



Determination of Virulence Factors and Biofilm Formation Among Isolates of Vulvovaginal Candidiasis

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Context: Under morphogenesis-inducing conditions, *Candida* spp. begins to undergo yeast-to-hypha switch. This shift from commensal to pathogenic state is dependent on several virulence factors. **Aim:** To find out whether the isolated *Candida* spp. were pathogens causing vulvovaginal candidiasis or mere bystanders. **Settings and Design:** Cross-sectional observational study conducted on 275 symptomatic hospital patients in Tripura between August 2012 and April 2015. **Subjects and Methods:** Discharge was collected from patients and identified by Grams staining and wet mount test. Culturing was done in Sabouraud dextrose agar followed by speciation. To test for virulence factors, assays for adherence, plasma coagulase, phospholipase, lipase, protease, hemolysin, and biofilm formation were carried out. **Statistical Analysis Used:** Significance between two groups was compared using one-way analysis of variance along with Tukey test, and Chi-square 2×2 contingency table at 95% confidence interval. **Results:** Fifty-six *Candida* spp. could be isolated in the study which was used for further virulence tests. One hundred percent of isolates expressed adherence. Among other virulence factors, maximum virulence 25 (45%) was shown through protease production. Hemolysin production and biofilm formation were the second most 22 (39%) expressed virulence factors. In a comparison of virulence factors between biofilm-forming isolates and planktonic cells, significant difference was seen for plasma coagulase and hemolysin production. **Conclusions:** All the isolates expressed one or more virulence factors. Adherence was expressed in all isolates but highest number was observed for *Candida albicans*. Furthermore, *C. albicans* strain number was highest for protease, hemolysin and coagulase expression and biofilm formation. *Candida krusei* isolates were the least in number for expressing any of the virulence factors. Significantly higher number of biofilm forming isolates produced hemolysin and coagulase in comparison to planktonic cells.

Key words: Biofilm, non-albicans *Candida* species, virulence factors, vulvovaginal candidiasis

INTRODUCTION

Genital/vulvovaginal candidiasis (VVC) is an infection associated with dermatitis of the vaginal mucosa commonly caused by an excessive growth of one of the various species of *Candida*. In women, VVC is characterized by vulvar pruritus, urinary frequency and urgency, thrush patches on the vulva and sometimes a “cottage cheese-like” vaginal discharge. Though rare, men may be infected with genital candidiasis and experience balanitis and balanoposthitis.¹

Candida is found as a commensal in the mouth, gastrointestinal tract, and vagina. However, under

morphogenesis-inducing conditions such as high estrogen levels, elevated vaginal pH, diabetes mellitus, weakened immune system, use of corticosteroids or concurrent antibiotics, and benign colonized *Candida* spp. begin to undergo yeast-to-hypha switch.² *Candida* infections can also be passed from person to person through sexual routes.

This shift from commensal to pathogenic state is dependent on several virulence factors. Although not yet defined, virulence factors are generally considered as those that interact directly with host cells causing damage.³ Morphological transition between yeast and hyphal forms, expression of adhesins and

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invasins on the cell surface, production of tissue-damaging hydrolytic enzymes (e.g., proteases, phospholipases and hemolysins), phenotypic switching and thigmotropism are the commonly known virulence factors.⁴

Besides these virulence factors, formation of biofilms is also a critical feature for the development of clinical infection. The growth of microorganisms when associated with a surface is called biofilm where the organisms grow as a community rather than as separate surface adherent cells. Biofilm communities may be formed on host tissues or medical devices and are more resistant to antifungal drugs than cells growing in suspension (called planktonic cells). The reasons for increased tolerance to drugs are biofilm structural complexity, presence of extracellular matrix, and biofilm-associated up-regulation of efflux pump genes.⁵

In North East India, fungal disease incidences are high; our previous study from Tripura showed VVC rates at 25% and as one of the most common finding among STIs.⁶ Studies from India on virulence factors expressed by *Candida* species isolated from VVC subjects are few,⁷ therefore this study was taken up with the aim to find out whether the isolated *Candida* spp. from symptomatic patients were pathogens causing VVC or mere bystanders.

SUBJECTS AND METHODS

The study was cross-sectional observational, conducted in a tertiary hospital in Tripura between August 2012 and April 2015 post-clearance from Institutional Ethical Committee. Study subjects comprised excessive vaginal discharge cases, cases with vaginal pruritus or frequent micturition, within the reproductive age of 18–49 years. Those on concurrent antibiotic use, menstruating, pregnant, or with any intrauterine device were excluded from the study. After obtaining informed consent, details were recorded in a structured questionnaire about the subject's demographic profile, routine hygiene practices, and sexual history. Discharge was collected using cotton swabs from male and female subjects in duplicate.

Candida species identification and culture

Direct Grams-stained smears were observed for presence of polymorphonuclear leukocytes and fungal elements. Wet mount was done with 10% potassium hydroxide (KOH). Discharge samples were cultured in Sabouraud dextrose agar (SDA) with and without chlorhexidine. Yeast identification was done through colony characteristics, Grams stain reaction, and sugar assimilation test. Further, *Candida* speciation was confirmed by germ tube test and cultures in corn meal agar and HiChrome agar (HiMedia Laboratories, Mumbai, India). Strains of *Candida albicans* (ATCC® 90028™), *Candida*

glabrata (ATCC® 90030™), *Candida krusei* (ATCC® 6258™) and *Candida tropicalis* (ATCC® 750™) were used as quality controls.

Test of virulence

Adherence assay was carried out as per the protocol of Jain *et al.*⁸ Briefly, vaginal epithelial cells (VECs) from three healthy women volunteers with no history of recent illness were collected. Before preparing for the experiment, each sample was Grams stained to confirm the presence of ample, healthy epithelial cells. The samples were then washed with phosphate-buffered saline (pH 7.2), pooled and cell count adjusted to 1×10^5 cells/ml. Similarly, stationary phase cells of *Candida* were selected, washed and adjusted to 10^8 cells/ml. Equal proportions (200 µl) of VECs and test culture were incubated at 37°C for 1 h. The preparation was then washed and the pellet was Grams stained and about 100 epithelial cells observed under oil immersion field. The ratio of VECs to which fungal cells have attached to the total number of cells counted was noted and percentage adherence calculated. The number of yeast cells attached per VEC was also noted and their average calculated.

Virulence assays for plasma coagulase, phospholipase (Pz), protease (Prz), and hemolysin (Hz) production were carried out as per the methods of Deorukhkar and Saini.⁷ Assessment of biofilm formation was carried out as per the protocol of Yigit *et al.*⁹ In brief, single colony from SDA of the test sample was inoculated in saline and incubated at 37°C for 24 h. To 5 ml of sabouraud dextrose broth containing 8% glucose 500 µl of the overnight grown culture was added and incubated at 37°C for 48 h with no agitation. Next, the culture broth was gently aspirated out, and the tubes were washed with distilled water and stained with 2% crystal violet for 10 min. A visible, uniform, violet coloration in the form of a film at the bottom wall of the tube was indicative of positive result. For lipase assay, to the phospholipase test plates postincubation, 10 ml of saturated copper sulfate was flooded. Excess solution was drained off after 20 min. Bluish – Green-colored zone formed due to precipitation of insoluble copper salt of fatty acids was considered as positive for the depiction of lipase activity.

Statistical analysis

To minimize experimental error, each of the assays was conducted in duplicate on three separate occasions for every test isolate. Significant adherence differences between various *Candida* species were compared using one-way analysis of variance (ANOVA) and the species which differed was determined by Tukey test, respectively. It has been also tested that the significant effect difference for virulence expression between biofilm-forming isolates and planktonic cells was

determined by the equality of mean differences through Chi-square 2×2 contingency table at 95% confidence interval.

RESULTS

A total of 275 symptomatic cases were sampled, of which 56 *Candida* spp. could be isolated by culture which were then used for further virulence studies. *C. albicans* constituted of 22 (39%) isolates and the rest 34 (61%) non-albicans included 15 *C. tropicalis*, 12 *C. glabrata*, and 7 *C. krusei*. All the isolates expressed adherence as shown in Table 1. The highest percentage adherence was observed in isolates of *C. albicans*, which also showed the maximum number of fungal cell attachment to VECs [Table 2 and Figure 1]. As the box and whiskers plots [Figure 2a and b] show, for majority of

isolates percentage adherence range was 90–95%, with a mean of 89.52%. Numbers of fungal cells attached mostly were from 3 to 5.5. *C. glabrata* and *C. krusei* also showed high adherence mostly ranging between 50% and 95% in both. Fungal cells attached for *C. glabrata* varied from 1 to 4.5, whereas for *C. krusei* the cell count was between 2 and 4.5. *C. tropicalis* showed the widest variation, except for a few outliers ranging between 35% and 95% with least mean percentage adherence at 66.87%. Fungal cell attachment number was between 0.5 and 4. Significant difference was found between various *Candida* spp. for percentage of adherence as analyzed through one-way ANOVA. The significant difference ($P < 0.05$) has been found for *C. tropicalis* isolates by Tukey test.

Apart from adherence, as shown in Figure 3; of the total isolates, 27 (48%) expressed two or more of the six virulence factors tested, 18 (32%) expressed one factor, and 11 (20%) expressed none. Maximum virulence was shown through protease production [Table 1]. A total of 25 (45%) isolates were positive for Prz, of which the highest number of isolates 9 (41%) that produced protease were of *C. albicans*. However, higher percentage 7 (58%) of protease production was seen among the *C. glabrata* isolates. Hemolysin production and biofilm formation were the second most 22 (39%) expressed virulence factors. Hz production was maximally seen in *C. albicans* 9 (41%) and *C. tropicalis* 7 (47%). All isolates

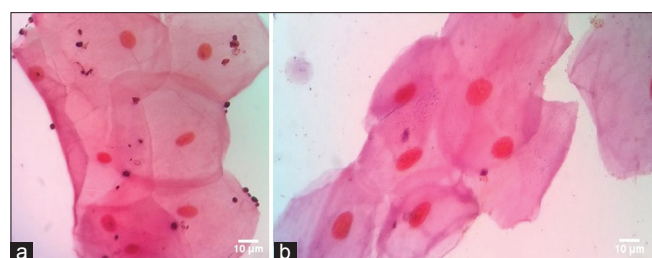


Figure 1: Adherence assay showing fungal cells adhering to vaginal epithelial cells. (a) Positive (b) negative control

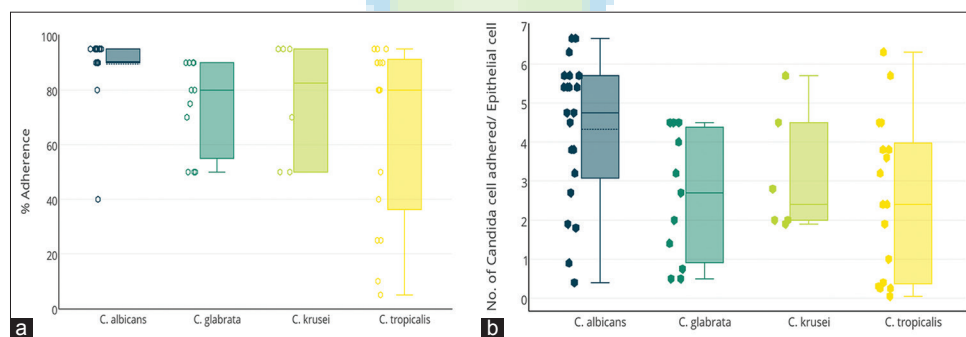


Figure 2: Box and whiskers plot of adherence assay. (a) % adherence, (b) average number of fungal cells adhered per vaginal epithelial cell

Table 1: Virulence expression by total number of isolates

	Total isolates (n=56) (%)	<i>Candida albicans</i> (n=22) (%)	<i>Candida tropicalis</i> (n=15) (%)	<i>Candida glabrata</i> (n=12) (%)	<i>Candida krusei</i> (n=7) (%)
Adherence assay	56 (100)	22 (100)	15 (100)	12 (100)	7 (100)
Phospholipase	10 (18)	3 (14)	3 (20)	2 (17)	2 (29)
Lipase	3 (5)	1 (5)	2 (13)	0	0
Protease	25 (45)	9 (41)	6 (40)	7 (58)	3 (43)
Hemolysin	22 (39)	9 (41)	7 (47)	4 (33)	2 (29)
Biofilm	22 (39)	10 (45)	7 (47)	3 (25)	2 (29)
Coagulase	10 (18)	5 (23)	5 (33)	0	0

Numbers in parentheses represent percentage of isolates expressing the specific factor

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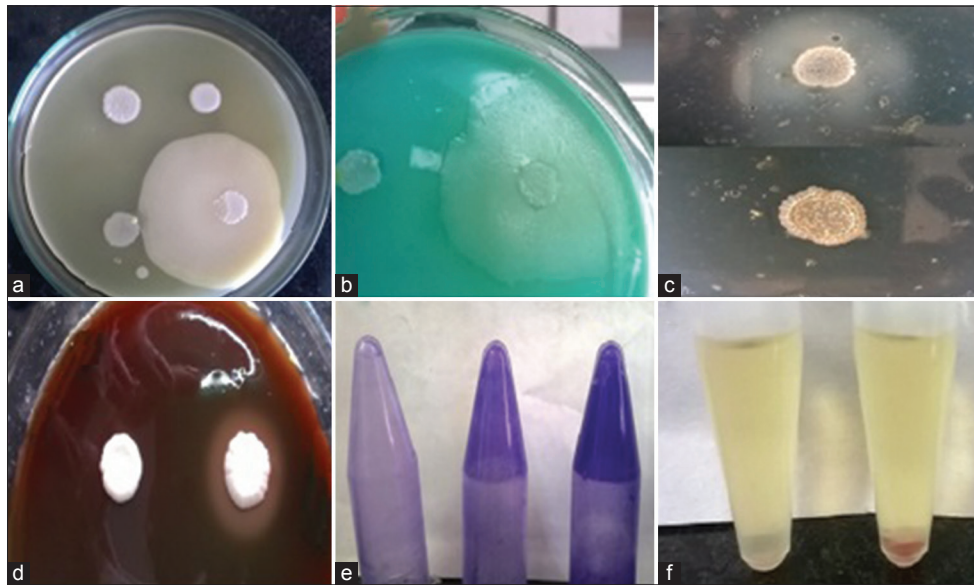


Figure 3: Various virulence factors exhibited; (a) phospholipase activity (positive: Lower right), (b) lipase activity (positive: Right), (c) protease activity (positive: Top), (d) hemolysin activity (positive: Right), (e) biofilm formation (positive: Right and middle), (f) coagulase activity (positive: Right)

Table 2: Expression of adherence factor in isolates

	Mean percentage adherence	Average number of <i>Candida</i> cells attached per VEC
<i>Candida albicans</i> (n=22)	89.52	4.32
<i>Candida glabrata</i> (n=12)	74.09	2.60
<i>Candida krusei</i> (n=7)	75.83	3.15
<i>Candida tropicalis</i> (n=15)*	66.87	2.61

*Significant difference found between various *Candida* spp. for percentage of adherence as analyzed through one-way ANOVA, Tukey test showed significant difference ($P<0.05$) for isolates of *Candida tropicalis*. VECs = Vaginal epithelial cells; ANOVA = Analysis of variance

that produced hemolysin were alpha hemolytic. Biofilm was formed mostly 10 (45%) by *C. albicans* isolates while among the non-albicans *Candida* species highest number of biofilm forming isolates were observed for *C. tropicalis* 7 (47%). However only a few of *C. glabrata* 3 (25%) and *C. krusei* 2 (29%) isolates were biofilm formers. Coagulase, phospholipase, and lipase productions were in fewer isolates, and no particular subspecies stood out as majority. Of the ten isolates that produced coagulase, four (three of *C. albicans*, one of *C. tropicalis*) were seen to give a positive result within 2 h of incubation, the other six were positive at 24 h of incubation.

Expression of virulence factors was compared between biofilm-forming isolates and those that did not form biofilm (planktonic cells) [Table 3]. Significant difference as calculated through Chi-square 2×2 contingency table was seen for plasma coagulase production. Nine (41%) biofilm producers produced coagulase as compared to 1 (3%) of planktonics. Significant difference was also seen for hemolysin

production, where 13 (59%) and 9 (26%) of biofilm producers and planktonics, respectively, produced hemolysin. On the other hand, protease production significantly reduced in biofilm producers 7 (32%), when compared to planktonics 18 (53%). Phospholipase and lipase production were comparable in both groups, and no significant difference was observed between biofilm producers and planktonic isolates both for percentage adherence to VECs and for fungal cell attachment number.

DISCUSSION

Upon entering a mammalian host, fungal cells change their nutritional requirement from saprophytic to parasitic. The success of infection depends on the cell's ability to adapt to the changing microenvironment. With environment changes such as elevated temperature and acidic pH, *Candida* starts to manifest virulence factors. Adhesion is the first step to infection in a host which helps the pathogen to avoid being washed away by mucosal secretions. Adhesins present on the surface of the pathogen helps in the step. It starts with nonspecific interactions such as van der Waals, Brownian movement, and hydrophobic and ionic interactions, and later becomes more permanent with receptor-ligand interactions.¹⁰ In our study, all the isolates showed adhesion property. *C. albicans*, known to be the most virulent species among *Candida*, showed highest percentage adherence as well as highest number of fungal cell attachment. Contrary to related studies,¹¹ percentage adherence and fungal cell attachment number were also high for *C. glabrata* and *C. krusei*. However, *C. tropicalis* showed

Table 3: Expression of virulence factors in biofilm forming and planktonic isolates

	Adherence assay		Phospholipase	Lipase	Protease	Hemolysin	Coagulase
	Mean percentage adherence	Mean cell attachment/VEC					
Biofilm formers (n=22)	74.88	3.19	4 (18)	1 (5)	7 (32)	13 (59)*	9 (41)*
Planktonic cells (n=34)	77.06	3.14	6 (18)	2 (6)	18 (53)*	9 (26)	1 (3)

*Significant effect found difference between biofilm formers and planktonic cells at 95% CI by the equality of mean differences through Chi-square 2×2 contingency table. CI = Confidence interval; VEC = Vaginal epithelial cell

the widest variation both in percentage adherence (35–95%) and cell attachment number (0.5–4) which was significantly different ($P < 0.05$) when compared with other *Candida* spp isolates. Adherence depends on several factors including the profile of cell wall proteins and cell surface physicochemical properties. What caused less number of *C. tropicalis* strains to show adherence needs further molecular studies.

The diversity in expression of various extracellular enzymes leads to exaggerated synergistic effect showing better adaptability of the fungi to its new found environment. In our study, we found 48% of the isolates exhibited two or more of the virulence factors, some strains exhibiting up to five of the six factors tested. An interesting finding was the expression of none of the other virulence factors in 20% of the isolates in spite of showing adherence. The isolates mostly were of *C. tropicalis* and *C. albicans* and plausible explanation for this may be that they were mere bystanders or fresh colonizers yet to manifest virulence. Although literature¹² suggests the appearance of lesion in the subject and presence of ample yeast forms on microscopic examination with 10% KOH are sufficient to confirm pathogenesis, it was not found applicable to the present study. Of the other virulence factors, the one found maximally was production of protease, an important enzyme having major role in adhesion and tissue penetration as also was found in a study by Kvaal *et al.*,¹³ next most abundant were hemolysin-producing and biofilm forming isolates. Hemolysin production was found in all the *Candida* species and all isolates produced alpha hemolysis, as was also observed in a study by Luo *et al.*¹⁴

Plasma coagulase produced by *Candida* species acts by converting prothrombin to thrombin by proteolysis. This in turn activates protease which converts fibrinogen to fibrin. Studies on virulence mechanism of *Candida* through coagulase activity are limited.⁷ However, studies show that in *Staphylococcus aureus* the fibrin clot coats the pathogen and protects it from phagocytosis and other host immune responses. In this study, its production was seen in *C. albicans* and *C. tropicalis* in 5 (23%) and 5 (33%) isolates, respectively, but not in isolates of *C. krusei* or *C. glabrata*, which was consistent with findings from other studies.¹⁵

Biofilm ecosystems have been reported to be more virulent than free-floating planktonic cells.¹⁶ Biofilm formation aids

in evasion from attack by the host immune factors, resulting in increased attachment, and invasion into tissues. Tissue invasion in turn heightens virulence and resistance to drugs. Several recent studies have reported increased production of phospholipase, protease, and adhesion in biofilm as compared to planktonic cells.^{16,17} This study however found no difference between phospholipase production and cell adhesion, whereas protease production was seen in reduced number of biofilm producing isolates than in planktonic isolates. On the other hand, significantly higher number of biofilm producers produced coagulase or hemolysin compared to planktonic cells.

CONCLUSIONS

Adhesion the first step to fungal infection in host was expressed in all isolates, however highest number was observed for *Candida albicans*, where as significantly lower number of *C. tropicalis* isolates showed mean percentage adherence as well as mean number of fungal cell attachment per vaginal epithelial cell. *C. albicans* strain numbered highest for protease, haemolysin, coagulase expression and biofilm formation. The study also showed significantly higher number of biofilm forming isolates produced haemolysin and coagulase in comparison to planktonic isolates, however biofilm forming isolates were significantly lower producers of protease in comparison to the planktonic isolates. In conclusion, all the strains included in the study expressed one or more virulence factor. *C. albicans* strains were the highest in number for expressing virulence factors. Similarly, a comparison between biofilm forming isolates and planktonic isolates showed biofilm forming isolates to be more virulent.

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Conflicts of interest

There are no conflicts of interest.

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