Development of simple and rapid identification system for *Candida* species isolated from clinical samples

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Abstract

A study was performed to develop a novel chromogenic media and PCR based molecular marker for the identification of Candida species. Totally, nine different new chromogenic media were tested against five species of Candida viz. C. albicans, C. glabrata, C. tropicalis, C. krusei and C. lusitaniae. The Sabouraud dextrose medium supplemented with methyl blue (0.025%) and phenol red (0.04%) [Chromogenic Candida Agar 7 (CCA7)] showed astonishing results by differentiating the five species of Candida [C. albicans (light blue), C. glabrata, (steel blue), C. tropicalis (violet blue), C. krusei (light purple) and C. lusitaniae (sky blue)]. To check the efficacy of CCA7 medium further, it was evaluated using five clinical strains of different species, which showed similar results as that with the standard strains. Interestingly, newly formulated medium showed differentiation of Candida species better than the commercial reference media. Efforts on development of new molecular marker for direct identification of Candida species using polymerase chain reaction were also carried out by designing primers for gene isocitrate lyase (ICL1) involved in the glyoxalate pathway that amplify 200-bp product in C. albicans and C. tropicalis, but not in other species tested.

However, a set of primers designed for INT1 gene involved in adherence and filamentation of organisms that were able to amplify 227-bp gene product only in the C. albicans. The similar patterns of PCR products were observed both in clinical isolates and standard Candida strains. Hence, newly developed chromogenic medium along with molecular marker is very useful in the rapid detection of commonly occurring C. albicans and other species from the clinical specimens in large numbers.

Keywords: *Candida*, chromogenic media, identification, *ICL1* and *INT1* genes, polymerase chain reaction.

Introduction

Candida species is responsible for the chronic superficial infections of skin, nail, mucosal surfaces and also causes life threatening systemic infections in immunocompromised individuals[26,41]. *Candida* is one of the dangerous opportunistic pathogen in HIV patients and it is responsible for increased morbidity and mortality. The HIV patients are vulnerable for oropharyngeal, vaginal and systemic candidiasis[26,34,36,41]. *Candida* vaginitis is the most common disease found in females, who visit to gynecology clinics[40]. More than ten species of *Candida* are known to cause infection in human, but predominately infected by *C. albicans*[25,36]. However, a shift in the spectrum of *Candida* species has been observed, with an increase of non-*albicans* species being found in clinical isolates[43].

An increasing number of reports have described the recovery of atypical, germ tube and chlamydospore positive oral *Candida* isolates from HIV-infected individuals in widely distant geographic locations[1,7]. *Candida* infections with less sensitivity to the antimycotics are increasing due to immunocompromisation, so that early and accurate identification of *Candida* species is vital for appropriate antifungal therapy. Since antifungal susceptibility testing procedures require more time, rapid species-level identification is important for prompt initiation of appropriate therapy.

A common method to diagnose the mycoses caused by the *Candida* is to study the wet smear of the samples collected using KOH in common diagnostic laboratories. Further specimen can be cultured on Sabouraud dextrose agar or using other specific medium for isolation of *Candida* strains. It can be identified using various physiological tests. They are labor intensive and consume several days and are expensive mycological media. The conventional methods of yeast identification which mainly consists of assimilation /fermentation characteristics, are reported to be cumbersome and often beyond the expertise range in non-specialized clinical microbiology laboratories and lead to ambiguous results[12].

Chromogenic medium is selective and deferential medium with the inclusion of chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colors according to the species. Even though, number of chromogenic media is available for identification of the *Candida* species in the developed countries, there is paucity of chromogenic media in the developing countries for common clinical laboratory use.

Furthermore, the currently available chromogenic media are very expensive and they cannot be easily adopted for routine use because of the poor paying capacity of the patients. Apart from physiological and agar based identification

system, molecular based rapid identification system is more promising in recent years. A number of procedures using PCR amplification techniques have been suggested for identification of *Candida* species[5,16,17,22,24,28]. The ribosomal genes are popular targets for PCR based systems for detection and identification of fungal pathogens[10,21]. However, identification of yeast on the species level is difficult to achieve due to limited variation of common yeast species on the 18S rDNA level[10,35].

PCR-based methods utilize several unique or multicopy molecular targets for the highly sensitive detection of *Candida* species in the culture or biological samples. Only a few methods for the detection and identification of several species by a single and direct PCR have been proposed[19]. The objective of the present study is to develop a novel and cost-effective differential chromogenic media to distinguish *Candida* species and confirm the result through molecular marker using polymerase chain reaction (PCR).

Material and Methods

***Candida* strains:** *Candida* cultures obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India include *Candida albicans* NCIM 3074, *C. tropicalis* NCIM 3118, *C. glabrata* NCIM 3248, *C. krusei* NCIM 3129 and *C. lusitaniae* NCIM 3484 used as reference strains. A set of clinical isolates *viz. C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. lusitaniae* was isolated from oral cavities of HIV patients and another set from vaginal region. These samples were collected from patients of Tiruchirappalli, India. All the *Candida* strains were cultured on Sabouraud dextrose agar at 37 °C.

Chemicals and media: Nine chromogenic *Candida* agar (CCA) media were prepared using basal medium Sabouraud dextrose agar (2% dextrose, 1% peptone and 2% agar and pH was adjusted to 5.6). The three chromogenic compounds *viz.* methyl blue (0.025%), phenol red (0.04%) and methylene blue (0.02%) were added separately to the basal medium. Six novel chromogenic media containing mixture of chromogens such as methyl blue (0.025%) and neutral red (0.02%), methyl blue (0.025%) and phenol red (0.04%), methylene blue (0.02%) and phenol red (0.04%), methyl blue (0.02%) and methylene blue (0.02%), phenol red (0.02%) and methyl violet (0.02%), methyl blue (0.02%) and methyl violet (0.02%) were also prepared separately. The concentrations of the chromogens were determined by initial preliminary screening with *Candida* strains. The chromogenic medium, HiChrome *Candida* agar obtained from the Himedia Pvt. Ltd., Mumbai, India was used as reference medium for comparison.

Evaluation of chromogenicity of medium: The reference *Candida* strains are separately streaked on chromogenic media. Five oral isolates and five vaginal isolates were also separately streaked on individual test medium. After the inoculation, cultures were incubated at 30°C for five days. Colony development pattern and chromogenicity of each strain were recorded daily. Chromogenicity of reference strains and clinical isolates was studied with unblinded fashion in parallel with the commercial media (HiChrome Candida agar) for direct comparison. The chromogenicity of methyl blue with phenol red supplemented media (CCA7) was further evaluated by incubating at three different temperatures (25°C, 30°C and 37°C).

Molecular identification of *Candida* species

Isolation of genomic DNA from *Candida*: Genomic DNA from the *Candida* strains was extracted by the modified method of Sambrok and Russell[38]. Briefly, *Candida* isolates were cultured on Sabouraud dextrose agar for 48 h at 30°C. Few colonies were picked from the plates and suspended in sterile water. It was washed briefly in sterile distilled water. 500 µl of cold lysis buffer [containing 2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris–HCl pH 8.0 and 1 mM EDTA] was added to the cells collected by centrifugation at 7000 rpm for five minutes and mixed. The cell suspension was treated with lysozyme (10 µl of 10 mg/ ml) for 10 minutes at 37°C, then with proteinase K (3 µl of 10mg/ ml) for one hour at 50 ºC.

Further, 200 µl of potassium acetate and 500 µl of buffered phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) were added and vortexed for 20 secs at maximum speed. After centrifuging for 10 minutes at 9000 rpm at 4°C, the upper phase was pipetted into one ml of ice cold isopropanol and mixed gently to precipitate the DNA. The precipitated nucleic acids were collected by centrifugation and washed with 70% ethanol to remove proteins. The pellet was redissolved in 50 µl TE buffer, 3 µl RNAse (10 µl/ml) was added and then it was incubated at 37°C for five min. The concentration of DNA was estimated using UV-visible spectrophotometer (Jasco, Japan) at wavelength of 260 nm.

Primers: The nucleotide sequences of *INT1* (Intigrin-like protein involved in the adherence and filamentation) genes and *ICL1* (isocitrate lyase is key enzyme involved in glyoxalate pathway) were obtained from the KEGG database (http://www.genome.jp/kegg/) and submitted to primer3 software (http://frodo.wi.mit.edu/) to design primers. The forward primer 5' AGG CAA CTC CTA AAG CGT CA 3' and reverse primer 5' CGT CGT ATG AAG TGG TGG TG 3' were designed to amplify the *INT1* gene. In addition, the forward primer 5' GGC CAA GGA ATT GAC TGG TA 3' and reverse primer 5' CTT TAA CAC CGT CGG CAA AT 3' were designed to amplify *ICL1* gene. Oligonucleotide primers for polymerase chain reaction (PCR) were obtained from Alpha DNA (Canada).

Polymerase chain reaction: The DNA extracted from each *Candida* strain was subjected to PCR amplification. The PCR was performed in a reaction volume of 50 µl containing 0.2 mM of deoxynucleoside triphosphate mix (dATP, dTTP, dGTP and dCTP), buffer mix containing 50 mM KCl, 10mM $MgCl_2$, 25 mM Tris-HCl pH 9, 0.1% Triton X-100, 25 pM

of each primer, 2.5 U of *Taq* DNA Polymerase (Promega, USA) and 20 ng of Candidal DNA. Reaction mixtures were subjected to 35 cycles following the denaturation at 94°C for one min, primer annealing at 58°C for one min, extension at 72°C for one min and elongation at 72°C for 10 min using automated thermal cycler (Eppedorff, Germany). The size and purity of the PCR products were tested by electrophoresis in 1.5% agarose gel and stained with ethidium bromide.

Results

The *Candida* strains obtained from the NCIM (Pune, India) were used as standard reference strains for chromogenicity and molecular studies. Oral *Candida* isolates of HIV patients and vaginal isolates of patients in gynecological clinics were used as test strains. All the clinical isolates used in this study were initially identified by conventional physiological tests such as germ tube formation in bovine serum, chlamydospores formation on corn meal agar in addition to auxanotropy of clinical isolates with 12 sugars by fermentation (data are not given).

All *Candida* strains did grow on the test media, except three formulated chromogenic media (CCA3, CCA5 and CCA6). The distribution of the colony colors on various newly formulated chromogenic media and commercial media within each yeast species is listed in table 1. Generally, color of the colonies intensified as incubation time increased while peaking at 48 hours. Media with single chromogen differentiated only few species of *Candida* forming different colored colonies (Table 1). However, chromogenic media with combination of chromogens showed better differentiation of *Candida* species than with single chromogen. Among the newly formulated chromogenic media, color development of the *Candida* strains varied with species. The current study utilizes the HiChrome *Candida* agar which is a commercial chromogenic medium, is used to identify *C. albicans* (light green), *C. tropicalis* (light blue), *C. krusei* (white fuzzy) and *C. glabrata* (light pink). Interestingly, among the new media formulations with chromogenic dye namely methyl blue (0.025%) and phenol red (0.04%) CCA7 showed better differentiation among the five species of *Candida viz*., *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. lusitaniae*. The species divergence on the novel chromogenic medium (CCA7) was expressed in the form of different distinct colors.

Both clinical and reference strains of *Candida* such as *C. albicans*, *C. glabrata*, C. *tropicalis*, *C. krusei* and *C. lusitaniae* showed light blue, steel blue, violet blue, light purple and sky blue colors respectively on CCA7 media (Figure 1A). The novel chromogenic medium CCA7 differentiated more number of species (five species) (Figure 1A) than the commercial medium (Figure 1B). The commercial chromogenic medium showed differentiation of only four *Candida* species *viz. C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*. Other chromogenic media supplemented with methyl blue and methyl violet (CCA9) showed differentiation of *C. tropicalis*, *C. glabrata* and *C. lusitaniae* but *C. krusei* was not differentiated from *C. albicans* (Table 1).

Except chromogenic media CCA7, none of the other chromogenic media showed the differentiation of all five *Candida* species. Interestingly, clinical isolates also showed similar coloration as compared to the standard strains on the novel chromogenic medium (CCA7) (Figure 2). The implicit method proved efficacy of the newly formulated media for identification of *Candida* species and also for the clinical specimens. Further, chromogenicity of methyl blue and phenol red supplemented medium remains constant even in the three different temperatures tested (25°C, 30°C and 37°C).

Table 1
Color variation among different *Candida* species on novel chromogenic media (CCA- Chromogenic Candida Agar)

Code	Chromogen/s	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. krusei*	*C. lusithaniae*
CCA1	Methyl blue (0.02)	Dark blue	Dark blue	Steel blue	White	Steel blue
CCA2	Phenol red	White	White	Pale yellow	Pale yellow	Pale orange
CCA3	Methyl voilet (0.02)	Sandy brown	Sandy brown	Sandy brown	White	Sandy brown
CCA4	Bromophenol blue (0.02)	White	White	White	Pink	White
CCA5	Methylene blue (0.02)	White	White	White	White	White
CCA6	Methyl blue and (0.02) bromophenol blue (0.02)	Steel blue	Dark blue	Steel blue	White	Steel blue
CCA7	Methyl blue and Phenol red	Slate blue	Sky blue	Violet blue	Dark sea green	Indigo
CCA8	Methylene blue and phenol red	Parrot green	Parrot green	Parrot green	No growth	No growth
CCA9	Methyl blue and Methylene blue	Sky blue	Sky blue	Slate blue	No growth	No growth
CCA10	Phenol red and methyl violet	Pale yellow	Light orange	Pale yellow	Pale yellow	Pale yellow
CCA11	Methyl blue and methyl violet	Violet blue	Steel blue	Light blue	Violet blue	Slate blue
HiChrome Candida agar		Light green	Light blue	Light pink	White fuzzy	White

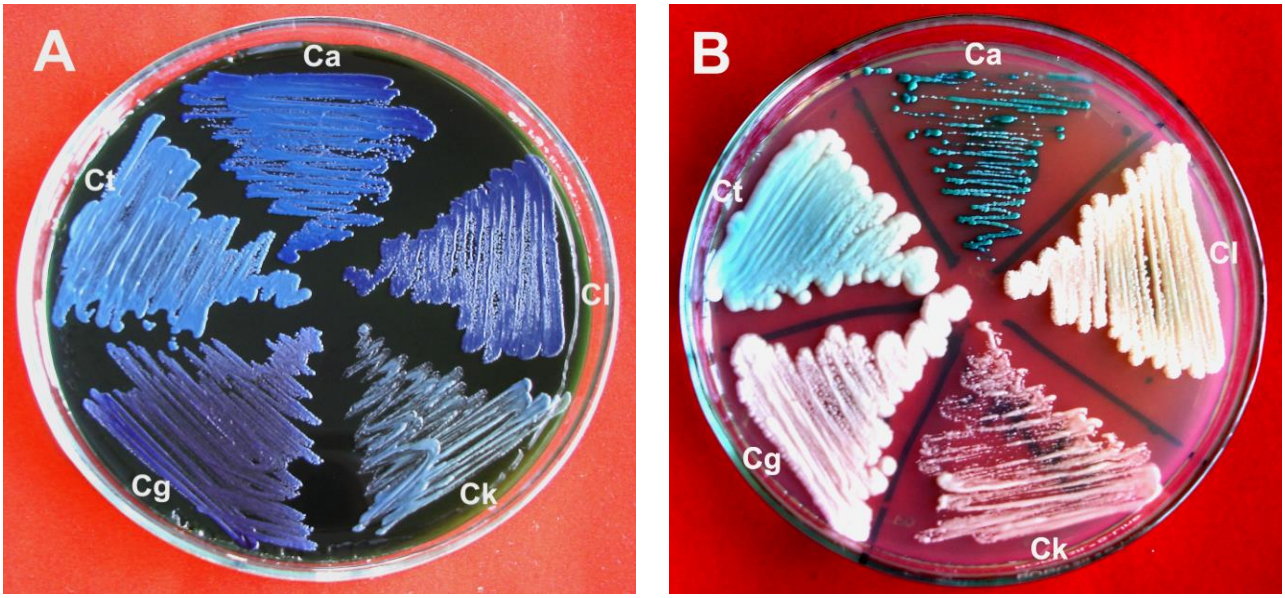

Figure 1: Comparison of chromogenicity of different *Candida* species grown on (A) CCA7 and (B) Hichrome Candida agar at 37°. *C. albicans* NCIM 3074 (Ca); *C. lusitaniae* NCIM 3484 (Cl); *C. krusei* NCIM 3129 (Ck); *C. glabrata* NCIM 3236 (Cg) and *C. tropicalis* NCIM 3118 (Ct)

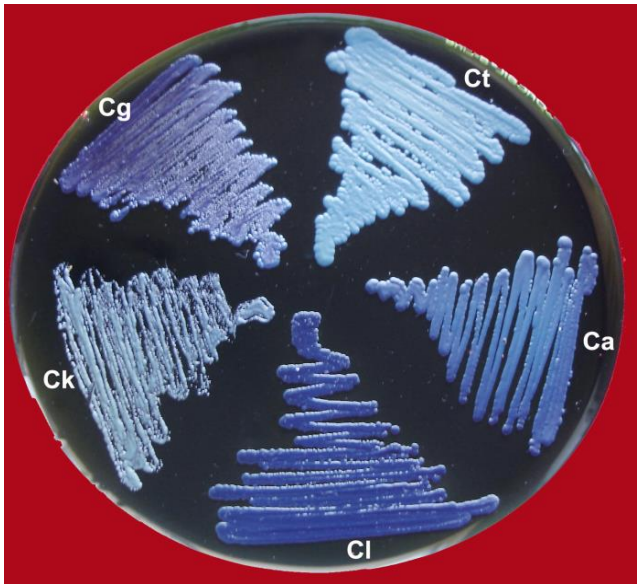

Figure 2: Chromogenicity of different clinical strains of *Candida* grown on CCA7 at 37°C. *C. albicans* (Ca); *C. lusitaniae* (Cl); *C. krusei* (Ck); *C. glabrata* (Cg) and *C. tropicalis* (Ct)

Molecular identification of *Candida* species: To develop a precise and rapid approach for identification of *Candida* species, pair of oligonucleotide primers was designed for PCR amplification. The primers of *INT1* gene were able to detect only *C. albicans*. The DNA pattern of PCR product by using *INT1* primer shows a prominent PCR product of 227-bp only in the *C. albicans* strains, it was absent in other three species of *Candida* which is used in the present study (Figure 3). The PCR products obtained with different strains of *C. albicans* showed identical banding pattern irrespective of the source of the strains.

Another pair of primers for the gene *ICL1* is able to amplify in the *C. albicans* and *C. tropicalis*, but they are not in other three species (*C. glabrata*, *C. krusei* and *C. lusitaniae*). The DNA patterns of the PCR product in the agarose gel of gene *ICL1* obtained from *Candida* strains are well documented (Figure 4). PCR amplicons of 200-bp were obtained from *C. albicans* and *C. tropicalis* of NCIM and clinical strains when tested repeatedly. Hence, primer *ICL1* gene is a good marker which can be used to detect the *C. albicans* and *C. tropicalis* in the clinical samples.

Discussion

The mycoses caused by *Candida* species have an important infective complication in immunocompromised patients. *Candida* species are threatening women at least once in a life time by causing valvovaginal candidiasis (VVC) or recurrent valvovaginal candidiasis (RVVC). The emergence of resistance against fluconazole and amphotericin B

necessitated a rapid identification of *Candida* to species level. It is of clinical relevance to find appropriate antifungal therapy. The conventional methods of identification are time consuming and require manipulation of human or animal serum. Alternative methods for quick identification of *C. albicans* include the use of chromogenic media as well as rapid detection by using molecular probes[4,15].

The use of newly formulated chromogenic media is proved to be a better approach for traditional identification methods of clinical *Candida* isolates than the commercial media. Traditional methods used for the identification of clinical isolates of *Candida* are mainly based on phenotypic characteristics and therefore can lead to inconsistent results[18]. Application of the CHROMagar was introduced for rapid identification of *C. albicans, C. tropicalis, C. krusei* and *C. glabrata* from clinical specimens[33]. The identification of *Candida* species from vaginal infections using chromogenic media was also reported[29]. The CAD2 is a modified chromogenic medium developed by the Eraso et al[11] for the identification of *C. albicans* (cobalt blue), *C. dubliniensis* (turquoise blue), *C. tropicalis* (pinkish blue) and *C. krusei* (blue gray).

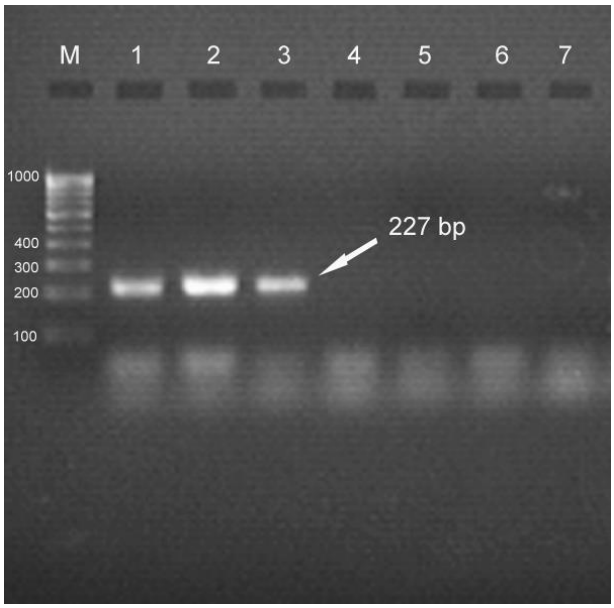

Figure 3: Gel electrophoresis of PCR products amplified with *INT1* primers in *Candida* strains.
Lane M, molecular marker (100-bp DNA ladder); Lane 1 *C. albicans* NCIM 3074; Lane 2 *C. albicans* oral isolate; Lane 3 *C. albicans* vaginal isolate; Lane 4, *C. tropicalis* NCIM 3118; Lane 5 *C. glabrata* NCIM 3236; Lane 6 *C. krusei* NICM 3129; Lane 7, *C. lusitaniae* NCIM 3484

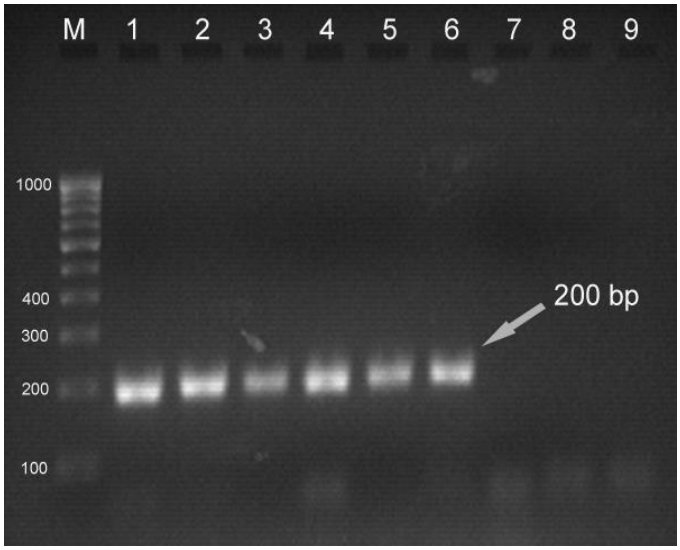

Figure 4: Gel electrophoresis of PCR products amplified with *ICL1* primers in *Candida* strains.
Lane M, molecular marker (100-bp DNA ladder); Lane 1, *C. albicans* NCIM 3074; Lane 2 *C. albicans* oral isolate; Lane 3 *C. albicans* vaginal isolate; Lane 4, *C. tropicalis* NCIM 3118; Lane 5 *C. tropicalis* oral isolate; Lane 6 *C. tropicalis* vaginal isolate Lane 7, *C. glabrata* NCIM 3236; Lane 8 *C. krusei* NCIM 3129; Lane 9 *C. lusitaniae* NCIM 3484

Murray and co-investigators[27] emphasized usefulness of chromogenic media for the identification of *Candida* species over the routine media. The CHROMagar *Candida* is one such media used for identification of the *C. albicans* (green colonies), *C. tropicalis* (steel blue colonies) and *C. krusei* (fuzzy, rose colored colonies)[30]. Though there are many commercially available chromogenic media to differentiate *Candida* species in the mixed cultures, false-positive and false negative results in these media favor need of novel chromogenic media[39]. The present study is focused on formulating different chromogenic media for identification and differentiation of *Candida* species.

Among these media, methyl blue with phenol red (0.025% and 0.04%) showed interesting results by differentiating the five *Candida* species. Further, studies were made on clinically relevant species (*C. albicans*, *C. tropicalis*, *C. krusei*, *C. lusitaniae* and *C. glabrata*) which are commonly occurring in immunocompromised patients. The newly formulated media showed better differentiation among standard strains of *Candida* by developing various colors than the commercial medium. Interestingly, the novel chromogenic media effectively differentiated the clinical isolates than the commercial medium.

Rapid identification of *C. krusei* and *C. glabrata* isolates with chromogenic media has a special importance because *C. glabrata* is less sensitive than the other species to ketoconazole and fluconazole and *C. krusei* exhibits innate resistance to fluconazole[8]. However, identification of *C. albicans* and *C. tropicalis* is also very essential, because most of candidal mycoses are caused by *C. albicans* and followed by the *C. tropicalis*[2]. There are number of chromogenic media available for the isolation and presumptive identification of *C. albicans* based on the pigmentation of the developing colonies due to different enzyme activities among *Candida* species[3,31]. However, they are expensive to adopt in routine laboratory analysis particularly in the developing countries due to the economy of the poor people.

Therefore, present study was focused on development of novel chromogenic media for routine laboratories in cost effective manner by using methyl blue with phenol red as chromogenic compound that showed better results and it could differentiate the five species of *Candida.* The new culture media can be prepared easily being cost effective in the clinical laboratory, whereas commercial media are expensive and availability may also be some time limited especially in developing countries.

There is a report on use of Sabouraud dextrose agar supplemented with methyl blue alone for the identification of *C. albicans*[44] and also use of fluorogenic substrate for the identification of *Candida* species[37]. But, in the present study, combination of chromogens was used to draw better differentiation of *Candida* species. Due to increased incidence of immunocompromised patients and development of drug resistant strains of *Candida,* it is also necessary to detect species using molecular probes after identification with traditional methods for effective antifungal therapy. Molecular methods particularly PCR better suit for the rapid identification of *Candida* species in clinical isolates and also confirm the species identified based on chromogenic compounds.

The earlier studies emphasise the use of species-specific molecular probes that can differentiate closely related species as well as novel strains[5,19]. However, these techniques with currently available marker have some limitations because of the emergence of new species of *Candida* in immunocompromised individuals leading to the requirement of differentiation of existing species from the emerging species.

The PCR based procedures for direct identification of *Candida* species have been reported[9,20,32,42]. However, most epidemiological studies available so far tend to rely on phenotype-based methods of discrimination due to the costs and technical efforts necessary for applying molecular tests to a larger scale of isolates. Present study deals with development of two potential molecular markers, which are easily adaptable to common laboratory and do not require any advanced technical skills. Its application can also be extended with modifications for use in diagnosis of various types of mycoses in patients. These approaches only require electrophoresis of PCR products on an agarose gel stained with ethidium bromide. The molecular marker gene *ICL1* involves in the glyoxalate pathway and helps to escape from host defense system for pathogenic yeast[23] and *INT1* gene product contributes to *C. albicans* adherence and filamentous growth as well as intestinal colonization[6,13,14].

Uses of these genes have advantages over the other because both the genes targeted here are directly involved in the pathogenesis. PCR based identifications of *Candida* species using the *SAP1, SAP2, SAP3*, *DAP2*[5], *ALS*[16], tRNA[9] and 28S rRNA[17] genes were already reported by various researchers elsewhere. The current study demonstrated that the direct PCR method was capable of detecting the *C. albicans* and *C. tropicalis* quickly using novel gene marker *ICL1*. The specific detection of the *C. albicans* is possible using *INT1* gene. This provides an opportunity for the early adjustment of antifungal therapy particularly immunocompromised patients. However, it is essential to evaluate these chromogenic media and molecular markers in large number of samples to validate the method.

Conclusion

In conclusion, *Candida* colonies grown on medium supplemented with methyl blue and phenol red (CCA7) show specific colors than the commercial medium. The new chromogenic CCA7 medium showed better performance over the commercial medium for identification of *Candida* species of clinical relevance (*C. tropicalis*, *C. glabrata*, *C. krusei* and *C. lusitaniae*). Moreover, CCA7 is a cost effective

medium and can be easily prepared in the common clinical laboratories.

Molecular marker *INT1* can be used to detect the *C. albicans* alone, while *ICL1* can be used to detect the both *C. albicans* and *C. tropicalis* from clinical source. *C. tropicalis* can be confirmed if the DNA amplified for *ICL1* but not in the *INT1* gene.

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