Rapid molecular differentiation and genotypic heterogeneity among *Candida parapsilosis* and *Candida orthopsilosis* strains isolated from clinical specimens in Kuwait

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Recent molecular studies have led to the recognition of three distinct species within the *Candida parapsilosis* complex, namely *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis*. As currently available yeast identification systems fail to differentiate these species, there is a paucity of information on their occurrence in different geographical regions. This study describes a simple PCR-based protocol for rapid discrimination among *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* strains by using primers derived from unique sequences within the internally transcribed spacer 1 (ITS1)–5.8 rRNA–ITS2 region. Retrospective analysis of 114 *C. parapsilosis*-complex isolates recovered from clinical specimens in Kuwait identified 109 as *C. parapsilosis*, five as *C. orthopsilosis* and none as *C. metapsilosis*. The results were further validated by PCR-RFLP patterns of the secondary alcohol dehydrogenase gene fragment. DNA sequencing of the ITS region and the D1/D2 regions of the 28S rRNA gene confirmed the species-specific identification of all five *C. orthopsilosis* strains. The amplicon length of the intergenic spacer between the 28S and 5S rRNA genes (IGS1) was also species-specific, and PCR-RFLP analyses of the IGS1 region identified two distinct genotypes among the five *C. orthopsilosis* strains, which corresponded with the ITS region sequence data. The three bloodstream *C. orthopsilosis* strains were confined to a single genotype. Among 81 randomly selected *C. parapsilosis* strains, two genotypes were detected by IGS1 region analyses, indicating limited genotypic heterogeneity among *C. parapsilosis sensu stricto* strains. As far as is known, this is the first report on the identification of *C. orthopsilosis* from a bloodstream infection in the Arabian Gulf region.

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INTRODUCTION

Although *Candida albicans* is the most commonly isolated *Candida* species from bloodstream infections, *Candida parapsilosis* has recently emerged as an important nosocomial pathogen causing invasive candidiasis in critically ill hospitalized patients and low-birth-weight infants (Pappas *et al.*, 2003; Almirante *et al.*, 2006; Bassetti *et al.*, 2006; Pfaller & Diekema, 2007; Yong *et al.*, 2008). In Kuwait, *C. parapsilosis* is the second most frequently isolated *Candida* species from bloodstream infections, in both adult and paediatric patients (Mokaddas *et al.*, 2007; Asadzadeh *et al.*, 2008; Al-Sweih *et al.*, 2009). The species is frequently found on the skin and thus can be transmitted exogenously on the hands of healthcare professionals (Bonassoli *et al.*, 2005). Infections usually occur in association with catheter/

prosthetic devices, where *C. parapsilosis* is capable of forming biofilms (Weems, 1992).

It has been known for some time that *C. parapsilosis* represents a 'complex' of genetically different strains/species and consists of at least three genetically distinct groups (I, II and III) (Lin *et al.*, 1995; Roy & Meyer, 1998). Detailed molecular analyses including multilocus sequence typing studies have recently recognized *C. parapsilosis* groups II and III as separate species, *Candida orthopsilosis* and *Candida metapsilosis*, within the *C. parapsilosis* complex, whilst group I isolates have been proposed to retain the name *C. parapsilosis* (Tavanti *et al.*, 2005). Although the exact prevalence of *C. orthopsilosis* and *C. metapsilosis* infections remains unknown, several recent studies indicate a significant contribution of these two newly described species towards human diseases, including bloodstream infections (Lasker *et al.*, 2006; Kocsube *et al.*, 2007; Tavanti *et al.*, 2007; Yong *et al.*, 2008; Gomez-Lopez *et al.*, 2008; Lockhart *et al.*, 2008a). A major impediment of

Abbreviations: IGS, intergenic spacer; ITS, internally transcribed spacer.

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the lack of this information is the application of tedious and complex techniques that are required for the identification of *C. orthopsilosis* and *C. metapsilosis* strains among clinical isolates (Tavanti *et al.*, 2005; Kocsube *et al.*, 2007; Yong *et al.*, 2008). In this study, we have developed a simple PCR-based method for rapid identification of clinical isolates of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* by using species-specific primers derived from unique sequences within the internally transcribed spacer (ITS) 1 and ITS2 of the ITS1–5.8S rRNA–ITS2 region. Using this protocol, we analysed 114 phenotypically identified isolates of *C. parapsilosis* recovered from various clinical specimens and the results of these studies are presented here.

METHODS

Reference strains, clinical isolates and DNA isolation. The reference or well-characterized clinical strains of *Candida* and other fungal species were obtained from the American Type Culture Collection (ATCC) or the Centraalbureau voor Schimmelcultures (CBS). These included *C. parapsilosis* (ATCC 22019), *C. orthopsilosis* (ATCC 96139), *C. metapsilosis* (ATCC 96143), *Lodderomyces elongisporus* (clinical isolate), *C. albicans* (ATCC 76615), *Candida dubliniensis* (CD36), *Candida tropicalis* (ATCC 750), *Candida glabrata* (ATCC 15545), *Candida krusei* (ATCC 6258), *Candida haemulonii* (CBS 5149), *Aspergillus fumigatus* (CBS 113.26/ATCC 1028), *Aspergillus flavus* (CBS 113.2), *Cryptococcus neoformans* (ATCC 90112), *Trichosporon asahii* (CBS 2530) and *Trichosporon jirovecii* (CBS 6864). A total of 114 randomly selected *C. parapsilosis* strains isolated from the bloodstream ($n=66$) and other clinical specimens ($n=48$) from 1996 to 2007 in Kuwait and identified by CHROMagar *Candida* and the VITEK 2 yeast identification system (bioMérieux) were analysed. The cultures were grown on Sabouraud dextrose agar

(Difco; Becton-Dickinson) and the genomic DNA from reference and clinical *Candida* species and other fungal pathogens was isolated as described previously (Ahmad *et al.*, 2002; Khan *et al.*, 2008a).

Primer construction and PCR amplification. Nine primer pairs were synthesized for pan-fungal, *C. parapsilosis*-complex or *Candida* species-specific amplification of various target sequences (Table 1). The primer pair CPDET and CTS1R (Ahmad *et al.*, 2002) was used to confirm that all clinical isolates used in this study belonged to the *C. parapsilosis* complex. The species specificity of the primer pairs CPAF+CPAR, CORF+CORR and CMEF+CMER for *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, respectively, was indicated by BLAST searches (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). PCR amplification of the secondary alcohol dehydrogenase (*SADH*) gene followed by *Ban*I digestion of amplicons to generate three unique RFLP patterns for the three species of the *C. parapsilosis*-complex strains was also carried out by using the SADHF1+SADHR1 (Tavanti *et al.*, 2005) or SADHF2+SADHR2 primers (Table 1). The pan-fungal primers ITS1+ITS4 (Ahmad *et al.*, 2005) and NL-1+NL-4 (Kurtzman & Robnett, 1997) were used for PCR-based sequencing of the ITS (ITS1–5.8S rRNA–ITS2) region and the D1/D2 regions of the 28S rRNA gene, respectively. The primers IGSF and IGSR were designed for PCR-RFLP analyses of the intergenic spacer (IGS1) between the 28S and 5S rRNA genes from *C. parapsilosis*-complex isolates.

PCR amplification of various gene targets was carried out as follows. The reactions in a total volume of 50 μ l contained 1 $\times$ AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase (Applied Biosystems), 10 pmol each of forward and reverse primer (Table 1), 2 μ l genomic DNA and 0.1 mM dNTPs. The cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 63 °C (for the CPDET+CTS1R, CPAF+CPAR, CORF+CORR and CMEF+CMER primer pairs), 60 °C (for the ITS1+ITS4 and NL-1+NL-4 primer pairs) or 50 °C (for the SADHF1+SADHR1 or SADHF2+SADHR2 primer pairs) for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The reactions for amplification of the IGS1 region in a final volume of

Table 1. Specific features and sequences of the PCR primers used in this study

Pair no.	Primer	Target gene	Direction	Species specificity	Sequence (5'→3')	Amplicon size (bp)
1	CPDET	ITS2	Forward	<i>C. parapsilosis</i> complex	ACAAACTCCAAAACCTCTTCCA	88
	CTS1R	28S rRNA	Reverse		TCTTTTCCTCCGCTTATTGATATG	
2	CPAF	ITS1	Forward	<i>C. parapsilosis</i>	TTTGCTTTGGTAGGCCCTTCTA	379
	CPAR	ITS2	Reverse		GAGGTCGAATTTGGAAGAAGT	
3	CORF	ITS1	Forward	<i>C. orthopsilosis</i>	TTTGGTGGCCACGGCCT	367
	CORR	ITS2	Reverse		TGAGGTCGAATTTGGAAGAATT	
4	CMEF	ITS1	Forward	<i>C. metapsilosis</i>	TTTGGTGGGCCCACGGCT	374
	CMER	ITS2	Reverse		GAGGTCGAATTTGGAAGAATGT	
5	SADHF1	<i>SADH</i>	Forward	<i>C. parapsilosis</i> complex	GTTGATGCTGTTGGATTGT	717
	SADHR1	<i>SADH</i>	Reverse		CAATGCCAAATCTCCCAA	
6	SADHF2	<i>SADH</i>	Forward	<i>C. parapsilosis</i> complex	TGGATTGTGGGGAYAATTATGT	310
	SADHR2	<i>SADH</i>	Reverse		GTCAATACRGCATCAGTTGAAGC	
7	ITS1	18S rRNA	Forward	Pan-fungal	TCCGTAGGTGAACCTGCGG	Variable
	ITS4	28S rRNA	Reverse		TCTTTTCCTCCGCTTATTGATATG	
8	NL-1	28S rRNA	Forward	Pan-fungal	GCATATCAATAAGCGGAGGAAAAG	Variable
	NL-4	28S rRNA	Reverse		GGTCCGTGTTTCAAGACGG	
9	IGSF	28S rRNA	Forward	Pan-fungal	CAACGGAGTATTGTAAGCAGTA	Variable
	IGSR	5S rRNA	Reverse		GGCTTAATCTTTGAGACAAGCAT	

50 µl contained 15 µl 3.3 × *r**Th* DNA polymerase buffer, 5 µl 10 × magnesium acetate, 2 U *r**Th* DNA polymerase (Applied Biosystems), 10 pmol each of IGS1F and IGS1R primers, 2 µl genomic DNA and 0.1 mM dNTPs. The cycling conditions comprised one cycle of 95 °C for 3 min and 96 °C for 1 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min, with a final extension period of 10 min at 72 °C. Inclusion of the spike to 96 °C for 1 min during denaturation of the genomic DNA resulted in improved amplification of the IGS region, as has also been observed previously for higher G+C DNA regions (Ahmad *et al.*, 2007). For species-specific detection of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* strains, PCR products (20 µl) were run on 2% (w/v) agarose gels, as described previously (Ahmad *et al.*, 2004). An amplicon was obtained only with the corresponding species-specific primer pair. For other PCRs, a sample (5 µl) was run on a 2% (w/v) agarose gel to confirm amplification of a DNA fragment of the expected size. The remaining PCR-amplified fragments were purified by using QIAquick PCR purification columns (Qiagen) according to the kit instructions. The purified amplicons were either digested with restriction enzymes to generate RFLP patterns (for the *SADH* gene and the IGS1 region) or subjected to DNA sequencing.

RFLP analyses of the *SADH* gene fragment. *SADH* amplicons obtained with the SADHF1+SADHR1 or SADHF2+SADHR2 primers from reference and clinical isolates were digested with *Ban*I. The reactions in a final volume of 25 µl contained 10 µl purified amplicon, 2.5 µl 10 × NE Buffer 4, 2 µl (10 U) *Ban*I (New England Bio-Laboratories) and 10.5 µl sterile distilled water and were incubated at 37 °C for 3 h. The products were separated on 2% agarose gels to generate RFLP patterns. The 717 bp amplicons obtained with the SADHF1+SADHR1 primers yielded two fragments of 521 and 196 bp, a single undigested fragment of 717 bp, and four fragments of 375, 189, 93 and 60 bp from *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, respectively (Tavanti *et al.*, 2005). The 310 bp amplicons generated with SADHF2+SADHR2 designed in this study should yield two fragments of 166 and 144 bp, a single undigested fragment of 310 bp, and two fragments of 268 and 42 bp from *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, respectively.

RFLP analysis of the IGS1 region DNA fragment. Genotypic heterogeneity among clinical *C. parapsilosis* or *C. orthopsilosis* strains was determined by restriction digestion of IGS1 region amplicons with a battery of frequently cutting restriction enzymes to generate RFLPs. The restriction digestions containing 10 µl of the purified amplicon, 2.5 µl appropriate 10 × NE Buffer, 2 µl (10 U) of each restriction enzyme (*Dde*I, *Rsa*I, *Hinf*I, *Msp*I or *Alu*I; New England Bio-Laboratories) and 10.5 µl sterile distilled water were carried out at 37 °C for 4 h and the products were separated on 1% agarose gels.

DNA sequencing of the ITS region and the D1/D2 region of the 28S rRNA gene. The results of species-specific identification of clinical isolates were confirmed by DNA sequencing of the ITS region and the D1/D2 region of the 28S rRNA gene and both strands were sequenced. The amplicons obtained with the ITS1+ITS4 primers were sequenced by using ITS1FS, ITS2, ITS3 and ITS4RS as sequencing primers as described previously (Ahmad *et al.*, 2005; Al-Sweih *et al.*, 2005; Al-Obaid *et al.*, 2006). The amplicons obtained with the NL-1+NL-4 primers were sequenced by using NL-1, NL-2A, NL-3A and NLR3R as sequencing primers as described previously (Khan *et al.*, 2008b). Reverse complements were generated and aligned with forward sequences using CLUSTAL W (<http://www.ebi.ac.uk/clustalw>) during sequence assembly (Khan *et al.*, 2008b). BLAST searches were performed for species identification. Pairwise comparisons and multiple sequence alignments were also performed with CLUSTAL W.

RESULTS AND DISCUSSION

Species-specific amplification of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*

The species specificity of the CTS1R+CPDET primers for detection of DNA from *C. parapsilosis*-complex strains has been described previously (Ahmad *et al.*, 2002). PCR amplification with the CPAF+CPAR primers (Table 1) yielded an amplicon of ~379 bp with DNA from the reference strain of *C. parapsilosis* (Fig. 1, lane 1), but not from *C. orthopsilosis* (Fig. 1, lane 4) or *C. metapsilosis* (Fig. 1, lane 7), as expected. Similarly, PCR amplification with CORF+CORR and CMEF+CMER yielded amplicons of the expected size (367 and 374 bp, respectively) only with DNA from *C. orthopsilosis* (Fig. 1, lane 5) and *C. metapsilosis* (Fig. 1, lane 9), respectively, but not from the other two *C. parapsilosis*-complex species. No amplicon was obtained with any of the three primer pairs using DNA isolated from reference strains of other *Candida* or other common fungal species (data not shown). These results established the specificity of the three primer pairs for species-specific detection of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. PCR with *C. parapsilosis*-complex-specific (CPDET+CTS1R) primers (Ahmad *et al.*, 2002) yielded an amplicon of ~90 bp from all clinical *C. parapsilosis* isolates (*n*=114) identified by phenotypic methods (CHROMagar *Candida* and VITEK 2 yeast identification system) (data not shown). In uniplex PCR with primers CPAF+CPAR, CORF+CORR or CMEF+CMER (Table 1), five and 109 isolates yielded an amplicon with *C. orthopsilosis*-specific (Fig. 1b, lane 2) and *C. parapsilosis*-specific (Fig. 1b, lanes 4, 7 and 10) primer pairs, respectively, whilst no isolate yielded an amplicon with *C. metapsilosis*-specific primers.

Identification based on PCR-RFLP analyses of the *SADH* gene

To validate the results of our protocol, PCR-RFLP analyses of the *SADH* gene were also carried out as described by Tavanti *et al.* (2005). *SADH* amplicons obtained with primers SADHF1+SADHR1 from the reference *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* strains yielded RFLP patterns as described previously (Tavanti *et al.*, 2005). However, a PCR product was obtained only from 75 isolates, whilst five strains did not yield an amplicon during initial analyses of 80/114 *C. parapsilosis*-complex strains isolated in Kuwait. The latter five isolates were easily amplified with *C. parapsilosis*-complex-specific or species-specific primers (Table 1) and were identified as *C. parapsilosis* strains, indicating that lack of amplification was most likely due to differences in primer-binding sequences in the *SADH* gene. These isolates also could not have belonged to group IV advocated by Iida *et al.* (2005) or *L. elongisporus* (Lockhart *et al.*, 2008b) due to differences in the target sequences of the CPAF and CPAR primers. Lockhart *et al.* (2008a) also reported recently that 47/1976 *C. parapsilosis*-complex strains isolated from diverse

Fig. 1. (a) Agarose gel of PCR-amplified products obtained from *C. parapsilosis* (ATCC 22019) (lanes 1–3), *C. orthopsilosis* (ATCC 96139) (lanes 4–6) and *C. metapsilosis* (ATCC 96143) (lanes 7–9) reference strains amplified with primers CPAF + CPAR (lanes 1, 4 and 7), CORF + CORR (lanes 2, 5 and 8) and CMEF + CMER (lanes 3, 6 and 9). (b) Agarose gel of amplicons from four representative clinical isolates amplified individually with primers CPAF + CPAR (lanes 1, 4, 7 and 10), CORF + CORR (lanes 2, 5, 8 and 11) and CMEF + CMER (lanes 3, 6, 9 and 12). As shown in lanes 1–3, 4–6, 7–9 and 10–12, each isolate yielded an amplicon with only one of the three species-specific primer pairs. Thus the isolate in lanes 1–3 was identified as *C. orthopsilosis*, whilst the isolates in lanes 4–6, 7–9 and 10–12 were identified as *C. parapsilosis*. Lane M is a 100 bp DNA ladder and the positions of migration of the 100, 300 and 600 bp fragments are indicated.

geographical locations around the world could not be speciated, probably due to lack of amplification of the *SADH* gene with the SADHF1 + SADHR1 primers. Thus a new primer pair (SADHF2 + SADHR2) was designed for *SADH* gene amplification. The target region of the *SADH* gene was carefully selected within the region originally amplified by Tavanti *et al.* (2005) to contain variable patterns of *Ban*I restriction sites in the resulting amplicons from *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, as

described in Methods. The shorter *SADH* gene fragment (~310 bp) obtained with the SADHF2 + SADHR2 primers from the reference *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* strains yielded species-specific RFLP patterns, as described in Methods. The *SADH* gene was amplified with the SADHF2 + SADHR2 primers from all of the 114 phenotypically identified *C. parapsilosis*-complex isolates (data from six isolates are shown in Fig. 2a, lanes 1–6). The *Ban*I-generated RFLP patterns identified 109 isolates (data from four isolates are shown in Fig. 2b, lanes 1–4) with two fragments of 166 and 144 bp as *C. parapsilosis* and five isolates (data from two isolates are shown in Fig. 2b, lanes 5 and 6) with the original undigested fragment of ~310 bp as *C. orthopsilosis*.

DNA sequencing studies

Direct DNA sequencing of the ITS region was carried out to confirm the identification of all five *C. orthopsilosis*

Fig. 2. Agarose gel of amplicons of the *SADH* gene using primers SADHF2 + SADHR2 (a) and RFLP patterns of the amplicons from six selected clinical *C. parapsilosis*-complex strains (b). The digested fragments in lanes 1–4 are characteristic for *C. parapsilosis*, whilst those in lanes 5 and 6 are characteristic for *C. orthopsilosis*. Lane M is a 100 bp DNA ladder and the positions of migration of the 100, 300 and 600 bp fragments are indicated.

Table 2. Nucleotide differences within the ITS region (ITS1–5.8S rRNA–ITS2) among clinical *C. parapsilosis* and *C. orthopsilosis* isolates in Kuwait in comparison with reference strains

<i>Candida</i> sp.	Isolate no.	ITS region length (bp)	No. of nucleotide differences compared with reference strain:		
			<i>C. parapsilosis</i> ATCC 22019	<i>C. orthopsilosis</i> ATCC 96139	<i>C. metapsilosis</i> ATCC 96143
<i>C. parapsilosis</i>	113/98	431	0	23	44
	119/98	431	0	23	44
<i>C. orthopsilosis</i>	301/97	422	28	9	31
	304/97	422	28	9	31
	709/04	425	27	11	28
	1056/04	422	28	9	31
	1782/06	425	27	11	28

strains (isolates 301/97, 304/97, 709/04, 1782/06 and 1056/04). Two distinct genotypes were identified. The ITS region sequences of three *C. orthopsilosis* isolates (301/97, 304/97 and 1056/04) were identical and differed at three nucleotide positions with the sequences from the other two isolates (insertion of T in isolates 709/04 and 1782/06). A pairwise comparison of ITS region sequences showed fewer differences with the corresponding sequence from the reference *C. orthopsilosis* strain than with sequences from reference *C. parapsilosis* or *C. metapsilosis* strains (Table 2). Four distinct genotypes among the clinical *C. dubliniensis* strains were also identified recently based on ITS region sequences (Gee *et al.*, 2002; Al-Sweih *et al.*, 2005). The D1/D2 region sequences of two *C. orthopsilosis* isolates (301/97 and 709/04) representing the two ITS region-based genotypes exhibited a single nucleotide difference with each other and were nearly identical (zero or one nucleotide difference) with the sequence from the reference *C. orthopsilosis* strain. Two randomly selected *C. parapsilosis* strains were also sequenced in the ITS region (isolates 113/98 and 119/98) and D1/D2 region (isolates 113/98 and 426/04). The ITS and D1/D2 region sequences of both *C. parapsilosis* isolates were identical and showed 100 % identity with the corresponding sequences from the reference *C. parapsilosis* strain.

Thus species-specific amplification of the ITS region and PCR-RFLP analyses of the *SADH* gene were concordant for all clinical isolates in Kuwait and identified 109 isolates as *C. parapsilosis* and five isolates as *C. orthopsilosis*. None of the *C. parapsilosis*-complex strains in Kuwait was identified as *C. metapsilosis*. Our results are in agreement with recent reports showing that some clinical isolates identified as *C. parapsilosis* by conventional carbon-assimilation methods, such as the VITEK 2 Yeast ID system, are in fact isolates of *C. orthopsilosis* (Tavanti *et al.*, 2007; Gomez-Lopez *et al.*, 2008; Lockhart *et al.*, 2008a). The absence of *C. metapsilosis* among 114 *C. parapsilosis*-complex strains in Kuwait also supports the infrequent occurrence of this species, particularly among bloodstream isolates (Kocsube *et al.*, 2007; Tavanti *et al.*, 2007). The exact prevalence of *C. orthopsilosis* and *C. metapsilosis* strains among clinical *Candida* species isolates or candidaemia cases is not known, as only a few studies have been carried out in the last couple of years. A comparison of the prevalence of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* among phenotypically characterized *C. parapsilosis*-complex strains in various studies is presented in Table 3. Only two studies detected the presence of both of the newly described species, i.e. *C. orthopsilosis* and *C. metapsilosis*, among *C. parapsilosis*-complex strains (Gomez-Lopez *et al.*,

Table 3. Occurrence of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* among phenotypically identified *C. parapsilosis*-complex strains in various studies

Total no. of isolates tested	No. (%) of isolates identified as:			Reference
	<i>C. parapsilosis</i>	<i>C. orthopsilosis</i>	<i>C. metapsilosis</i>	
27	20 (74.1)	7 (25.9)	0 (0)	Tavanti <i>et al.</i> (2005)
290	277 (95.5)	13 (4.5)	0 (0)	Tavanti <i>et al.</i> (2007)
26	24 (92.3)	0 (0)	2 (7.7)	Kocsube <i>et al.</i> (2007)
78	67 (85.9)	5 (6.4)	6 (7.7)	Gomez-Lopez <i>et al.</i> (2008)
1929*	1762 (91.3)	117 (6.1)	34 (1.8)	Lockhart <i>et al.</i> (2008a)
114	109 (95.6)	5 (4.4)	0 (0)	This study

*Sixteen isolates were identified as *L. elongisporus*.

Fig. 3. Representative agarose gel of restriction digestion of IGS1 region amplicons with *RsaI* from seven selected *C. parapsilosis* strains (a) and with *HinfI* from the five *C. orthopsilosis* strains (b). In (a), the RFLP patterns in lanes 1–4 were classified as pattern A, whilst those in lanes 5–7 were classified as pattern B. In (b), the RFLP patterns in lanes 1, 2 and 4 were classified as pattern C, whilst those in lanes 3 and 5 were classified as pattern D. Lane M, *BstEII*-digested λ DNA ladder, with fragment sizes indicated (bp).

2008; Lockhart *et al.*, 2008a). Similar to our results, Tavanti *et al.* (2005, 2007) also did not detect any *C. metapsilosis* strains, whilst Kocsube *et al.* (2007) identified 7.6% (2/26) of *C. parapsilosis*-complex isolates as *C. metapsilosis* but did not find any *C. orthopsilosis* strains (Table 3). It is not known at present whether *C. orthopsilosis* and *C. metapsilosis* occupy any particular niche in the human body.

Genotypic heterogeneity among clinical *C. orthopsilosis* isolates

The genotypic heterogeneity among 81 randomly selected *C. parapsilosis* and all five *C. orthopsilosis* strains was studied further by PCR-RFLP analyses of the IGS1 region. Only *RsaI* and *HinfI* yielded discriminatory RFLP patterns of the IGS1 region among *C. parapsilosis* and *C. orthopsilosis* strains, respectively. All 81 *C. parapsilosis* strains yielded an IGS1 amplicon of ~2200 bp (data not shown). Forty-nine isolates yielded one pattern (designated pattern 'A') (Fig. 3a, lanes 1–4), whilst 32 isolates exhibited a second pattern (designated pattern 'B') (Fig. 3a, lanes 5–7) during RFLP analyses of IGS1 with *RsaI*. Almost equal numbers of *C. parapsilosis* strains isolated from the blood yielded pattern A (23/47, 49%) and pattern B (24/47, 51%). However, the vast majority (26/34, 76%) of strains isolated from other body sites yielded pattern A (Table 4). All five *C. orthopsilosis* strains yielded a shorter (~1700 bp) IGS1 region amplicon (data not shown). Thus the length of the IGS1 region was also species-specific. Other yeasts such as *Trichosporon* species also exhibit IGS1 length variations (Sugita *et al.*, 2002; Rodriguez-Tudela *et al.*, 2007). Three *C. orthopsilosis* isolates (Fig. 3b, lanes 1, 2 and 4) yielded one pattern (designated pattern 'C'), whilst two isolates (Fig. 3b, lanes 3 and 5) exhibited a second pattern (designated pattern 'D') during *HinfI*-generated RFLP analyses (Table 4). Application of tedious and technically demanding amplified fragment length polymorphism analyses has also recently shown genotypic heterogeneity and identified four major clusters among 13 *C. orthopsilosis* strains from Italy (Tavanti *et al.*, 2007). All three *C. orthopsilosis* strains yielding the pattern C RFLP also

Table 4. Genotypic heterogeneity among 81 randomly selected *C. parapsilosis* and all five *C. orthopsilosis* strains isolated in Kuwait by PCR-RFLP patterns of the 28S–5S rRNA IGS region

<i>Candida</i> sp.	No. of isolates	Source of isolation	IGS region length (bp)	No. (%) of isolates with PCR-RFLP	
				First pattern	Second pattern
<i>C. parapsilosis</i> *	47	Bloodstream	~2200	A: 23 (49)	B: 24 (51)
	34	Other source	~2200	A: 26 (76)	B: 8 (24)
<i>C. orthopsilosis</i> †	3	Bloodstream	~1700	C: 3 (100)	D: 0 (0)
	2	Other source	~1700	C: 0 (0)	D: 2 (100)

*Only restriction enzyme *RsaI* yielded the two discriminatory patterns.

†Only restriction enzyme *HinfI* yielded the two discriminatory patterns.

exhibited the same genotype based on ITS region sequences and were isolated from the bloodstream (Table 4). Whether these findings have any significance with regard to the virulence of this genotype remains to be determined. Although only two *C. parapsilosis* strains were sequenced, the PCR-RFLP results of the IGS1 region were consistent with earlier observations showing greater sequence variations among *C. orthopsilosis* than *C. parapsilosis* clinical isolates (Tavanti *et al.*, 2005, 2007).

In conclusion, we have described a simple amplification protocol for rapid discrimination among *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* clinical isolates. We have also reported the occurrence of *C. orthopsilosis* among clinical isolates of the *C. parapsilosis* complex from the Arabian Gulf region for the first time. Three out of five *C. orthopsilosis* strains were isolated from the bloodstream and belonged to a single genotype. The absence of *C. metapsilosis* indicates that this species probably has a limited clinical significance.

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REFERENCES

- Ahmad, S., Khan, Z., Mustafa, A. S. & Khan, Z. U. (2002). Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. *J Clin Microbiol* **40**, 2483–2489.
- Ahmad, S., Mustafa, A. S., Khan, Z., Al-Rifaiy, A. I. & Khan, Z. U. (2004). PCR-enzyme immunoassay of rDNA in the diagnosis of candidemia and comparison with amplicon detection by agarose gel electrophoresis. *Int J Med Microbiol* **294**, 45–51.
- Ahmad, S., Al-Mahmeed, M. & Khan, Z. U. (2005). Characterization of *Trichosporon* species isolated from clinical specimens in Kuwait. *J Med Microbiol* **54**, 639–646.
- Ahmad, S., Jaber, A.-A. & Mokaddas, E. (2007). Frequency of *embB* codon 306 mutations in ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. *Tuberculosis (Edinb)* **87**, 123–129.
- Almirante, B., Rodriguez, D., Cuenca-Estrella, M., Almela, M., Sanchez, F., Ayata, J., Alonso-Tarres, C., Rodriguez-Tudela, J. L. & Pahissa, A. (2006). Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. *J Clin Microbiol* **44**, 1681–1685.
- Al-Obaid, I., Ahmad, S., Khan, Z. U., Dinesh, B. & Hejab, H. M. (2006). Catheter-associated fungemia due to *Exophiala oligosperma* in a leukemic child and review of fungemia cases caused by *Exophiala* species. *Eur J Clin Microbiol Infect Dis* **25**, 729–732.
- Al-Sweih, N., Ahmad, S., Khan, Z. U., Khan, S. & Chandy, R. (2005). Prevalence of *Candida dubliniensis* among germ tube-positive *Candida* isolates in a maternity hospital in Kuwait. *Mycoses* **48**, 347–351.
- Al-Sweih, N., Khan, Z. U., Khan, S. & Devarajan, L. (2009). Neonatal candidemia in Kuwait: a 12-year study of risk factors, species spectrum and antifungal susceptibility. *Mycoses* **52** (in press). doi:10.1111/j.1439-0507.2008.01637.
- Asadzadeh, M., Al-Sweih, N. A., Ahmad, S. & Khan, Z. U. (2008). Antifungal susceptibility of clinical *Candida parapsilosis* isolates in Kuwait. *Mycoses* **51**, 318–323.
- Bassetti, M., Righi, E., Costa, A., Fasce, R., Molinari, M. P., Rosso, R., Pallavicini, F. B. & Viscoli, C. (2006). Epidemiological trends in candidemia in intensive care. *BMC Infect Dis* **6**, 21.
- Bonassoli, L. A., Bertoli, M. & Svidzinski, T. I. (2005). High frequency of *Candida parapsilosis* on the hands of healthy hosts. *J Hosp Infect* **59**, 159–162.
- Gee, S. F., Joly, S., Soll, D. R., Meis, J. F., Verweij, P. E., Polacheck, I., Sullivan, J. D. & Coleman, D. C. (2002). Identification of four distinct genotypes of *Candida dubliniensis* and detection of microevolution in vitro and in vivo. *J Clin Microbiol* **40**, 556–574.
- Gomez-Lopez, A., Alastruey-Izquierdo, A., Rodriguez, D., Almirante, B., Pahissa, A., Rodriguez-Tudela, J. L., Cuenca-Estrella, M. & Barcelona Candidemia Project Study Group (2008). Prevalence and susceptibility profile of *Candida metapsilosis* and *Candida orthopsilosis*: results from population-based surveillance of candidemia in Spain. *Antimicrob Agents Chemother* **52**, 1506–1509.
- Iida, S., Imai, T., Oguri, T., Okuzumi, K., Yamanaka, A., Moretti-Branchini, L. M., Nishimura, K. & Mikami, Y. (2005). Genetic diversity of the internal transcribed spacers (ITS) and 5.8S rRNA genes among the clinical isolates of *Candida parapsilosis* in Brazil and Japan. *Nippon Ishinkin Gakkai Zasshi* **46**, 133–137.
- Khan, Z. U., Ahmad, S. & Theyyathel, A. M. (2008a). Detection of *Aspergillus fumigatus*-specific DNA, (1–3)- β -D-glucan and galactomannan in serum and bronchoalveolar lavage specimens of experimentally infected rats. *Mycoses* **51**, 129–135.
- Khan, Z. U., Ahmad, S., Mokaddas, E., Chandy, R., Cano, J. & Cano, J. (2008b). *Actinomyces elegans* var. *kuwaitiensis* isolated from the wound of a diabetic patient. *Antonie van Leeuwenhoek* **94**, 343–352.
- Kocsube, S., Toth, M., Vagvolgyi, C., Doczi, I., Pesti, M., Pocsi, I., Szabo, J. & Varga, J. (2007). Occurrence and genetic variability of *Candida parapsilosis* sensu lato in Hungary. *J Med Microbiol* **56**, 190–195.
- Kurtzman, C. P. & Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* **35**, 1216–1223.
- Lasker, B. A., Butler, G. & Lott, T. J. (2006). Molecular genotyping of *Candida parapsilosis* group I clinical isolates by analysis of polymorphic microsatellite markers. *J Clin Microbiol* **44**, 750–759.
- Lin, D., Wu, L. C., Rinaldi, M. G. & Lehman, P. F. (1995). Three distinct genotypes within *Candida parapsilosis* from clinical sources. *J Clin Microbiol* **33**, 1815–1821.
- Lockhart, S. R., Messer, S. A., Pfaller, M. A. & Diekema, D. J. (2008a). Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. *J Clin Microbiol* **46**, 2659–2664.
- Lockhart, S. R., Messer, S. A., Pfaller, M. A. & Diekema, D. J. (2008b). *Lodderomyces elongisporus* masquerading as *Candida parapsilosis* as a cause of bloodstream infections. *J Clin Microbiol* **46**, 374–376.
- Mokaddas, E. M., Al-Sweih, N. A. & Khan, Z. U. (2007). Species distribution and antifungal susceptibility of *Candida* bloodstream isolates in Kuwait: a 10-year study. *J Med Microbiol* **56**, 255–259.
- Pappas, P. G., Rex, J. H., Lee, J., Hamill, R. J., Larsen, R. A., Powderly, W., Kauffman, C. A., Hyslop, N., Mangino, J. E. & other authors (2003). A

prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin Infect Dis* **37**, 634–643.

Pfaller, M. A. & Diekema, D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**, 133–163.

Rodriguez-Tudela, J. L., Gomez-Lopez, A., Alastruey-Izquierdo, A., Mellado, E., Bernal-Martinez, L. & Cuenca-Estrella, M. (2007). Genotype distribution of clinical isolates of *Trichosporon asahii* based on sequencing of intergenic spacer 1. *Diagn Microbiol Infect Dis* **58**, 435–440.

Roy, B. & Meyer, S. A. (1998). Confirmation of the distinct genotype groups within the form species *Candida parapsilosis*. *J Clin Microbiol* **36**, 216–218.

Sugita, T., Nakajima, M., Ikeda, R., Matsushima, T. & Shinoda, T. (2002). Sequence analysis of the ribosomal DNA intergenic

spacer 1 regions of *Trichosporon* species. *J Clin Microbiol* **40**, 1826–1830.

Tavanti, A., Davidson, A. D., Gow, N. A. R., Maiden, M. C. J. & Odds, F. C. (2005). *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J Clin Microbiol* **43**, 284–292.

Tavanti, A., Hensgens, L. A. M., Ghelardi, E., Campa, M. & Senesi, S. (2007). Genotyping of *Candida orthopsilosis* clinical isolates by AFLP reveals genetic diversity among independent isolates and strain maintenance within the same patient. *J Clin Microbiol* **45**, 1455–1462.

Weems, J. J., Jr (1992). *Candida parapsilosis*: epidemiology, pathogenicity, clinical manifestations, and antimicrobial susceptibility. *Clin Infect Dis* **14**, 756–766.

Yong, P. V., Chong, P. P., Lau, L. Y., Yeoh, R. S. C. & Jamal, F. (2008). Molecular identification of *Candida orthopsilosis* isolated from blood culture. *Mycopathologia* **165**, 81–87.