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PCR-RFLP ASSAY IDENTIFICATION OF NON-ALBICANS CANDIDA SPECIES BY MSPI RESTRICTION ENZYME.

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ABSTRACT

Introduction- Non albicans Candida (NAC) are most commonly isolated from various clinical specimens. The conventional methods use for the identification are time consuming and difficult to perform.

Aim- To identify NAC species from various clinical specimens using polymerase chain reaction (PCR) and Restriction fragment length polymorphism (RFLP) with MspI restriction enzyme.

Materials and methods – By using universal primers, ITS1 and ITS4, we could amplify ITS1 and ITS2 rDNA region in 79 NAC isolates.

Result- in our study we successfully identified all isolates using MspI restriction enzyme separately. C.tropicalis was identified as the most common species (74.68%) followed by C.parapsilosis, C.glabrata, C.krusei and C.guilliermondii.

Conclusion- PCR-RFLP method by using ITS1 and ITS4 primers and the restriction enzyme is rapid and reliable method in clinical laboratories for identification of medically important NAC species.

KEYWORDS : PCR-RFLP, Non-albicans Candida (NAC), MspI enzyme

Introduction

Candida species are the common cause of fungal infections worldwide. These are the third most dominant cause of healthcare-related infections [1].

C. albicans is the most common species which is isolated in humans; however, the increasing of non-*albicans Candida* (NAC) species has been recognized significantly during the last two decades [2–4].

NAC is a heterogeneous group of *Candida* species and cause similar clinical manifestation as in C.albicans but differ in virulence factors, epidemiology and most importantly the antifungal drug susceptibility pattern. It has been observed that most of the NAC infections are caused by *C. glabrata*, *C. tropicalis, C. parapsilosis, C. dubliniensis, C. guilliermondii, C. krusei*, and *C. kefyr* [5–7].

Conventional tests used in identifying these fungi are sometimes laborious and misidentification of emerging species may occur in some instances (8, 9)

Although, the conventional tests are relatively inexpensive, these tests fail to identify some emerging fungi with ambiguous phenotypic profiles in clinical mycological analyses. Recently, molecular techniques have been used to distinguish and characterize a wide range of pathogenic fungi including *Candida* species where the conventional phenotypic test has failed to detect and identify the fungal species involved (8,10).

NAC are significantly varied in their prevalence as per country and species identification also has an important role in formulation of local therapeutic guidelines (11). Identification of *Candida* up to species level is important for accurate treatment as not all species respond to the same treatment (12, 13)

The objective of this study was to analyze the phenotypic and genotypic features of Candida isolates.

Material and Methods

1 Study Design

This was a laboratory-based prospective cross-sectional study on *Candida* species isolated from clinical specimens.

2 Study Site

The present study was conducted in the department of Microbiology, Subharti Medical College, and Meerut (UP).

79 Candida species were isolated from the clinical samples like urine, sputum, high vaginal swab (HVS) and blood received from various intensive care units, wards and outpatient department. The samples analyzed were mostly from all ages attending the hospital from January 2017 to December 2019.

Control strains used in the study, which were provided by PGI, Chandigarh are listed in table 1.

Table 1:-Control strains used in the study

S. No	*Candida species*	Control Strains
1	***C.albicans***	ATCC 90028
2	***C.glabrata***	ATCC 2001
3	***C.tropicalis***	ATCC 0750
4	***C.krusei***	ATCC 6258
5	***C.guilliermondii***	ATCC 6260
6	***C.parapsilosis***	ATCC 22019

Identification methods

The clinical specimens were processed for the isolation of Candida species using standard mycological methods. Direct mount were made to study the yeast morphology. Gram staining was performed from direct specimen and the specimens were inoculated on Sabourauds Dextrose Agar slants, incubated at 37°C for 24 hours. Germ-tube identification was also performed to rule out C.albicans if any and all isolates was used for molecular analysis. ATCC 90028 C albicans control was also used in the PCR-RFLP assay to rule out any C albicans in the study.

DNA extraction:

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

PCR assay

PCR amplification was done in a final volume of 100 µl. Each reaction consists of 1 µl 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 µM, each deoxynucleoside triphosphate (dNTP) at 0.1 mM, 10 µl of 10X PCR buffer, and 2.5 U of Taq DNA polymerase. The amplification was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, extension at 72°C for 1min, with a final extension step of 72°C for 7 minutes and amplified products were visualized by 1.5 %(w/v) agarose gel electrophoresis in TAE buffer and stain with ethidium bromide visualized under UV light and photographed.

RFLP analysis

Digestion was performed by incubating a 20µl aliquot of PCR product with 10 U of MspI in a final reaction volume of 25µl at 37°C for 2hr. Restriction fragment were separated by 1.8% agarose gel electrophoresis in TAE buffer for approximately 45 min at 100V and visualized by staining with ethidium bromide.

Results

We got the successful PCR amplification of ITS-1 and ITS-2 region of 79 isolates using primers ITS-1 and ITS-4 using a single PCR product

of approximate 510-870 bp (table-2, fig-1). Restriction enzyme MspI was used for RFLP technique as done by Mirhendi. *et.al* (14). Digestion of ITS region of NAC species by MspI generated 2 bands for C.tropicalis, C.glabrata, C.krusei and 3 bands for C.guilliermondii. However PCR and digestion products for C.parapsilosis were of the same size and it had no recognition site for MspI enzyme. We identified five Non Albicans Candida species and C.tropicalis was identified as the most common species (74.68%) followed by C.parapsilosis, C.glabrata, C.krusei and C.guilliermondii (table-3). The banding patterns are different for each Candida species and hence it was easy to differentiate them (fig -2).

Table-2: Various sizes of ITS1-ITS4 PCR products for Candida species before and after digestion with Msp1.

Candida species	ITS1-ITS4 size	Restriction products size
C.tropicalis	524	340,184
C. albicans	535	297,238
C. parapsilosis	520	520
C.krusei	510	261,249
C.glabrata	871	557,314
C.guilliermondii	608	371,155,82

Fig-1- PCR products from Candida species. Lane 1 - NC (Negative Control), Lanes 2-ATCC C.albicans, Lanes 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.

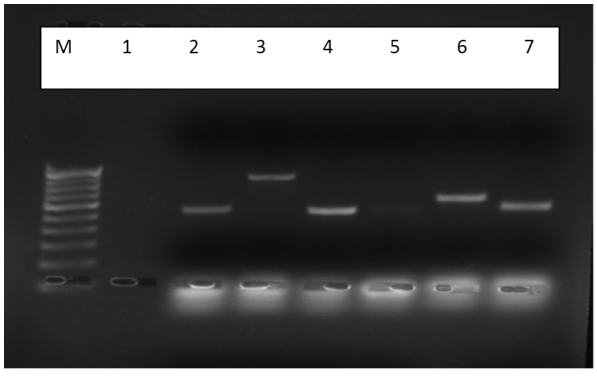
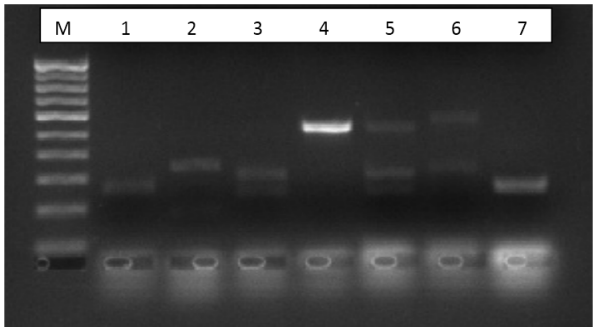


Table 3:- Identification of NAC species by PCR-RFLP amplification and RFLP analysis with MspI enzyme.

NAC species	Number	%
C. tropicalis	59	74.68%
C. parapsilosis	10	12.6%
C. glabrata	06	7.59%
C. krusei	03	3.79%
C. guilliermondii	01	1.26%
Total	79	100

Fig-2 Candida strains with the enzyme MspI. Lanes 1and7: 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



DISCUSSION

In the present study, by using ITS1 and ITS4 primers we successfully amplified the fragments of 510-879 bp of the ITS1and ITS2 rDNA region from genomic DNA of 79 strains of Candida species. Currently the phenotypic methods may take 48-72 hrs to diagnose Candida isolates. NAC are becoming resistant to fluconazole, therefore identification up to species level has a direct impact on choice of antifungal treatment. Higher incidences of NAC ranging from 54-74% have been seen in various studies. (14, 15, 16)

In the present study C.tropicalis was the most common isolated NAC species. Factors like increased use of antifungal drugs, use of broad spectrum antibiotics, long term use of catheters and increase in the number of immunocompromised patients contribute to the emergence of NAC species (17).The increased isolation rates of NAC species with a moderate shift in the antifungal susceptibility profile underlines the need of early and accurate diagnosis of infecting Candida species. It is suggested that increase isolation and complete identification of Candida species in all microbiology laboratories is done so that the epidemiology, emergence and spread of Non-albicans Candida could be revealed (18).

Molecular techniques are better substitute for the identification and diagnosis of Candida spp., because of high power diagnosis being rapid and easy. PCR methods can detect small amount of DNA and can do early detection of pathogenic fungi which may lead to proper antifungal treatment that may improve chances of survival. These methods can detect the presence of fungi with high degree of specificity and sensitivity (19). It is reported that RFLP- like techniques use universal primers to identify different fungi (19). PCR-RFLP assay has an advantage over other molecular technique, such as RFLP with genomic DNA and electrophoretic karyotyping, is being simple and quick (20).

It is suggested that increase isolation and complete identification of Candida species in all microbiology laboratories is done so that the epidemiology, emergence and spread of Non-albicans Candida could be revealed.

Conclusion

In our study, among NAC species C.tropicalis was most commonly isolated from the various clinical specimens. So, the accurate identification for Candida species is essential for the selection of appropriate antifungal drug. PCR-RFLP method by using ITS1 and ITS4 primers and the restriction enzyme is rapid and reliable method in clinical laboratories for identification of medically important NAC species.

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