



Original Research Article

Molecular Confirmation of *Candida* Species Using Self Designed Primers by PCR

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ABSTRACT

Keywords

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Corn Meal
and
Tween Agar

The genus *Candida* comprises more than 300 species of which over 40 are pathogens and have been associated with life-threatening infections in humans, especially those with an impaired immune system. Various clinical samples were collected from 200 patients visiting the Meenakshi medical college and hospital in Kanchipuram, Tamil nadu. Such as urine, sputum, throat swabs, vaginal swabs. During Jan 2012 to Jan 2013, all patients were immunocompromised. Among the 200 samples 51 isolates of *Candida* species identified by Culturing on Sabouraud's Dextrose Agar (SDA), Culturing on CHROMagar *Candida*, Germ-Tube test, Culturing on Corn Meal Tween 80 Agar (CMA). Among the 51 *Candida* isolates 5 *Candida* species were identified. Followed by antifungal susceptibility pattern was done using 5 antifungal drugs. After phenotypic identification self designed primer was designed, for identification of *Candida* species by molecular method.

Introduction

The genus *Candida* comprises more than 300 species [1] of which over 40 are pathogens [2] and have been associated with life-threatening infections in humans, especially those with an impaired immune system. However, in recent years, the taxonomy of the most important *Candida* species such as *Candida albicans*, *C. parapsilosis*, *C.guilliermondii* and *C. glabrata* has undergone significant changes due to the description of new closely related species and therefore they are, nowadays, recognized as ' cryptic species complexes '

[3 – 6]. In 1995, a group of Irish researchers described for the first time a new pathogenic species called *Candida dubliniensis* which shares several phenotypic and genotypic characteristics with *C. albicans* and it is easily misidentified as such [3].

Material and Methods

Collection of samples

Various clinical samples were collected from 200 patients visiting the Meenakshi

medical college and hospital in Kanchipuram, Tamil Nadu. Such as urine, sputum, throat swabs, vaginal swabs. During Jan 2012 to Jan 2013. All patients were immunocompromised.

Phenotypic identification of *Candida* isolates

A- Culturing on Sabouraud's Dextrose Agar (SDA)

All samples were cultured onto Sabouraud's Dextrose Agar (SDA) (HiMedia, Mumbai, India) plates supplemented with 0.05% (W/V) chloramphenicol (7). Cultures were incubated at 37°C for 24-48 hours after which the growing fungi were purified and kept in slants for further phenotypic and molecular studies.

B- Culturing on CHROMagar *Candida*

Chromogenic media contain chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colours (8). CHROMagar *Candida* Differential agar (HiMedia) is a selective and differential medium, which facilitates rapid isolation and presumptive identification of some yeasts from mixed cultures. The medium contained (g/L): agar 15; peptone 10.2; chromogenic mix 22; chloramphenicol 0.5; pH: 6.1. According to the manufacturer 47.7 grams of the powdered medium were slowly dispersed in 1 liter of sterile distilled water and brought to a boil by repeated heating until complete fusion of agar grains. The medium was cooled in a water bath to 45-50°C, with gentle stirring, then poured into sterile Petri dishes and allowed to solidify. Separate colonies from all

Candida isolates on SDA were subcultured onto CHROMagar *Candida* and incubated at

37°C for 48 hr. Presumptive identification was done based on colony colour of the growing *Candida* strains.

C- Germ-tube test

Small inoculum of suspected *Candida* cultures were inoculated into 1 ml of human serum in a small tube and incubated at 37°C for 2 hours. After incubation, a loop-full of culture was placed on a glass slide, overlaid with a cover-slip and examined microscopically for the presence or absence of germ-tubes. Formation of germ tubes was seen as long tube like projections extending from the yeast cells with no constriction or septa at the point of attachment to the yeast cells. The germ tube is indicative of *C. albicans* and *C. dubliniensis* (7).

D- Culturing on Corn Meal Tween 80 Agar (CMA)

Chlamydospore formation by certain *Candida* species (*C. albicans* and *C. dubliniensis*) is encouraged by culturing on CMA. This test is negative with other *Candida* species. All yeast isolates were subcultured on SDA and in glycerol water (15% V/V) and kept under low temperature for further molecular and in vitro antifungal sensitivity test (9,10)

Antifungal susceptibility test

The disc diffusion test was performed according to the procedure described in the Clinical and Laboratory Standard Institute (CLSI, 2004). Cell suspensions of individual *Candida* strains were prepared in 2 ml sterile 0.85% saline solution. The turbidity was adjusted to yield 0.5 McFarland standard (approximately 5×10^3 cells/ml). Five kinds of antifungal agents obtained from HiMedia Company in India were tested. The interpretative breakpoints of these antifungal agents were done (11) as shown in table 1.

Candida species confirmation by self designed primers

A specific self designed primer were used. The primer designed from ITS region of sequenced gene with the help of bioinformatics friends. Total DNA was extracted as described by philippsen et.al (1991). Pure fast - * fungal genomic DNA purification kit was used for DNA extraction. After extraction of DNA is checked by loading in 1% agarose gel and 1 µl of extracted DNA is used for PCR amplification, Master mix 25µl, primer (F) – 1 µl, primer R - 1µl, genomic DNA – 1 µl, water – 22 µl, total volume – 50 µl was prepared, after that initial denaturation 94°C for 3 min. denaturation 94°C for 1 min. annealing 55°C for 1 min. extension 72°C for 5 in has been settled. After amplification, the amplicon loaded in to 2% agarose gel, result will be taken from gel documentation system.

Results and Discussion

Of the 200 immunocompromised patients 100 males and 100 were females. Among this 51 patients (25.5%) by positive culturing on Sabouraud's agar medium. Characteristics of yeasts cultured on SDA, CMA and CA Candida media and identification after phenotyping

Self designed primers for Candida species:

Pichia guilliermondii

Product:315bp
GCATCGATGAAGAACGCAGC
GTTTGGTTGTTGTAAGGCCGGG

Candida dubliniensis

product: 102bp
5'-TGAAAGCGCATGGGCGGTGTTA-3' /
5'-ACCTACAGCCAACCATCCACGG-3'

Candida parapsilosis –

Product: 300bp
CPA1 GCCAGAGATTAAGTCAACCAA
CPA2
CCTATCCATTAGTTTATACTCCGC

Candida tropicalis-372 bp

CTR1 CAATCCTACCGCCAGAGGTTAT
CTR2 TGGCCACTAGCAAATAAGCGT

Candida albicans

Product size:273bp
5'-TTTATCAACTTGTCACACCAGA-3'
5'-ATCCCGCCTTACCACTACCG-3'

Yeasts are common fungal agents affecting humans. They cause diseases with severity ranging from benign to potentially life-threatening infections, with the most commonly yeasts being the *Candida* species. *Candida albicans* remains the predominant species causing over half of all the yeast infection cases in the world(12). Increase in the prevalence of yeast infections caused by *non-albican Candida* such as *Candida glabrata*, *Candida kursei*, *Candida tropicalis* and *Candida parapsilosis* have been reported in many parts of the world(13). Results obtained in this study establish several points pertinent to the prevalence of Candidiasis in immunocompromised patients. Five *Candida* species (*Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii* and *Candida dubliniensis*) were isolated from the various clinical samples of immunocompromised patients. In my study *Candida* species are identified by CHROM agar, biochemical reactions and polymerase chain reaction(PCR). After phenotypic identification, *Candida* species was confirmed by the self designed primers.

Table.1 Interpretative breakpoints of antifungal agents: Zone of activity in mm

Antifungal agents (abbreviations)	Concentration /disc	Sensitive	Intermediate	Resistant
Amphotericin-B(AM-B)	100U	≥15	10-14	<10
Nysitatin(NYS)	100U	≥15	10-14	<10
Fluconazole(FLU)	10ug	≥19	15-18	≤14
Ketoconazole (K ET)	10ug	≥28	21-27	≤20
Itraconazole(ITR)	10ug	≥23	14-22	≤13

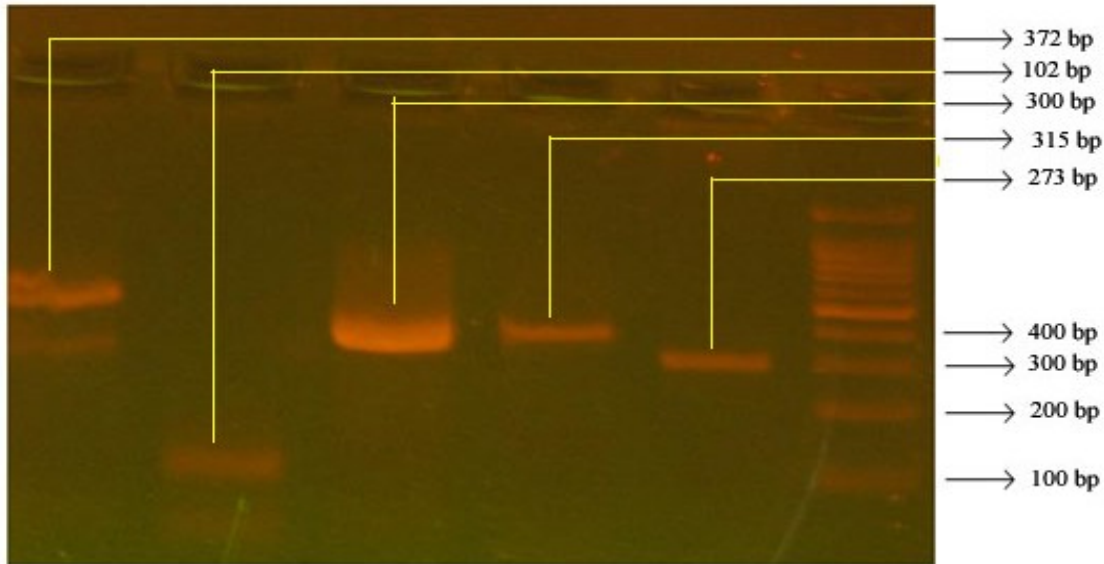
Table.2 The table shows (2&3) characteristics of Candida species and antifungal susceptibility of Candida species

S.No	Growth on SDA	Colour in chrom agar	Chlamydo-spore on CMA	No.of isolates	Identification of species
1.	Budding cells & hyphae	Green	+	36	Candida albicans
2.	Budding cells & hyphae	Blue	-	11	Candida tropicalis
3.	Budding cells & hyphae	Creamy pink	-	2	Candida guilliermondii
4.	Budding cells & hyphae	Cream	-	1	Candida parapsilosis
5.	Budding cells & hyphae	Dark green	++	1	Candida dubliniensis

Table.3 Antifungal susceptibility test

Drugs used	Sensitivity	Intermediate	Resistant
Amphotericin -B	26	16	9
Fluconazole	8	3	40
Itraconazole	-	-	51
Ketaconazole	-	4	47
Nystatin	44	7	-

Gel image of Candida species



This self designed primer confirmed by the sequencing. Among five self designed primers only *Candida albicans* and *Candida tropicalis* primers are sequenced. Because, the highest rate of isolates are *Candida albicans* followed by *Candida tropicalis*. Polymerase chain reaction is very less time consumable than sugar fermentation and sugar assimilation.

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