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Molecular detection of Candida species by Restriction Fragment Length Polymorphism (RFLP) analysis of PCR from HIV infected persons

Sandhya Rani T*[1], Srikumar R[2], Prabhakar Reddy E[3], Latha S[4]

[1]Research Scholar, Department of Microbiology, Sri Lakshmi Narayana Institute of Medical Sciences, Affiliated to Bharath Institute of Higher Education, Pondicherry-605502, India
[2]Centre for Research, Sri Lakshmi Narayana Institute of Medical Sciences, Affiliated to Bharath Institute of Higher Education, Pondicherry-605502 , India
[3]Department of Biochemistry, Sri Lakshmi Narayana Institute of Medical Sciences, Affiliated to Bharath Institute of Higher Education, Pondicherry-605502, India
[4]Department of Physiology, Sri Lakshmi Narayana Institute of Medical Sciences, Affiliated to Bharath Institute of Higher Education, Pondicherry-605502, India

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ABSTRACT

Oral Candidiasis an important opportunistic fungal infection in HIV patients. This is the first sign of HIV infection. It can spread from the mouth through the pharynx to the oesophagus. Oral Candidiasis is most regularly complicated with *oesophagal Candidiasis,* which may restrain nourishment and result in weight reduction, alarming the overall health of the HIV tainted persons. In present study, we have a tendency to determine in Candida species by blend of Restriction Fragment Length Polymorphism (RFLP) examination of PCR from HIV tainted people and furthermore we demonstrated that phenotypic and genotypic identification method was vital to work out the species. We recognized seventy-two Candida confines acquired from a hundred and fifty patients contaminated with HIV by utilizing PCR-RFLP assay. All inclusive primers for the internal transcribed spacer (ITS) area (ITS1– ITS4) of the fungal DNA qualities were utilized for this measure. We distinguished the diverse Candida spp. By utilizing the limitation enzyme MspI. Here most of the isolates were obtained from male patients 40(51%). *Candida albicans* was the recurrent species segregated (46.8%) pursued by *C. tropicalis* (30.3%), *C. glabrata* (7.59%), *C. parapsilosis* (6.3%) *C.dubulinesis* (6.3%) along with *C. krusei* (2.5%) detected successfully. PCR- RFLP is a high definite, sensitive, quick, reliable technique and also applicable method for fungal detection in clinical laboratory for identification of medically important *Candida* spp.

*Corresponding Author

Name: Sandhya Rani T
Phone: 7397446315
Email: sandhya.micro86@gmail.com

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INTRODUCTION

Oral Candidiasis a standout amongst the most widely recognized astute mycological infections in HIV patients. The range of *Candida* infection is starting from symptomless colonization to oropharyngeal candidiasis (OPC), esophagitis, onychomycosis, vulvovaginitis, cutaneous candidiasis and systemic candidiasis inclusive of candidemia. It has been seen that just about all HIV tainted individuals are colonized with *Candida* (Brandolt *et al.*, 2017). Oropharyngeal candidiasis (OPC) is the first sign of HIV

infection. It can spread from the mouth through the pharynx to the oesophagus.

Candida albicans is as often as possible segregated species as a pathogen of the oral mucosa, Non *albicans* species are ensnared with more prominent recurrence of entrepreneurial pathogens related with disorders especially in immunocompromised hosts (Repentigny *et al.*, 2002). OPC mainly occurs when the patients having CD4 counts less than 200 and additionally increased plasma HIV RNA levels. Which is undoubtedly correlated among oral *Candida* carriage just as with oral Candidiasis in HIV patients (Liu *et al.*, 2006). These results may propose that a decline in oropharyngeal *Candida* carriage and Oral Candidiasis in HIV can be accomplished by starting patients on exceptionally dynamic antiretroviral therapy (HAART) without the requirement for explicit antifungal treatment. Oral Candidiasis is usually complicated with oesophagal Candidiasis which may restrain sustenance utilization and result in weight loss, undermining the overall health of the HIV infected persons.

Nowadays, oral Candidiasis may cause the improvement of azoles resistant because of delayed utilization of antifungal agents and may incline to a move in non-*albicans* species related with unmanageable and repetitive diseases (Hamza *et al.*, 2006). Moreover, various procedures dependent on phenotypic qualities, for example, segregation dependent on colony colour on CHROM agar Candida medium, assimilation profiles and spore formation for identification of Candida species, they are not fully reliable. During this study, we have tendency to determine in *Candida* species by sequence of Restriction Fragment Length Polymorphism (RFLP) analysis of PCR from HIV infected persons and furthermore we demonstrated that phenotypic and genotypic identification method was necessary to work out the species.

MATERIALS AND METHODS

The present study has been endorsed by the Research and Ethical Committee of Medical Microbiology and Immunology Department, Faculty of Medicine at Sri Lakshmi Narayana Medical College Pondicherry. Patients who have utilized antifungal medications were prohibited from the study. Every one of the patients signed an informed consent form to participate in the study.

Specimen collection

10 ml of sterile phosphate-buffered saline was given to the patients and asked them to wash their mouth for sixty seconds and then expectorate into the given container. Every sample was instantly taken to the laboratory; vortex mixed followed centrifugation at 6000 rpm for 10 min. The pellet acquired from the rinse sample was re-suspended in 1 ml of sterile phosphate buffered saline. A hundred microliters of the re-suspended specimens were cultured on Sabouraud Dextrose Agar and incubated at 37 °C for 72 h. Separates on SDA were distinguish as Candida by colony morphology, 10%KOH and Gram staining.

Phenotypic identification

Candida isolates were speciated phenotypically by Germ Tube Test (GTT). For determination and identification of multiple species used CHROME-agar Candida on the reported of colony colour for 48h at 37^0C and other discriminative tools chlamydospore formation on cornmeal agar. The isolated Candida was put away in glycerol broth at 70^0C for additional process by molecular techniques.

Molecular detection

Restriction Fragment Length Polymorphism (RFLP) examination of PCR was one of the highest quality level technique for identification of Candida species in the present study (Santos *et al.*, 2010).

DNA Extraction

DNA eradication was performed utilizing the conventional bead beater method. A loopful of fresh pure colonies of Candida was suspended in 100 μl STES buffer [200 mM Tris–HCl (pH 7.6), 100 mM EDTA (Ethylenediaminetetraacetic acid), 0.1% SDS(sodium dodecyl sulfate)] and 40 μl of TE (Tris–EDTA) buffer [10 mM Tris–HCl (pH 8), 1 mM EDTA], 120 μl phenol: chloroform mixture (1:1 V/V) and 0.3 g sterile zirconium beads (0.1 mm diameter; Bio Spec-Products)were added. The samples were homogenized using a mini bead beater (model 3110BX; Bio Spec Products) at 480 rpm for 5 min. The upper aqueous phase (100 μl) was transferred to a sterile microcentrifuge tube and DNA was precipitated in the presence of 220 μl cold ethanol (100%) and 10 μl of 3 M sodium acetate at -20 °C for 18 h. The solution was centrifuged at (13,000 rpm)15,493$\times g$ for 12 min and the DNA pellet was air dried and dissolved in 30 μl TE buffer. Extracted DNA samples were stored at -20 °C until used.

PCR Analysis

The ITS-1 and ITS-2 regions of Candida spp. Were amplified using universal primers; ITS-1(50- TCC GTA GGT GAA CCT GCG G-30) and ITS-4 (50- TCC TCC GCT TAT TGA TAT GC-30). Initial Denaturation for 5 mins at 94°C: This progression warms the double-stranded DNA template strand to the purpose where

ever the strands begin denaturing and the hydrogen bonds are broken between the nucleotide base pairs. Denature thirty seconds at 94°C: Continuing denaturation of double-stranded DNA. Anneal primers for forty-five seconds at 58°C.The forward and reverse primers are steady among this temperature vary to strengthen to every one of the single-stranded DNA template strands. The DNA polymerase is likewise steady enough to now tie to the primer DNA sequence. Broaden DNA for one minute at 72°C: The emerald polymerase has an ideal temperature around 70-75°C so this progression empowers the DNA polymerase to synthesize and extend the new target DNA strand precisely and quickly. Rehash stages two to four for forty cycles. Last Extension for seven minutes at 72°C: The last extension to fill-in any projecting ends of the freshly synthesized strands. Intensified PCR products were kept running on 2% agarose gel electrophoresis and envisioned by UV transilluminator. Presently the DNA polymerase is likewise steady enough to tie to the primer (Mirhendi S et al.,2006).

RFLP analysis

RFLP is done by using MSP1 restriction enzyme.5'...CC G G...3',3'...G G CC...5'

1 μL MspI enzyme 5000 units (BioLabs, England) and 2 μL enzyme buffer (NEB buffer 4) were added to 7 μL of each PCR product. Incubation at 37^0C for 16 h was done. Restriction fragments were separated by 3% agarose gel electrophoresis (Iwen *et al.*, 2002).

RESULTS AND DISCUSSION

Out of 150 HIV positive patients an authoritative analysis of Candidiasis was made in 79 patients. Different presentations of Candidiasis in HIV positive patients prohibit oropharyngeal candidiasis, oesophageal candidiasis, candidemia, pulmonary candidiasis, cutaneous candidiasis and candidal diarrhoea. Most typical presentation of oral candidiasis observed in 79(52.6%) patients and most of the isolates were obtained from male patients 40(51%) Figure 1.

Table 1 Shows *Candida albicans* was the commonest species segregated 37 (46.8%) pursued by *C. tropicalis* 24(30.3%),*C. glabrata* 6 (7.59%), *C. parapsilosis* 5(6.3%) *C.dubulinesis* 5 (6.3%) and *C. krusei* 2(2.5%). The separation rate of *non-albicans Candida* species was higher 42 (53.1%) as compared to *Candida albicans* 37(40.9%).

Candida is a universal fungus, which is severe as a disease in immuno compromised persons like AIDS-affected individuals with other clinical manifesta-

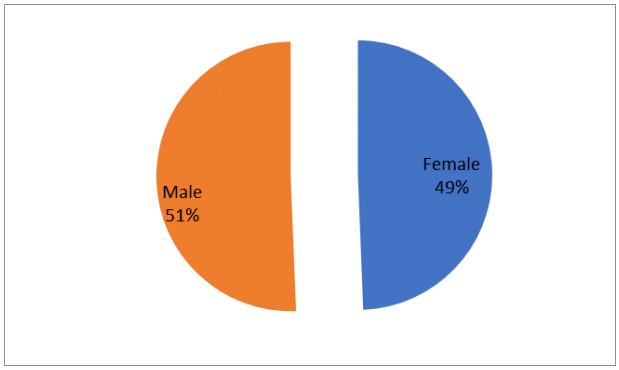

Figure 1: Gender conveyance of studied patients

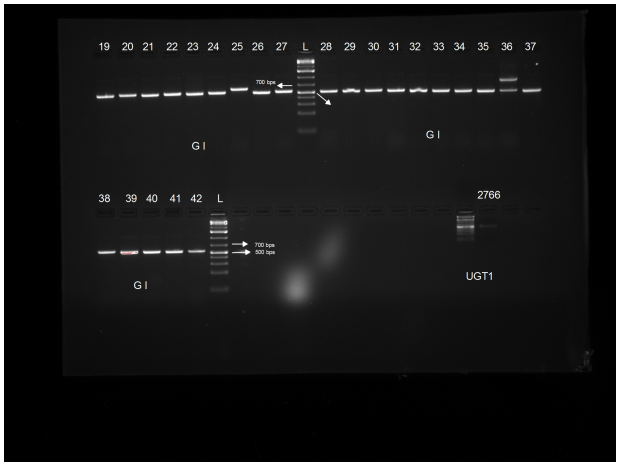

Figure 2: PCR derivates before processing with limitation enzymes

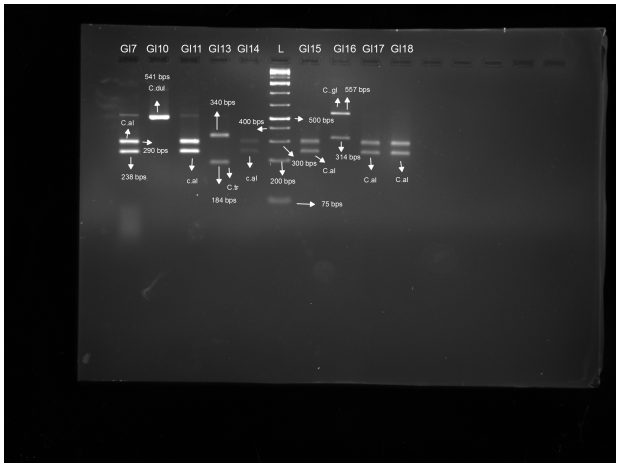

Figure 3: Restriction digestion of polymerasechain reaction derivates of Candida clinical segregates with the enzyme MSP1. Lanes-1: atomic weight marker (100-1000bp); GI7, GI11, GI14, GI15, GI17 & GI18: C.albicans (at238bp); GI10C. dubulinsis (at 541), GI13 C.tropicalis (at 184bp), GI16 C. glabrata (at557bp)

Table 1: Colony colour of Candida isolates on HicromeCandida differential agar

S.No.	Candida species	Colony colour on Hicrome Candida differential agar	No. of Candidaisolates (n=79)
1	Candida albicans	Light green	37 (46.8%)
2	Candida tropicalis		24(30.3%)
3	Candida glabrata	White to cream	6 (7.59%)
4	Candida.dubulinesis	Dark green	5(6.3%)
5	Candida parapsilosis	Light pink	5 (6.3%)
6	Candida krusei	Purple fuzzy	2(2.5%)

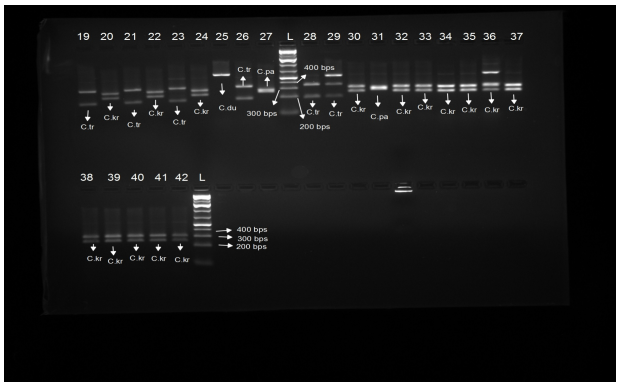

Figure 4: Restriction digestion of polymerase chain reaction derivates of Candida clinical segregates with the enzyme MSP1. Lanes-1: atomic weight marker (1001000bp); GI24: C.krusei (at261bp); GI31C.paralopsis (at 541)

tions. The treatment of Candidiasis is obstructed due to delays in analysis and absence of accurate demonstrative techniques for identification of species level.

The present study was intended to spot the spectrum of Candida species associated with HIV in a group of 79 patients presenting with oral Candidiasis and to evaluate their antifungal susceptibility pattern out of total. Rate of prevalence of HIV were 52.6% among the studied group. Out of the 150 oral wash isolates 40(51%). These reports are similar with the findings of K. P. Anwar,et al and V. Nissapatorn, C. K. e where they explained the static factors of the HIV positive people in the nation and features the way that males are at expanded risk in their employment and propensities which involve them to be increasingly transitory in contrast with females (Nissapatorn *et al.*, 2004; Khan *et al.*, 2012).

In the present study, 46.8% were C albicans were more frequent which is similar to the study by Thompson GR et al. and Campisi G, et al. 32% to 62% respectively (Thompson *et al.*, 2010).this condition may be due to CD4 counts and Anti-retroviral treatment of the patient, which is inversely related to the Candida infection.

The isolation rate of *non-albicans Candida* species was higher 53.1% as compared to *Candida albicans*.This results correlated with other studies, Anupriyawadhwa et al. found 40% of non-albican (Wadhwa *et al.*, 2007). The second most isolated non albicans *C. tropicalis* 24(30.3%), *C. glabrata* 6 (7.59%), *C. parapsilosis* 5(6.3%) *C.dubulinesis* 5 (6.3%) and *C. krusei* 2(2.5%) from oral candidiasis of HIV positive patients. The scale and distribution of Candidial infection may be depend on anatomical localization and environmental condition. *C. tropicalis C. glabrata* and *C. parapsilosis* are mostly isolated in the throat and vagina (Brandolt *et al.*, 2017).

Early detection of Candidiasis infection is mandatory to enable clinicians directly for best treatment choices and increment the possibility of patients to survive. The capacity of molecular techniques to recognize Candida species is far before to that of customary phenotyping methods (Shokohi *et al.*, 2011). Hence, nowadays, different types of molecular techniques have been connected for the detection of hereditary identification of Candida spp. Which incorporate standard PCR, multiplex PCR, PCR with species-specific probes, PCR-RFLP, real-time PCR, randomly amplified polymorphic DNA (RAPD)–PCR, DNA sequence analysis, and the mitochondrial large subunit ribosomal RNA (mtLsurRNA) mtLsurRNA gene Sequences (Resende *et al.*, 2004; Sugita and Nishikawa, 2004).

In present study, Candida spp.was recognized by PCR-RFLP technique by exploitation two widespread primers called as ITS1, ITS4 and the restriction enzyme MspI. This does not recognize C. albicans and C. dubliniensis as both are morphologically similar species of Candida (Mirhendi *et al.*, 2006). Isogai et al. and Williams et al. used restriction enzymes HaeIII HaeIII, DdeI, and BfaI for identification of clinically important Candida spp (Isogai *et al.*, 2010; Williams *et al.*, 1995). Evaluation of RFLPs obtained from the deoxyribonucleic

acid extraction of Candida species, the upside of PCR technique when contrasted with the phenotypic techniques. These studies recommended that PCR-RFLP is most helpful and dependable technique for detection of non-albicans species from HIV-positive patients in mycology laboratory. Many authors concern PCR-RFLP is one of the best molecular technique for the identification of Candida spp (Irobi *et al.*, 1999; Makimura *et al.*, 2006).

CONCLUSION

In conclusion, our study showed that C. albicans and non-albicans like *C. tropicalis* C. glabrata, C. parapsilosis along with *C. krusei* were the vital species segregated from HIV-confirmed patients may be due to misuse of drug and lack of sexual discrimination which are the main factors impact the wide-ranging of dispensation frequency of Candida spp. PCR-RFLP is a high definite, sensitive, quick, reliable technique and also applicable method for fungal detection in clinical laboratory for identification of medically important *Candida* spp. It is used for detection of mycological examinations in HIV-positive along with other immuno compromised patients for epidemiological studies.

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