



Construction and use of PCR primers from a 70 kDa heat shock protein gene for identification of *Candida albicans*

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Methods for detection of *Candida albicans* in culture or biological samples were developed by the use of polymerase chain reaction (PCR) with oligonucleotide primers from *C. albicans* 70 kDa heat shock protein gene (Cahsp70). The PCR amplifies a 335-base pair fragment which is then hybridized with a non-radioactive probe, leading to the specific identification of *C. albicans* and its differentiation from all other human pathogenic *Candida* and/or yeast species. *Candida albicans* could be rapidly detected in human urine and blood, with a sensitivity of 10 and 50 fungal cells per sample, respectively.

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KEYWORDS: *Candida albicans*, polymerase chain reaction, molecular diagnosis, epidemiology.

INTRODUCTION

Candida albicans is the primary agent of presently widespread opportunistic yeast infections.^{1–4} It is routinely identified following isolation in pure culture and examination of morphological, biochemical and serological properties. This examination usually requires sufficient cell viability, a few days period and, for the assessment of some morphological properties, a degree of subjectivity. Clinical problems arise for high-risk patients in the setting of neutropenia and invasive diagnostic procedures, where rapid and accurate detection of few *Candida* cells is extremely important.⁴ In some cases, the detection of even non-viable *Candida* cells in pathological materials may be of importance.¹

Rapid polymerase chain reaction (PCR)-based identification assays for several fungal groups including *Candida* spp.^{5–8} have been described. In general, the assay requires a combination of different PCR primers, restriction enzymes and hybridization probes, most often with radioactive tracers.

We have developed an approach for rapid *C. albicans* identification based on the amplification of specific sequences of the gene coding for one member of the 70 kDa heat shock protein family (CaHSP70).⁹ The relevant Cahsp70 gene contains not only sequences that are highly conserved between *C. albicans* and HSP70 genes of other organisms but also less conserved and variable sequences, inside and adjacent, respectively, to the open reading frame that may allow sufficient interspecies discrimination.¹⁰ Both cultures and biological samples containing low numbers of fungal cells were studied.

MATERIALS AND METHODS

Yeast strains

Candida albicans (47 isolates); *C. krusei* (7 isolates); *C. kefyr* (6 isolates) *C. parapsilosis* (7 isolates); *C.*

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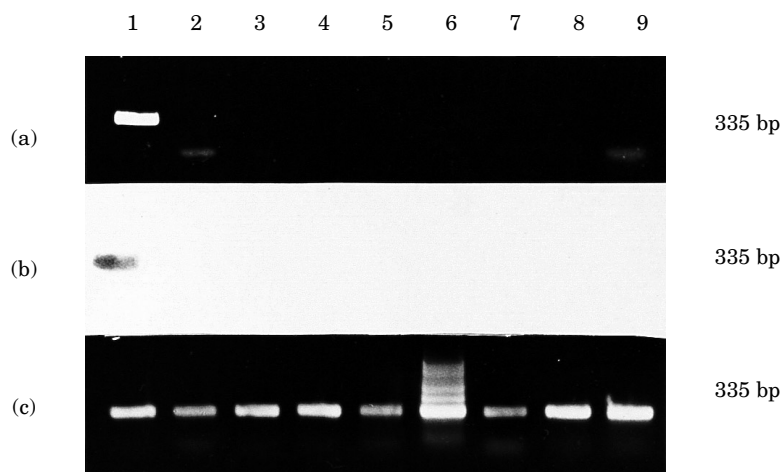


Fig. 1. *Candida albicans* identification based on PCR amplification of CaHSP70 gene fragment. (a) Ca2-Ca3 PCR amplification products of *C. albicans* (lane 1), *C. parapsilosis* (2), *C. glabrata* (3), *C. guilliermondii* (4), *C. krusei* (5), *C. kefyr* (6), *C. tropicalis* (7), *Rhodotorula rubra* (8) and *Cryptococcus neoformans* (9) purified DNAs electrophoresed on 1.5% agarose gel. (b) Southern blot of panel (a) samples hybridized with fluorescein-conjugate Ca11 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.

glabrata (6 isolates); *C. guilliermondii* (2 isolates); *C. tropicalis* (7 isolates); *Rhodotorula rubra* (2 isolates); *Cryptococcus neoformans* (2 isolates); and *Saccharomyces cerevisiae* (2 isolates) were used throughout this study. Table 1 summarizes the clinical source and some differential features of these isolates. All isolates had been 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 20C gallery system (Biomérieux, Marcy, l'Etoile, France) and agglutination with adsorbed anti-*Candida* sera (*Candida* check kit, latron, Tokyo, Japan) as previously described.^{11,12}

Sample preparation

Candida isolates were grown in YPD (yeast extract 10 mg ml⁻¹, peptone 20 mg ml⁻¹, dextrose 20 mg ml⁻¹) for 24 h in a shaker, at 28°C. The cells were washed by centrifugation twice (5000 rev min⁻¹, 15 min) with H₂O, then resuspended in SE buffer (1.2 M sorbitol, 0.1 M disodium EDTA, pH 8), supplemented with 0.1 mg ml⁻¹ proteinase K (Sigma, St. Louis, USA) and 0.05 M dithiothreitol (DTT). After incubation for 1 h at room temperature, the cells were washed in saline, rinsed in SE supplemented with 0.3 mg ml⁻¹ zymolyase (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), supplemented with

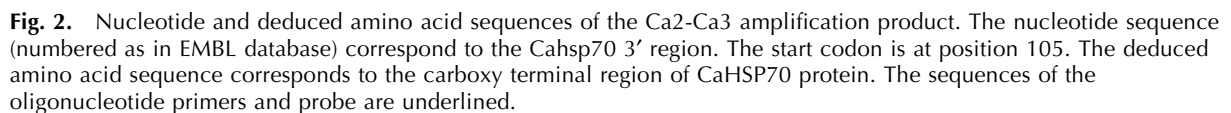
0.5% SDS, 0.1 mg ml⁻¹ Proteinase K, and incubated at 37°C overnight.

The DNA was extracted using phenol-chloroform, and after precipitation with 3 M Potassium acetate and three volumes of ethanol, the samples were centrifuged and rinsed in H₂O.

Cryptococcus neoformans was grown in LB medium (10 mg ml⁻¹ Tryptone, 5 mg ml⁻¹ NaCl, 5 mg ml⁻¹ yeast extract) 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 room temperature. After centrifugation, the pellet was dissolved in 0.04 M citric acid, 0.120 M Na₂HPO₄, 0.05 M EDTA pH 6, 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 spheroplasts were dissolved in 10 ml of NTE-SDS-Proteinase K solution as described above, and incubated at 37°C overnight. DNA extraction and precipitation were as described above. For PCR application to biological samples, human urine or blood samples (1 ml) were spiked with 5 to 1 × 10⁴ cells of *C. albicans* or *C. krusei*. The DNA was immediately extracted from these samples with DNA genomic Kit (Pharmacia Biotech, USA), eluted in 50 µl of H₂O, lyophilized, resuspended in 5 µl and used as template in PCR.

Primer selection

Database searches and sequence analysis were performed using the Apple FIIX computer system. For



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 dATP, dCTP, dTGP and dTTP (or 190 μ M dTTP, 10 mM DIG-11-dUTP for digoxigenin labelled PCR), 50 pmol of each primer and 1 U of *Taq* polymerase (Perkin Elmer Roche, Branchburg, NJ, USA). Samples of 100 ng of each yeast DNA (or their dilution or the DNA extracted from biological samples) were added and PCR was performed in an automated thermal cycler using the following protocol: initial denaturation at 94°C for 10 min followed by 40 cycles of denaturation at 94°C for 120 s; annealing at 60°C

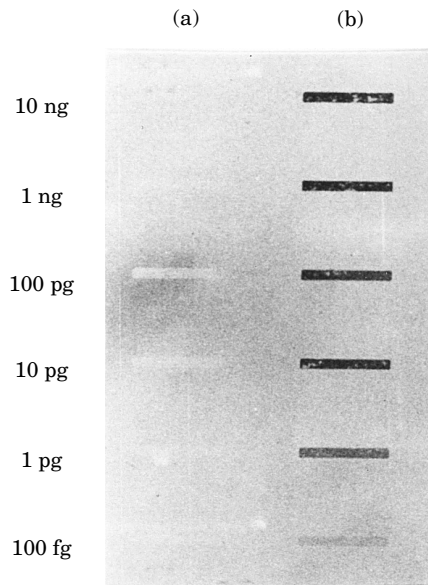


Fig. 3. Dot-blot of PCR amplification products for the detection of CaHSP70 gene in purified DNA. Ca2-Ca3 DIG-labelled PCR amplification products of *C. krusei* (a) and *C. albicans* (b) were dot-blotted onto nylon membrane, reacted with AP conjugate anti-DIG antibody and revealed with NBT-BCIP substrate. Different amounts of purified DNA (left) were used in PCR amplification.

for 120 s, extension at 72°C for 120 s and final extension step at 72°C for 10 min.

Control (negative) PCR reactions were performed by omission of either the polymerase or the template. In other controls, the specific primers were omitted. Positive PCR reaction control was done with cytochrome P450 lanosterol-14 α demethylase gene (L1A1) oligonucleotide primers (see above) instead of CaHSP70 primers (see also Table 2).

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 Vcm⁻¹ for 1 h, visualized using a u.v. transilluminator and blotted onto nitrocellulose membrane (Biorad, Richmond, CA, USA), as described by the manufacturer. The filters were hybridized with fluorescein conjugated Ca11 oligonucleotide probe and reacted with alkaline phosphatase (AP)-conjugated anti-fluorescein Fab fragment (1:5000 Boehringer Mannheim, Germany). The reaction was developed using AP substrates NBT or BCIP (Nitro Blue tetrazolium; 5-bromo-4-chloro-3-indolyl phosphate).

Sequence analysis

The Ca2-Ca3 amplified fragment was recovered from 1.5% agarose gel with Qiaquick gel extraction kit (Qiagen, Inc. Chatsworth, CA, USA) as from manufacturer's instructions. The fragment was sequenced by using amplycycle sequencing Kit (Perkin Elmer, Cetus).

Dot blot

A 25 μ l aliquot of each of the DIG-labelled PCR products was dot-blotted onto nylon membrane using Bio-dot apparatus (Biorad), reacted with AP conjugate anti-digoxigenin (DIG) antibody, and developed with AP-substrates.

Table 1. Species-specificities of genomic DNA PCR amplified with Ca2-Ca3 primers

Genomic DNA ^a	Source of isolation	Disease status	Number of strains tested	Number positive by PCR	% Positive
<i>C. albicans</i> serotype A	Vagina	Vaginitis and carriage	36	36	100
<i>C. albicans</i> serotype A	Oral cavity		4	4	100
<i>C. albicans</i> serotype A	Blood	Candidemia	4	4	100
<i>C. albicans</i> serotype B	Vagina	Vaginitis and carriage	5	5	100
<i>C. parapsilosis</i>	Vagina	Vaginitis and carriage	7	0	0
<i>C. tropicalis</i>	Vagina	Carriage	7	0	0
<i>C. krusei</i>	Vagina	Vaginitis and carriage	7	0	0
<i>C. kefyr</i>	Vagina		6	0	0
<i>C. glabrata</i>	Vagina	Vaginitis and carriage	6	0	0
<i>C. guilliermondii</i>	Vagina	Vaginitis	2	0	0
Other yeast species ^b			6	0	0

^a 0.1 μ g of purified DNA was PCR amplified as described in Material and Methods.

^b Including *Rhodotorula rubra*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, two isolates each.

Table 2. Sequence and localization of Ca oligonucleotides

Oligonucleotide	5' to 3' sequence	Localization	Gene
Ca2	GAAATGAAAGATAAGATTGGTGCA	1770–1793 ^a	CaHSP70 cds
Ca3	CCACAGTAAATTACCTATTTCTTCCTC	2104–2078 ^a	CaHSP70 non-cds
Ca11 ^b	FITC–TAGTGGTGCTTATGGT	1922–1937 ^a	CaHSP70 cds
P450 ₁	ATGACTGATCAAGAAATYGCTAA	1021–1043 ^c	cytochrome P-450-14 α demethylase (L1A1)
P450 ₂	TAACCTGGAGAAACYAAAAC	1370–1351 ^c	cytochrome P-450-14a demethylase (L1A1)

^a Numbered as in the EMBL database (accession number EMBL Z30210). The start codon of CaHSP70 is at position 105 and its stop codon is at nucleotide position 2073.

^b Ca11 is a Fluorescein isothiocyanate conjugate (FITC) oligonucleotide.

^c Sequence and base numbers of the L1A1 gene refer to the location within the published P-450 L1A1 gene sequence of *Candida albicans*.²⁵

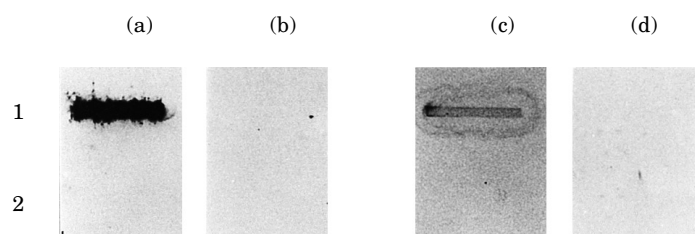


Fig. 4. PCR assay for detection of CaHSP70 gene in *Candida*-spiked biological samples by reverse dot-blot. Ca2–Ca3 DIG-labelled PCR amplification products of *C. albicans* (a and c) or *C. krusei* (b and d) (as a negative control) spiked human urine (a and b) or blood (c and d) were used as probes in hybridizations on *C. albicans* (1) and *C. krusei* (2) DNA dot-blotted onto nylon membranes.

Reverse dot blot

A 20 µg sample of *C. albicans* or *C. krusei* DNA was dot-blotted onto hybond-N membrane, denatured, neutralized and fixed according to the manufacturer's instructions. The filters were hybridized with Ca2–Ca3 DIG-labelled PCR product generated by amplification of biological samples, washed under stringent conditions and developed with NBT/BCIP chromogenic substrate after incubation with an AP-conjugate anti-DIG antibody DIG-DNA labelling and detection kit (Boehringer, Mannheim, Germany).

RESULTS

Design of the oligonucleotide primers

HSP are common and highly conserved genes among all living organisms. This homology is very extensive, so as to reach >90% in the aminoterminal coding region of *C. albicans* and *S. cerevisiae* HSP70. However, most of the substitutions are in the same domain (the C-terminal coding sequence). These sequence variations could confer species-specificity to selected oligonucleotides in PCR reaction.

Hence, for amplification of CaHSP70 gene, two

oligonucleotide primers (Ca2 and Ca3) were designed on the basis of the known sequence, their low homology to *S. cerevisiae* HSP70 genes, and their capacity to amplify the gene fragment which encompassed the C-terminal coding region of CaHSP70, with an expected size of 335 bp (Table 2).⁹

PCR detection of the CaHSP70 gene in *C. albicans* strains using purified genomic DNA

By using the above primers, 47 isolates of *C. albicans* from various sources were assayed by PCR for the presence of the target CaHSP70 gene sequence. Purified fungal genomic DNA (0.1 µg) was used as a template in these experiments. Under the reaction condition described above, an amplification product of the expected size (335 bp) was observed in all *C. albicans* isolates. The identity of this amplified product was confirmed by Southern blot hybridization (Fig. 1) and sequencing of the amplified fragment (Fig. 2). None of the other yeast isolates tested (Table 1) showed any amplification product under the conditions of the assay. No amplification product was observed in any of the control samples (see Methods). On the contrary, all yeast isolates gave a positive amplification product when oligonucleotide primers

of the cytochrome P450-14 α demethylase L1A1 gene, which has been shown to amplify DNA of *Candida* spp.,²⁴ was used as positive control.

Digoxigenin-labelled PCR

The experiments described above showed the specificity of our PCR approach to detect *C. albicans*. However, the method based on PCR alone was of low sensitivity if uncoupled from signal detection by hybridization. On the other hand, when hybridization with a radioactive or a fluoresceinated probe was used, the sensitivity increased but an approach based on PCR and Southern blotting hybridization required a minimum of 4 days. Thus, we explored the possibility to find an alternative, faster method with potentially easier implementation in the clinical laboratory. A PCR method, based on the use of DIG-labelled nucleotides in a dot-blot assay, requiring only 1 working day, was therefore addressed.

We assessed the sensitivity of this dot-blot method to detect CaHSP70 gene in *C. albicans* isolates by using, as a template, different amounts of purified genomic DNA from the strain ATCC 20955 (Fig. 3). A specific signal could be obtained using as little as 100 fg of DNA template, which approximates two equivalent diploid genomes of *C. albicans*.¹³ In these experiments, DNA of *C. krusei* (one of the PCR-negative species taken as control) gave signals only with 10 ng of DNA template (Fig. 3).

PCR detection of *C. albicans* in biological samples

Based on the data of the previous section, which demonstrated the high sensitivity of a rapid PCR assisted-amplification method with visualization of DIG-labelled amplicons in a dot-blot assay, we used the above method in an attempt to detect *C. albicans* in simulated biological samples. To ensure specificity to the dot-blot assay, we used as a probe DIG-labelled PCR product, generated by amplification of *Candida*-spiked biological samples with Ca2-Ca3 oligonucleotides, while *C. albicans* DNA was dot-blotted onto nylon membrane. As shown in Fig. 4 immobilized DNA from *C. albicans* but not that from *C. krusei* was specifically hybridized both in urine and blood samples. By this method, the lowest threshold detection of *C. albicans* burden was 10 and 50 cells ml⁻¹ in urine and blood, respectively.

DISCUSSION AND CONCLUSION

In this paper, we describe several PCR assays for detection of *C. albicans* CaHSP70 specific sequences. All these assays are based on the use of primers which specifically amplify a 335-bp fragment and detect DNA of representative, clinically-relevant *Candida* and other yeast species. The primers were able to amplify only from *C. albicans* DNA (100% positivity) whereas the DNA of all other yeast isolates belonging either to non-*albicans* species of *Candida* (35 strains) or other yeast genera (*Saccharomyces*, *Cryptococcus*, *Rhodotorula*) was not amplified.

Because of the relative insensitivity of the current microbiological, biochemical and serological diagnostic methods, and the length of time required to obtain results by these methods, the use of PCR for detection of pathogenic opportunistic yeasts has gained increased attention, and several methods have been proposed, both for genus and species discrimination.^{5-8,14-20,24}

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, in particular in the settings of critically-ill patients.²¹ Direct identification at species level is important since the different species of yeast pathogens may widely vary in their susceptibility to antimycotics and other therapeutic interventions, as well. Moreover, it is essential together with biotype determination for epidemiologic purposes.^{1,21,22} Although various methods have been proposed, none has yet gained a degree of routine acceptance. Most of the methods are indeed cumbersome, non-standardized and require radio-labelled probes for Southern blotting that greatly limits its potential acceptability in the clinical laboratory.^{7,17,19}

Among the various gene sequences used as amplicons for PCR, Matthews *et al.* resorted to those from a 90 kDa heat shock protein (hsp90), a protein whose degradation product of 47 kDa has been assumed to be an important protective antigen.^{18,20} To our knowledge, however, the present report is the first to show that sequences of a HSP70 gene can be used for specific PCR detection of *C. albicans*.

As for HSP90, HSP70 genes are largely homologous throughout all living organisms, thus they share most conserved sequences.^{9,10} In principle, therefore, HSP gene sequences would be unreliable for detection of micro-organisms at species level. There are, however, both inside the codifying HSP70 sequence and in closely adjacent regions, specific sequences that can be used for identification at single species level. An advantage of selecting HSP sequences is that these

genes are mostly expressed during stress, and it has been shown that they are usually increased in their expression during infection.¹⁰ Interestingly, the isolates of *C. albicans* studied here were from highly diverse clinical source, different serotype and biotype.^{12,22,23} This shows that the selected amplimers target a species-specific, biotype-indifferent region of *C. albicans* and thus can be used for rapid diagnosis of this fungus. It is also of some interest that our PCR method does not amplify *C. tropicalis* DNA, since it is known that this *Candida* species is very closely related to *C. albicans*.^{1,13}

Besides as a diagnostic tool for culture isolates of yeasts, PCR-based methods are expected to greatly strengthen the direct diagnosis of the fungal organism in biological samples. This would be of particular value in critically-ill patients where the decision to treat with an aggressive drug (such as, for instance, amphotericin B) must be balanced with the life-threatening conditions of the patient. For this decision, the selected method of identification requires a particularly high specificity and sensitivity, and rapid assessment. After several approaches with the use of either PCR coupled with Southern blotting or dot-blot methods, we found that the requirements above were well satisfied with reverse dot-blot, particularly for the detection of *C. albicans* in blood and urine. In fact, with the reverse dot-blot method described here, DNA from as few as 10 and 50 fungal cells could be specifically and reproducibly detected in human urine and blood, respectively. These figures compare quite well with the reported highest sensitivities of PCR of biological samples.⁸

Both the method of DNA extraction from the biological sample and the use of a relatively short DNA sequence (335 bp) as the target help explain the high sensitivity of *C. albicans* detection by our method. It should be noted, however, that our clinical specimens are simulated, and the method has not so far been employed with true clinical samples. Nonetheless, the whole technical conduct was highly reproducible in the simulated samples with constant detection of the threshold values of *Candida* cells. Finally, our method does not require the use of radioactive reagents, which are largely impractical for the clinical laboratory.

In summary, we have developed here several PCR methods based on Cahsp70 primers which could be useful either for epidemiological purpose or for clinical diagnosis of *C. albicans* in relevant biological samples such as blood and urine. Depending on the specific purpose, one or more of these methods could be selected. Particularly, our procedure of reverse dot-blot method may be considered as potentially relevant for its application in the clinical laboratory.

Further investigations with the use of clinical samples may help assess the real clinical significance of the proposed method, especially in the setting of critically-ill patients.

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