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Phenotypic and Virulence activity of *Candida* species from immunocompromised patients

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Candida species, immuno-compromised patients, Fungal infections

A B S T R A C T

Fungal infections are generally opportunistic. The extent and severity of Candida infections depend on the immune status of the host. Candida species have some virulence factors that facilitate proliferation; they may result in adhesion to the epithelium and invasion of the host tissue. It seems that extracellular hydrolytic enzymes play an important role in Candidal overgrowth. 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. All Candidal isolates processed for virulence activity of Phospholipase, proteinase, esterase, hemolytic activity and biofilm formation.

Introduction

Candida species are commonly seen yeasts that exist as an element of normal flora in the skin, mucosa, and gastrointestinal tract of humans (Hazen and Howell, 2003). Fungal infections are generally opportunistic. The extent and severity of *Candida* infections depend on the immune status of the host (Iven, 2011).

Candida spp. have some virulence factors that facilitate proliferation; they may result in adhesion to the epithelium and invasion of the host tissue play an important role in candidal overgrowth (Schaller *et al.*, 2005). The extracellular hydrolytic enzymes including secreted aspartyl proteinase and

phospholipases degrade immunoglobulins and proteins of the extracellular matrix; they also inhibit the phagocytosis of polymorphonuclear neutrophils and induce inflammatory reactions (Reynaud *et al.*, 2001; Hube and Naglik, 2001). Furthermore, the survival and ability of *Candida albicans* to establish infections within humans are mainly related to its ability to procure elemental iron through hemolysin production (Manns *et al.*, 1994; Yenişehirli *et al.*, 2010). There is an increasing amount of evidence linking lipases to microbial virulence (Willis *et al.*, 2001; Sardi *et al.*, 2011). It seems that extracellular hydrolytic enzymes been proposed that microbial extracellular lipases play various roles including the digestion of lipids for nutrient acquisition, adhesion to host cells and tissues, synergistic interactions with other enzymes, unspecific hydrolysis due to additional phospholipolytic activities, and the initiation of inflammatory processes by affecting immune cells and self-defense by lysing the competing microflora (Khedidja *et al.*, 2011; Trofa *et al.*, 2009).

Moreover, it has been reported that biofilm formation also plays an essential role in the pathogenicity of *Candida* spp. (Hazen and Howell, 2003; Ozkan *et al.*, 2005). It is also known that *Candida* spp. have the ability to grow in both aerobic and anaerobic conditions. Moreover, *Candida* spp. possess some adaptive mechanisms to survive in both situations (Sardi *et al.*, 2011; Akcağlar *et al.*, 2011). Although there are many studies about pathogenic fungi, it has been reported that studies on virulence factors are still needed (Akcağlar *et al.*, 2011).

In the present study, we aimed to investigate some virulence factors in *Candida* spp. isolated from patients with suspected invasive fungal infection. Thus, the identification of *Candida* species is very important in the diagnostic laboratory, because such identification shows prognostic and therapeutical significance, allowing the early and correct antifungal therapy (Godoy *et al.*, 2001; Milan and Zaror, 2004).

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, India, such as urine, sputum, throat swabs, and vaginal swabs during Jan 2012 to Jan 2013. All patients were immunocompromised.

Phenotypic identification of Candida isolates

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 (Bhavan *et al.*, 2010). 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.

Culturing on CHROM agar Candida

Chromogenic media contain chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colours (Pfaller *et al.*, 1996). CHROM agar Candida Differential agar (HiMedia) is a selective and differential medium, which facilitates rapid isolation and presumptive identification of some yeast 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 CHROM agar Candida and incubated at 37°C for 48 hr. Presumptive identification was done based on colony colour of the growing *Candida* strains.

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* (Bhavan *et al.*, 2010).

Culturing on Corn Meal Tween 80 Agar (CMA)

Chlamydospore formation by certain Candida species (*C. albicans* and *C. dubliniensis*) is a 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 (Koehler *et al.*, 1999; Ellis *et al.*, 2007).

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 $5x10^3$ cells/ml). Five kinds of antifungal agents obtained from HiMedia Company in India were tested. The interpretative breakpoints of these antifungal agents were done (Ellis, 2011) as shown in table 1.

Table.1 Interpretative breakpoints of antifungal agents

Zone of activity in mm

Antifungal agents (abrivations)	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

Determination of phospholipase activity

To determine phospholipase activity, the egg yolk agar method of Price *et al.* (1982), which was modified by Samaranayake *et al.* (1984), was employed. The culture medium consisted of 1 L of SDA (Himedia) containing 1 M NaCl, 0.005 M CaCl2, and 10% sterile egg yolk. Ten microliters of previously prepared yeast suspension was inoculated onto plates; these were then incubated at 37 °C for 5 days. The presence

of enzyme activity was determined by the formation of a precipitation zone around the yeast colonies (Price *et al.,* 1984).

Determination of proteinase activity

To determine proteinase activity, bovine-serum albumin agar defined by Staib (1965) was employed. The agar contained 0.1% KH_2PO_4, 0.05% $MgSO_4$, 2% agar and 1% bovine serum albumin (Himedia, India). The final pH was adjusted to 4.5. Ten micro liters of previously prepared yeast suspension was inoculated onto the plates; these were then incubated at 37°C for 10 days. The presence of proteinase activity was determined by the formation of a transparent halo around the yeast colonies (Price *et al.,* 1982)

Determination of hemolytic activity

To determine hemolytic activity, SDA (Himedia) containing 7% human blood and 3% glucose with a final pH adjusted to 5.6 ±0.2 was employed. Ten microliters of previously prepared yeast suspension was inoculated onto plates; these were then incubated at 37 °C for 48 h. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive hemolytic activity (Manns *et al.,* 1994).

Esterase activity

To determine esterase activity, Tween-80 opacity test medium was used. The test medium with a pH adjusted to 6.8 consisted of 1% peptone, 0.5% NaCl, 0.01% CaCl2, and 1.5% agar. After cooling the medium (50 °C), 0.5% of Tween-80 was added. Ten microliters of previously prepared suspension from each isolate was carefully deposited on the Tween-80 opacity test medium; this was then incubated at 37 °C for 10 days. Esterase activity was considered as positive in the presence of a halo to light around the inoculation site (Slifkin, 2000).

Biofilm formation

Sterile 96-well microplates were used to evaluate biofilm formation (Toledo-Arana *et al.,* 2001). By using a loop, a spot of each isolate was placed into tubes containing 2 mL of brain heart infusion broth (BHIB) medium with glucose (0.25%) and incubated at 37°C for 24 h. Then all tubes were diluted at a ratio of 1:20 by using freshly prepared BHIB. From this final solution, 200 µL was placed into the microplates, which were then incubated at 37°C for 24 h.

After incubation, the microplates were rinsed with PBS 3 times and then inverted to blot. Then 200 µL of 1% crystal violet was added to each well, followed by incubation for 15 min. After incubation, the microplate was again rinsed with PBS 3 times. Then 200 µL of ethanol:acetone mixture (80:20 w/v) was added to each well. They were read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Biorad) and the OD was recorded for each well. Two wells were used for each strain.

Staphylococcus aureus ATCC was employed as the control strain. Sterile BHIB without microorganism was employed as the negative control. The cutoff value was determined by arithmetically averaging the OD of the wells containing sterile BHIB and by adding +2 standard deviation. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative.

Result and Discussion

From the 200 immunocompromised patients 100 males and 100 were females collected the samples for this study. Among the 200,

51 (25.5%) were positive culturing on Sabouraud's agar medium. Table 2 showed Characteristics of yeasts cultured on SDA, CMA and CA Candida media and identification after phenotyping for the present study.

Table.2 Characteristics of yeasts cultured on SDA, CMA and CA Candida media and identification after phenotyping

S.No	Growth on SDA	Colour in Chrom agar	Chlamydospore 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	-

Table.4 Virulence factors

Virulence activity	Albicans	Non- albicans	Total no of isolates
Phospholipase activity	15	5	20
Protienase activity	20	2	22
Hemolysis activity	6	15	21
Esterase activity	6	2	8
Biofilm activity	5	12	17

Table 3 showed antifungal susceptibility test interpretation test for the Candida sps and the table 4 showed Virulence factors activity for the isolated Candida sps. In the present study, prevalence rate of *Candida* species were found to be 51 (25.5%) in that throat swab 5 (2.5%), urine 18(35.3%), sputum 19 (37.3%) and vaginal swab 9(17.6%). The prevalence rate of *Candida albicans* in India was found to be 37.5% and *non Candida albicans* 62.6% (Slifkin, 2000). In other study *Candida albicans* 49.3% and *non Candida albicans* 46.3% (Feglo1 and Narkwa, 2012). All *Candida* isolates

showed good growth on (SDA). Colonies were white to cream in colour, smooth, glabrous and yeast- like in appearance. Microscopic morphology showed spherical to subspherical budding yeast cells with several isolates producing pseudohyphae. Although simple and inexpensive, these criteria are not enough for identification for *Candida* species. In the present work germ-tube production was in 36/51 strains, which were identified as *Candida albicans* whereas the remaining strains are Siva S *et al.*,2010 failed to produce germ-tubes, being identified as non-*Candida albicans* This ratio is markedly lower than that reported by Kangogo *et al.* (2011), who found 112/130 (86%) of *Candida* species produced germ-tubes and were identified as *Candida albicans* and 18/130 (13.9%) were identified as non-*Candida albicans*.

The germ-tube production test has the advantage to be simple and efficient in the economical and fast identification of *Candida albicans* (Fisher and Cook, 1998; Lacaz *et al.,* 2002). Some authors evaluated sensitivity and specificity of the germ-tube test, finding results between 93 and 98.8%, and between 73.3 and 100%, respectively (Cambell *et al.,* 1998; Conceicao *et al.,* 2005 and Gatica *et al.,* 2002). All cultures of *Candida* grew well on chron meal agar were able to produce chlamydospores. *Candida albicans* and *Candida dubliniensis* are produced chlamydospores.

In the present study *Candida* species are identified by CHROM agar, chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colors (Pfaller *et al.,* 1996). All *Candida* species are grown in Chrom agar. The *Candida albicans* produce light green color, bluish gray color colonies are produced by *Candida tropicalis*, *Candida guillierrmondii* produced cream to pink color, *Candida parapsilosis* produced cream colr colonies and *Candida dubliniensis* produced dark green color colonies. In the prevalence rate of antifungal susceptibility testing for *Candida* species, nystatin was the most active drug against the majority of *Candida* strains. Althouh high proportion of *Candida* species was sensitive to amphotericin-B followed in descending order itraconazole, fluconazole.

Amphotericin-B effective against 100% of *Candida* isolates, 71.1% strains sensitive to ketaconazole, 61.4% to fluconazole and 47.1% to itraconazole. Nystatin was effective against 100% of *Candida* isolates (Silva *et al.,* 2010). In this study for amphotericin –B 51% sensitive, 31% intermediate and 18% resistance. Fluconazole 16% sensitive, 6% intermediate and 78.4% of strains resistance. No sensitive strains are seen in itraconazole and ketaconazole. 100% of strains are resistance for itraconazole. 8% of strains are intermediate and 92.1% resistance for ketaconazole and nystatin no resistance strains are seen 86.2% sensitive, 14% strains are intermediate.

So the present study nystatin was effective drug followed by amphotericin-B. Prevalence rate of the enzymatic activity of *Candida* species may vary depending on the species and source of isolates. 17(37.8%) expressed phospholipase activity, 25(55.6%) expressed proteinase activity, 1(2.2%) *Candida albicans* strain expressed esterase activity, 41(91.1%) strains are expressed hemolytic activity and biofilm formation was detected in 10 isolates (22.2%) (Mohammed S Alhussaini *et al.,* 2013). In the present study phospholipase activity 20(39.2%), proteinase activity 22(43.1%), esterase activity 8(16%), hemolytic activity 21(41.1%) and biofilm assay 17(33.3%).

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