47; 11, 1 kb DNA ladder.

that two of the isolates that belonged to *C. parapsilosis sensu lato* were members of the *C. metapsilosis* species (see Supplementary Fig. S1). In other studies, *C. metapsilosis* has been identified in the USA, Japan, Brazil, Norway and Belgium (Enger *et al.*, 2001; Rycovska *et al.*, 2004; Iida *et al.*, 2005; Tavanti *et al.*, 2005). To the best of our knowledge, the present study is the first report on the occurrence of this species in Central Europe. In addition, in this study, *C. metapsilosis* was isolated from a bloodstream infection for the first time. The results of the API 20C AUX tests also indicated that these two isolates belonged to the *C. metapsilosis* species, since in a similar manner to the results of Lin *et al.* (1995), the two isolates could grow on D-xylitol, in contrast to the *C. parapsilosis sensu stricto* isolates (data not shown).

Antifungal susceptibility tests

All isolates were susceptible to the antifungal drugs tested, with MICs $<1 \text{ mg l}^{-1}$ for amphotericin B and voriconazole, $<8 \text{ mg l}^{-1}$ for fluconazole and $<0.125 \text{ mg l}^{-1}$ for itraconazole. The lowest MIC values were obtained for itraconazole and voriconazole. According to earlier studies, the amphotericin B, fluconazole, itraconazole and voriconazole susceptibilities of *C. parapsilosis* isolates range from 0.03 to 2 mg l^{-1} , 0.06 to 16 mg l^{-1} , 0.03 to 2 mg l^{-1} and 0.02 to 1 mg l^{-1} , respectively (Lin *et al.*, 1995; Rex *et al.*, 1995; Pfaller *et al.*, 2001; Lu *et al.*, 2004; St-Germain *et al.*, 2001; Tortorano *et al.*, 2004, 2006; de Hoog *et al.*, 2000). The MIC values obtained for *C. metapsilosis* isolates fell within the range of MICs of *C. parapsilosis* isolates (data not shown). *C. metapsilosis* isolates exhibited lower mean MIC values for voriconazole and amphotericin B than *C. parapsilosis* isolates. Similarly, lower MIC values have been observed for amphotericin B in *C. metapsilosis* than in *C. parapsilosis* in earlier studies (Lin *et al.*, 1995).

Fig. 2. Neighbour-joining tree based on RAPD profiles of *C. parapsilosis sensu lato* isolates. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

However, further isolates should be examined to draw definite conclusions.

In conclusion, 9.6 % of *Candida* infections were found to be caused by *C. parapsilosis sensu lato* in our survey of two Hungarian hospitals. Two of these isolates were found to belong to the recently described *C. metapsilosis* species. This is believed to be the first report on the identification of *C. metapsilosis* from bloodstream infection. Further studies are in progress to identify the rest of the *Candida* isolates using molecular and phenotypic data, and to survey other Hungarian hospitals for the presence of *C. metapsilosis*.

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