

Prevalence of virulence factors and antifungal susceptibility testing of *Candida* species isolated from clinical specimens

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Abstract

Introduction: The main aim of our study is to isolate and identify *Candida* species from clinical samples and to detect the virulence markers and also to determine their susceptibilities to various antifungal agents.

Materials and Methods: Eighty *Candida* isolates from respiratory samples were included in the study. Detection of various virulence markers of *Candida* spp such as Phospholipase, Proteinase, Hemolysin and Biofilm production was carried out by Phenotypic methods. Antifungal susceptibility testing of the isolated yeasts for Amphotericin B, Itraconazole, Fluconazole, Caspofungin and Voriconazole was determined by E-test methods.

Results: In our study, most common spp isolated was *Candida albicans*(65%) followed by *Candida tropicalis*(15%) and *Candida glabrata*(10%).Prevalence percentage of virulence factors such as Hemolysin, Proteinase, Biofilm and Phospholipase production in *Candida* spp were 85%, 77.5%, 68.8% and 45% respectively. The susceptibility pattern of *Candida* isolates to antifungal agents showed highest resistant rate of 33.8% for Itraconazole and lowest resistant rate of 2.5% for Caspofungin.

Conclusion: Presence of virulence factors in *Candida* species might indicate invasiveness and its relation with infection. Hence the significance of virulence determination and antifungal susceptibility testing should be adopted as a routine procedure in the laboratory.

Keywords: *Candida* species, E-test, Hemolysin Biofilm formation, Proteinase, phospholipase activity.

Introduction

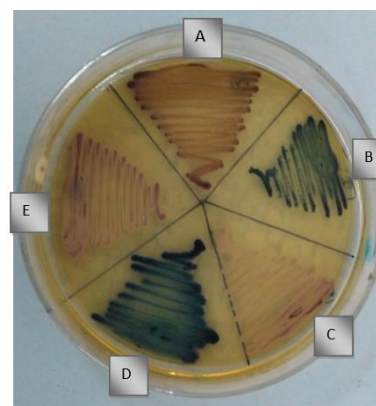
Most of the humans are colonized with *Candida* species and the leading one being *C.albicans*. Candidiasis is mainly an opportunistic infections affecting mucous membranes. Invasive Candidiasis can be very serious causing high mortality. Due to various risk factors, some adults develop Candidiasis and women are highly susceptible to genitourinary candidiasis as many of them carry *Candida* species in their vaginal tract. The new borns can develop oral Candidiasis or vulvo-vaginitis. Rarely Candidemia can progress to meningitis, leading to complications and death.^{1,2} The incidences of Candidiasis is on increase due to various risk factors like continuous catheterization, other prolonged invasive procedures, HIV infections, immunosuppressive therapy and other immunocompromised status. The aim of the study is to detect virulence factors and to determine the antifungal susceptibility pattern of isolated *Candida* spp from clinical samples.

Materials and Methods

A prospective study done in a tertiary care hospital included a total of 80 *Candida* species isolated from respiratory samples. All suspected yeast colonies were confirmed by Gram staining and further speciated by following test like germ tube test (GTT), microscopic morphological features on Nutrient deficient medium

like cornmeal agar (CMA), sugar assimilation and fermentation test.

Further speciation of *Candida* isolates was done by sub-culturing them on chromogenic medium (HiCHROM *Candida*; HiMedia, Mumbai, India) and incubated at 37°C for 48 h. Presumptive identification of *Candida* species was done by noting the color of the colonies as per the manufacturer's Instructions in HiCHROM medium (*C. albicans*-green, *Candida tropicalis* - blue, *C. krusei* - pink colonies with matt surface, *C. parapsilosis* - cream to pale pink). (Fig. 1)



A: *Candida glabrata*, B: *Candida albicans* C: *Candida parapsilosis* D: *Candida tropicalis*
E: *Candida krusei*

Fig. 1: Growth of *Candida* species on chromogenic medium

Detection of Virulence factor of candida

The virulence factors such as Phospholipase, Proteinase, Hemolysin, and Biofilm production were detected in vitro by the following methods.

1. Detection of Biofilm production

Biofilm formation was detected by method proposed by Branchini *et al.*³ A loopful of colony from SDA plate was inoculated into 10ml of Sabouraud's liquid broth with 8% glucose supplemented. The tubes were further incubated at 37°C for 24 hrs. Following which the broth was aspirated from tube and walls were stained using safranin stain. Biofilm formation was graded as negative (0+) and 1+, 2+ and 3+ means weak, moderate and strong positivity.

2. Proteinase detection

The proteinase activity was detected by the slightly modified Staib *et al.*, method.⁴ The medium used was Bovine serum albumin medium. On the surface of the medium the wells were punched out and yeast suspension (approximately 10^8 yeast cells /ml) was inoculated into it. The plates were incubated for 48hrs at 37°C. After incubation, the plates were fixed using 20% trichloroacetic acid following which staining done by 1.25% amidoblack. Further decolourisation of the stained plates were done by 15% acetic acid. The diameter of unstained area surrounding the well is measured. The proteinase activity (Pz) is the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. If the value of Pz = 1, indicates absence of proteinase activity and low Pz means high proteinase enzyme activity.

3. Detection of Phospholipase activity

Phospholipase activity of Candida species was detected by modified method of Samaranayake *et al.*⁵ The medium used is egg yolk agar medium. The wells were punched out on the surface of the well and 10 μ l aliquots of the yeast suspension was inoculated in the corresponding wells. The plates were further incubated for 48 hrs at 37°C for. The phospholipase activity (Pz value) is the measure of ratio of the diameter of the well to the diameter of the precipitation zone around the well. The value of Pz = 1, means phospholipase activity is absent and low value of Pz means more production of phospholipase enzyme. (Fig. 2)

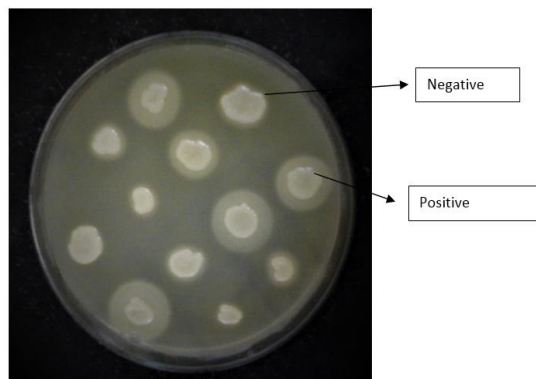


Fig. 2 Phospholipase production of Candida spp on egg yolk agar

4. Detection of Haemolysin activity

It was detected by inoculating the colony in SGA blood plate with 3% glucose added. The plate was further more incubated for 2 days at 37 degree C in 5% CO₂. Haemolytic index (Hz value) is measure of ratio of the diameter of the colony to that of diameter of translucent zone of haemolysis (in mm).^{6,7}

Antifungal susceptibility testing

Antifungal susceptibility testing of the isolated yeasts was done by E-test methods (Fig. 3) for Amphotericin B, Itraconazole, Fluconazole, Caspofungin and Voriconazole. Interpretation of susceptibility was performed by applying the breakpoints criteria defined by the CLSI in 2012^[8] as well as EUCAST.⁹

The MIC range of drugs used in this study were Amphotericin B (0.002 to 32 μ g/ml), Fluconazole (0.016 to 256 μ g/ml), Voriconazole (0.002 to 32 μ g/ml), Itraconazole (0.002 to 32 μ g/ml) and Caspofungin (0.002 to 32 μ g/ml).



Fig. 3: Antifungal susceptibility testing of Candida spp by E-test method

Results

In the current study, the most common candida species isolated was *Candida albicans* (65%), which was followed by *C. tropicalis* 12(15%), *C. glabrata* 8(10%), *C.parapsilosis* 4(5%) and *C. krusei* 4(5%) (Table 1)

Detection and prevalence of virulence factors in *Candida spp* showed 85% of isolates were positive for Hemolysin and 77.5% for Proteinase followed by positivity of 68.8% for Biofilm and 45% for Phospholipase. (Table 2)

Testing for antifungal susceptibility pattern showed resistant rate of 33.8%(27/80) for Itraconazole, 13.7% (11/80) for AmphotericinB, 5 % (4/80) for Fluconazole & Voriconazole and 2.5% (2/80) for Caspofungin. (Table 3)

Table 1: Percentage of *Candida* species isolated from clinical samples

Candida species	No (%)
<i>C. albicans</i>	52 (65%)
<i>C. tropicalis</i>	12 (15%)
<i>C. glabrata</i>	8 (10%)
<i>C.parapsilosis</i>	4 (5%)
<i>C. krusei</i>	4 (5%)
Total	80

Table 2: Prevalence of virulence factors in *Candida spp*

Virulence factors	Positive	Negative
Biofilm	55(68.8%)	25(31.2%)
Hemolysin	68(85%)	12(15%)
Phospholipase	36(45%)	44(55%)
Proteinase	62(77.5%)	18(22.5%)

Table 3: Antifungal susceptibility pattern of *Candida spp*.

Antifungal agents	Sensitive	Resistant
Amphotericin B	69(86.2%)	11(13.7%)
Itraconazole	53(66.2%)	27(33.8%)
Fluconazole	76(95%)	4(5%)
Caspofungin	78(97.5%)	2(2.5%)
Voriconazole	76(95%)	4(5%)

Discussion

Though more than 80 *Candida* species are described, only about 12-15 species can cause diseases in humans and most common is *C.albicans* followed by *C.tropicalis*, *C.glabrata*, *C.krusei*, *C.kefir*, *C.guilliermondii*, *C.parapsilosis* and recently *C.dublinsiensis*. *Candida albicans* and other species can cause vulvo-vaginitis, oral candidiasis, UTI, esophagitis (mostly among HIV patients), meningitis, Candidemia and other opportunistic infections The risk factors for the development of *Candida* infections and various types of disease are: Prolonged antibiotic therapy, HIV

infection and other Immunocompromised status, prolonged catheterization, various other invasive procedures, artificial organs and other plastic/metal implants

In our study, the most predominant isolate of candida species was *Candida albicans*(65%), which is followed by non albicans *Candida spp*. which accounts for 35%.This is well comparable to previous other studies where among all candida spp, *C. albicans* was the most predominant isolate from the clinical samples.^{10,11}

But there are few other studies, which have proven that non albicans *Candida* species is emerging and showing increased prevalence.^{12,13} There are many types of virulence factors described for Candidiasis. The leading ones are slime production and adhesion factors. The others being hemolysis, phospholipase and proteinases.

The present study done on detection and prevalence of virulence factors in *Candida spp* showed 85% of isolates were positive for Hemolysin and 77.5% for Proteinase followed by positivity of 68.8% for Biofilm and 45% for Phospholipase. A similar study conducted by Deepa et al¹⁴ showed that the prevalence of virulence factors in *Candida spp* such as hemolysin, proteinase, biofilm & phospholipase were 63%, 86.8%,78.9% and 52.6% respectively much comparable to our study.

In our study, haemolysin was the major virulence factor expressed by *Candida* isolates 85% irrespective of its species in comparable to study by Ghosh et al.¹⁵ Several other studies have shown 60.9%,52.6%,33% positivity of phospholipase activity.^{10,14,16} In our study we have recorded 45% phospholipase activity by *Candida spp*.

In current study 77.5% of *Candida spp* were positive for Proteinase production. In concurrence with this report, 75%, 86.8% percentage of protease enzyme production have been reported in other studies.^{10,14} In the present study 68.8% of *Candida* isolates were biofilm producers. Study by Jose et al., have reported similar percentage.¹⁰

Due to rapid advancement in complicated surgeries and many invasive procedures, *Candida* species invade blood stream and later affect many organs of the body. *Candida* species which develop drug resistance in due course invade bloodstream can lead to high mortality upto 35%. During the past two decades, the invasive *Candida* infections increased by about 20%.

E-test strips are commercially available for amphotericin B, fluconazole itraconazole, flucytosine, voriconazole, posaconazole, and caspofungin. One of the most remarkable advantage of E-test method is its user-friendly. In addition, irrespective of the test medium used, E-test has once been found to be most reliable and effective method when compared to other

method like reference microdilution method for detection of resistance to amphotericin B in *Candida*.¹⁷

In the present study on antifungal susceptibility pattern of *Candida* spp showed high resistant rate of 33.8% for Itraconazole followed by Amphotericin B (13.7%). Among 80 *Candida* isolates, resistant rate of 5% for Fluconazole & Voriconazole followed by 2.5% for Caspofungin.were recorded. In this study we found that Caspofungin, fluconazole and voriconazole continue to be active against most isolates of *Candida*. We have also observed that resistance to fluconazole predicted resistance to voriconazole well agreed with study by Lyon *et al.*¹⁸

Detection of virulence factors like haemolysin proteinase, phospholipase, and biofilm production will help us to understand the relationship between the infection and *Candida* species isolated. In recent years, a gradual increase in the fungal diseases and the widespread use of empirical antifungals caused the emergence of resistant strains of fungi. Thereby, the need for in vitro antifungal susceptibility testing are increasing nowadays to select appropriate and effective antifungal therapy. Treatment should be started only to those who are having clinical symptoms and after confirming with laboratory diagnosis. Hence, the significance of virulence determination in rational antifungal therapy cannot be ignored and should be adopted as a routine procedure in the laboratory.

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