ORIGINAL RESEARCH

A COMPARISON OF HICROM AGAR AND PCR - RFLP FOR THE IDENTIFICATION OF *CANDIDA* SPECIES

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ABSTRACT

Aim: The study's aim was to compare the effectiveness of HiCHROMagar and PCR-restriction fragment length polymorphism (PCR-RFLP) in identifying *Candida* species in order to ascertain the advantages and drawbacks of each technique.

Methods: One hundred and twenty five *Candida* strains were isolated from different clinical specimens from patients admitted to different wards of Era's Lucknow Medical College. The *Candida* isolates were from urine, Sputum, Blood, vaginal swab, pus and nail. Clinical samples werecultured on Sabouraud-dextrose agar (SDA) with chloramphenicol for 48 h at 37 °C.to obtain *candida* colonies. Wet mount,Gram staining, germ tube, sugar fermentation and assimilation were performed for all isolates. *Candida* isolates on SDA were subcultured on HiCrome Candida Differential agar (HiMedia, Mumbai, India) for 24-48 hours; plates were incubated at 37 °C and was re-evaluated by employing digestion of the ITS1–5.8SrDNA–ITS2 region using Msp I restriction enzyme for RFLP and universal primers internal transcribed spacer 1(ITS1) and ITS4 for PCR amplification**.**

Results: Out of 125 patients 58.4% were female and remaining was male. The mean age of the patients was 44.76 years. Most of the sample collected from the urine..*Candida* isolates identified as *C.albicans* based on colour (light green) by HiCrome agar were in agreementwith PCR-RFLP while , the identifcation of the non albicans *Candida species (C. krusei, C. glabrata ,C.parapsilosis* and *C. tropicalis*) by colour code on HiCrome agar showed a discrepancy with PCR–RFLP.

Conclusion: Hi chrome media has the benefit of being inexpensive and less challenging than other molecular methods, but it fail to clearly identify some uncommon *candida* species

while RFLP-PCR employing ITSI and ITS4 primers and restriction enzyme is a trustworthy, and useful approach for identifying medically significant *Candida* spp. in clinical laboratories
Keywords: PCR-RFLP, HiCROMagar, *Candida* species.

Introduction

Candida species often live as commensals on mucosal membranes in healthy people and may be found in around 50% of the population in a non-virulent state. [1] These species, however, may become pathogenic if the host's natural flora is disturbed or immunity is compromised. Among human pathogenic fungus, the genus *Candida* is the most common cause of systemic life-threatening infections in hospitalized patients.[2]*Candida* species have been identified as the fourth most prevalent pathogen responsible for nosocomial bloodstream infection in hospitalized patients.[3] This genus contains at least 30 medically significant species that are implicated in human candidiasis. [4]

Due to the rise of immunosuppressive circumstances, the incidence of infections caused by the genus *Candida* has significantly grown during the last few decades. Furthermore, formerly assumed to be non-pathogenic organisms are now known to be opportunistic pathogens.[5,6] The present alterations in candidiasis epidemiology show a shift in the predominance of *Candida* species, with a decreased percentage of *C. albicans* and a rise in non- albicans *Candida* species. [7] According to a recent assessment, *C. albicans* accounted for more than 80% of all *Candida* isolates recovered from nosocomial yeast infection in the 1980s. *C. albicans* presently accounts for less than half of all *Candida* blood isolates.[9] Because *Candida* species differ in their sensitivity to antifungal drugs, early and precise identification of the species may aid in the discovery of an effective treatment for candidiasis. [10] On the other hand, it is necessary to identify the primary source of the illnesses and decide if they are endogenous or acquired exogenously from other patients or health care professionals. [11,12] As a result, reliable identification of strains at the species and sub-species levels is critical for epidemiological investigations and outbreak management.

There are several tests available for identifying *Candida* species, which may be split into two categories: phenotypic and genotypic. Time-consuming phenotypic assessments include yeast colony morphologies on malt extract agar or chromogenic culture medium, sugar absorption and fermentation tests, and commercial kits (e.g., API). Furthermore, since these methodologies rely on the varied expression of phenotypic features, the findings might be inconsistent.

Genotypic tests, on the other hand, are more accurate and less susceptible to fluctuations owing to growth conditions and phenotypic flipping. The most well-known molecular techniques include specific primers in polymerase chain reaction (PCR) and multiplex PCR, specific probes for each species, PCR-restriction fragment length polymorphism (PCR-RFLP), sequencing of specific regions of the genome, real-time PCR, and PCR-fragment size polymorphism (PCR-FSP). [13,14] Several investigations have shown that PCR-RFLP is simple, fast, affordable, and extremely useful methods for differentiating *Candida* species.[15]

Materials and Methods

One hundred and twenty five Candida strains were isolated from different clinical specimens from patients admitted to different wards of Era's Lucknow Medical College during the

period from August 2019 to September 2020.The *Candida* isolates were from urine, Sputum , Blood , vaginal swab , pus and nail.

Conventional methods

Clinical samples werecultured on Sabouraud-dextrose agar (SDA) with chloramphenicol for 48 h at 37 °C.to obtain *candida* colonies. Wet mount, Gram staining, germ tube ,sugar fermentation and assimilation were performed for all isolates.

Species Identification by HiCrome Candida Differential Agar - *Candida* isolates on SDA were subcultured on HiCrome Candida Differential agar (HiMedia, Mumbai, India) .For 24-48 hours; plates were incubated at 37 °C. The colonies were recognised based on their distinctive morphology and colour in accordance with the media's chromogenic scheme. *C. albicans* and *C. dubliniensis*(green), *C. tropicalis*(metallic blue*), C. krusei*(purple) *, C. parapsilosis* (pink,) and *C. glabrata* (cream to white)

Species Identification by PCR-RFLP

DNA Extraction DNA isolation was performed from all the clinical isolates and standard strains by using Fungal DNA isolation kit (Qiagen).

PCR Assay – The master mix was prepared containing 25 ul of PCR mix (Qiagen),1 ul of forward primer ITS-1 (forward 5' TCC GTA GGT GAA CCT GCG G-3' and ITS4 reverse (reverse 5 TCC TCC GCT TAT TGA TAT GC-3')),1 ul of template DNA and the volume made up of 50 ul with sterile nuclease-free water. The reaction mixture was kept in the thermocycler (BIORAD), the program was performed as follows: Initial denaturation at 94 °C for 2 minutes, denaturation at 94 °C for 30 sec, annealing at 56°C for 30 sec, extension at 72 °C for 1 minute. Amplified products were visualized by 1% agarose gel electrophoresis in TBE buffer (20 mmol/l EDTA, 10 mmolTris boric pH 8). Gel was stained with ethidium bromide (0.5 µg/ml) and photographed by ultraviolet photography. And it yielded a unique PCR product size of approximately 510–881 bp.

Restriction fragment length polymorphism analysis Digestion of amplified products was performed using restriction enzyme MspI. MspI makes DNA cleave where there is a CCGGsequence. The digestion of ITS region of Candida spp. by MspI enzyme created two bands for *C. albicans,* C. glabrata, *C. tropicalis and C. krusei* . For *C. parapsilosis,* the size of the PCR and digestion product was similar. The restriction patterns of each *Candida* spp. were perfectly specific. The sizes of amplified DNA fragments were identified by comparison with molecular size marker DNA (100-bp DNA ladder).[15]

Results

Out of 125 patients 58.4% were female and remaining was male. The mean age of the patients was 44.76 years (Table -1). Most of the sample collected from the urine (44%)followed by sputum (29.6%), Blood (10.4%) pus(8%) vaginal swab (5.6%) and nail (2.4%)(Table -2). Based on the colour code on HiCrome agar, 48 of the 125 candida isolates were identified as *C. tropicalis ,*35 *as C. albicans*, 20 as *C. glabrata*, 10 as *C.parapsilosis* ,9

as *C. krusei* and3 unidentified (Fig. 1).By using the PCR-RFLP technique, all of the isolates were further genotypically identified. Using universal primers, the ITS1 and ITS4 successfully amplified the ITS region of 125 clinical isolates from the examined *Candida* spp and it produced a distinctive PCR product with a size of roughly 510-881 bp. (Table -3, Fig.2& 3)

According to Table 4, all *Candida* isolates identified as *C.albicans* based on colour (light green) by HiCrome agar were in agreement withPCR-RFLPwhile , the identifcation of thenon albicans *Candida species (C. krusei, C. glabrata ,C.parapsilosis* and *C. tropicalis*) by colour code on HiCrome agar showed a discrepancy with PCR–RFLP.The minimum time required for the identification of 10 sample of *candida* species in our study included 3 days for HiCROMagar and approx. 2 days for PCR- RFLP (Table -5)

Discussion-

Rapid and precise identification of clinical isolates of Candida species may alter the mortality rate, cost of therapy, and length of hospitalisation for invasive infections. The chromogenic medium HiCrome Candida Differential Agar and PCR-RFLP were evaluated in this research for their performance, accuracy and speed, in identifying Candida species. We discovered that, although being a simple , HiCrome Candida Differential was unable to distinguish different species. As a consequence, some investigations have shown disagreement between the findings of molecular and phenotypic testing. [16,17]

CHROMagar, a chromogenic medium, has been offered for both isolation and identification of Candida species, based on the pigmentation of the colonies with distinct hues, which is due to varied enzyme activity in *Candida* species. In compared to other standard tests, such as API systems and the Vitek 2 ID system, this approach has the benefit of being less costly and less complex. However, this approach takes longer than molecular testing such as PCR-RFLP. By presenting distinct coloured colonies on a plate at the same time, this medium may identify the existence of mixed cultures. However, since CHROMagar is only intended to differentiate threespecies (*C. albicans* [Light green],*C. tropicalis* [metallic blue]*C. krusei* [purple with fuzzy border]), it cannot accurately identify additional species.[18] Such species misclassification was also documented in the research conducted by Estrada et al. and Souza et al.[19,20]The detection of fungal infections using molecular biology tests is more reliable than standard phenotyping procedures. The common and some uncommon or unusual pathogenic *Candida* were distinguished in the PCR-RFLP test by the size of the fragments generated from the restriction digestion of the PCR products by an enzyme[21]. The PCR-RFLP method takes less amount of time as standard phenotypic conventional procedures, and it is far more sensitive in identifying all species of *Candida*.When compared to HiCrome Candida differential agar, the sensitivity of PCR-RFLP was shown to be 100%.

Conclusion- For the preliminary identification of some frequently encountered *Candida* spp., HiCrome Candida Differential Agar is a quick and reliable approach (*C. albicans , C. krusei*)but PCR-RFLP tests are more effective in identifying different *Candida* species compared to HiCrome Candida Differential Agar.Therefore, a molecular method with great discriminatory power, such as PCR-RFLP, might be highly suggested for identifying *Candida* species.

Abbreviations- PCR–RFLP: polymerase chain reaction-restriction fragment length polymor- phism; ITS: internal transcribed spacer;

Conflict of interest: None

Authors' contributions:SK, drafted the manuscript, gathered information from theliterature, performed the experiments, designed the figures & tables and wrote thepaper. MS and VK supervised and reviewed the manuscript**.**

Ethical : Ethical committee of ERA medicalcollege, approved the study protocol.

Availability of Data and Materials –All the data is available with the corresponding author Ethics statement.

Table 1 Age and Gender distribution of patients

Gender	Number	Percentage
Male	52	41.6
Female	73	58.4
Age in Mean	44.76	

Table 2- Sample for identification

Specimens	Number	Percentage
Urine	55	44
Sputum	37	29.6
Blood	13	10.4
Pus	10	8
Vaginal swab	7	5.6
Nail	3	2.4

Table 3 -Size of ITS1-ITS2 products for*Candida* spp. prior to and following MspI digestion.

Candida species	**Size of ITS -1 and ITS-4**	**Size of restriction product bp**
C.tropicalis	**524**	(340, 184 bp),
C.albicans	**535**	(297, 238 bp)
C.glabrata	**871**	(557, 314 bp)

C.krusei	510	(297, 238 bp)
C.parapsilosis	520	(520 bp).
C. guillermondii	608	(371, 155 , 82 bp)
C. rugosa	881	(320,561 bp)

Table 4 -Disagreement between PCR RFLP and HiCrome agar in the speciation of *Candida* **isolates**

Candida species	Color on HiCrome media as directed by the manufacturer/ (no of isolates showing the respective colony colour)	Results of PCR-RFLP are in agreement with HiCrome agar.	Discrepancy between PCR-RFLP and HiCrome agar
C.tropicalis	Metallic blue 48	43 (89.58%)	C.rugosa 5 (10.41%)
C.albicans	Light green 35	35 (100%)	0
C.glabrata	Cream to white 20	17 (85%)	C.parapsilosis 3 (15%)
C.Krusei	purple with fuzzy border 9	9 (100%)	0
C.parapsilosis	Pink 10	9 (90%)	C.glabrata 1 (10%)
unidentified	Dark Pink 3	0	C.guillermondii 3(100%)

Table 5. Time taken for identification of *Candida* **species**

Methods	Steps involved	Time (hr)	Total time
HiCrome Candida differential agar	Growth on SDA media Growth on HiCrome	24 -hr 24-48	3 days
PCR –RFLP	Growth on SDA media	24 hr	
	DNA extraction PCR RFLP with gel electrophoresis	4 hr 3 hr 2 hr	Approx 2 days

Fig 1: HiCrome™ Candida Differential Agar showing candida species.

a)   b)
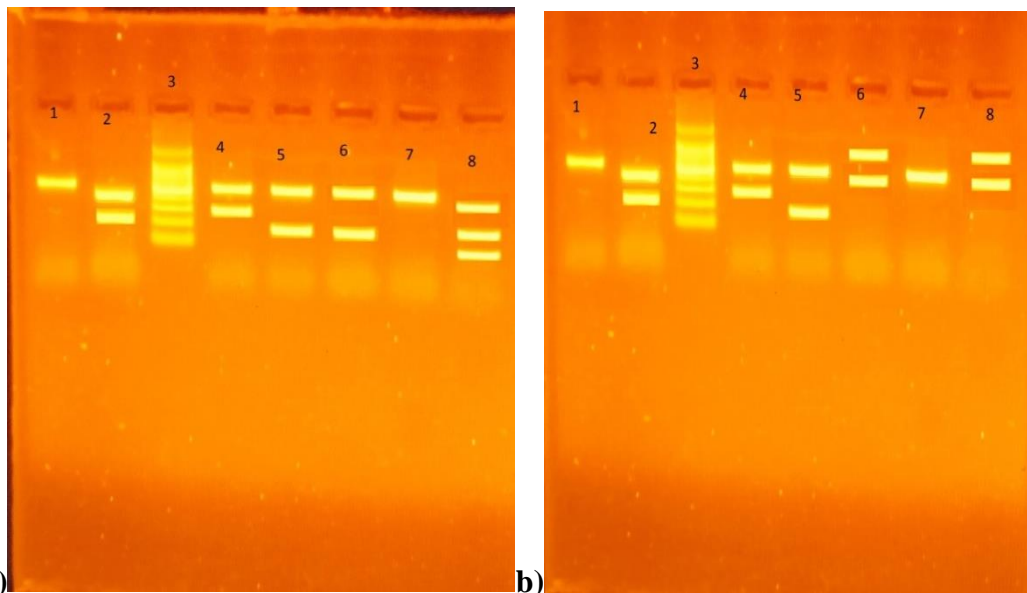
Fig 2. a) Restriction digestion of PCR products of *candida* isolates with the enzyme *MSP*I .Lane 1 *C.parapsilosis* (ATCC 22019),Lane 2 *C.albicans* isolate , Lane 3 – molecular marker (100bp), lane 4 *C.glabrata,* Lane5 *C. tropicalis* (ATCC 0750), Lane 6 *C. tropicalis* isolate ,Lane 7 *C.parapsilosis* isolate , Lane 8 *C.guilliermondii.*b)) Restriction digestion of PCR products of *candida* isolates with the enzyme *MSP*I *C.parapsilosis* isolate Lane 2 *C.albicans* isolate , Lane 3 –molecular marker (100bp)lane4 *C.glabrata,* Lane5 *C. tropicalis* isolate Lane 6 and 8 C. rugosa,Lane 7 *C.parapsilosis* isolate .

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