MYCOLOGY

Single primer pair for PCR identification of *Candida parapsilosis* group I isolates

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A DNA fragment, isolated from a genomic DNA mini-library of *Candida parapsilosis* group I reference strain ATCC 22019, was sequenced and characterised. The fragment was first probed by Southern blotting against a pool of DNA from several yeasts. The hybridisation tests revealed that the probe was specific for strain ATCC 22019 and 49 (90.74%) of 54 *C. parapsilosis* clinical and soil isolates that were attributed to *C. parapsilosis* group I by the electrophoretic images of their restriction fragment length polymorphisms (RFLPs) and electrophoretic karyotype (EK). The remaining five negative isolates, according to the same criteria, were attributed to group II (one isolate) and III (four isolates). Two primers were selected from the probe DNA sequence and a PCR-based test was developed for specifically detecting *C. parapsilosis* group I isolates, which represent the majority of the common clinical isolates. The PCR assay confirmed the Southern hybridisation results. This PCR assay could be a simple and reproducible tool for the rapid, sensitive and species-specific identification of *C. parapsilosis* major group I isolates.

Introduction

In recent years, *Candida parapsilosis* has become increasingly important as a cause of fungaemia or endocarditis [1–4] affecting neutropenic and severely debilitated patients receiving parenteral nutrition [4–6]. Roles for *C. parapsilosis* have been reported also in human vaginitis [7] and lesions of the skin and in cases of onychomycosis [4]. Furthermore, this species produces a biofilm [8, 9], which has been suggested as a virulence marker for bacteria [10]. *C. parapsilosis* isolates have been classified into three genotypically heterogeneous groups by restriction fragment length polymorphism (RFLP) analysis [11]. This grouping was confirmed by random amplified polymorphic DNA (RAPD) analysis [12]. Subsequently, three genetically distinct groups were detected among clinical isolates by

iso-enzyme profile analysis and comparison of the internal transcribed spacer 1 (ITS1) region sequence flanking the 5.8S RNA gene [13]. Moreover, DNA–DNA re-association and DNA relatedness [14] have confirmed grouping and have affirmed definitively that these groups represent three different species.

Clinically, significant yeasts such as *C. parapsilosis* are commonly identified by standard biochemical kits, which analyse sugar fermentation or assimilation [15, 16]. Several authors have reported incomplete or incorrect identification of *C. parapsilosis* isolates from human specimens in studies comparing different commercial biochemical diagnostic kits [17–20]. This situation highlights the need to distinguish isolates belonging to the formally named species *C. parapsilosis* by rapid, sensitive and reproducible methods. Molecular methods, particularly PCR-based methods, seem to represent the best choice.

To address this need, a PCR-based tool has been developed for rapid, sensitive and species-specific detection of *C. parapsilosis* group I isolates, represented in the literature [13] and among common clinical isolates.

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Materials and methods

Organisms and culture conditions

Table 1 lists all the micro-organisms utilised in this work. The biochemical and pathogenic characters of the 46 clinical and soil isolates designated as SA, HEM and ISS have been described in previous studies [9, 21–23]. All the isolates were identified by standard established laboratory procedures as follows: morphological analysis on corn meal agar, germ-tube formation in serum, carbon source assimilation profile analysis by the ID 32C yeast identification system with an ATB reader (API Systems Vital, bioMérieux Italia, Rome, Italy) (Table 2), and agglutination tests with anti-*Candida* sera (*Candida* Check Kit, Iatron Laboratories, Tokyo, Japan). Yeast cells were grown to stationary phase in YPD medium containing yeast extract 1% w/v, peptone (Biolife Italiana, Milano, Italy) 2% w/v and D-glucose (Carlo Erba Reagenti, Milano, Italy) 2% w/v at 30°C in a horizontal shaking incubator (Gallenkamp). Bacteria used for cloning – *Escherichia coli* DH5 α (Stratagene, La Jolla, CA, USA) – were grown in LB medium with NaCl (Carlo Erba Reagenti) 1% w/v, peptone 1% w/v and yeast extract (Biolife Italiana) 0.5% w/v at 37°C in a horizontal shaking incubator.

DNA preparations

Yeast DNA was prepared as described previously [21]. Single colonies of each isolate were grown to stationary phase (as assessed by measurements of

optical density of cultures) in 10 ml of YPD medium at 30°C in 50-ml sterile disposable polypropylene tubes (30 × 115 mm). For spheroplast formation, the cells were washed in sterile distilled water and resuspended in 1.2 M D-sorbitol and 0.1 M EDTA, pH 7.5, with 25 μ l of Lyticase (4000 U/mg; Lyticase, Sigma) 5 mg/ml solution and incubated at 37°C for 1 h. The spheroplasts were centrifuged and incubated in lysis buffer (50 mM Tris-HCl, 20 mM EDTA and sodium dodecyl sulphate 1%, pH 7.5) for 30 min at 65°C. Then 1.5 ml of 5 M potassium acetate were added and the mixture was kept on ice for 1 h and then centrifuged. The pellet was discarded and the supernate was treated for DNA purification [24]. Plasmid mini-preparations from bacterial cells were performed with Qiagen DNA plasmid extraction kits according to the manufacturer's instructions (Qiagen GmbH, Hilde, Germany).

Construction of *C. parapsilosis* genomic mini-library

Genomic DNA (10 μ g) from *C. parapsilosis* ATCC 22019 was digested with *Hind*III (Boehringer Mannheim GmbH, Mannheim, Germany) and electrophoresed. The small-size portion of the resulting fragments, ranging in size from 564 to 2000 bp, was purified from an agarose 0.8% w/v gel (BioRad, Segrate, Milano, Italy) with a Jet-Sorb gel extraction kit (Genomed GmbH, Bad Oeynhamen, Germany) and ligated into the unique *Hind*III cloning site of p-Bluescript SK[–] (Stratagene) previously treated with calf intestinal alkaline phosphatase (CIP) (Stratagene) [25]. The ligation mixture was used for transforming *E. coli* DH5 α (Stratagene) competent cells in a Gene-Pulser (BioRad). Ampicillin-resistant transformants were selected on AMP (Boehringer Mannheim GmbH) 50 μ g/ml agar plates supplemented with X-gal (Bromo-4-chloro-3-indolyl- β -D-galactopyranoside) 40 μ g/ml and IPTG (isopropyl- β -D-galactopyranoside) (Boehringer Mannheim GmbH) 40 μ g/ml for white/blue phenotype screening of putative positive clones (50–100 total colonies for plates). Cells from white colonies were screened further by PCR with the M13 universal forward and –20 reverse primers (Stratagene) homologous to the polylinker region of p-Bluescript to assess the presence of the recombinant fragments. The white colonies containing recombinant plasmids, with *C. parapsilosis* inserts, were pooled to constitute the genomic mini-library.

Agarose plug preparation and electrophoretic karyotyping

Agarose plugs were prepared from all the yeast isolates (ATCC or clinical strains) as reported previously [23]. Samples were examined for electrophoretic karyotypes by pulsed-field electrophoresis with a CHEF Mapper (BioRad) [26]. The electrophoretic running conditions were: a pulse-time from 60 s to 500 s at 120 V constant voltage for 72 h in agarose 1% w/v gel (BioRad),

Table 1. Micro-organisms used in this study

Species	Source	Strain designation
<i>C. albicans</i>	ATCC	ATCC 10261 ATCC 90029
<i>C. krusei</i>	ATCC	ATCC 6258
<i>C. tropicalis</i>	ATCC	ATCC 750
<i>C. glabrata</i>	ATCC	ATCC 90030
<i>Blastoschizomyces capitatus</i>	ATCC	ATCC 62963 ATCC 62964
<i>Saccharomyces cerevisiae</i>	YPH*	YPH 499 YPH 501
<i>C. parapsilosis</i>	ATCC	ATCC 22019 ATCC 96140 ATCC 96144
	CpP†	CpP1, CpP2, CpP3, CpP6, CpPFA, pPGH, CpP5, CpPO
	ISS‡	Soil isolates (3)
	SA	Vaginal isolates (20)
	HEM	Blood isolates (23)
<i>E. coli</i>	...	DH5 α †

ATCC, American Type Culture Collection.

*From Stratagene.

†*C. parapsilosis* isolates from Pisa University, Pisa, Italy: CpP 1, 2, 3, 6, FA, GH from Neonatology Care Unit, CpP1 and CpP2 isolated from the same patient, conjunctival buffer and blood, respectively; CpP5 from a patient in the Infectious Disease Unit; CpPO from a wash-basin in the Ophthalmology Department.

‡Isolates ISS, SA and HEM were analysed previously for genetic and phenotypic characters [9, 22, 23].

Table 2. Attribution of the ID 32C code to *C. parapsilosis* isolates

ID 32C Code	Soil isolates	Vaginal isolates	Blood isolates	ATCC strains	Pisa strains
55473503-17	ISS 4879, ISS 4884	SA 17, SA 19, SA 23, SA 28, SA 36, SA 38, SA 42, SA 197, SA 297, SA 355, SA 365, SA 381, SA 392, SA 393, SA 447, SA 461	HEM 1, HEM 2, HEM 3, HEM 4, HEM 9, HEM 10, HEM 11, HEM 12, HEM 13, HEM 14, HEM 15, HEM 18, HEM 19, HEM 20, HEM 21, HEM 23, HEM 24, HEM 25, HEM 26	ATCC 22019, ATCC 96140	CpP6
55473503-13					FA
55473503-15		SA 139	HEM 8, HEM 17		
55473507-17			HEM 22		
55473507-13			HEM 16		
55477503-15		SA 383			
55477503-17		SA 342			GH
55413507	ISS 4888	SA 25			
55443501-15				ATCC 96144	CpPO, CpP1, CpP2, CpP3, CpP5

Isolates found to be negative in hybridisation or PCR tests, or both, are shown in bold type.

running buffer $0.5 \times$ TBE, 45 mM Tris base, 45 mM boric acid and 1 mM EDTA at a constant temperature of 14°C. Images of the gels were obtained by Gel-Doc 2000 (BioRad). CHEF gels, previously treated for 10 min with 0.2 M HCl, were denatured in 0.5 M NaOH, 1.5 M NaCl, neutralised in 1.5 M NaCl, 0.5 M Tris-HCl, 0.1 mM EDTA, pH 7.2, and transferred to a nylon Hybond-N membrane (Amersham Italia, Milan, Italy) in $10 \times$ SSC, 1.5 M NaCl, 165 mM tri-sodium citrate 2-hydrate, pH 7, for Southern hybridisation [25].

HindIII restriction endonuclease digestion

For each isolate, 5–6 µg of DNA were digested with *HindIII* (Boehringer Mannheim GmbH) and electrophoresed in agarose 1% w/v gel (BioRad) in $1 \times$ TBE buffer, 89 mM Tris-base, 89 mM boric acid and 2 mM EDTA. Gels were photographed by Gel-Doc 2000, denatured in 0.5 M NaOH, 1.5 M NaCl, neutralised in 1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.2, and transferred to a nylon Hybond-N membrane in $10 \times$ SSC, 1.5 M NaCl, 0.165 M tri-sodium citrate 2-hydrate, pH 7, for Southern hybridisation [25].

Southern blot hybridisation analysis

Positive clones from the constructed genomic mini-library were used in hybridisation experiments to test their species-specificity for *C. parapsilosis* group I isolates. Plasmids containing fragments were digested with *HindIII* to excise the inserts and these, after electrophoresis, were purified from agarose (BioRad) 0.8% w/v gel in a Jet-Sorb gel extraction kit (Genomed GmbH). The fragments were radiolabelled with [α - 32 P]dCTP (Amersham Italia) by random primer extension reaction with a Rediprime II labelling kit (Amersham Italia) as recommended by the manufacturer and used in high stringency hybridisation conditions to probe the electrophoretic karyotype (EK) and the restriction endonuclease analysis (REA) constructed Southern blots for all strains. One of the

fragments, found to be specific for *C. parapsilosis* group I, was selected for sequence analysis because it had a short sequence and appeared not to be repetitive, but was a single copy sequence and did not correspond to the size of *HindIII* mtDNA fragments analysed previously [21]. The plasmid containing this fragment was named pBHCpC1 (p-Bluescript *HindIII* *C. parapsilosis* clone 1).

Sequence analysis

The complete DNA sequence of the CpC1 fragment was obtained by using the M13 universal forward and –20 reverse primers reading the p-Bluescript (SK-) plasmid sequence from the polylinker. The primers were end-labelled (10 pmol) with [γ - 32 P]ATP and T4 polynucleotide kinase (HT Biothechnology, Cambridge) and used in PCR sequence reactions in a Thermo SequenaseTM cycle sequencing kit (Amersham Pharmacia Biotech, Cleveland, OH, USA). To complete the sequence, new oligodeoxynucleotides (Gibco, Life Technologies Italia, San Giuliano Milanese, Milano Italy) were selected from the sequenced regions of the fragment: Fw1 (5'-GTG AGA GAT GTT TGT AAT TGC GAG AAC C-3', bp 103–130), Rv1 (5'-GCA TTA TTC GGA CGT TCA ACC CG-3', bp 775–753), Rv2 (5'-CCC TAA CAG TAA TAA CGC TC-3', bp 514–495), Rv3 (5'-GCT CAA GCA ATG GAA GAC AGG TTG G-3', bp 664–639).

Characterisation of CpC1

The DNA fragment was sequenced and characterised (Fig. 1) and found to be 800 bp in size. This sequence, checked by Blast v. 2 search [27], did not exhibit homology with other known sequences. The sequence was analysed with OMIGA v.1.1.3 (Oxford Molecular, Oxford). A GA repeat and possible TATA and CCAAT boxes were found (these sites are underlined in Fig. 1), but no relevant ORFs were found. The sequence was submitted to GenBank: accession no. AF207607.

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1-   AAGCTTTATG AAATATTATT ATTACATGTG CAATTAATAC GTAATAAACC GGAAAAAAG
61-   GGGAGAAAGT AGATTCAATT TTGTGTGGGG GGAGAAAGAG GAGTGAGAGA TGTTTGTAAT
121-  TGCAGAAACC AAATGGAATA ACTTTTGGTT GTTCCAATTA CTATGACATT ATCATCAAGC
181-  TATGACACTC TTTGAAAGTT CGTCAATTGA GAGACTCTAT GTCTCATTAT AATAGAAGAT
241-  GCATTTCGGT AGGTTAAAAA GTATGCTGTA AGATTAGGTT GGTACTGCAT ATAAACTTTT
301-  GTTGGTCCTT TTTTTTTTTT CATTTCGTGA GACACAAAGA TTGTGTTACT TAGGCAGATG
361-  AAGTTGATAA AGAATCAATT GACAAGAGGT AAAGATGTGG TGAGTGATAG TACCTCCCGT
421-  GTACTACTCA AAGAAGCCTT CATGTAAAAA GACCATGAAT TAAAGTGATT CTTGACTATT
481-  CAATCGTGTT CATTGAGCGT TATTACTGTT AGGGTATTTA TTTACGAAGT TTTAAATGAT
541-  CTTGTGAGTG TTATATACTA ATTGTCCACA AATACACTAC CTTTTCGTAA ATTCACAATT
601-  TGCATGTGAA AAAATAAACC ATACTCATTG CTTTTCGAAC CAACCTGTCT TCCATTGCTT
661-  GAGCAACAGA TCTATTCAAA TATTGATCAG CAAGAGTTGT TCACATAATT CACCACTACT
721-  TTTTCTAAT TCAGAAGTAT TGAAGTATGT TACGGGTTGA ACGTCCGAAT AATGCAGGAC
781-  AAATAAACC CTTTAAGCTT

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Fig. 1. The complete 800-bp DNA sequence of the CpC1 fragment. Underlined text indicates the CCAAT and TATA hypothetical boxes and the GA repeat.

PCR analysis

DNA from each isolate (100 μ g) was added to a PCR master mix consisting of 5 μ l of 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂, 0.5 M KCl, 1% Triton X-100, 0.1% w/v as stabilizer), 7 μ l of a mixture of 178 μ M deoxynucleoside triphosphates (equimolar concentration of each dATP, dCTP, dGTP and dTTP), 50 pmol of the primers Fw1 and Rv1 and 1 U of Super *Taq* (HT Biotechnology) adjusted to a final volume of 50 μ l with sterile distilled water. PCR was performed in a Techne Thermal Cycler (Progene Techne, Duxford Cambridge) with 3 min initial denaturation at 94°C and 25 cycles as follows: 1 min at 94°C, 1 min at 65°C and 1 min and 30 s at 72°C. The last cycle was followed by a supplemental 7 min extension at 72°C and then cooled to 10°C. PCR products (6.5 μ l) were electrophoresed in agarose 1.2% w/v gel (BioRad) in 1 $\times$ TBE buffer and images were captured by Gel-Doc 2000. Gels denatured with 0.5 M NaOH, 1.5 M NaCl and neutralised with 0.5 M Tris

HCl, 1.5 M NaCl and 0.1 mM EDTA, pH 7.2, were transferred on to Hybond-N nylon membrane by capillarity (10 $\times$ SSC). The internal primer Rv2 was end-labelled (300 pmol) with [γ -³²P]ATP with T4 polynucleotide kinase (HT Biotechnology) and used as a hybridisation probe to control the species-specificity of the PCR test confirming the presence or absence of the expected fragments.

Results

Southern blot analysis

Hybridisation with the probe CpC1 on to Southern blots of EK and REA was positive for strain ATCC 22019, and for 49 (90.74%) of 54 *C. parapsilosis* clinical and soil isolates (Figs. 2 and 3). ATCC 96140 and ATCC 96144 reference strains of *C. parapsilosis* group II and III were not detected. The five negative isolates in the *C. parapsilosis* pool were strains

Fig. 2. CHEF gel and hybridisation results obtained with representative isolates. The ATCC representative strains of the three groups are compared with several of the *C. parapsilosis* isolates screened (see Table 1 for complete list). The arrow indicates the chromosomal band positive to hybridisation. Note the faint lane of strain SA 197, which corresponds to a faint hybridisation band.

Fig. 3. *Hind*III RFLP gel and hybridisation results of representative isolates of the whole pool tested (see Table 1 for complete list). The size marker is λ /*Hind*III.

HEM16, HEM22, SA139, SA383 and CpP5. All the other species of *Candida* or yeasts tested and DNA from *Homo sapiens* gave negative results (Fig. 3). The positive hybridisation signal detected by the probe on to Southern blots of CHEF gels, under the electrophoretic conditions employed, seems to correspond to the third major chromosomal band of all the positive isolates. Hybridisation of the probe on REA Southern blots revealed a positive band of 800 bp for the expected isolates (Fig. 3). By comparison of EK or *Hind*III REA profiles, or both, it was possible to attribute strain HEM 22 to group II and strains SA 139, SA 383, HEM 16 and CpP5 to group III.

PCR analysis

Primers Fw1 and Rv1 were selected for PCR analysis. The expected fragment of 673 bp was detected only for strain ATCC 22019 (Fig. 4) and 49 (90.74%) of 54 clinical and soil isolates, confirming the Southern hybridisation experiments. Strains SA 139, SA 383, HEM 16, HEM 22 and CpP5 gave negative results (Fig. 5). The two strains ATCC 96140 and ATCC 96144, representative of *C. parapsilosis* groups II and III respectively, and the other *Candida* or yeast species

and human DNA also gave negative results (Fig. 4). The results of PCR tests were confirmed by oligo-hybridisation with the primer Rv2 internal to the sequence of Fw1-Rv1 fragment (Fig. 4).

Discussion

C. parapsilosis is an ever-increasing cause of fungaemia in patients with various disorders [1–5] or undergoing hyperalimentation therapy employing central venous catheters (CVC) [1, 6]. In some epidemiological studies this fungus represented the most prevalent cause of candidaemia with respect to other *Candida* spp. [1]. The species *C. parapsilosis* is normally identified by commercial biochemical kits. Several authors have reported misidentification of *C. parapsilosis* from clinical specimens in studies comparing these different kits [17–20]. This species can be misidentified as other *Candida* spp. or as a yeast belonging to another genus [17–20]. Moreover, the formally named species *C. parapsilosis* consists of three distinct genetic groups [13, 14] represented by three ATCC strains (22019, 96140 and 96144, respectively). *C. parapsilosis* isolates can be assigned

Fig. 4. Representative PCR test. (a) ATCC strains representative of the three groups of *C. parapsilosis*, the negative control (Nc) and several of the *C. parapsilosis* isolates screened are evident. (b) The same PCR on ATCC strains of other *Candida* or *Blastoschizomyces capitatus* isolates; the relative negative control is shown in (a). The size marker is $\Phi \times 174/\text{HaeIII}$.

to one of the three groups by sequencing the internally transcribed spacer region 1 (ITS1) flanking the 5.8S RNA gene [13], or by analysing the nuclear DNA base composition, DNA–DNA re-association and DNA RFLPs [14]. The comparison of DNA relatedness has clarified that the three groups are three distinct species, with <25% DNA identity between them [14]. In the light of this information it is paramount that clinically significant yeasts are identified accurately and that the characterisation of *C. parapsilosis* strains at a biotype and clonal level will be very useful for epidemiological studies. Molecular methods [13, 14] appear to discriminate between the three groups, but do not appear to be suitable for routine use. The PCR test described here represents a reproducible and, compared with the previously reported molecular methods [13, 14], a more rapid and easier tool for identifying *C. parapsilosis* group I isolates. The PCR test has enabled the reproducible identification of all isolates belonging to *C. parapsilosis* group I. Most clinical isolates appear to be incorporated in this group, accounting for 90.74% in the present study and for 73.33% of *C. parapsilosis*

isolates analysed by Lin *et al.* [13]. The former study found that group III contained more isolates (four isolates) with respect to group II (one isolate), unlike the reported data of Lin *et al.* [13] (10 isolates in group II and 2 isolates in group III). This finding could suggest regional or local differences in the distribution of these two minor groups. The development of a PCR test for complete identification of all the three groups (species) is necessary for a correct diagnosis and characterisation of pathological characteristics.

The present study did not analyse phenotypic or epidemiological differences among the isolates, being concerned only with diagnostic aspects. On the basis of the data reported previously regarding the ISS, SA and HEM isolates of the pool analysed [9, 22] it can be concluded that no specific differences exist that indicate more pathogenicity in a particular group with respect to the others. However, group I is the only group that includes isolates that are not slime producers (all the isolates designed ISS and the majority of the SA isolates) [9], the four isolates of groups II and III

Fig. 5. All the 54 clinical and soil isolates of *C. parapsilosis* analysed are represented in these three panels; the relative negative control (Nc) is shown in (a). The size marker is $\Phi \times 174/HaeIII$.

were able to grow in agar plates supplemented with 5-fluorocytosine (25 μ l/ml) [22] and strains HEM 16 and HEM 22 (group III and II, respectively) were non-proteolytic for acid (aspartyl) proteinase secretion [22]. The three groups should be analysed further to determine their peculiar epidemiological and pathogenic characteristics.

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