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IDENTIFICATION OF CANDIDA SPECIES BY PHENOTYPIC AND PCR-RFLP ASSAY

Microbiology

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ABSTRACT

Non-albicans Candida (NAC) is known as the most common cause for the opportunistic infections.. In the present study we identify Candida species isolated from various clinical specimens by using phenotypic and genotypic method. A total of 100 samples were examined by conventional methods and the genotypic analysis was done by using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) technique. The internal spacer region (ITS) of the fungal rRNA genes was recruited for PCR amplification of target sequences and MspI enzyme was used to digest PCR amplicons. By using genotypic method, we successfully identified all clinical isolates of Candida. Among them C.tropicalis was identified the most common species followed by C.albicans, C.parapsilosis, C.glabrata, C.krusei, C.guilliermondii. PCR-RFLP using ITS1 and ITS4 primers and restriction enzyme MspI is a simple, rapid, and cost-effective method for the differentiation between Candida species that is applicable in clinical laboratories.

KEYWORDS

Candida species, MspI enzyme, PCR-RFLP.

INTRODUCTION

Out of 200 species from Candida genus, 30 species have been isolated from human infections, and the number is increasing(1). Candida is the normal flora of the skin and the mucosal surfaces of the body such as mouth, vagina, intestine and respiratory system of human body. If it gets the favorable conditions, it proliferates and creates pseudohyphae which are capable of causing disease. Despite C.albicans have been reported as the most important causative agent of this family, other species such as C.tropicalis, C.parapsilosis, C.glabrata, C. krusei and C.guilliermondii are also isolated from clinical samples. However many comprehensive studies have shown the significant growth of infections related to other non-albicans Candida (NAC) species (2-5). In few available studies from India, C.tropicalis has been the most common species of Candida isolated from blood (6, 7)

Identification of yeast pathogens by traditional method requires several days and specific mycological media. These methods are thus time consuming and labour intensive. Several brands of chromogenic media contain substrates that react with enzymes secreted by the target microorganisms to yield colonies of varying colors (8). Recently molecular techniques have provided alternative methods for diagnosis and identification of pathogenic fungi, including Candida species (9, 10). Use of genotypic methods are required for accurate identification of Candida at species level, especially in epidemiological studies to assess the transmission routes as well as to determine appropriate antifungal drugs (11-13).

Here, we report the application of rapid PCR-based technique using a one-enzyme restriction fragment length polymorphism (RFLP), for discrimination of medically important Candida species from different clinical isolates.

MATERIALS AND METHODS

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

The present study was carried out between Jan 2017 to Dec 2018 in the department of Microbiology, Chatrapati Shivaji Subharti Hospital, and Meerut. A total of 100 consecutive Candida isolates from various clinical specimens like urine, blood, sputum and HVS were included in the study. These specimens were processed for the isolation of Candida species using standard mycological methods (14). 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 test was done and the positives identified were either C.albicans or C.dubliniensis. C.albicans was further identified by growth at 45°C and Chlamydospore formation on Corn meal agar (15). All the 100 isolates were subjected to sugar fermentation and sugar assimilation test. Concurrently Candida species were inoculated on Chrom agar and incubated at 37°C for 24 hours and the species were identified by type and color of the colonies on CHROM agar media as per manufacturer's instructions.

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 (polymerase chain reaction) assay

The amplification of PCR was carried out in a final volume of 100 µl (16). 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. Initial denaturation step was done at 94°C for 5min 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 Tris acetate (TAE buffer) and stain with ethidium bromide visualized under UV light and photographed.

RFLP (Restriction fragment length polymorphism) 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.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 software (IBM Corporation, SPSS inc., Quarry Bay, Hong Kong). The continuous variables were expressed in number (%).

Results-The work included total 100 non repeat clinical isolates of Candida species, in which 27 (27%, out of them 16%-male neonates and 11% female neonates) clinical isolates were collected from neonates (age range of 0-3 months), 30 (30%) were collected from females and 43 (43%) were collected from males. The age range of the adult patients was 22-85 years. The clinical specimens from which these Candida species were isolated included urine 54 (54%), blood 37 (37%), sputum 7 (7%) and high vaginal swab (HVS) 2(2%) as mentioned in table-2.

Table 2:- Distribution of clinical specimens included in the studyy

Clinical specimens	Neonates	Female	Male	Total No. (100)
Urine	---	22	32	54
Blood	27	06	04	37
Sputum	---	---	07	7
HVS	---	02	---	2
Total (%)	27 %	30 %	43 %	100 %

HVS: High vaginal swab

All Candida isolates were first identified by phenotypic method and then genotypic method, the distribution of Candida isolates is mentioned in the table-3 and figure-1(a, b).

Table 3:- Distribution of isolates of various Candida species by Phenotypic (PM) and Genotypic method (GM).

Candida species	Phenotypic method (No.)	Genotypic method (No.)
C. tropicalis	61	59
C. albicans	15	21
C. parapsilosis	09	10
C. guilliermondii	08	01
C. glabrata	04	06
C. krusei	03	03
Total (%)	100 %	100 %

Figure-1(a): Distribution of isolates of various Candida species by Phenotypic Method.

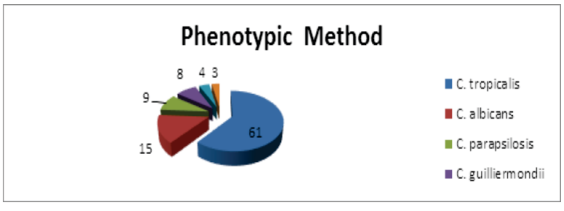
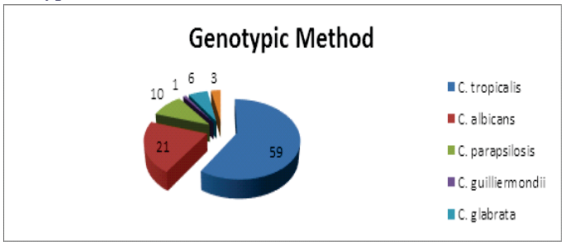


Figure-1(b): Distribution of isolates of various Candida species by Genotypic Method.

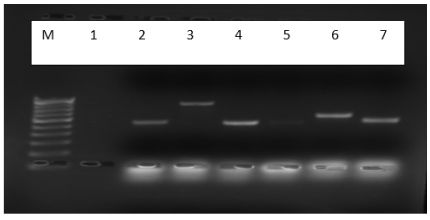


To confirm the identity of the Candida species the ITS1-5.8ITS2 regions of fungal rRNA genes were amplified with universal primers. The PCR products were digested with one restriction enzyme, Msp1, allowed discrimination of medically important Candida species. PCR assay successfully amplify the ITS region of all 100 isolates by using universal primers ITS-1 and ITS-4 providing a single PCR product of approximately 510-870bp (table-4, figure-2).

Table-4, Sizes of ITS1-ITS4 PCR products for Candida species before and after digestion with Msp1.

Candida species	ITS1-ITS4 sizes	Restriction products sizes
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-2, 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.



The restriction enzyme MspI was used for RFLP technique as done by Mirhendi et al, it produced two bands for C. albicans, C.tropicalis, C.glabrata and C.krusei, and three bands for C.guilliermondii except C.parapsilosis as there were no recognition site for this enzyme, and its PCR and digestion product is of same size. The bands pattern produced by RFLP technique is different from each other and hence it is easy to distinguish from one another (fig-3).

Fig-3 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 mark

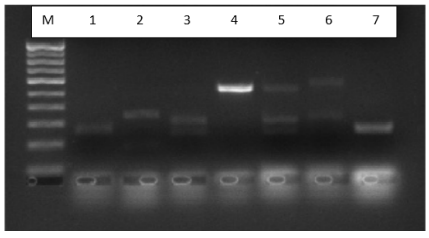


Table:-5 Species wise distribution of Candida isolates misidentified by Phenotypic Method

Candida spp.	Phenotypic method (PM) (No.)	Genotypic method (GM)(No.)	Similar Identification by PM & GM (No.)	Misidentification by Phenotypic method (No.)
C. tropicalis	61	59	53	08
C. albicans	15	21	14	01
C. parapsilosis	09	10	07	02
C. guilliermondii	08	01	01	07
C. glabrata	04	06	04	00
C. krusei	03	03	02	01
Total (%)	100 %	100 %	81%	19 %

As seen in table-5, 81% of Candida isolates was identified by both phenotypic and genotypic method and 19% of Candida isolates was misidentified by the phenotypic method which show a high significant variation between the phenotypic and genotypic method.

Identification shown by genotypic method was significantly higher for C. albicans than the phenotypic method.

DISCUSSION

Nowadays, the opportunistic pathogen Candida causes a life threatening infection especially in immunocompromised patients [16]. The early diagnosis of fungal infections is necessary for the decrease of mortality rate. Now days, Candida species have been identified by

traditional techniques such as germ-tube production and biochemical tests. These methods need to purify the target organisms that are laborious and very time consuming. Molecular techniques have high discrimination power which is helpful in species identification especially in the epidemiological studies to evaluate route of transmission, as well as to choose appropriate antifungal drugs. Several molecular methods are currently in use [16-20]. 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 [21]. The ITS1 and ITS2 regions are surrounded by 5.8SrDNA gene. These regions are used for diagnosis, identification, and taxonomy of medically important fungi. Universal primers are used as a beneficial approach for clinical microbiological diagnosis[20,21,22].

It is reported that RFLP- like techniques use universal primers to identify different fungi. (21). PCR and RFLP method has good advantage over the other molecular technique, such as RFLP with genomic DNA and electrophoretic karyotyping, is being simple and quick (23).Irobi et al, (24) also used RFLP method, to differentiate C.albicans, C.tropicalis, C.dubliniensis, and C.krusei from 114 isolates.

McCullough et al,(25) used two molecular methods to identify unusual strains of C.albicans and to compare them with standard strains of C. dubliniensis and type I C. stellatoidea.

Mirhendi et al (26) by using universal primers (ITS1 and ITS2) were able to amplify the ITS regions of all yeast tested, and then PCR amplicons were digested with MspI. Finally they could successfully identify the medically important Candida spp.

In our study, restriction digestion of the ITS amplification product with MspI produced the specific pattern for each species. Similar to other papers, the same results were obtained by CHROMagar and other phenotypic methods in detecting different Candida spp.

The results of genotypic method when compared with phenotypic method, we observed the total 19 misidentification of Candida isolates by phenotypic method which is highly significant. Due to this finding we got a reason to apply molecular methods for the determination and identification of medically important Candida species in clinical laboratories.

Analysis of RFLP derived of DNA for different Candida spp. has the good advantage of being an easy, fast and reliable in comparison with the phenotypic method, which is insensitive, lacks reproducibility and standardization and besides have limited availability (18). We did not identify any C.dubliniensis between all isolates suspected to dubliniensis.

In Conclusion, PCR-RFLP is an easy, fast and highly valuable method which can be used in routine diagnostic laboratories to speciate Candida isolates from various clinical specimens. The rapid and easy method of Candida speciation will help clinicians to decide on empirical therapy in Candidiasis before antifungal susceptibility results are available.

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