



**DETECTION OF VIRULENCE FACTORS IN *CANDIDA* ISOLATED FROM
DIFFERENT CLINICAL SPECIMENS**

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ABSTRACT

Candida species are emerged as successful pathogens in both invasive and mucosal infections. Various virulence factors have contributed to their pathogenicity. The purpose of this prospective study was to isolate, speciate, and determine virulence factors of *Candida* species isolated from various clinical specimens. Total of 136 *Candida* were isolated from various clinical specimens, identified up to species level by standard mycological techniques. All isolates were tested in vitro for biofilm formation (BF), proteinase (SAPs), phospholipase (PL), and haemolysin (HF) activity. One thirty six *Candida*, were isolated from different clinical samples. 71(52%) were *C.albicans* and 65(47%) non-*albicans Candida* (NAC) species. Biofilm formation was detected in 86(63%), proteinases activity (SAPs) in 90(66.2%), phospholipases (PL) activity in, 94((69.11%) and haemolysins (HF) activity was demonstrated in 123(90%). From the present study it can be established, that both *C.albicans* and NAC species are causing candidiasis and form biofilm, produce extracellular phospholipases, proteinases and haemolysins.

Key words: *Candida albicans*, Haemolysins, NAC species, Phospholipases, Proteinases

1. INTRODUCTION:

Fungal diseases gained clinical importance in the second half of the last century, primarily due to advances in medical technologies. With the remarkable modern advances in medicine, there has been an increase in the number of immune compromised individuals who need extensive care in hospitals [1]. This has resulted in increase in the incidence of fungal infections, especially those due to *Candida* species. *Candida* species exist in as part of an individual's normal mucosal micro flora and can be detected in approximately 50% of the population in this form. However, if the balance of the normal flora is disrupted or the immune defences are compromised, *Candida* species can invade mucosal surfaces and cause disease manifestations [2]. As the important opportunistic fungal pathogen *Candida* infections have increased significantly worldwide, with *C.albicans* responsible for most of these infections [3]. Although *C.albicans* remains the most common causative agent of both superficial and deep fungal infections, an increasing incidence of non-*albicansCandida* (NAC) species such as *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. parapsilosis*, also been documented in the last few years [4]. The transition of *Candida* from a harmless commensal to disease causing pathogen depends on the immune system of the host

and virulence factors of *Candida* [5]. Growing number of treatment failures, associated mortality, and shift to more resistant isolates advocate the need for species identification in *Candida*.

Several virulence factors contribute to the pathogenicity of *Candida* species including the ability to adhere to epithelial cells, production of hydrolytic enzymes [5] acquisition iron through haemolytic factor [6] and biofilm formation, which consists of a complex community of cells embedded in a matrix of extracellular polysaccharides that also promotes resistance to antimicrobial agents [7].

Biofilms are the structured microbial communities that are attached and encased in a matrix of exopolymeric material, and are important for the development of clinical infection. The most external layers of *Candida* cells are crucial for the adherence to host surface, thereby playing a pivotal role in the pathophysiology of candidiasis [8]. The benefits of forming a biofilm include protection from the environment, nutrient availability, metabolic cooperation, and acquisition of new genetic traits [9].

Secretaryaspartyl proteinases are capable of degrading epithelial and mucosal barrier proteins, such as collagen, keratin and mucin, as well as antibodies, complement and cytokines. Cloning and

disruption of the genes for these enzymes have shown their involvement in *Candida* virulence [10].

The extracellular phospholipases of *C.albicans* have important role in the pathogenesis of infections and invasion to mucosal epithelia. In addition, several studies have shown that clinical isolates of *C.albicans* have higher levels of extracellular phospholipase activity [11].

Haemolysin is another putative virulence factor thought to contribute to *Candida* species pathogenesis. In particular, the secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis [12].

Invasive or mucocutaneous candidiasis is frequently caused by *C. albicans*. Due to its clinical prevalence this species is the best characterized among the genus *Candida*. Non-*albicans Candida* species have been increasingly identified as infectious agents; however its respective virulence traits are poorly described [13].

Determining exactly how this transformation from commensal to pathogen takes place and how it can be prevented is a continuing challenge for the medical mycology field. However, the current understanding of virulence factors in *Candia* is limited due to various reasons, such as different species of *Candida* differ

in their virulence and expression of these virulence factors has been studied mainly in *C. albicans*, than in other *Candida* species [14].

Earlier, studies have described correlation between source of isolate, species and elaboration of either SAPs or PL [4, 5, 6], However, few have looked at combined determination biofilm formation and enzyme activities. Due to the paucity of studies in our setting, the present study investigated biofilm formation (BF), proteinase (secretory aspartyl proteinases, SAPs), phospholipase (PL), haemolysin (haemolytic factors HF) activity of *Candida* species isolated from different clinical samples.

2. AIMS AND OBJECTIVES:

The aim of this study was to evaluate the spectrum of *Candida* species in clinical infections. It was also aimed at in vitro detection of virulence factors such as biofilm formation, proteinase, phospholipase, and haemolysin activities.

3. MATERIALS AND METHODS:

A total of 136 *Candida* isolated from various clinical specimens such as high vaginal swabs (HVS), clean catch midstream urine ,catheterised urine, blood, respiratory samples (sputum, broncoalveolar lavage (BAL), tracheal aspirates), and oropharyngeal swabs (OPS) submitted to the microbiology laboratory from various clinical departments of J. J

.M. Medical college were included in the study. The specimens were processed according to the standard microbiological procedures. Cultured on to blood agar, MacConkey's agar, and Sabouraud's dextrose agar (SDA), when fungal culture was requested. Incubated at 37°C for 24-48 hours. All suspected yeast colonies were confirmed by Gram's staining and further speciated by conventional methods such as germ tube test (GTT), microscopic morphology on cornmeal agar (CMA), and sugar assimilation, fermentation tests. Standard ATCC strains, *C.albicans* (ATCC 90028, 10231), *C.parapsilosis* (ATCC2201), were used throughout the study as quality control strains. All yeast isolates that grew on sheep blood agar and SDA were sub-cultured on to chromogenic medium (HiCHROM *Candida*; HiMedia, Mumbai, India) and incubated at 37°C for 48 h. The isolates grew well and developed characteristic coloured colonies after incubation. Presumptive identification was made by observing the colour of the colonies as per the manufacturer's instructions (*C. albicans*-green, *C.tropicalis* - blue, *C. krusei* - pink colonies with matt surface, *C. parapsilosis*-cream to pale pink, *C.glabrata* white to cream). The virulence factors such as biofilm formation, proteinases, phospholipases, haemolysins activities were tested in vitro.

3.1. Biofilm formation:

Biofilm formation was determined by visual test tube method with little modification [4]. The isolate was inoculated into polystyrene test tube containing 5 ml of Sabouraud's dextrose broth supplemented with 8% glucose. The test tube was incubated at 37°C for 48 hours. After incubation the broth from the test tube was gently aspirated using Pasteur pipette and washed twice with phosphate buffered saline to remove non-adherent cells. The test tube was stained with 2% safranin for 10 minutes. Excess of stain was removed by washing with phosphate buffered saline and test tube was examined for the presence of adherent layer. The isolate was considered positive for biofilm formation when a visible film was seen on the wall and bottom of the test tube. The formation of ring at the liquid interface was not considered as an indication of biofilm production [4].

3.2. Preparation of yeast suspension:

Yeast suspension was prepared from the isolates. A small amount of stock culture was inoculated on Sabouraud's dextrose agar with chloramphenicol using sterile loop and incubated at 37°C 24-48 hours. The yeasts were harvested by washing with sterile saline and suspended in sterile saline. Turbidity was adjusted to McFarland tube 2 scale, the final

suspension was adjusted to contain (1×10^8 to 5×10^8 CFU/mL) yeast cells/ml.

3.3. Determination of proteinase activity:

Candida isolates were tested in vitro for extracellular proteinase activity in terms of bovine serum albumin (BSA) degradation [15]. Sixty millilitres of solution containing 0.04 g $MgSO_4 \cdot 7H_2O$, 0.5g K_2HPO_4 , 1 g NaCl, 0.2 g yeast extract, 4 g glucose and 0.5 g BSA was prepared, and the pH was adjusted to 4 with 1 M HCl. The solution was sterilized by filtration and mixed with 140 ml molten agar. Twenty millilitres of this medium was poured into Petri dishes. Standard inoculum of test and control *Candida* isolates, an aliquot of 10 μ l containing 10^8 yeast cells were spot inoculated onto the plates. The plates were incubated at 37°C for 5 days. The plates were then fixed with 20% trichloro acetic acid and stained with 1.25% amido black. Decolourisation was performed with 15% acetic acid. Degradation of the protein was seen as opaqueness of the agar, corresponding to a zone of proteolysis around the colony which could not be stained with amidoblack. The assay was done in duplicate on two different occasions for each *Candida* isolate. Proteinases activity (Pr) was determined as the ratio of the colony to the diameter of the proteolytic unstained zone. A Prz value of 1 signifies

no activity, and less than one ($Prz < 1$) means proteinases activity. The lower the Prz value, the higher the enzymatic activity. Reference strains of *C.albicans* (ATCC 10231) served as positive control [15].

3.4. Determination of phospholipase activity:

The *Candida* phospholipase activity was detected by measuring the size of precipitation zone after the growth on egg yolk agar [6]. The egg yolk medium consisted of 13.0 g Sabouraud's dextrose agar (SDA), 11.7 g NaCl, 0.11 g $CaCl_2$ and 10% sterile egg yolk (all in 180 ml distilled water) First, the components without the egg yolk were mixed and sterilized, then 20 ml sterile egg yolk emulsion (HI Media Mumbai) was added to the sterilized medium [6]. Standard inoculum of the test and control *Candida* isolates, 10 μ l, of 10^8 yeast cells were deposited onto the egg yolk agar medium and left to dry at room temperature. Each culture was then incubated at 37°C for 48 hours. The assay was conducted in duplicate on two separate occasions for each *Candida* isolate. Phospholipases activity of the isolate was considered positive when a precipitation zone was visible around the colony on the plate. The value of phospholipases activity (Pz) was measured by the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. A Pz value of 1 denotes no activity, and less

than one ($Pz < 1$) indicated the phospholipases activity. The lower the Pz value, the higher the enzymatic activity. Reference strain of *C. albicans* (ATCC10231) served as positive control [16].

3.5. Determination of haemolysin activity:

Haemolysin activity was evaluated with a blood plate assay [12]. Media was prepared by adding 7 ml fresh sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3 % (w/v). The final pH of the medium was 5.6 ± 0.2 [6]. Standard inoculum of both the test and the control *Candida* isolates $10\mu\text{l}$ with 10^8 yeast cells in saline was deposited onto the medium. The plate was then incubated at 37°C in 5% CO_2 for 48 h. The ratio of the diameter of the colony to that of the translucent zone of haemolysis (in mm) was used as the haemolytic index (Hz value) to represent the extent of haemolysin activity by different *Candida* isolates [15]. The assay was conducted in duplicate on two separate occasions for each yeast isolate. Reference strains of *C. albicans* (ATCC 90028), *C. parapsilosis* (ATCC 2201), served as a positive and negative controls.

4. RESULTS:

Table 1, and Table 2, shows *Candida* species distribution and virulence factors activity in different clinical samples.

Total of 136 *Candida* isolates were isolated from various clinical specimens. *C. albicans* 52.2% and 47.8% NAC species, Among the NAC species, *C. tropicalis* (25%) was the most common isolate, followed by *C. krusei* (9.5%) and *C. parapsilosis* (8 %). *C. glabrata* (5.1%).

Biofilm was detected both in *C. albicans* and NAC species. Among the 136 isolates of *Candida*, 63.2% were found to be biofilm producers. *C. tropicalis* was the most common biofilm producer 73.5%, followed by *C. krusei* 69.2% *C. albicans* 59.2%, *C. parapsilosis* 63.6%, and *C. glabrata* 42%.

The proteinase activity was detected in 66.2%, *C. albicans* 80.3% was the most common proteinase producer followed by NAC species 50.8%. Among NAC species *C. Tropicalis* 64.7% was the highest proteinase producer followed by *C. krusei* 61.5%, *C. parapsilosis* 27.2 % and all *C. glabrata* were negative for proteinase activity.

Phospholipase activity was detected in 69.1 %, *C. albicans* 87.3 % showed highest phospholipase activity, and in NAC species it was 49.2 %. Among NAC species, it was more noted in *C. tropicalis* 67.6% followed by *C. krusei* 53.8 %, whereas in *C. Parapsilosis* 18.1% and all *C. glabrata* were negative for phospholipase activity.

Out of 136 *Candida* isolates 90.4% were positive for haemolysin activity. In

C.albicans it was positive in 97.2% and in NAC species it was positive in 83.1%. All *C. parapsilosis* were negative for haemolysin activity. Maximum proteinase 80.3%, phospholipase 87.3%, and

haemolysin 97.2% production was seen in *C.albicans* isolates. Whereas biofilm formation was noted more in NAC (67.7%) species. It was comparatively lower in *C.albicans* (59.2%).

Table 1: Production of different virulence factors with respect to different *Candida* species and site of infection

Sl No	Species	Total(%)	BF (%)	PTA (%)	PLA (%)	HEA (%)
1	<i>Candidaalbicans.</i>					
	HVS (49)	30(61.2)	17(56.7)	26(86.7)	27 (90)	30(100)
	Urine (45)	16(35.5)	11(68.8)	10(62.5)	12(75)	14(87.5)
	Blood (22)	9(40.9)	5(55.6)	7(77.8)	8(88.9)	9(100)
	RES (17)	13(76.4)	7(53.8)	11(84.6)	12(92.3)	13(100)
	OPS (3)	3(100)	2(66.7)	3(100)	3(100)	3(100)
	Total No:136	71(52.2)	44(59.2)	57(80.3)	60(87.3)	69(97.2)
2	<i>Candidatropicalis.</i>					
	HVS (49)	6(12.2)	3(50)	3(50)	4(66.7)	6(100)
	Urine (45)	23(51.1)	18(78.3)	16(69.6)	16(69.6)	23(100)
	Blood (22)	4(18.1)	3(75)	3(75)	3(75)	4(100)
	RES (17)	1(5.8)	1(100)	-	-	1(100)
	OPS (3)	-	-	-	-	-
	Total No:136	34(25)	25(73.5)	22(64.7)	23(67.6)	34(100)
3	<i>Candidakrusei.</i>					
	HVS (49)	4(8.1)	3(75)	2(50)	2(50)	4(100)
	Urine (45)	3(6.6)	2(66.7)	1(33.3)	1(33.3)	3(100)
	Blood (22)	3(13.6)	2(66.7)	3(100)	2(66.7)	3(100)
	RES (17)	3(17.6)	2(66.7)	2(66.7)	2(66.7)	3(100)
	OPS (3)	-	-	-	-	-
	Total No:136	13(9.5)	9(69.2)	8(61.5)	7(53.8)	13(100)
4	<i>Candidaparapsilosis.</i>					
	HVS (49)	4(8.1)	3(75)	1(25)	1(25)	-
	Urine (45)	3(6.6)	2(66.7)	2(66.7)	1 (33.3)	-
	Blood (22)	4(18.1)	2(50)	-	-	-
	RES (17)	-	-	-	-	-
	OPS (3)	-	-	-	-	-
	Total No:136	11(8)	7(63.6)	3(27.2)	2(18.1)	-
5	<i>Candida glabrata.</i>					
	HVS (49)	5(10.2)	2(40)	-	-	5(100)
	Urine (45)	-	-	-	-	-
	Blood (22)	2(9)	1(50)	-	-	2(100)
	RES (17)	-	-	-	-	-
	OPS (3)	-	-	-	-	-
	Total No:136	7(5.1)	3(42.8)	-	-	7(100)
6	Cumulative Percentage.					
	HVS	49	28(57.1)	32(65.4)	34(69.4)	45(91.8)
	Urine	45	33(73.3)	29(64.4)	30(66.7)	40(88.9)
	Blood	22	13(59.1)	13(59.1)	13(59.1)	18(81.8)
	RES	17	10(58.8)	13(76.5)	14(82.4)	17(100)
	OPS	3	2(66.7)	3(100)	3(100)	3(100)
	Total No	136	86(63.2)	90(66.2)	94(69.1)	123 (90.4)

BF: Biofilm formation, PTA: Proteinase activity, PLA: Phospholipase activity, HEA: Haemolytic activity HVS: High vaginal swab, RES: Respiratory samples, OPS: Oropharyngeal swabs

Table 2: Production of different virulence factors in Non *albicans Candida* species

Sl No	Species	Total	BF (%)	PTA (%)	PLA (%)	HEA (%)
<i>NonalbicansCandida</i>						
1	HVS (49)	19(38.8)	11(57.9)	6(31.6)	7(36.8)	15(78.9)
2	Urine (45)	29(64.4)	22(75.9)	19(65.5)	18(62.1)	26(89.7)
3	Blood (22)	13(59.1)	8(61.5)	6(46.2)	5(38.5)	9(69.2)
4	RES (17)	4(23.5)	3(75)	2(50)	2(50)	4(100)
5	OPS (3)	-	-	-	-	-
	Total No:136	65(47.8)	44(67.7)	33(50.8)	32(49.2)	54(83.1)

5. DISCUSSION:

Fungi, once considered microbiological curiosities without pathogenic potential, have emerged as important opportunistic human pathogens [17]. Opportunistic fungal pathogens are becoming increasingly important cause of community acquired and nosocomial infections. The yeasts of the genus *Candida* are the most important opportunistic fungal pathogens associated with the rising incidence in immunocompromised individuals [17].

The pathogenicity of *Candida* depends on several putative virulence factors, including germination, adherence to host cells, phenotypic switching and production of extracellular hydrolytic enzymes. [10]. Among these virulence traits of *Candida* species, the present study targeted biofilm formation, extracellular proteinase, phospholipase, and haemolysin activities in *Candida* species isolated from various clinical samples.

The present study demonstrates higher isolation rates of *C. albicans* 71 (52.2%) than NAC65 (47.8%) possibly due to predominance of *C. albicans* in mucosal infections and colonization. NAC were isolated predominantly from urine and blood from invasive infections probably due to the involvement of cases from high risk patient care areas. Similar observation was also reported by Saherand Ziab *et al.*,

C. albicans (61%) than NAC species [18]. Nerurkar *et al.*, also reported the most commonly isolated species was *C. Albicans* (61.4%) [19]. Whereas in a study conducted by Vijaya *et al.*, non-*albicans Candida* predominated (54.1%) over *C. Albicans* (45.9%) [20]. Lower prevalence rates (39.5% and 25.3%) of *C. albicans* was also reported by Mokaddas *et al.*, [21] and Chakrabarti *et al.*, [22] respectively. In our study *C. tropicalis* was the major isolate among NAC species followed by *C. krusei*, *C. parapsilosis*, and *C. glabrata* these rates are similar to those reported by Binesh and Kalyani, among NAC spp. *C. Tropicalis* (53.4%) was the main isolate and majority were isolated from urine (63.9%) [23].

We report a higher proportion of *C. albicans* in vvc (61.2%), respiratory infections (76.4%) and oral candidiasis (100%). It is in contrast to the observations indicating the changing trends in aetiologies with a shift towards NAC species [24]. Geographic differences and increased use of self-medications with antifungal agents might have contributed to up surge of infections by more resistant NAC species [25]. However various studies worldwide have shown a predominance of *C. albicans* in mucosal infections and colonization [6, 12].

While the predominant species recovered from urine (64.4%) and blood

samples (59.1%) were NAC species, perhaps due to the involvement of cases from high risk patient care areas. These findings are consistent with study of Jain *et al.*, who revealed that NAC species, especially *C. tropicalis* (52.9%) predominated in urine samples [26]. Our observation of higher percentage of *C. tropicalis* isolation in urine could be due to previous history of treatment with broad spectrum antibiotics, urinary catheter or total parenteral nutrition, other co-morbid conditions such as diabetes mellitus and malignancy. These results are nearly similar to those reported by Mohandas and Ballal the proportion of such infections due to NAC species is persistently rising [27].

Biofilm production is considered as one of the most potent pathogenic traits attributed to treatment failures and recurrent infections. Reports have showed that majority of biofilms forming *Candida* isolates were from urine samples, followed by blood [28].

Our clinical isolates of *Candida* showed highest percentage of biofilms forming *Candida* from urine (73.3%) and oral samples (66.7%) followed by blood stream (59.1%), respiratory samples (58.8%) and high vaginal swabs (57.1%) infections. BF was demonstrated more frequently in *C. tropicalis* (73.5%) followed by *C. krusei* (69.2%) and

C. parapsilosis (63.6%), *C. albicans* (59.2%), and the least producer was *C. Glabrata* (42.8%), but a study done by Mohandas and Ballal reported that BF most frequently occurred in isolates of *C. krusei* followed by *C. tropicalis* [4]

Secretary as partyl proteinases are secreted by pathogenic species of *Candida* in vivo during infection. Secretary as partyl proteinases (Saps) are responsible for the adhesion, tissue damage, and invasion of host immune responses. Proteinases fulfill a number of specialized functions during the infective process, they include digesting molecules for nutrient acquisition, digesting or distorting host cell membranes to facilitate adhesion and tissue invasion, and digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack by the host [10].

In the present study the proteinase positive isolates were 90 (66.2%). All three *C. albicans* isolated from oral candidiasis samples demonstrated proteinase activity 100%, followed by (76.5%) in respiratory sample (65.3%) in vvc and (64.4%) in urine, (59.1%) in blood stream isolates. Proteinase activity was high *C. albicans* (80.3%) followed by in *C. Tropicalis* (64.7%), *C. Krusei* (61.5%), and *C. parapsilosis* 27%. which is similar to the observation of Sachin CD *et al.*, [15].

Phospholipases are a group of enzymes produced by *Candida* species that primarily help in digesting the phospholipids of the host cells leading to cell lysis [11]. *C.albicans* is the major producer of phospholipases, whereas a less proportion of non-albicans *Candida* produce this enzyme.

In our study, the majority 87 % of phospholipase producers were *C. albicans*, whereas (49.2) % of non-*albicans Candida* were positive for phospholipases. Phospholipase production was predominately seen in isolates from oral candidiasis (100%), followed by respiratory samples (82.4%), vvc (69.3%), urine (66.7%) and in blood stream infections it was (59.1%). Previous studies have reported phospholipase activity in 30 to 100% of *Candida* isolates [4, 6, 30].

Haemolysin secretion followed by iron acquisition facilitates deeper tissue invasion by *Candida*. Studies on the activity of haemolysin in *Candida* are limited. We noted all produced beta haemolysis and haemolysin activity was higher in *C.albicans* (97.2%) All *C. parapsilosis* were negative for haemolytic activity. Manns *et al.*, first reported that a complement mediated haemolysis was induced by *C. albicans*, defined the condition under which *C.albicans* can display haemolytic activity [29]. Luo *et al.*

demonstrated the variable expression of haemolysin by different *Candida* species, Moreover, Luo *et al.*, suggested that haemolysis can be categorized into complete (beta), incomplete (alpha) or no haemolysis (gamma) [12]. Yigit & Aktas reported that sheep blood SDA is most sensitive and appeared to give the best indication of haemolytic activity of *Candida* species compared to rabbit blood SDA and human blood SDA [30].

The present study involved *Candida* isolates from invasive and mucosal infection sites and studied the production of important virulence determinants, especially among less explored NAC species. More sensitive methods are required to detect certain enzymes like phospholipases in these isolates. Exploring genetic mechanisms of virulence in NAC species would assist in understanding the spectrum of disease and changing trends in disease epidemiology.

6. CONCLUSION:

The keen concern in *Candida* species is a reflection on the incidence of *Candida* infections. Species of *Candida* other than *C. albicans* which were earlier considered as less or non-virulent are now implicated in human diseases. In our study biofilm formation, extracellular hydrolytic enzyme activities were shown by *C. albicans* and in NAC species. Detection of

virulence factors like biofilm phospholipase, proteinase and haemolysin will help to provide sufficient data in the establishment of a relationship between the infection and *Candida* species isolated.

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