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

Selvaraj Stephen[1], Pramodhinii S.[2,*], Shanmugam J.[3], Bhuvaneshwar D.[4]

[1]Professor, [2]Associate Professor, [3]Emeritus Professor, [4]M.Sc Student, Dept. of Microbiology, Mahatama Gandhi Medical College and Research Institute, Shree Balaji Vidhyapeeth (Deemed University), Puducherry, India

***Corresponding Author:**
Email: pramodhinis@gmail.com

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.

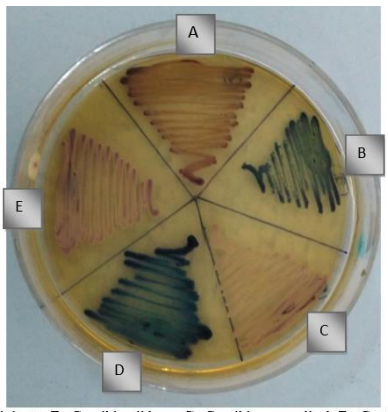
Introduction

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 develope 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% trichloracetic 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 medim.The wells were punched out on the surface of the well and 10 µl 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)

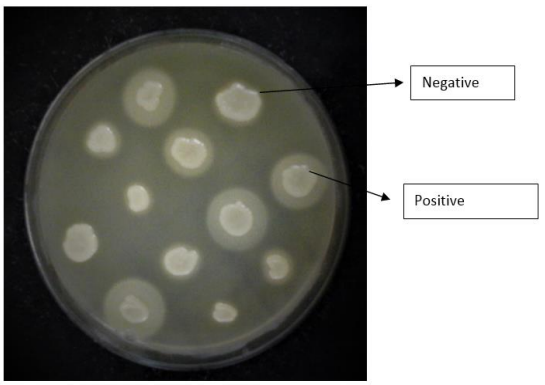


Fig. 2 Phospholipase production of Candida sppon egg yolk agar

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 µg/ml), Fluconazole (0.016 to 256 µg/ml), Voriconazole (0.002 to 32 µg/ml), Itraconazole (0.002 to 32 µg/ml) and Caspofungin (0.002 to 32 µg/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 (%)
C. albicans	52 (65%)
C. tropicalis	12 (15%)
C. glabrata	8 (10%)
C.parapsilosis	4 (5%)
C. krusei	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 C*andida* 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.dubliniensis. 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 Immuncompromised status, prolonged catheterization, various other invasive procedures, artiificial 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 hemolysisn, 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 al[14] 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.

References

1. Farrugia MK, Fogha EP, Miah AR, Yednock J, CarlPalmer H, Guilfoose J. Candida meningitis in an immunocompetent patient detected through (1→3)-beta-d-glucan. Int. J Infect Dis. 2016; 51:p 25-6.
2. Voice RA1, Bradley SF, Sangeorzan JA, Kauffman CA. Chronic candidal meningitis: an uncommon manifestation of candidiasis. Clin Infect Dis. 1994 Jul;19(1):p 60-6.
3. Branchini ML, Pfaller MA, Rhine- chalk berg J, Frempong T, Isenberg HD. Genotype variation and slime production among blood and catheter isolates of *C parapsilosis*. J Clin Microbiol. 1994;32:452–6.
4. Staib F. Serum-proteins as nitrogen source for yeast like fungi. Sabouraudia. 1965;4:187–93.
5. Samaranayake YH, Dassanayake RS, Jayatilake JA, Cheung BP, Yau JY, Yeung KW, et al. Phospholipase B enzyme expression is not associated with other virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. J Med Microbiol. 2005;54:583–93.
6. Sacristan B, Blanco MT, Galan-Ladero MA, Blanco J, Perez-GiraldoC,Gomez-Garcia AC. Aspartyl proteinase, phospholipase, hemolytic activities and biofilm production of *Candida albicans* isolated from bronchial aspirates of ICU patients. Med Mycol 2011;49:94-7.
7. Shehabi AA, NazzalSA,Dajani N. Putative Virulence Factors of Candida Species Colonizing Respiratory Tracts of Patients. MicrobEcol Health Dis 2004;16:214-7.
8. Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard-Third Edition. CLSI document M27-A3. Wayne: Clinical and Laboratory Standards Institute; 2008.
9. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W. EUCAST technical note on the EUCAST definitive document EDef 7. 2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect. 2012;18:E246–E247.
10. Jose NC, Mudhigeti N, Asir J, Chandrakesan SD. Detection of virulence factors and phenotypic characterization of Candida isolates from clinical specimens.Journal of Current Research in Scientific Medicine 2015;1(1):27-31.
11. Khan PA, Fatima N, Nabeela, Jahan S, Khan HM, Malik A.Antifungal Susceptibility Pattern of Candida Isolates from a Tertiary Care Hospital of North ndia: A Five Year Study. Int. J. Curr. Microbiol. App. Sci 2015;1:177-81.
12. Manchanda, V., Agarwal, S., Verma, N.2011. Yeast identification in routine clinical microbiology laboratory and its clinical relevance. Indian J. Med. Microbiol.,29(2):172.
13. Ragini A.K., SandhyaBelawadi, GaytriDevi, Indumal. 2001. Characterisation and antifungal susceptibility testing for Candida in a tertiary care hospital. J. Health Sci. Res., 2(2):1-12.
14. Deepa K, Jeevitha T, Michael A. Invitro evaluation of virulence factors of candida spcies isolated from oral cavity. J Microbiol. Antimicrob 2015;7(3):28-32.
15. Ghosh RR, Ghosh M, Chatterjee M, Banerjee M. In vitro demonstration of potential virulence determinants among clinical isolates of various Candida species and its clinical implication in a Teaching Hospital in Eastern India. Indian J. Med. Microbiol. 2016; 34(23):406-7.
16. Sachin C.D, Ruchi K, Santosh S. In vitro evaluation of proteinase, phospholipase and haemolysin activities of Candida species isolated from clinical specimens. Int J Med Biomed Res 2012;1(2):153-7.
17. Peyron F, Favel A, Michel-Nguyen A, et al. Improved detection of amphotericin B-resistant isolates of Candida lusitaniae by Etest. J Clin Microbiol 2001; 39: 339-42.
18. Lyon GM, Karatela S, Sunay S, Adiri Y. Antifungal Susceptibility Testing of Candida Isolates from the Candida Surveillance Study. J Clin Microbiol 2010:48(4);1270–5.

How to cite this article: Stephen S, Pramodhinii S, Shanmugam J, Bhuvaneshwar D. Prevalence of virulence factors and antifungal susceptibility testing of Candida species isolated from clinical specimens. Indian J Microbiol Res 2018;5(1):106-109.