

In vitro evaluation of virulence factors associated with clinical isolates of *Candida* species from patient in tertiary care hospital in Central India

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Abstract

Background: Adherence to host tissues, morphological changes and secretion of extracellular hydrolytic enzymes are important virulence attributes of *Candida* species. These enzymes play pivotal roles in pathogenicity of *Candida* infection. **Aim:** The present study was conducted with an aim to determine in *vitro* phospholipase, haemolysin, Coagulase activities and biofilm production of *Candida* species isolated from various Clinical samples. **Material and Method:** A total of 156 *Candida* species isolated from various clinical specimens were identified up to species level by standard mycological techniques and was tested for extracellular hydrolytic enzyme activity and Biofilm formation. **Results:** 156 *Candida* species were isolated from 915 clinical specimens. Biofilm production was shown by 77 candidal strains (49.35%), phospholipase activity was observed by 74 candidal strains (47.43%), haemolysin activity was observed by 66 candidal strains (42.30%) and Coagulase production in 38 of *Candida* (24.35%) isolates. **Conclusion:** Both *C. albicans* and Non-albicans *Candida* (NAC) species produce extracellular hydrolytic enzymes. Since these enzymes are important to understand the co-relation between the species and infection, their detection is extremely important in the management of infections caused by various *Candida* species.

Keywords: Extracellular hydrolytic enzymes, phospholipase, haemolysin, Coagulase non-albicans *Candida* species.

Introduction

The incidence of invasive fungal infection has increased steadily over the past two decades. With recent advances in medical and surgical intervention and increasing population of immunocompromised patients, the diversity and list of fungal pathogens continue to grow [1]. The virulence attributes of *Candida* includes hyphal switching, germination, surface recognition and production of extracellular hydrolytic enzyme [2]. Among these, extracellular hydrolytic enzymes play an important role in adherence and infection of *Candida* [3]. Recently, microbial biofilms have gained prominence because of the increase in infections related to indwelling medical devices. The advantages of forming a biofilm for the organism include protection from the environment, nutrient availability, metabolic cooperation, and acquisition of new genetic traits [4].

Research on *Candida* pathogenicity has been focused on hydrolytic enzymes, such as proteinases, phospholipases and lipases. Little information concerning *Candida* coagulase and hemolytic activities was found in a recent literature search.

Hemolytic and coagulase activities in *Candida* spp. are virulence factors less well-studied and their importance to pathogenicity require new and more rigorous studies [5].

The present study was conducted with an aim to determine in *vitro* phospholipase, haemolysin, Coagulase activities and biofilm production of *Candida* species isolated from various Clinical samples.

Aim: To determine the virulence factors of *Candida* species isolated from various clinically suspected samples of Candidiasis.

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Materials and Methods

Study design- Hospital based cross sectional study.

Study setting- Department of Microbiology, Ruxmaniben Deepchand Gardi Medical College (RDGMC) and Chandrikaben Rashmikant Gardi Hospital (CRGH) and Research Centre, Surasa, Ujjain.

Study subjects- Clinically suspected candidiasis among patients admitted in C.R.Gardi Hospital.

Study samples- Clinical samples from suspected patients of candidiasis including-urine, blood, vaginal swab, oral swab, sputum, stool, lip scraping, pus, CSF, central line tip, eye swab and umbilical swab.

Study period- One and a half year from January 2013 to June 2014.

915 Clinical samples from suspected patients of candidiasis received in Laboratory. 156 *Candida* species were isolated from 915 clinical specimens.

The study was approved by the Institutional research and ethics committee. Speciation of *Candida* isolates were done by evaluating the germ tube formation, assimilation, and sugar fermentation test. Culture on cornmeal agar was for demonstration of chlamydo spores while culture on candid chromagar was for the identification of the species. 156 isolates were screened for extracellular enzymes production and Biofilm formation.

Determination of virulence factor

- 1) Phospholipase enzyme production
- 2) Haemolytic enzyme production
- 3) Coagulase production
- 4) Production of biofilm

1) Phospholipase enzyme production: The phospholipase activity of *Candida* isolates was detected by the method of Samaranyakeet al. [6] (color plate-1)

Preparation of media- The egg-yolk medium consisted of 13.0g Sabouraud's dextrose agar (SDA), 11.7g sodium chloride, 0.11g Calcium chloride and 10% sterile egg yolk in 184ml distilled water. First, the components without the egg yolk were mixed and sterilized (autoclave). After autoclaving the egg yolk was centrifuged at 500 rpm for 10 minute at room temperature and 20ml of the supernatant was added to the sterilized medium.

Yeast suspension preparation- 10^8 yeast cells/ml was prepared in sterile saline

Procedure- Extracellular phospholipase activity was detected by inoculating 5 μ l of yeast suspension onto the surface of the egg-yolk medium and left to dry at room temperature. *Candida albicans* 90028 was used as Positive control. Culture plate was incubated at 37°C for 2 days.

Calculation of the zone of phospholipase activity (Pz) was performed as follows:

$$Pz = \frac{\text{Colony Diameter}}{\text{Colony Diameter} + \text{Zone of precipitation}}$$

- **Pz ratio of one-** no activity: negative (–)
- **Pz ratio of less than one** – indicated the phospholipase activity.

- a) 0.99-0.9 as weak (+)
- b) 0.89-0.8 as mild (++)
- c) 0.79-0.7 as relatively strong (+++)
- d) <0.69 (++++) as very strong positive

The lower the Pz value, higher the phospholipase activity [7, 8].

Haemolytic enzyme production: Haemolysin activity was assessed by the method of Manns et al. [9] on blood agar plate. (color plate-2)

Media preparation- SDA supplemented 7% sheep blood and 3% glucose with a final pH adjusted to 5.6 ± 0.2 was used.

Procedure- 10 μ l of previously prepared yeast suspension was inoculated onto plates and incubated at 37 °C in 5%CO₂ for 48 h.

Interpretation- Beta (β)-haemolysis was indicated by a clear, colourless zone surrounding the colony of the organism and alpha (α)-haemolysis showed a greenish zone. Gamma (γ) haemolysis was indicated by an absence of any apparent haemolysis or discolouration. To represent the extent of haemolysin activity by different *Candida* isolates [Haemolysin activity (Hz) was determined by calculating the ratio of the diameter of the colony to that of the translucent zone of haemolysis (in mm). The assay was conducted on three

separate occasions for each isolate. *C. albicans* ATCC 90028 was used as positive control. One strain each of *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus sanguis*, which induce beta and alpha haemolysis, were used as positive controls. [5,10,11].

3) Coagulase production of *Candida* spp: was done by EDTA- Sheep plasma by a tube test method 0.1 ml of an overnight culture of each isolate in Sabouraud's dextrose broth was inoculated into a tube containing 500 µl of EDTA- Sheep plasma. The tubes were incubated for 4 h at 35°C. The presence of a clot that could not be resuspended by gentle shaking indicated positive coagulase test. If no clot formed, the tube was reincubated & re-examined after 24 h. *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 were used as positive and negative controls. [5,12] (color plate-3)

4) Detection of biofilm production – Biofilm formation by *Candida* isolates was assessed by the tube method as described by et al. A loopful of yeast from

overnight SDA culture plates was inoculated in 10ml Sabouraud's dextrose broth in polypropylene falcon tube and incubated for 48h at 35°C. After 48h, broth was aspirated gently and washed with distilled water twice and then stained with 1% safranin for 10 min after which they were examined on walls for the presence of an adherent layer. Biofilm producer *Staphylococcus epidermidis* ATCC 35984 was used as a positive control. Each isolate was tested twice and read independently by two different observers. [5,13] (color plate- 4)

Interpretation: Biofilm formation was considered positive when a film lined the wall and bottom of the tube. Ring formation at the liquid interface was not considered as an indication of biofilm production [8, 10].

Biofilm production was scored as

- 0..... non adherent (NA),
- +..... weak adherent (WA)
- ++..... Moderate adherent (MA)
- +++..... strong adherent (SA)

Result

Table-1: Phospholipase activities of different *Candida* species (n=156).

Species	No of isolates	Phospholipase enzyme Production
<i>C.tropicalis</i>	73	43 (58.90%)
<i>C.albicans</i>	58	21(36.20%)
<i>C.parapsilosis</i>	15	7(46.66%)
<i>C.kefyr</i>	3	0
<i>C.guilliermondii</i>	3	1(33.33%)
<i>C.krusei</i>	2	2(100%)
<i>C.dubliensis</i>	2	0
Total	156	74(47.43%)

In present study *C.tropicalis* shows the maximum Phospholipase activity. Phospholipase activity was seen in 54.08% NAC species and 36.20% *C.albicans* species.

Table-2: Extracellular hydrolytic activity of different *Candida* species.

Species	No of isolates	Haemolysin Production	Coagulase Production (Sheep Blood)
<i>C.tropicalis</i>	73	37(50.68%)	21(28.76%)
<i>C.albicans</i>	58	27(46.55%)	15(25.86%)
<i>C.parapsilosis</i>	15	1(6.66%)	2(13.33%)
<i>C.kefyr</i>	3	0	0
<i>C.guilliermondii</i>	3	0	0
<i>C.krusei</i>	2	0	0
<i>C.dubliensis</i>	2	1(50%)	0
Total	156	66 (42.30%)	38(24.35%)

D) Haemolysin activity (Hz) - Among all the isolates, 66 (42.30%) expressed hemolytic activity. Beta hemolysis was seen. The hemolytic index values ranged from 0.32–0.86.

Haemolysin production was more in NAC (39.79%). Haemolysin production was not noted in *C.kefyr*, *C.krusei* & *C.guilliermondii*.

II) Coagulase production- Maximum Coagulase production was seen in *C.tropicalis* (28.76%) isolates in sheep plasma

Table-3: Slime production by *Candida* species (n=156)

Species	No of Isolates	No of positive isolates	Weakly adherent (1+)	Moderately adherent (2+)	Strongly adherent (3+)
<i>C.tropicalis</i>	73	42 (57.53%)	13	12	17
<i>C.albicans</i>	58	24 (41.37%)	14	2	8
<i>C.parapsilosis</i>	15	8 (53.33%)	2	3	3
<i>C.krusei</i>	2	1 (50%)	0	1	0
<i>C.guilliermondii</i>	3	1(33.33%)	0	1	0
<i>C.kefyr</i>	3	0	0	0	0
<i>C.dubliensis</i>	2	1(50%)	0	1	0
Total	156	77(49.35%)	29	20	28

C.tropicalis (57.53%) was the most common biofilm producer

Discussion

We have done study on selective virulence factors such as phospholipase, hemolysin, coagulase enzyme production and biofilm.

Out of 156 *Candida* species studied, phospholipase activity was seen in 74 (47.43%) isolates. Previous studies have reported phospholipase activity in 30 to 100% of Candidal isolates. Similar observations were found in other Indian studies [14]. Vinita et al study indicates that even though all the isolated strains were pathogenic, not all strains of *Candida* produced phospholipase as virulent factors. The virulence of *Candida* species is attributed not to a single factor but to a combination of several factors, like proteinase, phospholipase, biofilm production etc. Phospholipase positivity among non-*albicans* *Candida* strains was reported by Vinita et al [14] and Sachin et al [11]. In present study among the NAC species *C. tropicalis* showed maximum phospholipase production (58.90%), so it means it is more invasive among various isolates. Similar observations were found in other Indian studies [15]. In our study Phospholipase was the major virulent factor expressed by *C. tropicalis* isolates. Researchers have reported contradictory findings regarding phospholipase activity in *C. tropicalis*. Investigators like Thangam et al [16] reported high phospholipase activity in *C. tropicalis* isolates among NAC spp. while others like Samaranayake et al [17] reported no activity. These inconsistencies in observations may be due to biological differences among the isolates tested [15].

Most of the studies on exoenzymes are focused on phospholipases and secreted aspartyl proteinases (Sap). Coagulase production and haemolytic activity of are the least studied [12].

In present study haemolysin activity was detected in 42.30% isolates of *Candida* species. Study by Sachin et al [1] in 2012 noted (51.8%) haemolytic activity and 48% in *C. tropicalis*. In our study all isolates showed β type of haemolysis and *C. tropicalis* showed maximum haemolytic activity (50.68%). Haemolysin is another putative virulence factor thought to contribute to candidal pathogenesis. In particular, the secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis [5].

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In the present study 24.35% isolates were coagulase test positive when tested with sheep plasma. None of these *Candida* isolates reacted positively in human plasma. In the study by Sachin et al [11] in 2013 reported 30.1% isolates were reported coagulase positive. Coagulase binds plasma fibrinogen and activates a cascade of reactions that induce clotting of plasma. Rodrigues et al. [18] reported high coagulase activity in *C.tropicalis* (82.6%). Variations in coagulase production by *Candida* spp may be related to their pathogenicity. It is concluded that the laboratory detection of coagulase activity in clinical isolates of *Candida* spp may help the diagnosis of *Candida*-related infections.

Among the three virulence factors, Phospholipase (47.43%) was the most common virulent factor followed by Haemolysin production (42.30%) and Coagulase production (24.35%) in the present study.

NAC species 53/98 (54.08%) produced biofilm in comparison to *C. albicans* 24/58 (41.37%) showing relatively lower slime production. Also strong biofilm production was seen in cases of *C. tropicalis* compared to weak biofilm production that was seen in *C. kefyr*, *C. krusei*, and *C.guilliermondii*.

Similar observation was seen in study by Yigit *et al.*[5] Studies, investigating biofilm production among *Candida* species detected higher levels of positivity among non-albicans *Candida* strains (51-60%) than that of *C.albicans* strains (8-12%) [8]. Biofilms are surface associated microbial communities which are firmly fixed within an extracellular matrix [19]. It limits the penetration of an antifungal agent through the matrix and protects the yeast cells from host immune response.

Virulence of *Candida* depends on the type of species and the clinical sample from which it has been isolated. Hence, significance of virulence determination in rational antifungal therapy cannot be ignored and should be adopted as a routine procedure in the laboratory.

Conclusion

In conclusion, it was seen that NAC spp. had greater biofilm-forming ability when compared to *C. albicans* species especially *C. tropicalis* showed the highest ability to produce phospholipase, haemolysin, Coagulase and biofilms. Species of *Candida* other than *C. albicans* which were previously considered as less or non virulent are now implicated in human disease. In our study extracellular hydrolytic enzyme activities was shown by *C. albicans* and NAC species. Detection of virulence factors like phospholipase, haemolysin, Coagulase and Biofilm formation will help to provide sufficient data in the establishment of a relationship between the infection and *Candida* species isolated.

This study will provide data about distribution of various *Candida* species and their Virulence markers among patients visiting our tertiary care hospital and our geographical area specially in and around Ujjain district (M.P.).

Detection of Virulence factor**Color plate-1****Phospholipase enzyme Production****Color plate -2****Haemolysin enzyme production**

Color plate -3



Positive

Coagulase production



Negative

Color plate-4 Biofilm formation

a) Non-adherent b) weakly-adherent c) Moderately-adherent d) Strongly adherent

a

b

c

d



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