

Construction and use of PCR primers from a 65 kDa mannoprotein gene for identification of *C. albicans*

S. Arancia, S. Sandini, A. Cassone, F. De Bernardis, R. La Valle*

Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy

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Abstract

A method for detection of *Candida albicans* in biological samples (blood, serum, urine) was developed by the use of polymerase chain reaction (PCR) amplification of a DNA fragment of a gene coding for a 65 kDa mannoprotein of *C. albicans* (CaMP65). The PCR amplifies a 220 bp fragments whose specificity for *C. albicans* was demonstrated by Southern blot with a non-radioactive probe, leading to the differentiation from all other yeast species or human and bacterial DNA. The sensitivity of this assay was 5–10 *C. albicans* cells per milliliter of biological sample.

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1. Introduction

The incidence of systemic candidiasis continues to increase worldwide in immunocompromised patients [1,2], being a particularly severe infectious complication in neutropenic patients undergoing antineoplastic chemotherapy or bone marrow transplant. In particular, candidemia has risen to become the fourth most frequent cause of blood stream infection, with a mortality rate of about 50% [3–5]. It requires an aggressive chemotherapeutic treatment, the outcome of which is at least partially dependent on early and accurate diagnosis. Diagnosis of invasive candidiasis is, however, very difficult [6–8]. In fact, most clinical signs and symptoms of disseminated candidiasis are non-specific and cultures are often negative or become positive too late. Blood cultures were cited as being negative in 56% of necropsy proven cases of disseminated candidiasis [9]. Consequently, many cases of invasive candidiasis continue to be diagnosed only by postmortem. Moreover, the use of invasive diagnostic techniques may not be permitted by the underlying conditions of these patients. Finally, there are also difficulties in discriminating invasive candidiasis from candidal colonization.

For all the reasons listed above, there is an increasing interest in the use of molecular-based methods of the diagnosis and prophylactic treatment of these infections. In fact, a rapid and accurate detection of few *Candida* cells in biological samples is extremely important. In some cases, the detection of even non-viable *Candida* cells in pathological materials may be of importance. The molecular-based technology that has the greatest potential to decrease the time required to diagnose fungal infections and, therefore, reduce the mortality associated with systemic candidiasis is based on the use of polymerase chain reaction (PCR). This technique can detect low amount of microbial nucleic acid in blood or tissue. During the last years, numerous studies have been performed to investigate the potential value of PCR in the diagnosis of invasive candidiasis, with variable results in terms of diagnostic accuracy [10–18]. In this study, we have used a rapid method for extraction of DNA from biological samples (urine, blood, serum) spiked with very low numbers of *Candida* cells. We are here proposing a rapid and highly sensitive method for *Candida albicans* identification based on PCR amplification of a 220 bp DNA fragment of a gene coding for a 65 kDa mannoprotein of *C. albicans* [19]. Previous studies have shown the relevance of this immunodominant antigen expressed in healthy human subjects [19,20]. The sensitivity of this assay was 5–10

* Corresponding author. Tel.: +39-6-49902808; fax: +39-6-49387112.
E-mail address: rlavalle@iss.it (R. La Valle).

Candida cells ml⁻¹ of biological sample. The specificity was demonstrated by Southern blot and sequence analysis of PCR product from different *Candida* species.

2. Materials and methods

2.1. Microbial strains

C. albicans, *C. krusei*, *C. kefyr*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, *C. tropicalis*, *Saccharomyces cerevisiae*, *Rhodotorula rubra*, *Cryptococcus neoformans*, *Aspergillus fumigatus* (as shown in Table 1) and different bacteria were used throughout this study. All yeast isolates were identified according to the established taxonomic procedures, inclusive of morphology on corn meal agar with 0.1% tween 80, germ tube test in serum, assimilation of carbon sources in the API 20 gallery system (Biomérieux, Marcis, l'Etoile, France) as previously described [21,22].

Bacterial isolates were identified by API system.

2.2. DNA preparation

Candida isolates were grown in YPD (yeast extract 10 mg ml⁻¹, peptone 20 mg ml⁻¹, dextrose 20 mg ml⁻¹ Becton Dickinson and Co., Sparks, MD 21152, USA) for 24 h in a shaker, at 28 °C.

The cells were washed twice by centrifugation (5000 rev min⁻¹, 15 min) with H₂O, then resuspended in SE buffer (1.2 M sorbitol, 0.1 M disodium EDTA, pH 8.0), supplemented with 0.1 mg ml⁻¹ proteinase K and 0.05 M dithiothreitol (DTT) (Sigma, St Louis, USA). After incubation for 1 h at room temperature (r.t.), the cells were washed in saline, rinsed in SE supplemented with 0.3 mg ml⁻¹ zymolase (Seikagaku, Tokyo, Japan) and incubated at 37 °C for 1 h. Finally, the cells were centrifuged at 1500 rev min⁻¹, 30 min and the protoplasts rinsed in 10 ml of NTE (0.1 M NaCl, 0.02 M Tris-HCl, 0.001 M EDTA, pH 7.5) (SIGMA), supplemented with 0.5% SDS, 0.1 mg ml⁻¹ proteinase K, and incubated at 37 °C overnight (o.n.).

Table 1

Genomic DNA	Number of strains tested	Number positive by PCR	% Positive
<i>C. albicans</i>	33	33	100
<i>C. parapsilosis</i>	9	0	0
<i>C. glabrata</i>	17	0	0
<i>C. guilliermondii</i>	6	0	0
<i>C. krusei</i>	8	0	0
<i>C. kefyr</i>	9	0	0
<i>C. tropicalis</i>	25	0	0
<i>S. cerevisiae</i>	4	0	0
<i>Rhodotorula</i>	1	0	0
<i>C. neoformans</i>	7	0	0
<i>A. fumigatus</i>	1	0	0

The DNA was extracted using 1 volume of phenol–chloroform, and after adding 0.1 volume of 3 M potassium acetate and 3 volumes of ethanol, the samples were precipitated o.n. at –20 °C, then centrifuged and rinsed in H₂O.

C. neoformans was grown in LB medium (10 mg ml⁻¹ tryptone, 5 mg ml⁻¹ NaCl, 5 mg ml⁻¹ yeast extract) (Becton Dickinson and Co.) at 30 °C with aeration to stationary phase (5 × 10⁷ cells ml⁻¹), centrifuged, resuspended in a solution containing 10 mM Tris, 50 mM EDTA, 10 mM DTT and incubated for 20 min under slow agitation, at r.t. After centrifugation, the pellet was dissolved in 0.04 M citric acid, 0.120 M Na₂HPO₄, 0.05 M EDTA pH 6.00, 1.2 M sorbitol, 0.005 M DTT, 2.4 mg ml⁻¹ Novozym 234 (Seikagaku) and incubated for 2 h at 37 °C. The cells were centrifuged at 1500 g for 10 min and the spheroplast were dissolved in 10 ml of NTE-SDS-proteinase K solution as described above and incubated at 37 °C o.n.

DNA extraction and precipitation were as described above for *Candida*. *Aspergillus* was grown at 30 °C for 24 h in 10 ml of YPD, centrifuged at 7000 g. The growth medium was carefully removed and the mycelia placed on nylon membrane. After rinsing with several volumes of sterile cold water, the mycelium (2 g) was transferred to a mortar. After addition of 1 g of clean glass beads and 10 ml of TE buffer (Tris 50 mM, EDTA 20 mM) pH 8.0, the mycelia were ground to the homogeneity and 4 ml of suspension were transferred to a 50 ml glass tube, and added with 5 ml of H₂O and 9 ml of phenol. After repeated shaking the sample was spun down at 7000 g for 10 min at r.t. The supernatant was then transferred into a new tube for DNA precipitation. This was done with 2.5 volumes of cold ethanol (–70 °C) for 1 h, centrifuged and the pellet was resuspended in H₂O.

For PCR application to biological samples, 1 ml of urine, blood and serum from a healthy human donor was spiked with 1 to 10 cells of *Candida* spp. The DNA was extracted from these samples using the GFX genomic and blood DNA purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. The DNA was eluted in 20 µl of H₂O and used as template in PCR.

2.3. Patient sera

Sequential antigenomic sera from three patients with underlying hematological malignancies and affected by histologically proven invasive candidiasis caused by *C. albicans* and five serum samples from healthy subjects were used to test the validity of the assay.

2.4. Primer selection

The two CaMP65-specific primers (Table 2) were chosen in the 5' region of CaMP65 that, among the sub-family

Table 2
Sequences and localization of Ca oligonucleotides

Oligo-nucleotide	5' to 3' sequence	Localization ^a	Gene
Ca 62	TCAGTGTGACAGAAAC	+221 +205	Ca MP65
Ca 64	ATGTTATTCAAGTCTTTC	+1 +18	CaMP65
Ca 63	ATGTTGATGATGTTGATG	+64 +80	CaMP65
P450-1	ATGACTGATCAAGAAATYGC	1021 – 1043	L1A1
P450-2	TAACCTGGAGAAACYAAAA	1370 – 1351	L1A1

^a Base numbers of the CaMP65 and L1A1 gene refer to the location within the published CaMP65 and P-450 L1A1 gene sequence of *Candida albicans*, respectively.

genes retrieved for coding sequence, is less conserved region of the gene [19].

They amplify a 220 bp DNA segment coding for the amino-terminal fragment of CaMP65 protein. As positive PCR controls we used P450-1 and P450-2, which amplify a fragment of cytochrome P450 lanosterol-14 α -demethylase gene (L1A1) [13,23].

2.5. Polymerase chain amplification reaction (PCR)

The PCR reaction was performed in a volume of 25 μ l containing 50 mM KCl, 50 mM Tris pH 9, 2 mM MgCl₂, 200 μ M of deoxi-nucleotide, 50 pmol of each primer and 1 U Taq polymerase (Perkin Elmer Roche, Branchburg, NJ, USA).

Samples were added and PCR was performed in an automated thermal cycler (Perkin Elmer 9600) using the following protocol: one cycle with initial denaturation at 94 °C for 3 min, annealing at 59 °C for 1 min and extension at 72 °C for 1 min and 30 s; 40 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and 30 s. Control PCR reactions were performed by omission of template (negative) or by using (positive) cytochrome P450 gene (L1A1) oligonucleotide primers instead of CaMP65 primers. P450 oligos were used as previously described [13].

2.6. Southern blotting

After PCR, the 25 μ l reaction samples were electrophoresed on a 1.5% agarose gel with 5 μ g ml⁻¹ ethidium bromide. The gels were run at 10 V cm⁻¹ for 1 h, blotted onto nylon membrane (Hybond N, Amersham Pharmacia Biotech Inc.) as described by the manufacturer. The filters were hybridized with digoxigenin (DIG) labelled Ca63 oligonucleotide and reacted with anti-DIG AP-conjugate (1:5000). The reaction was developed using AP substrates NBT-BCIP (Nitro blue tetrazolium and 5-bromo-4chloro-3-indolyl phosphate Sigma).

3. Results

3.1. PCR detection of the MP65 gene in *Candida* spp

Various isolates of *Candida* species and other yeasts (Table 1) were assayed by PCR for the presence of the target CaMP65 gene sequence. Purified fungal genomic DNA (0.1 μ g) was used as template in these experiments. Under the reaction conditions described, an amplification product of the expected size (220 bp) was observed in all *C. albicans* isolates only, as none of all other *Candida* spp. tested (Fig. 1(a)) or other yeast, bacterial, murine, human and protozoan DNAs (not shown) displayed any amplification product. The identity of the CaMP65 amplified product obtained with DNA extracted from *C. albicans* cells was confirmed by Southern blot hybridization by using a DIG-labelled specific oligonucleotide probe (Fig. 1(b)). On the contrary, all *Candida* isolates showed positive amplification product when oligonucleotide primers of P450-1 and P450-2 (Fig. 1(c)) were used as positive control.

To further confirm the specificity of the reaction, the amplified fragment was purified and sequenced, revealing the already known CaMP65 sequence [19].

3.2. Sensitivity of *C. albicans* DNA detection in simulated biological samples

The experiments described above showed the specificity of the method on purified genomic DNA. We used the same PCR assay to detect *C. albicans* in simulated samples (blood, serum, urine), by spiking each sample with known numbers of fungal cells, then extracting the DNA as described in Section 2. By this method, the lowest threshold detection of *C. albicans* burden was 5 cells ml⁻¹ in each biological material, and it was possible to achieve the result in less than 4 h (Fig. 2(a)–(c)).

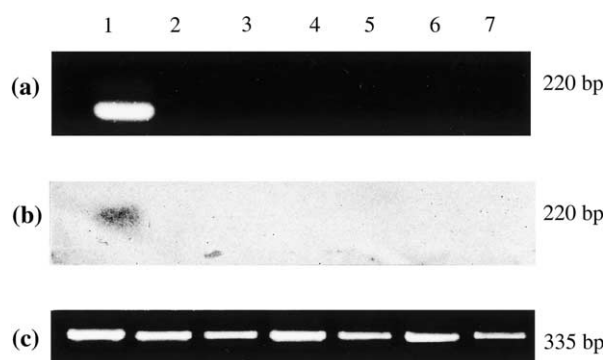


Fig. 1. *Candida albicans* identification based on PCR amplification of CaMP65 gene fragment. (a) Ca62–Ca64 PCR amplification products of *C. albicans* (lane 1), *C. parapsilosis* (2), *C. glabrata* (3), *C. guilliermondii* (4), *C. krusei* (5), *C. kefyr* (6), *C. tropicalis* (7). (b) Southern blot of panel (a) samples hybridized with digoxigenin labelled Ca63 oligonucleotide as described in Materials and Methods. (c) L1A1 gene PCR amplification products of purified DNA as in (a) electrophoresed on 1.5% agarose gel.

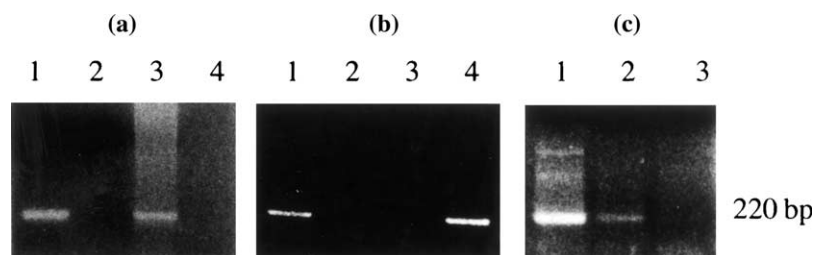


Fig. 2. PCR assay for detection of CaMP65 gene in *C. albicans* in simulated biological samples. (a) *Blood*. 100 ng of *C. albicans* 5314 DNA as positive control (lane 1), blood without cells (2), blood with 10 cells of *C. albicans* (3), H₂O as negative control (4). (b) *Serum*. 100 ng of *C. albicans* 5314 DNA as positive control (lane 1), serum without cells (2), H₂O as negative control (3), serum with 10 cells of *C. albicans* (4). (c) *Urine*. urine with 100 cells of *C. albicans* (lane 1), 10 cells (2), 5 cells (3).

3.3. PCR detection and Southern blotting of *Candida* DNA in clinical samples

Based on the data of the previous sections, which demonstrated the high sensitivity of a rapid PCR method on spiked, simulated biological samples, we used the above procedure in an attempt to detect *C. albicans* in clinical samples. Eight different sera from three patients with histologically proven invasive candidiasis and from five healthy subjects [24,25] were assayed by PCR for the presence of the target CaMP65 gene sequence.

As shown in Fig. 3(a), each serum from patients with invasive candidiasis gave an amplification product of the expected size. In contrast, no amplification was observed with the serum from all the healthy subjects examined. The amplicon identity was confirmed by Southern blot hybridization. (Fig. 3(b)).

4. Discussion and conclusions

The rising number of invasive candidiasis is directly related to the number of iatrogenically compromised hosts who are at greatest risk for these infections. Candidemia is the fourth commonest cause of nosocomial bloodstream infection [3,5]. Mortality rates among patients with

candidemia remain high, with estimates varying from 38 to 80% [5]. Early initiation of antifungal therapy is critical in reducing the mortality of disseminated candidiasis [16]. Therefore, the aim for molecular-based diagnosis tests is to provide evidence of infection more rapidly than occurs with conventional methods, thus obtain a positive results in patients with candidemia whose blood cultures remain consistently negative.

As emphasized elsewhere [10–13] the use of PCR for detection of pathogenic opportunistic yeasts provide a potentially more specific sensitive and rapid test respect to the current microbiological, biochemical and serological diagnostic methods. Consequently, several methods have been proposed both for genus and species identification. A prerequisite for the use of this technique in the routine clinical laboratory, however, is its fastness, more sensitivity and specificity.

PCR application to the clinical diagnostic laboratory may be particularly relevant for the rapid and sensitive detection of *C. albicans*, which remains the most prevalent pathogenic *Candida* species. Direct identification at species level is also important since the different *Candida* species may widely vary in their susceptibility to antimycotics.

In this paper, we describe a PCR assay for detection of *C. albicans* MP65, a recently cloned gene encoding a mannoprotein of important biological significance [19]. The primers were able to amplify *C. albicans* DNA (100% positivity) with a band of expected size (220 bp), whereas the DNA of all other yeast isolates belonging either to non-*albicans* species of the genus or other fungal genera (*Saccharomyces*, *Cryptococcus*, *Rhodotorula*, *Aspergillus*) were not amplified. At the same time, no amplicons were detected with several bacterial and human templates.

The method described in this study is simple, rapid, sensitive and reproducible. The total time required for the procedure is less than 4 h, and a high methodological sensitivity (5–10 cells for ml of biological sample) was observed in simulated clinical samples. Although this sensitivity limit was estimated in spiked, simulated samples, it favourably compares with detection limit of other PCR-based methods [10–13]. In particular, it appears to be more sensitive than our previously proposed method based on detection of *C. albicans* heat shock protein of 70 kDa [14].

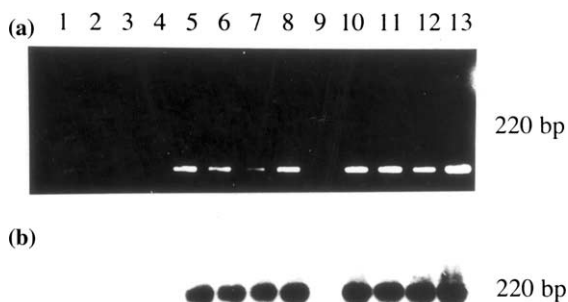


Fig. 3. *Candida albicans* identification based on PCR amplification of CaMP65 gene fragment in clinical samples. (a) Ca62–Ca64 PCR amplification product of *C. albicans* from sera in healthy subject (lane 1–4), sera from patients (5–8), sera in healthy subjects (9), sera from patients (10–13). (b) Southern blot of panel (a): the samples were hybridized with digoxigenin labelled Ca63 oligonucleotide as described in Section 2.

Additionally, no appreciable sensitivity differences were noticed among the different biological samples (urine or blood or serum). Importantly, the method gave 100% positivity with clinical sample from patients with proven invasive candidiasis without positive blood cultures by *C. albicans*. Although the numbers are certainly few (three patients), it is of relevance that *C. albicans* MP65 DNA was detected in all sequential serum samples from the three patients and we found some correlation between the value of mannemia previously detected in the sera and PCR positivity [24,25] (data not shown).

In summary, we have developed a PCR method based on MP65 primers which has shown to be prospectively useful for clinical diagnosis of *C. albicans* in relevant biological samples such as blood, serum and urine. Further investigation with a high number of samples from patients with candidiasis may help assess the real clinical significance of the proposed methods for aiding patient management in cases where invasive candidiasis is suspected.

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