

PCR- Detection of *Candida albicans* in Blood Using a New Primer Pair to Diagnosis of Systemic Candidiasis

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Abstract

The opportunistic pathogen *C.albicans* is able to cause disseminated infections in immunocompromised patients. Microbiological methods for the diagnosis of invasive candidiasis have many problems including low sensitivity, requirement to invasive clinical sampling such as biopsies or multiple blood cultures and need to expertise laboratory stuff. Since PCR has proven to be a powerful tool in the early diagnosis of several infectious diseases, we applied this approach as a rapid and sensitive method in detection of *C.albicans* cells in blood samples, for establishment a clinically useful method in diagnosing systemic candidiasis. DNA were extracted from blood samples seeded by serially diluted *C.albicans* cells, by omitting WBC and RBC followed by enzymatic breaking of fungal cell wall and phenol – chlorophorm extraction and alcohol precipitation of DNA. A new primer pair was designed for PCR-amplification of a part of ribosomal RNA gene. The primer set was able to amplify all medically important *Candida* species. When PCR was performed for detection of purified DNA, the sensitivity of the method was about 1 picogram fungal DNA, whereas the sensitivity for detection of *C.albicans* blastospores inoculated in blood was as few as 10 cell per 0.1 ml of blood. This method could be sensitive and useful for early and rapid diagnosis of systemic *Candida* infections and to simultaneous detection and speciation of *Candida* species by PCR-RFLP method.

Keywords: *Candida albicans*, PCR detection, Candidiasis

Introduction

Disseminated candidiasis is an important infectious complication in patients who have undergone cardiac or abdominal surgery or in patients who are severely granulocytopenic as a result of therapies for bone marrow transplantation or cancer (1, 2). Candidiasis continues to rise in the growing at-risk patient population (3, 4). During the period of 1986-1990 *Candida* species emerged as the sixth most common nosocomial pathogens in a hospital-wide survey. Among the nosocomial bloodstream infections, *Candida* species ranked fourth hospital-wide. *Candida* species accounted for 10.2% of all cases of septicemia and for 25% of all urinary tract infections in intensive care unit. The risk of candidemia is especially high in patients with acute leukemia. In one cancer hospital, the incidence was 2.9% with a mortality rate of 50% (5). Clinical diagnosis of disseminated candidiasis is difficult because the clinical signs and symptoms are usually nonspecific. Although two or more positive blood cultures are often used to identify disseminated disease, standard blood culturing methods have low sensitivity and can require 2-5 days for detection and even longer for species identification(6). To shorten the time required to obtain an accurate diagnosis independent of functioning immune system, laboratory test have been developed to detect circulating *Candida*

cell wall mannan (7, 8), enolase (9), D-arabinitol (6,7) or β -glucan (10) for rapid diagnosis of disseminated candidiasis. However the sensitivity of these tests varies among investigators and is reported to range from 22 to 100% (6). A more-sensitive method for the earliest possible diagnosis of systemic candidiasis and then starting antifungal therapy is essential for more prognoses. The development of DNA-based methods for detection of *Candida* spp. provides an alternative and potentially more sensitive means to diagnosis systemic candidiasis. PCR technology was recently adapted to amplify *Candida albicans* DNA, facilitating its detection in clinical samples especially in blood (11-17). In the present study, a new primer pair was designed for PCR amplification of complete part of 5.8 S rRNA gene, complete parts of adjacent internal transcribed spacer (ITS1 and ITS2) and partial parts of 18s and 28s rRNA genes (Fig1). PCR was applied invitro for amplification and detection of the target gene for medically important *Candida* species including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. guilliermondii* and *C. kefyr*, and exvivo for detection of *Candida albicans*, (the most important species) in blood samples seeded by serially diluted *C. albicans* cells.

Materials and Methods

Candida organisms. *C. albicans* (ATCC10261) was used for both invitro and exvivo experiments, whereas *C. tropicalis* (ATCC750), *C. glabrata* (ATCC90030), *C. krusei* (ATCC6258), *C. guilliermondii* (ATCC9058), *C. parapsilosis* (ATCC22019) and *C. kefyr* (TIMM0300), were used for only invitro experiments. Preparation of *Candida* cells: *Candida* cells were cultured (48h at 28°C) on glucose (4%) - pepton (1%) - agar (1.5%), were washed in saline (0.9%) and stored at 20°C until use. DNA extraction from *Candida* cells: DNA extraction was performed by glass bead method briefly, 200 microlitre of lysing buffer (10 mM Tris, 1mM EDTA, pH =8, 1% SDS, 100 mM NaCl, 2% Triton X-100), 200 microlitre of phenol chloroform (1:1) solution and 200 microlitre of 0.5 mm diameter glass beads, were added to a loopful of fungal cells in a 1.5 ml tube. After 5 minutes vigorous shaking and 5 minutes centrifugation in 10000 rpm, the supernatant were isolated and DNA was precipitated by 0.1 volume 3M sodium acetate (pH 5.2) and 2.5 volume cold isopropanol, centrifuged and washed by 70% ethanol. The pellet resuspended in 100 microliter TE buffer (10 mM Tris, 1mM EDTA pH 8) and stored at -20°C until use. Preparation of blood samples seeded with *C. albicans* blastospores: blood samples (normal human whole blood containing 0.1M EDTA) were seeded by given numbers of 0, 10¹, 10², 10³ and 10⁴ of *C. albicans* cells which had been enumerated microscopically with a hemocytometer. Preparation of template DNA from *C. albicans* suspended in blood: This was most problematic stage in our experiment. DNA extraction was performed as described by Buchman et al (11), with slight modification. Briefly, blood specimens were lysed by adding an equal volume of blood cells buffer (1% Tween 20, 1% Triton X-100, 1% NP-40 and 0.05M Tris pH 7.5). After micro-centrifugation, the pellets were resuspended in half-strength buffer and micro-centrifuged again. Pellet was washed twice in 0.05M Tris (pH 7.5) and 0.01M magnesium chloride and resuspended in the same solution that contain deoxyribonuclease I to a final concentration of 10 µg/ml and incubated for 15 minutes at 37°C, added 0.01M EDTA and heated at 85°C for 30 minutes. The pellet containing yeast, were resuspended in 300 µl of a solution that contained 0.3 mg/ml Zymolyase, 0.05M Tris (pH 7.5), 0.01M EDTA and 0.2% 2-mercaptoethanol and incubated for 60 minutes at 37°C. The specimens were solubilized by addition of 0.1% SDS and 15 µg/ml proteinase K. After incubation for 5 minutes at 37°C, the specimens were boiled for 5 minutes, and extracted with an equal volume of

Phenol/chloroform/isoamyl alcohol. Nucleic acid which partitioned into the aqueous phase was precipitated from ethanol by addition of 0.1 volume 3M sodium acetate and 1.5 volumes isopropanol, washed with 70% ethanol and vacuum dried. Primer designing: Forward (out F 5-CTG GTC AAG TGA CGA TGA TGA AC-3') and everse (out R: 5-GGT CAT GCA ATG CCA TGT CC-3') primers were designed considering rDNA sequences of medically important *Candida* species, derived from Gene-Bank. Data were analyzed by DNAsis software (Hitachi Japan). PCR: Each PCR assay contained 5µl of reaction buffer, 100µM DNTP, 2.5U *Taq* polymerase, 50 Pmol of each primer and DNA template solution. The volume was made up to 50µl with distilled water. Each mixture was heated to 94°C for 5 minutes and PCR performed under the following conditions: 94°C for 1 min, 55°C for 2 min. and 72°C for 1 min, 30 cycles. Thermal cycles were terminated by polymerization at 72°C for 10 minutes. Agarose gel electrophoresis: PCR product (10 µl) was electrophoresed in agarose 1.5% gel stained with ethidium bromide (0.5µg/ml) and visualized by transilluminator and photographed.

Results

Detection of target segment in different *Candida* species: using the new designed primer pair, a product of about 550-900 bp was amplified from 7 medically important *Candida* species including: *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. kefyr* (Fig 2), but not form human WBC. No amplification was observed without templates. The size for each *Candida* tested, was exactly the same as we expected according to data recorded in Gene-Bank. There was slight different in the PCR product length among *C. albicans*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* (fig2).

Sensitivity of detection of purified DNA by PCR.

The sensitivity of the PCR assay was evaluated with serially diluted (from 1µg to 100fg) of DNA extracted from *C. albicans*. Our primer set was able to amplify as low as 1 picogram of *C. albicans* purified DNA (Fig3).

Sensitivity of detection of *C. albicans* cells in blood.

PCR was performed with the template extracted from blood samples containing *C. albicans* cells. The detection limit was as few as 10 *C. albicans* cells by ethidium bromide staining (Fig 4). There was not any amplified product form blood samples without *C. albicans* cells (negative control).

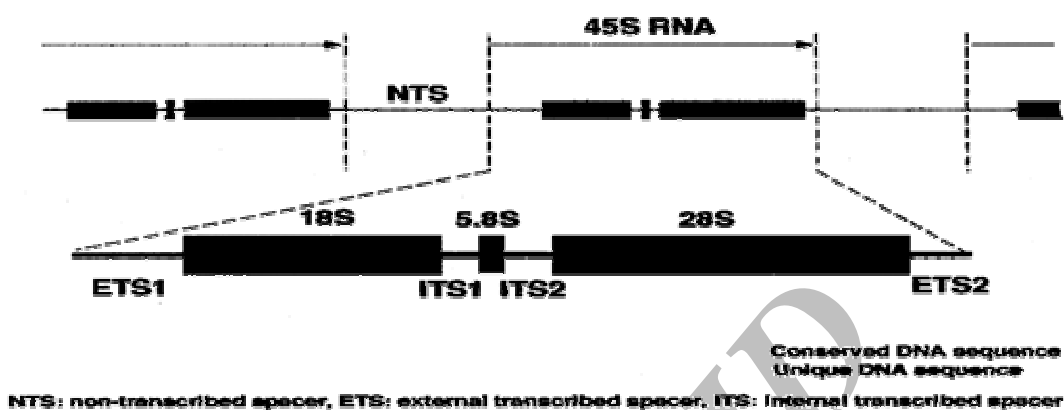


Fig. 1: Ribosomal RNA genes (rDNA) as the target of primer designing and PCR

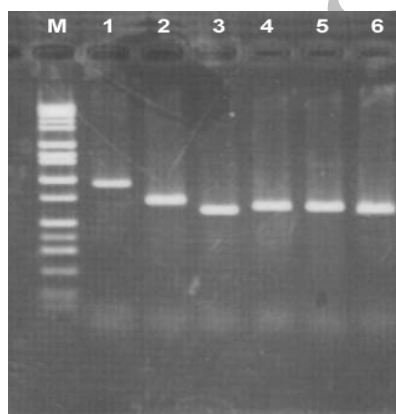


Fig. 2: PCR products from six medically important *Candida* species amplified by the designed primers; Lanes 1-6: *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. albicans*, *C. tropicalis* and *C. parapsilosis*, respectively; Lane M: 100 bp ladder molecular size marker

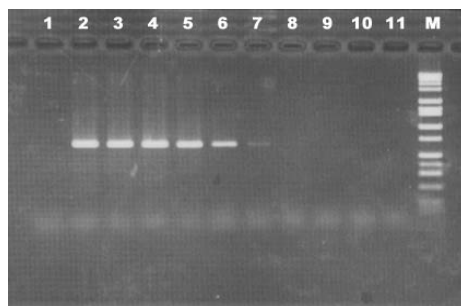


Fig. 3: PCR products of serially diluted DNA, extracted from *C. albicans* cultured cells, amplified by the designed primer set. Lane 1: dd water template control; Lanes 2-11: logarithmic dilution from 100ng to 0.1fg of *C. albicans* DNA, respectively; Lane M: 100bp ladder molecular size marker.

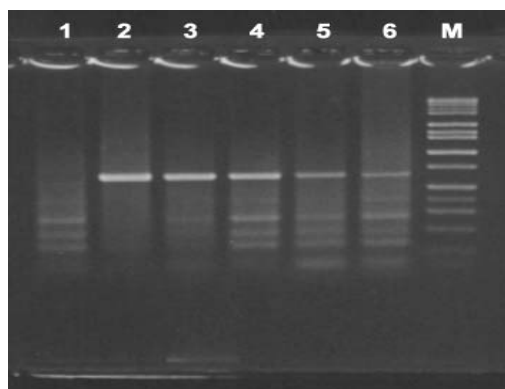


Fig. 4: PCR products of DNA extracted from *C.albicans* cells suspended in blood; Lane 1: Whole blood without *Candida* cells; Lane 2 – 6: Blood samples containing 100000, 10000, 1000, 100, and 10 *C.albicans* cells, respectively; Lane M: 100bp ladder molecular size marker.

Discussion

In this study we have designed a primer pair which is conserved in rRNA gene. Using these primers we have established a sensitive PCR for in-vitro and ex-vivo detection of *C. albicans* DNA in blood samples contained logarithmic numbers of *C. albicans* cells. The sensitivity of the PCR, with both purified DNA and yeast cells contained in blood, was comparable with the sensitivity of previously reported PCR methods for *Candida* species. The lower limit of PCR detection of fungi in the experiment of Makimura et al. (18) was 1 pg of purified genomic DNA and 100 *Candida albicans* cells. The level of sensitivity by the primers used by Holmes et al. (13) was 15 ± 5 cells of *C. albicans* in blood. Buchman (11) is the first investigator who worked on detection of *C. albicans* in blood. They established a technique to extraction of DNA from *Candida* cells contained in blood. The sensitivity of their method was 10 organisms in 100 μ l specimen. We used Buchman's method for fungal DNA preparation from blood because it seems that their technique may be highly efficient. The primer pair used in this study has been designed with considering rDNA sequence of most medically important *Candida* species. These primers were able to amplify a 550-900 bp fragment in different *Candida* species, but do not amplify any fragment belonging to human DNA. Moreover the primers are adjacent to the ITS1 and ITS2 regions and it is possible to use these 2 primer sets in a nested PCR for achieving more sensitivity. We already described a PCR-RFLP system based on ITS1-ITS2 sequences for identification of *Candida* species (19) so it could be possible using a nested-PCR RFLP system for simultaneous detection and identification of clinically important *Candida* species. Actually we have a plan to use this approach for molecular diagnosis of systemic candidiasis in animal modeling and clinical human samples. It seems

that molecular biological approach may be rapid and sensitive methods for *Candida* and candidiasis in diagnostic goals.

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