Direct colony polymerase chain reaction for rapid identification of yeasts isolated from blood specimen

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ABSTRACT

Context: Bloodstream infections (BSIs) caused by yeasts have an increasing frequency due to the growing population of immunosuppressed individuals. Among yeasts, Candida remains the most prevalent species with the increase in the incidence of non-albicans Candida species. Apart from Candida, other yeasts are also involved in causing BSI. High mortality associated with Candida and other yeast infection can be reduced by prompt and appropriate antifungal therapy. Hence, rapid identification and speciation of yeasts isolated from blood play a significant role in the management of the patients. Since conventional methods used for speciation of Candida and other yeasts are laborious, time-consuming and often unclear, rapid and accurate molecular techniques are required. **Materials and Methods:** Instead of using purified genomic DNA as template for polymerase chain reaction (PCR), we used yeast colony and cell suspensions in water and 0.10M potassium hydroxide as template for PCR. *Candida albicans,* Trichosporon and *Cryptococcus neoformans* were used as reference strains. Further, a total of 100 yeast isolates were also tested. All reactions were performed using the universal fungal primers ITS1 and ITS4; the PCR products were then digested with restriction enzyme (Msp1). **Results:** Direct colony PCR (DCPCR) produced sharp and distinct bands compared to the cell suspensions with the reference strains. All the 100 clinical isolates tested also produced distinct bands. **Conclusion:** DCPCR approach not only reduces the DNA template preparation time but is also easy, rapid and reduces the cost of PCR.

Key words: Candidemia, direct colony polymerase chain reaction, non-albicans Candida, restriction fragment length polymorphism

INTRODUCTION

Yeast infections, candidiasis, in particular, are a cause of increasing mortality and morbidity in immunocompromised patients.[1] They cause a wide range of clinical conditions in humans ranging from mild superficial infections to severe invasive disease.[2] For therapeutic, prognostic and epidemiological reasons, it is necessary to accurately identify the etiological agent.[3] The conventional methods of identification have low sensitivity, specificity and take three or more days.[4] Nowadays, nucleic acid-based detection methods are widely being used for identification of yeast infections, of which polymerase chain reaction (PCR) is widely used and has high sensitivity, specificity and is simple to perform.[5]

Like other nucleic acid-based techniques, PCR also requires purified DNA. Numerous methods are available for the purification of DNA, from indigenous to commercial methods. The conventional methods include enzymatic method, bead beating and using chaotropic agents.[6] These methods are time-consuming, laborious, use toxic chemicals such as Phenol and Chloroform and might release low-quality DNA.[7] The available commercial kits are expensive and not affordable by all laboratories.

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Colony PCR is a technique where the DNA extraction step is omitted, and the yeast cells are directly suspended into the PCR reaction mix and used for amplification. In this study, we evaluated colony PCR, in identifying important yeasts such as Candida*,* Cryptococcus and Trichosporon. In addition, a significant number of clinical isolates were tested for the efficiency of the technique.

MATERIALS AND METHODS

This study was carried out in the Mycology Division, Department of Microbiology, Sri Ramachandra Medical College and Research Institute, Chennai, Tamil Nadu, for one year from July 2013 to June 2014. Blood specimen positive for yeast growth was taken into the study. The 100 yeast isolates obtained during this period was used in the study.

Reference strains

Candida albicans ATCC 90028, *Trichosporon asahii* MTCC 6179 and *Cryptococcus neoformans* clinical isolate, which were confirmed by gene sequencing, were used as reference organisms to access the proficiency of colony PCR in this study.

Preliminary identification of clinical isolates

All the 100 clinical isolates obtained during the one-year duration were identified using automated culture identification system, Vitek2 (BioMerieux, USA). All these isolates were subjected to colony PCR after standardisation with reference organisms.

Colony polymerase chain reaction

Preparation of DNA for colony polymerase chain reaction

Three different approaches were used to prepare DNA for colony PCR. First, cell suspension was prepared by suspending a loopful of culture in 1 ml sterile distilled water. Second, a loopful of culture was suspended in 1 ml of 0.10M potassium hydroxide (KOH). Both these suspensions were boiled for 1 min to lyse the cell wall and release the genomic DNA. This crude DNA served as the template for PCR. Third, the yeast cells directly from Sabouraud's dextrose agar plate were used as template.

Polymerase chain reaction

Direct colony PCR (DCPCR) was performed in a total reaction volume of 50 µl containing 25 µl of 2× PCR master mix (GeNei, Bengaluru, India), 50 pmol ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and 50 pmol ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Sigma-Aldrich). To the reaction mix, 1, 2 and 5 µl of both water and KOH cell suspensions were added in corresponding tubes. In one tube, a speck of an isolated colony was suspended into the PCR reaction mix with a sterile micropipette tip. These served as the template. The volume was made up to 50 µl using nuclease-free water. In all PCR runs, positive and negative controls were included in the study. PCR reaction mix without culture was used as negative control, and DNA extracted from *C. albicans* ATCC 90028 was used as positive control.

The amplification parameters consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min. An initial denaturation of 94°C for 10 min and a final extension at 72°C for 10 min were also included in the study.

Restriction fragment length polymorphism

Restriction enzyme digestion was done for all the PCR products. The reaction mix contained 0.5 µl (10U) Msp1 enzyme (New England Biolabs, England), 2 µl of restriction buffer 4 (New England Biolabs, England) and 10 µl of amplicon obtained from DCPCR using ITS 1 and ITS 4 primers. The reaction volume was made up to 20 µl using nuclease-free water. The reaction mix was incubated at 37°C for 1 h.[8,9]

Amplicon detection

Ten microlitres of the PCR product and RFLP product were electrophoresed in 1.5% and 2% agarose gel in 1× TAE buffer, respectively, stained with ethidium bromide (10 mg/ml) and visualised under ultraviolet illumination.

On standardisation of with the reference strains, the technique was further extended and tested on 100 clinical yeast isolates.

RESULTS

The study was conducted from July 2013 to June 2014. One hundred yeast isolates obtained during this period were used in this study. Out of the 100 isolates, 29 were *C. albicans*, 57 were *Candida tropicalis*, 12 were *Candida parapsilosis* and 1 *Candida krusei* and *Candida glabrata*. There was no difference between the identification of isolates by Vitek2 and PCR-RFLP.

All the reference strains tested produced amplicons for the PAN fungal primers, ITS 1 and ITS 4. No false positive or false negative results were observed.

Amplicons were present in all the different volumes of cell lysates used. However, the amplicons produced by water suspension and direct colony were intense compared to the bands produced by KOH suspension. Among the various

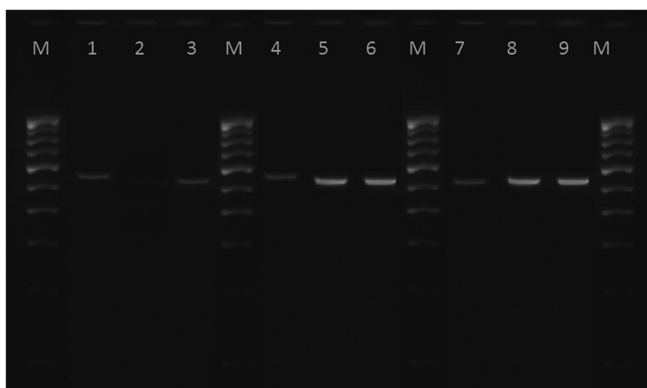

Figure 1: Polymerase chain reaction results of potassium hydroxide, water extract and direct suspension. Lane M - 100 bp ladder; Lanes 1–3 - 1, 2, 5 μl potassium hydroxide extract; Lanes 4–6 - 1, 2, 5 μl water extract; Lane 7 - Direct suspension

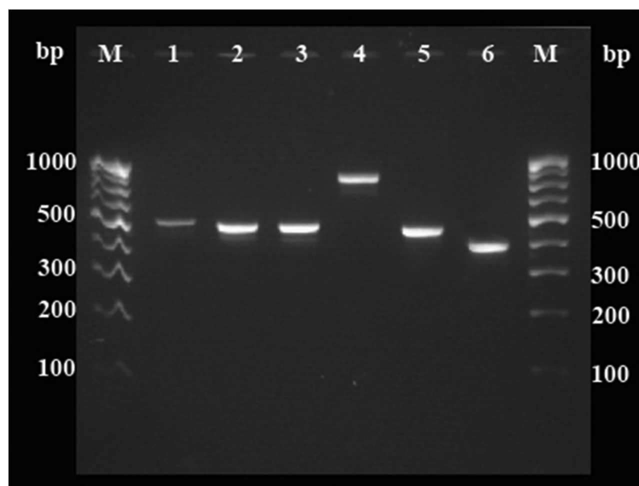

Figure 2: Direct colony polymerase chain reaction of representative species isolated. Lane M - 100 bp DNA marker; Lane 1 - *Candida albicans*; Lane 2 - *Candida tropicalis*; Lane 3 - *Candida parapsilosis*; Lane 4 - *Candida glabrata*; Lane 5 - *Candida krusei*; Lane 6 - Unidentified species

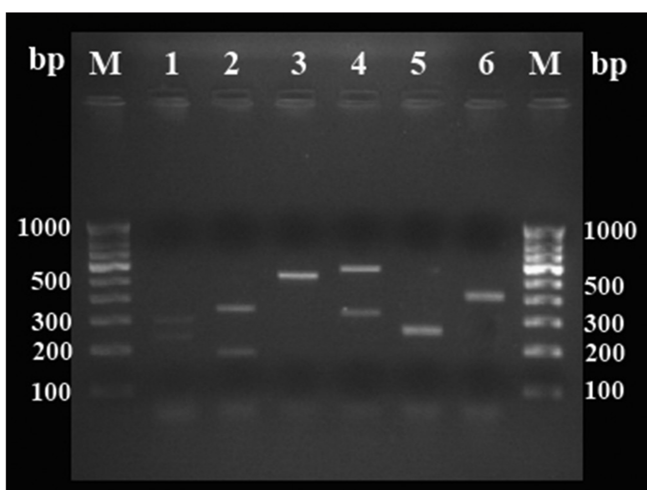

Figure 3: Restriction fragment length polymorphism pattern of representative species isolated. Lane M - 100 bp DNA marker; Lane 1 - *Candida albicans*; Lane 2 - *Candida tropicalis*; Lane 3 - *Candida parapsilosis*; Lane 4 - *Candida glabrata*; Lane 5 - *Candida krusei*; Lane 6 - Unidentified species

volumes of cell lysate added, 2 μl produced distinct and bright bands than 1 and 5 μl cell suspension [Figure 1]. The addition of direct colony also produced bright and distinct bands compared to other cell suspensions. This DCPCR was tested in 100 clinical isolates.

DCPCR produced amplicons with PAN fungal primers for all the 100 clinical isolates tested. A single band was seen for all the 100 isolates of which, 81 bands were sharp and 19 were faint bands, but still clear and visible. The PCR bands were of different sizes ranging from 350 to 850 bp approximately [Figure 2], and the RFLP banding pattern differed depending on the yeast species [Figure 3]. PCR-RFLP was able to identify five medically important Candida species. The different species obtained by PCR-RFLP are listed above.

DISCUSSION

Early and rapid identification of invasive yeast infections is hindered by the lack of sensitive and specific assays. Although techniques are available for rapid nucleic acid amplification and identification, the extraction and purification of DNA still remains a problem in developing countries like India, requiring time, high cost and workforce.[7] Here, we studied a simple, rapid and sensitive method to amplify DNA directly from yeast cells. Among the three DNA preparations tested, the addition of colony directly to the PCR reaction mix produced better results than the water suspension and KOH suspension. This even simplifies the method, as there will be no need of preparation of cell suspensions. DCPCR was applicable to three different yeasts which were tested and could be applied for all clinical isolates, as most of the bloodstream infections are caused by Candida*,* Cryptococcus and Trichosporon only. Furthermore, the technique was rapid and took only 4–6 h, whereas Vitek2 took a minimum of two days. In addition, the technique showed 100% efficiency when tested with clinical isolates with no false positive or false negative reactions. Although the omission of DNA extraction procedure compromised the availability of large amount of DNA, there was sufficient DNA available for the PCR reaction to produce positive results. This could be due to the fact that numerous cells were used in the reaction and each cell harbours multiple copies of the targeted RDNA gene (50–100 copies/cell).[10] RFLP analysis was done to check whether the cell debris from the PCR reaction inhibited the RFLP reaction. The RFLP bands were as clear and intense as the PCR bands showing that the cell debris in the PCR reaction mix did not interfere with the RFLP as well.

Earlier, Lau *et al*. and Mirhendi *et al*. have shown that colony PCR would be a rapid and convenient method for amplification of yeast DNA.[10] The method is less time intensive and additional steps are not required; further, it might serve as an alternative to the conventional DNA extraction procedure.

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Conflicts of interest

There are no conflicts of interest.

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