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Research Article

Biofilm formation among *Candida albicans* isolated from vagina

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Abstract

Purpose: Study was conducted in a rural tertiary care hospital with a purpose to demonstrate the biofilm forming abilities of *C. albicans* isolated from cases of vulvovaginal candidiasis and asymptomatic carriers.

Material and Methods: *C. albicans* was isolated and identified by standard laboratory techniques. Biofilm formation in vitro was tested using the 96 well microtitre plate method with crystal violet staining.

Results: Overall rate of Candida isolation in study subjects was 40%. Candida isolation in cases was 56% and that of controls was 24%. *C. albicans* was the predominant species in cases and controls (71.45% and 91.67%). Biofilm formation was seen in 40% versus 27.27% in cases and controls.

Conclusion: *C. albicans* isolated from cases and controls demonstrated similar biofilm forming abilities. Other virulence factors may be more significant than biofilm formation in pathogenesis of VVC. Further studies are needed to investigate the issue on larger sample size.

Keywords: Candida albicans, Biofilm formation, Vulvovaginal candidiasis

1. Introduction

In women of reproductive age group, *Candida albicans* can be present as colonizer in (17% to 30%) or is the leading cause of vulvovaginal candidiasis (VVC) in (85-90%)[1] of cases which will affect 75% of all women at least once in their lifetime and about 5-8% of women experience recurrent VVC[2]. Among many cases of vaginitis, VVC is the second most common after bacterial vaginosis and diagnosed in up to 40% of women with vaginal complaints in primary care setting. In United States only, vaginitis related health care costs are estimated at $1.8 billion annually.[3]

Therefore, it is important to understand fungal factors that contribute to its immunopathology. Several properties of *candida albicans* have been proposed to play major roles in causing disease and include morphogenesis, secreted aspartyl Proteinases (SAPs) and biofilm formation.[1] These traits may facilitate asymptomatic colonization or symptomatic infection[1]. *Candida albicans* secreted aspartyl proteinases(SAP) has been extensively reviewed.[4] and showed that *candida albicans* isolated from symptomatic patients with vaginal candidiasis are significantly more proteolytic than isolates from asymptomatic vaginal carriers.[4]

Candida albicans has ability to form biofilms on biotic and abiotic surfaces. Biofilms confer properties of increased adhesion, recalcitrance to clearance by the host immune system and enhanced antimicrobial resistance[5]. Studies have demonstrated wide variations in biofilm formation among *candida albicans* causing invasive and non invasive infections and among natural populations isolated from oral cavity, environment and vagina of candidiasis patients.[6,7] Therefore, this study was undertaken to investigate in vitro biofilm forming abilities of *Candida albicans* isolated from patients of VVC(cases) and asymptomatic carriers(controls).

2. Material and Methods

The present study was conducted in the Department of Microbiology and Obstetrics and Gynecology at Mahatma Gandhi Institute of Medical Sciences, Sewagram, Wardha, Maharashtra, a rural tertiary care hospital in central India in May and June 2014. The study was commenced after the approval of Institutional Ethical Committee. Informed consent of the patients was taken in local language in the prescribed format, before registering them in the study.

2.1 Study Subjects

Women attending Gynaecology and Obstetrics outpatient unit of Kasturba Hospital of Mahatma Gandhi Institute of Medical Sciences, Sewagram

2.2 Study population

Women with clinically diagnosed vulvovaginal candidiasis were registered as cases and healthy women not having vulvovaginal candidiasis were consequently registered as controls in the study. A total of 100 subjects were included (cases: 50 and controls: 50). Inclusion criteria for cases of VVC were married women of all age groups, attending Gynaecology clinic with complaints of white discharge per vaginum, clinically on per speculum examination presence of curdy white discharge and women consenting for vaginal swab and for controls criteria were women not complaining of white discharge per vaginum and not having clinical VVC but attending gynaecological outpatient unit for any other complaint such as routine antenatal care. Women with clinically diagnosed vulvovaginal candidiasis on antifungal treatment were excluded.

2.3 Specimen collection

Under all aseptic precautions, with the sterile cotton swab stick specimen from vaginal canal was collected in a sterile container and immediately transported to the laboratory. If there was delay in transportation, specimen was refrigerated at 4^0 C. Sample preparation: The cotton end of each swab was inserted into 0.5 ml of sterile water in test tube and mixed vigorously for 30 sec and following procedures were performed. Gram stain of sample was examined under microscope. For the isolation of candida, specimen was inoculated on SDA with antibiotics and was incubated at 37^0C for 48 hours. *Candida albicans* was identified by standard laboratory techniques and in vitro biofilm formation was detected using microtitre plate method.[7]

2.4 Preparation of inoculum and biofilm formation process

All strains were first streaked on yeast extract peptone dextrose (YEPD) agar and incubated at 37 0 C for 48 hrs. For each strain a large loop of actively growing cells was transferred to sterile yeast nitrogen base broth. After incubation at 37^0 C for 24 hrs, the cells were centrifuged and washed twice with PBS by vortexing and centrifuging at 5000g for 5 min. The washed cells were then re suspended in 1ml YNB broth. These cells suspension were used to grow biofilm. For each strain 100 ul of suspension was inoculated in to individual wells of 96 well flat bottom microtitre plates. Three repeats were performed. YNB broth containing no inoculums was used as negative control. The plates were incubated at 37^0 C for 90 min. Supernatant was discarded and wells were gently washed twice with PBS. For biofilm growth 100 ul of fresh YNB broth was added to each well. The plates were covered, wrapped with parafilm and incubated at 37^0 C for 48 hrs. After that washing with PBS buffer was done. 100 ul of 1% crystal violet (CV) was added to each well and incubated at 37^0C for 20 min. Then 150 ul of 95% ethanol was added and 100 ul of each mixture was transferred to new micro titre plate. The absorbance for each well was determined using microplate reader at A570. Similarly, wells with only YNB broth but no microbe were used as negative controls. Positive control strain of Staphylococcus epidermidis ATCC 35984 was also included. Cut off Optical Density (ODc) values were calculated by following formula. ODc = Average OD of negative control + 3 x S.D. of negative control and biofilm formation was categorized as <2x = Negative/weak, 2x to 4x = Moderate and >4x= strong[8]. Data Analysis: The results were entered into excel sheet and was analysed using SPSS software.

3. Results

All the women in the study were married with the mean age of 28 .2 years. The most common age group was 26 to 30 years (71 %) in both cases and controls. The pregnant population in the study was 32 (32 %) with 19 (44%) in the control group and 13 (26 %) in the cases. The total positivity of Gram stain was 37(37 %) in the study population.

Table 1: Culture positivity of Candida in study population

Culture	Cases (%)	Controls (%)	Total
Positive	28(56.00%)	12(24.00%)	40
Negative	22(44.00%)	38(76.00%)	60
Total	50(100.00%)	50(100.00%)	100

The overall rate of culture positivity was 40/100(40.00%). The rate of isolation of Candida from cases was 28(56%) and that of controls was 12 (24%) (Table1). Predominantly, *Candida albicans* 20(71.43%) was isolated in cases and 11(91.67%) in controls. Further biofilm formation was studied in these *candida albicans* strains.

Table 2: Biofilm formation among *Candida albicans* strains

Biofilm formation	Isolated from cases	Isolated from control	Total
Positive	08(40.00%)	03(27.27%)	11(35.48%)
Negative	12(60.00%)	08(72.73%)	20(64.52%)
Total	20(100.00%)	11(100.00%)	31(100.00%)

The rate of biofilm formation among the *candida albicans* isolated from cases was 40.00% as against 27.27% in controls (Table 2). The difference however, was not statistically significant (p value >0.05).

Table 3: Details of biofilm forming *C. albicans* strains

Strain No	Mean+ S.D.	Subject	Remark
2	1.22	case	Moderate
9	1.25	case	Moderate
15	3.49	case	Strong
17	3.77	control	Strong
27	1.10	case	Moderate
30	0.730	case	Moderate
31	0.900	case	Moderate
33	2.13	case	Strong
34	5.13	control	Strong
35	8.13	control	Strong
36	11.13	case	Strong

Optical Density of cut off (ODc): = 0.360, 2x= 0.720, 4x= 1.440

Biofilm forming abilities of *C. albicans* varies from 0.730 to 11.13 in cases of VVC and 3.77 to 8.13 in case of asymptomatic carriers. Though the *C. albicans* strains are moderate to strong biofilm producers, assay does not revealed significant differences in biofilm formation abilities among two groups of *C. albicans*(p>0.05).

Table 4: Clinical characteristics of the study subjects from whom biofilm producing *candida albicans* was isolated

Parameter	Cases								Controls		
Patient Id no	73	93	6	70	4	5	32	10	38	33	34
Age	25	27	25		30	29	36	27	20	25	29
Socioeconomic status	M	M	M	M	M	M	M	M	M	M	M
Pregnancy	Y	N	N	N	N	Y	N	Y	Y	Y	N
Gravida	1	N	N	N	N	2	N	N	1	N	N
Married	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Gestational age	22	N	N	N	N	33	N	N	37	N	N
Preterm pain	Y	N	N	N	N	N	N	N	N	N	N
PROM	N	N	N	N	N	N	N	N	N	N	N
DM	N	N	N	N	N	N	N	N	N	N	N
Anaemia	Y	Y	Y	N	N	Y	N	Y	N	N	Y
SCD	N	N	N	N	N	N	N	N	N	N	N
UTI	Y	N	N	N	N	Y	N	N	N	N	N
PID	N	N	N	N	N	N	Y	N	N	N	N
H/O Vaginitis	N	N	N	N	Y	N	N	N	N	N	N
H/O antibiotic	N	N	N	N	N	N	N	N	N	N	N
H/O antifungaL	N	N	N	N	N	N	N	N	N	N	N
H/O steroids	N	N	N	N	N	N	N	N	N	N	N
HIV	N	N	N	N	N	N	N	N	N	N	N
Neutropenia	N	N	N	N	N	N	N	N	N	N	N
KOH/Gram	+	+	+	+	+	+	+	+	-	-	-
culture	+	+	+	+	+	+	+	+	+	+	+
Sporadic/Re	s	s	s	s	s	s	s	s	s	s	s

Y= Yes, N= No, S= Sporadic, Re= recurrent, PID= pelvic inflammatory disease, UTI= urinary tract infection, M- Middle income status; SCD= sickle cell disease, DM= diabetes mellitus, PROM= premature rupture of membrane

All the women showing isolation of *candida albicans* with biofilm formation was married, sexually active, in the age group of 26- 30 years and belonged to the middle income group. All biofilm forming *candida albicans* isolates showed positivity on Gram staining whereas none of these isolates from control group showed Gram stain positivity. Anaemia was found in 5 (62.55 %) in cases in 1 (33.33%) of the controls. No other predisposing factor studied showed positive correlation in biofilm forming *candida albicans* isolates (Table4).

4. Discussion

Candida albicans an opportunistic polymorphic fungus and resident of normal vaginal microbiota, is the leading causative agent of vulvovaginal candidiasis and presents major quality of life issues for women worldwide[3]. Our study has reported Gram stain positivity of 37% which is higher than other studies; wherein 9.05% to 27.84% positivity has been reported[9]. The rate of colonization with *Candida albicans* in asymptomatic women was 24% and is comparable with studies of Lelic *et al*[10] and Paulo Giraldo *et al*[11]. Candida culture positivity in cases was 56% and the predominant species was *C. albicans* ((71.43% in cases and 91.67% in controls). Our study reported higher rates of Candida isolation in VVC than other studies in India[12,13] and other country[14]. Our results are comparable with a study from Kenya where candidiasis on culture was found to be 42.7%. * *Candida albicans* as a predominant species was also reported by these studies. However S. Mohanty *et al*[13] have reported C. glabrata as most common (50.4%) followed by *C. albicans* (35.1%) and Varsha Kumari *et al*[9] had reported 45.07% C. parapsilosis, 32.40% *C. albicans* and 22.53% C. glabrata.

Virulence factors are expressed by *C. albicans* on mucosal surfaces and have been shown to play a role in infection[15]. These include ability to evade host defences, adherence, biofilm formation on host tissues and medical devices and the production of tissue damaging hydrolytic enzymes such as proteases, phospholipases and haemolysins. In our study, overall rate of biofilm formation among *C. albicans* was 35.48%. 40% of *C. albicans* isolated from VVC and 27.27% from controls were biofilm producers. However, this finding was not statistically significant (p> 0.05). Our results are comparable to study from north India where in authors reported 11/23(47.82%) biofilm formation among *C. albicans* isolated from cases of VVC[9]. Data on candidal biofilm formation obtained by comparison of a large group of isolates from healthy and HIV infected individuals could not demonstrate significant differences in biofilm forming abilities between the isolates of recovered from HIV infected individuals and those recovered from HIV free individuals[15].

Our study results also reported no significant differences in biofilm forming abilities of *C. albicans* between the isolates from cases of VVC and asymptomatic carriers. In a study on biofilm formation in Candida strains isolated from urine, authors concluded that biofilm forming Candida isolates are stable but has highly variable characteristic of individual Candida strains that does not appear to be associated with specific condition or characteristic in the host[16].

A study by Leighann Sherry *et al*[17] also noted that biofilm formation is variable among *C. albicans* isolates. Jong Hee Shin *et al*[6] study found no significant differences in biofilm formation by bloodstream isolates of *C. albicans* than those obtained from other sites. In contrast biofilm positivity for non albicans candida obtained from bloodstream was significantly higher than of isolates from other sites. Our study results have not found significant association between biofilm formations by *C. albicans* with clinical characteristics. The study "*candida albicans* forms biofilm on the vaginal mucosa" [19] had demonstrated the biofilm formation on vaginal mucosa in vivo and ex vivo in murine mouse models and had raised an important question, does the biofilm play a role in the host response and / or pathogenesis of disease. Therefore, the results of our study strengthens the evidence by earlier study that *C. albicans* possess mechanisms other than biofilm production to establish infection and perhaps other virulence factors are more important for the pathogenicity of *C. albicans*. Pathogenesis of clinical VVC may be dependent on the more significant factors like secretion of proteinases which act by cleaving proteins on host epithelium resulting in mucosal structural damage and enhanced fungal burden and are significantly higher in cases of *C. albicans* isolated from the VVC than in asymptomatic carriers[3]. Further studies comparing the biofilm formation and other virulence markers are needed in cases and controls to investigate the issue in larger sample size.

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