

Original Research Article

# Speciation and Detection of Virulence Factors in Various *Candida* Isolates

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## ABSTRACT

**Background:** The last few decades have evidenced a rampant increase in the number and severity of *Candida* infections. Although non albicans *Candida* species are on a rise, *Candida albicans* continues to rank first among all *Candida* species isolated. Virulence attributes of *Candida* include adherence to host tissue, production of phospholipase, proteinase, coagulase, hemolysin and others. Recently biofilm production is also accounting for its heightened virulence.

**Objectives:** The present study was initiated to speciate and detects different virulence factors of various *Candida* isolates.

**Materials and Methods:** 92 *Candida* isolates from 103 clinical specimens like pus (20), vaginal discharge (22), urine(28), blood(16), cerebrospinal fluid (CSF) (02), gastric aspirate(02), bronchial brush sampling (02) were included and processed as per standard protocol. The speciation was done using CHROM agar *Candida* (Hi Media) and further confirmed by germ tube test and chlamydo-spore formation in cornmeal agar. The virulence factors detected were biofilm by tube and microtitre plate method, phospholipase in egg yolk agar, Coagulase by slide and tube method and hemolysin production. Additionally antifungal susceptibility was done using Fluconazole and Voriconazole discs by Kirby Bauer method as per CLSI guidelines.

**Results:** Among the 92 *Candida* isolates, 46(50%) were *C. albicans*, 20(21.7%) *C. tropicalis*, 10(10.9%) *C. glabrata*, 9(9.8%) *C. krusei* and 7(7.6%) *C.parapsilosis*.43 isolates were strong biofilm producers, 34 expressed phospholipase, 44 were coagulase positive and 31 showed hemolysis. *C. albicans* was the most virulent of all the species followed by *C. tropicalis*, *C. glabrata* and *C.parapsilosis*.49 isolates were resistant to both Fluconazole and Voriconazole among which 21 were *C. albicans*, 15 *C. tropicalis* and 09 *C. glabrata*.

**Conclusion:** The above results emphasize the need of future studies on the expression of virulence factors and their resistance profile which in turn may open up new options for therapeutic interventions.

**Key words:** Speciation, virulence factors, *Candida* isolates, Chrom agar.

## INTRODUCTION

During the last decade, *Candida* infections have substantially increased and have emerged as a significant cause of morbidity and mortality worldwide.

*Candida* species have the potential to invade all host organs and cause severe systemic infections. Although *C.albicans* is the organism most often associated with serious fungal infections, we are witnessing a

progressive shift in the epidemiology of candidiasis featured by a predominance of non albicans *Candida*.<sup>[1]</sup>

*C.albicans* is present in the skin, mouth, vagina and intestinal tract of healthy humans. These are opportunistic pathogens causing a spectrum of diseases ranging from superficial mycoses too disseminated fatal infections.<sup>[2]</sup> The virulence attributes of *Candida* species include adherence to host tissues, secretion of hydrolytic enzymes like phospholipases and proteinases, production of hemolysin, coagulase, phenotypic switching.<sup>[3]</sup>

The emergence of antifungal resistance as well as the advent of new antifungal drugs has led to renewed interest in antifungal susceptibility testing.<sup>[4]</sup> According to the CDC guidelines, broth dilution method remains the gold standard.<sup>[5]</sup> Recent efforts have led to more simple agar based methods like E test and disk diffusion that give results compatible with the CDC.<sup>[6]</sup> Fluconazole and Voriconazole have been used widely in the treatment of candidiasis. Injudicious use of these drugs have led to the development of resistance among *Candida* species mainly, the non albicans group.<sup>[7,8]</sup>

In the present study, an attempt was made to isolate, speciate and determine virulence markers of various *Candida* species and to know there in vitro susceptibility to Fluconazole and Voriconazole by disk diffusion method.

## MATERIALS AND METHODS

The present study was conducted in J.J.M Medical College, Davanagere from August 2012 to January 2013. 92 *Candida* isolates from a total of 103 clinical specimens including pus, vaginal discharge, urine, blood, CSF, gastric aspirate and bronchial brush were included in the study.

The clinical specimens were first inoculated into blood agar and nutrient agar. *Candida* isolates were identified based on colony morphology on nutrient and blood agar after 24 hours incubation at 37<sup>0</sup>C, gram staining, germ tube and chlamyospore

formation on cornmeal agar. For speciation, the *Candida* colonies from blood agar were inoculated on Hi Chrom *Candida* agar and kept at room temperature. They were examined for growth daily for a maximum of seven days.

Colonies from Chrom agar were subcultured into Sabouraud dextrose agar (SDA) with blood and 3% glucose which was also used to detect hemolysis. Colonies from blood agar were inoculated into egg yolk agar for phospholipase production and Sabouraud dextrose broth for biofilm detection, detection of coagulase and antifungal susceptibility testing.

### A. SPECIATION BY CHROM AGAR:

CHROM agar *Candida* (Hi Media, Mumbai) was prepared according to manufacturer's instructions. Colonies of *Candida* from blood agar were inoculated on CHROM agar and examined for growth daily at room temperature for seven days. Based on the colour obtained various *Candida* isolates were speciated.

### B. VIRULENCE MARKERS:

#### (I) Detection of Hemolysin

Hemolysin production was detected on SDA with blood agar with 3% glucose by the method described by *Manns et al*<sup>[9]</sup> and *Luo et al*<sup>[10]</sup>

The media was prepared by adding 7 ml sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3%(w/v) and a final pH of 5.6. Colonies were inoculated on the media and incubated at 37<sup>0</sup>C for 48 hours. After incubation plates were examine for zone of hemolysis around colonies. The hemolytic index (Hz) was calculated as the ratio of the diameter of the colony to that of the translucent zone of haemolysis (in mm). The assay was conducted in duplicate on three separate occasions for each yeast isolate tested, one strain each of *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus sanguis*, which induce beta and alpha haemolysis, were used as positive controls.

## **(II) Determination of Phospholipase**

Phospholipase production was detected by measuring the size of zone of precipitation around the colonies on Egg Yolk agar by the method described by Samaranyake et al. [11] The egg yolk medium consisted of 13.0 g SGA (Oxoid), 11.7 g NaCl, 0.11 g CaCl<sub>2</sub> and 10% sterile egg yolk (all in 184 ml distilled water). First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500 g for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium. Standard inocula of the test strains were spot inoculated and left to dry at room temperature.

Each culture was then incubated at 37 °C for 48 h, after which the diameter of the precipitation zone around the colony (an indicator of phospholipase activity) was determined. Phospholipase activity (P<sub>z</sub> value) was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone (in mm). [12] The assay was conducted in duplicate on three separate occasions for each yeast isolate tested. A P<sub>z</sub> value of 1 denoted no phospholipase activity, P<sub>z</sub> value less than 1 indicated phospholipase activity. The lower the P<sub>z</sub> value, the higher was the phospholipase activity.

## **(III) Detection of Biofilm**

Biofilm production was detected by tube and plate methods. The tube method followed was a modification of method described by Branchini et al [13] A loopful of organisms from CHROM Agar plate was inoculated into tube containing 10 ml Sabouraud Dextrose broth supplemented with glucose (Final concentration 8%). The tubes were then incubated at 37 °C for 24 hrs after which the broth was aspirated out and the walls of the tubes were stained with 1% crystal violet. Tubes were then kept still for 7 minutes. Crystal violet then was removed by washing and tubes were examined for biofilm production. Slime production was scored by two observers

simultaneously twice each to reduce as much as possible intra and inter observer's difference. It was scored as weak positive (1+), moderate positive (2+), or strong positive (3+).

The quantitative method of adherence to polystyrene plates proposed by Christensen et al for coagulase negative *Staphylococci* [14] was also used in the present study, with modifications like a longer incubation period (24 hrs instead of 18 hrs), and determination of optical density in dry plates and plates washed with 95% ethanol using filters of 492 and 540 nm. The isolates were classified into three categories: non-adherent- optical density ≤ 0.111; weakly adherent- optical density > 0.111 or ≤ 0.222; strongly adherent-optical density > 0.222. When the cut-off corresponded to non-adherent, the isolates were classified as negative, and as positive when the cut-off corresponded to weakly or strongly adherent.

## **(IV) Determination of Coagulase**

*Candida* isolates were screened for coagulase production by the classical tube method. [15] 0.1 ml of an overnight culture of the test strains were aseptically added to tubes containing 0.5ml of rabbit plasma. The tubes were incubated at 37°C for 4 hours. Presence of a clot which could not be re suspended on gentle shaking was considered a positive coagulase test. The tubes showing no clot after 4 hours of incubation were kept at room temperature and examined after 24 hours. *Staphylococcus aureus* ATCC 25923 and *Streptococcus epidermidis* ATCC 14990 were used as positive and negative controls respectively.

## **(V) Antifungal Susceptibility Testing**

Disk diffusion assay by Kirby Bauer method was used to test all *Candida* isolates. [16] Fluconazole (25µg) and Voriconazole discs (1µg), kindly supplied by Hi Media were used. Initially, a yeast inoculum suspension adjusted to match a 0.5 Mc Farland density standard was prepared. A sterile cotton swab moistened with the inoculum suspension was used to

applied to a 90 mm diameter plate containing Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue. The plates were allowed to dry for 5-15 minutes before a 1µg/ml fluconazole disk and a voriconazole disc were placed in the center of the agar. The plates were incubated for 18-24 hours at 35-37° C and the slowly-growing isolates could be read after 48 hours incubation. The zones of inhibition were interpreted as follows. Fluconazole: zone of inhibition: ≥19mm sensitive, resistant:≤14mm, dose dependent susceptibility:15-18mm Voriconazole: zone of inhibition: ≥17mm

sensitive , ≤13mm resistant and 14-16mm dose dependent susceptible.

**RESULTS**

We isolated 92 *Candida* isolates out of 103 clinical specimens like pus (20), vaginal discharge (22), urine(28), blood (16), CSF(02), gastric aspirate (02), bronchial brush(02). Among the species isolated 46(50%) were *C.albicans*, 20 (21.7%) were *C.tropicalis*, 10 (10.9%) *C.glabrata*, 9(9.8%) *C.krusei* and 7 (7.6%) were *C.parapsilosis*. (TABLE 1)

**TABLE 1: SPECIATION OF CANDIDA**

COLOUR	SPECIES	NUMBER	PERCENTAGE
Green	<i>C.albicans</i>	46	50
Dark blue to blue grey with dark halo in agar	<i>C.tropicalis</i>	20	21.7
Pink or purple	<i>C.glabrata</i>	10	10.9
Pale pink/purple (rough with spreading edge)	<i>C.krusei</i>	09	9.8
White to pale pink	<i>C.parapsilosis</i>	07	7.6

Among the virulence factors tested, we observed that 34(37%) isolates expressed phospholipase, 43 (46.73%) isolates produced biofilm, 44(47.82%) isolates produced coagulase and 31(33.69%) isolates produced hemolysin.(TABLE 2)

**TABLE 2: VIRULENCE MARKERS**

SPECIES	PHOSPHOLIPASE	BIOFILM	COAGULASE	HEMOLYSIN
<i>C.albicans</i>	16	19	30	19
<i>C.tropicalis</i>	06	15	04	06
<i>C.glabrata</i>	04	06	07	04
<i>C.krusei</i>	03	01	02	01
<i>C.parapsilosis</i>	02	02	01	01

On testing antifungal susceptibility we found that 29(31.5%) isolates were sensitive to both Fluconazole and Voriconazole, 14(15.2%) isolates were resistant to Fluconazole and sensitive to Voriconazole and 49(53.3%) isolates were resistant to both Fluconazole and Voriconazole. (TABLE 3)

**TABLE 3: ANTIFUNGAL SUSCEPTIBILITY**

SPECIES	S TO F+ V	R TO F S TO V	R TO F + V
<i>C.albicans</i>	20	05	21
<i>C.tropicalis</i>	00	07	13
<i>C.glabrata</i>	01	03	06
<i>C.krusei</i>	00	00	09
<i>C.parapsilosis</i>	00	00	07

S:sensitive R:resistant F:Fluconazole V:Voriconazole

**DISCUSSION**

*Candidas* have been recognized as major opportunistic pathogens recently

emerging as nosocomial pathogens. The present era is witnessing a rise in non albicans *Candida* in comparison to *C.albicans* in clinical scenario. In the present study, *C.albicans* accounted for 50% of isolates. Our findings are in accordance with that of Baradkar V P et al [17] and that of Odds CF and Bernaerts R. [18] 21.7% were *C.tropicalis*, 10.9% were *C.glabrata*, 9.8% were *C.krusei* and 7.6% were *C.parapsilosis*.

Virulence factors of *Candida* species have been of great interest. One of the important factors contributing to virulence of *Candida* is production of extracellular hydrolytic enzymes like phospholipase, proteinase and lipases. They facilitate the penetration into the host organism and

counteract its defense system. In this study phospholipase activity was detected in 34 isolates accounting for 37% with *C.albicans* being the predominant species. Previous studies have reported phospholipase activity in 30 to 100% of *Candida* isolates from various groups of patients. [12,19] In a study done by Kaur R et al, [20] 73.78% of isolates were found to produce phospholipase with non albicans *Candida* being the predominant isolate.

*Candida* produces large quantities of viscid slimy material in glucose containing solutions. [21] Evidence suggests that biofilms have dramatically reduced susceptibility to antifungal drugs. [22] Consequently, biofilm related infections are inherently difficult to treat and to fully eradicate with normal treatment regimens. [23]

In our study, 43 *Candida* isolates i.e.46.73% showed potent biofilm formation. *Candida albicans* showed maximum biofilm production when compared to non albicans isolates. Our findings are consistent with a similar study in Turkey [24] who reported biofilm production in 48% isolates. Deorurkhkar and Saini [25] reported 38.8% isolates having biofilm forming capacity. A higher value was reported by other researchers ranging from 60-85%. [20,26]

There is limited data on hemolytic activities in *Candida*. The secretion of hemolysin followed by iron acquisition facilitates invasion of host tissue. [9] In our study, we reported 31 isolates showing hemolysis accounting for 33.69%. Similarly a study on *C.glabrata* by Deorurkhkar and Saini [25] reports 24.5% isolates having hemolytic activity.

Similar to *Staphylococcus*, coagulase is also one of the hydrolytic enzymes of *Candida*. Very few reports are available on coagulase activity in *Candida*. [27,28] In the present study we report 44 *Candida* isolates showing coagulase production. In our study, most of the strains of *C.glabrata*(70%) and *C.albicans* (65.2% ) were able to induce clot formation at 24 hours. Lower values were

recorded in *C.krusei*(22%), *C.tropicalis* (20%) and *C.parapsilosis*(14%). This is in accordance with a similar study in Portugal. [28]

Disk diffusion methods for antifungals are not standardized and a direct correlation between the susceptibility pattern and clinical outcome has not been established. [29] However, considering that disk-diffusion assays are simple to perform and inexpensive, they may be a useful tool in large-scale surveys of clinical isolates to identify population distribution patterns of susceptibility to fluconazole. The disk diffusion test using 25µg fluconazole disks on an Mueller Hinton agar plate containing 2% glucose and 5mg methylene blue/ml is sufficiently reproducible and accurate to be used as a screening test. [30,31] Recently, this methodology has been used by different investigators for global surveillance fluconazole susceptibility. [32,33]

In our study, 29 isolates were found to be sensitive to both Fluconazole and voriconazole, 14 were resistant to Fluconazole and sensitive to Voriconazole and 49 isolates were resistant to both the drugs. Among the isolates resistant to both drugs, *C.albicans* accounted 43% followed by *C.tropicalis*(31%) and *C.glabrata*(26%). Various studies on resistance pattern of *Candida* to fluconazole report values ranging from 0 to as 70%. [24,25,26,29]

## CONCLUSION

The above results emphasize the need of future studies on the expression of virulence factors and their resistance profile which in turn may open up new options for therapeutic interventions.

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