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IP International Journal of Medical Microbiology and Tropical Diseases

Journal homepage: <https://www.ijmmt.org/>

Original Research Article

Identification of *Candida* species from blood samples of neonates by PCR-RFLP method in Western U.PGunjan Dutta^{1*}, Kamy Verma¹, Kanwaljit Kaur², Rajesh Bareja^{b3}¹Dept. of Microbiology, Saraswathi Institute of Medical Sciences, Hapur, Uttar Pradesh, India²Dept. of Pathology, Ajay Sangal Institute of Medical Sciences and Research and Aayushman Hospital, Shamli, Uttar Pradesh, India³Dept. of Microbiology, World College Of Medical Sciences & Research And Hospital (WCMSRH), Jhajjar, Haryana, India

ARTICLE INFO

Article history:

Received 14-05-2024

Accepted 12-07-2024

Available online 27-09-2024

Keywords:

Neonates

Candida

Candidemia

PCR-RFLP

ABSTRACT

Introduction: Correct identification of *Candida* species is necessary for actual therapy and epidemiology study. *Candida* species is the most frequent emerging cause of fungal infections globally. The most common cause of Candidemia worldwide is *Candida albicans*, however, non-*Candida albicans* (NCA) species like *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. guilleirmondii* being reported

Aim and Objective: To identify the different species of *Candida* from blood specimen of neonates by PCR-RFLP technique.

Materials and Methods : Total 27 *Candida* isolates were collected from blood samples of neonates. All the clinical specimens were inoculated for the isolation of *Candida* species using standard mycological techniques. The isolates were cultured on Sabouraud's Dextrose Agar (SDA) and further, they were identified by PCR and RFLP method.

Result: Molecular methods like PCR-RFLP were used in identifying six important *Candida* species (*C. tropicalis*, *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. guilleirmondii*) from blood. By using universal primers ITS-1 and ITS-4, PCR assay successfully amplified the ITS-1 and ITS-2 region of 27 isolates and the amplicon size of approximately 510-870 bp. This method is speedy, easy, and affordable which can be used in routine laboratory diagnostics for the quick isolation of *Candida* species from blood samples.

Conclusion: It is helpful for clinicians to start actual therapy in Candidemia patients before antifungal sensitivity results are obtainable.

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1. Introduction

Candida species is the most frequent emerging cause of fungal infections globally.¹ It is also the fourth most significant cause of blood stream infection (BSI) in the United States. Due to various multi-centric studies, the picture is not clear in India. It has been also seen that Candidemia is also linked with an elevated mortality rate, which ranges from 10-49%.² The most general cause of

worldwide Candidemia is *Candida albicans*, but it has been observed that non *Candida albicans* (NCA) species like *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. guilleirmondii* are additionally involved.²

The most frequent species of *Candida* has been isolated from blood in India is *C. tropicalis*.³ It has been observed from fewer studies that, fluconazole and other triazoles are resistant to *Candida* species are mostly *C. glabrata* and *C. krusei* whereas *C. parapsilosis* have been found susceptible to azole group of drugs.⁴ A few studies have also shown

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resistance to Amphotericin B and echinocandins.^{5,6}

Phenotypic methods used for the isolation of *Candida* species are sugar assimilation, sugar fermentation, Dalmou plate technique, culture characteristics on SDA or CHROM agar etc, but these methods require 48-72 hrs or even more for the identification. Quick identification and detection of *Candida* species is necessary for the suitable treatment and protection of patients who suffer from Candidemia.⁷

We used molecular methods like PCR-RFLP which are more sensitive, quick, smooth and price- effective for the diagnosis of different species of *Candida* from blood isolates of neonates.

2. Materials and Methods

This was a prospective cross-sectional study for which ethical clearance was taken.

Single *Candida* isolate per patient's specimen was comprised in the study to avoid duplication of isolates. Mixed cultures were excluded from the study. In Table1, six standard strains used in the study with the sizes of ITS1-ITS4 PCR products for *Candida* species before and after digestion with MspI restriction enzyme are listed, which were provided by PGI Chandigarh.

2.1. Clinical isolates

Entirely 27 successive blood isolates were collected of neonates from the Neonatal Intensive Care Unit (NICU) for two years period at the Microbiology laboratory, Subharti University, Meerut.

All the clinical specimens were cultivated for the isolation of *Candida* species using standard mycological techniques. The isolates were cultured on Sabouraud's Dextrose Agar (SDA) and further, they were used for molecular examination.

2.2. DNA isolation

By using a True-prep DNA extraction kit (Molbio Diagnostics Pvt Ltd) all the isolates was extracted for DNA. The eluted DNA was stored at -20°C until use. To confirm the presence of DNA, the gel electrophoresis was done.

2.3. PCR assay

Amplification for PCR was perform in a final volume of 100 μ l. Every reaction consists of 1 μ l of template DNA, each forward (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3' and reverse (ITS4, 5'TCC TCC GCT TAT TGA TAT GC-3') primer at 0.2 μ M, each deoxynucleoside triphosphate (dNTP) at 0.1 mM, 10 μ l of 10X PCR buffer, and 2.5 U of Taq DNA polymerase. The amplification was pursued by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, extension at 72°C for 1 min, with a final extension step of 72°C for 7 minutes. 1.5 % (w/v) agarose

gel electrophoresis in TAE buffer were used to visualize the amplified products and then they stain with ethidium bromide under UV light and photographed.

2.4. RFLP analysis and gel electrophoresis

To perform the digestion, a 20 μ l aliquot of PCR product was incubated with 10 U of MspI in a final reaction volume of 25 μ l for 2hr at 37°C. By 1.8% agarose gel electrophoresis in TAE buffer for approximately 45 min at 100V restriction fragments were separated and visualized by staining with ethidium bromide.

3. Results

By using universal primers ITS-1 and ITS-4, PCR assay successfully amplified the ITS-1 and ITS-2 region of 27 isolates and the amplicon size of approximately 510-870 bp (Figure 1) was obtained from medically important *Candida* species (*C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C.krusei* and *C.guilliermondii*). The restriction enzyme Msp I was used for the RFLP method in one study which was performed in Iran.⁸(Figure 2).

Digestion of ITS region by Msp I give rise to two bands in each species except for *C. parapsilosis* as it does not have any specific restriction site for Msp I. The pattern for bands was different for each *Candida* species and hence it was simple to differentiate them. All strains were compared with the control strains as shown in the table (Table 1). Dispersal of *Candida* species isolated from blood of NICU patients by PCR-RFLP are mentioned in (Table 2).

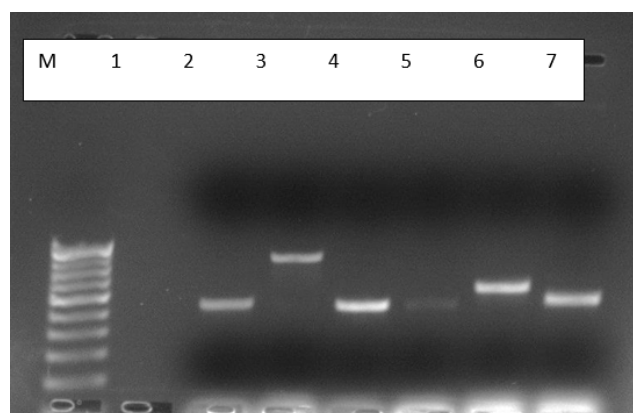


Figure 1: PCR products from *Candida* species. Lane 1- NC (Negative Control), Lanes 2- ATCC *C.albicans*, Lane 3-ATCC *C.glabrata*, Lane 4- ATCC *C.krusei*, Lane 5-ATCC *C.tropicalis*, Lane 6-ATCC *C.guilliermondii*, Lane 7: *C.parapsilosis*. Lane M: 100bp Molecular size marker

4. Discussion

For the handling of Candidemia and to lower the death rate related with it, early diagnosis of *Candida* from

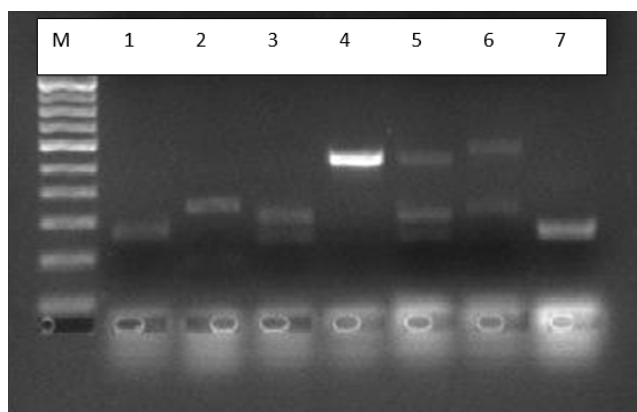


Figure 2: *Candida* strains with the enzyme MspI. Lanes 1 and 7: *C.krusei*, Lane 2: *C. tropicalis*, Lane 3: *C.albicans* Lane 4: *C.parapsilosis*, Lane 5: *C.guilliermondii*, Lane 6: *C.glabrata*, Lane M: 100bp Molecular size marker

Table 1: Sizes of ITS1-ITS4 PCR products for *Candida* species before and after digestion with MspI restriction enzyme.

<i>Candida</i> species	Size of ITS1-ITS4	Size of restriction product
<i>C.tropicalis</i>	524	340,184
<i>C. albicans</i>	535	297,238
<i>C. parapsilosis</i>	520	520
<i>C.krusei</i>	510	261,249
<i>C.glabrata</i>	871	557,314
<i>C.guilliermondii</i>	608	371,155,82

Table 2: Distribution of *Candida* species isolated from blood of NICU patients by PCR-RFLP.

<i>Candida</i> species	Number (%)
<i>C.tropicalis</i>	14
<i>C. parapsilosis</i>	06
<i>C. albicans</i>	04
<i>C.glabrata</i>	01
<i>C.krusei</i>	01
<i>C.guilliermondii</i>	01
Total	27

blood stream infections are very important. Currently to diagnose *Candida* up to species level by using phenotypic methods will take 48 to 72 hrs. Because of restrictions of conventional techniques, genotypic method mainly PCR is being operated for untimely detection of *Candida* from blood.

For the speedy detection of *Candida* species, many molecular methods such as DNA sequencing, RAPD (Random Amplified Polymorphic DNA), real time PCR etc. have been developed, but these methods require skilled workers and are more expensive.⁹

In our study, PCR-RFLP was performed for 27 *Candida* samples from the blood of neonatal intensive care unit. Universal primers, ITS1 and ITS2 were able to amplify the

ITS region of genomic DNA by PCR. RFLP was done for the amplified products with the help of MSP I restriction enzyme and all the isolates were correctly recognized up to the species level. Six *Candida* species were detected from NICU patient's blood samples (*C. tropicalis*, *C.parapsilosis*, *C.albicans*, *C.glabrata*, *C.krusei* and *C.guilliermondii*).

In our study, non *Candidaalbicans* (NCA) spp. were more commonly isolated than *Candida albicans*. The same finding also noticed in other studies done by Vaibhav Misra et.al.¹⁰ and Asifa Nazir et.al.¹¹ In epidemiological studies indicated that *Candida tropicalis* is involved in about 67-90% cases of candidemia.¹² In our study also *C.tropicalis* was the prominent one among other NCA species.

It has been found that PCR-RFLP is a quick and authentic technique to speciate *Candida* isolates in several studies also. In one research, MSP I enzyme with PCR-RFLP assay used for detection of medically essential fungi.¹³ Similar study from Iran, by Mirhendi et.al. also identified the six *Candida* isolates by PCR-RFLP.⁸

In other study of Iran, clinical isolates of *Candida* were identified by Real time PCR by High Resolution Melting Analysis (HRMA), and in one study of Vienna, various molecular methods are described for the diagnosis of *Candida* species.^{14,15}

In our study, we found PCR-RFLP to be an easy, speedy and affordable technique for the isolation of *Candida* species from blood isolates.

The whole procedure of PCR-RFLP will finish in approximately seven hours whereas conventional methods require 48-72 hours to speciate the isolates. This will help to start the actual therapy of Candidemia by clinicians before antifungal sensitivity reports are obtainable. For example *C.glabrata* is usually resistant to fluconazole, but it is safe and effective in invasive candidiasis treatment in infants.¹⁶ In one study, randomized controlled trials were done and based on that data the recommended antifungal were echinocandins as first line agent in older children and adults for Candidemia and disseminated candidiasis.^{17,18} Therefore, Candidemia sufferers can be treated by Amphotericin B and echinocandins.

5. Conclusion

PCR-RFLP has an advantage of high discrimination power, potential for wide range of speciation of *Candida* species and can be used for quality control of our phenotypic methods when these phenotypic methods fail to identify the species. It is also helpful for clinicians to start actual therapy in Candidemia patients before antifungal sensitivity reports are obtainable.

6. Ethical Approval

This study was conducted under Ethical committee certificate number-SMC/EC/2014/47.

7. Conflict of interest

There was no conflict of interest.

8. Source of Funding

None.

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Cite this article: Dutta G, Verma K, Kaur K, Bareja R. Identification of *Candida* species from blood samples of neonates by PCR-RFLP method in Western U.P. *IP Int J Med Microbiol Trop Dis* 2024;10(3):236-239.