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Comparative analysis of virulence factors & biotypes of *Gardnerella vaginalis* isolated from the genital tract of women with & without bacterial vaginosis

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***Background & objectives*: Bacterial vaginosis (BV) involves the presence of a thick vaginal multispecies biofilm, where *Gardnerella vaginalis* is the predominant species. The reason for an increase in the number of *G. vaginalis* which are usually present as normal flora of the female genital tract in cases of BV, is not known. Hence, the objective of the present study was to compare the biotypes and virulence factors of *G. vaginalis* isolated from the genital tract of women with and without BV.**

***Methods*: High vaginal swabs collected from 811 women of reproductive age were cultured. *G. vaginalis* isolates were biotyped and tested for adherence to vaginal epithelial cells, biofilm formation, agglutination of human red blood cells (RBCs), protease production, phospholipase production and surface hydrophobicity.**

***Results*: Of the isolates from women with BV, 83.3 per cent (60/72) showed good adherence, 78.4 per cent (58/74) produced biofilm, 82.9 per cent (63/76) produced phospholipase, 67.1 per cent (51/76) produced protease, 77.3 per cent (58/75) were positive for surface hydrophobicity and 61.6 per cent (45/73) were positive for haemagglutination of human RBC. In case of *G. vaginalis* from non-BV women, 25 per cent (15/60) isolates showed good adherence, 18.4 per cent (9/49) biofilm production, 35 per cent (21/60) phospholipase, 36.6 per cent (22/60) protease, 41.7 per cent (25/60) surface hydrophobicity and 10.1 per cent (6/59) agglutination of human RBCs. Maximum number of isolates belonged to biotypes 6, 2 and 3. Biotype 3 was more associated with non-BV rather than BV; biotype 6, 2 and 1 were more associated with cases of BV. Maximum virulence factors were expressed by biotypes 6, 2 and 1.**

***Interpretation & conclusions*: Virulence factors were more expressed by *G. vaginalis* isolates obtained from women with BV rather than from non-BV. Biotypes 6, 2 and 1 were more associated with cases of BV and expressed maximum virulence factors.**

Key words Adherence - bacterial vaginosis - biofilm - biotypes - *Gardnerella vaginalis* - haemagglutination - surface hydrophobicity

Bacterial vaginosis (BV) is the most common genital tract infection in women of reproductive age group. BV is characterized by a marked reduction in counts of lactobacilli which is frequently present as normal flora of the healthy vagina and an increase in numbers of anaerobic bacteria, including *Gardnerella*

vaginalis, *Atopobium vaginae*, *Mobiluncus* spp., *Bacteroides* spp., *Prevotella* spp. and various other anaerobes[1-3]. Being polymicrobial in nature, BV aetiology remains unclear. However, BV involves the presence of a thick vaginal multispecies biofilm, where *G. vaginalis* is the predominant species. Similar to what happens in many other biofilm-related infections, standard antibiotics like metronidazole are unable to fully eradicate the vaginal biofilm, which can explain the high recurrence rates of BV. BV is associated with a variety of obstetric and gynaecological complications such as preterm birth, low birth weight, postpartum endometritis and pelvic inflammatory disease. The factors which are associated with pathophysiology of BV are not completely known[1-5].

It is a known fact that the presence of virulence factors is one of the important determinants of the pathogenic potential of the organism. The various virulence factors of *G. vaginalis* are pili, microcapsule, surface hydrophobicity, adherence, vaginolysin, phospholipase C, protease and siderophores, sialidases and prolidases[6,7]. The reason for an increase in the number of *G. vaginalis* which are usually present as normal flora of the female genital tract in cases of BV is not known. In the present study, the virulence factors and biotypes of *G. vaginalis* isolated from the genital tract of women with and without BV were compared.

Material & Methods

This cross-sectional study was conducted in the department of Microbiology, Father Muller Medical College (FMMC), Mangaluru, India from January 2014 to December 2016. All consecutive women of 15-45 yr of age with complaints of vaginal discharge were included in the study. Women who were menstruating at the time of the specimen collection or were on medication for any bacterial, fungal, parasitic or viral infections for up to one month before the specimen collection were excluded. The study was approved by the Institutional Ethics Committee of FMMCH, Mangaluru, and written informed consents were obtained from the patients.

A detailed clinical history of each woman was taken, and their vaginal swabs were collected into sterile tube containing 0.5 ml normal saline and immediately transported to the microbiology laboratory. The vaginal swabs were subjected to wet mount preparation, Gram staining, *p*H determination and whiff test. BV cases were identified using Amsel's and Nugent's criteria[7-9]. Sample was inoculated onto human blood bilayer Tween 80 agar (Columbia blood agar base with 0.0075% Tween 80) with *G. vaginalis* selective supplement containing gentamicin sulphate 2 mg, nalidixic acid 15 mg, amphotericin B 1 mg (HiMedia Laboratories, Pvt. Ltd., Mumbai, India) and chocolate agar. The culture plates were incubated at 37°C for 48 h in candle jar which provided 5-10 per cent CO_2. Colonies of *G. vaginalis* appeared small, smooth, round and β-haemolytic. *G. vaginalis* was identified as Gram-variable coccobacilli by Gram stain, catalase and oxidase negative, fermented glucose, maltose and starch and did not ferment mannitol, lactose, xylose and sucrose variable, α glucosidase positive and β glucosidase negative[10,11]. The isolates were also tested for various virulence factors such as adherence to vaginal epithelial cells, biofilm formation, surface hydrophobicity, haemagglutination, phospholipase and protease production. All the tests were performed in triplicate. *G. vaginalis* ATCC 14018 was included as control with each test.

Evaluation of virulence factors: Vaginal swabs were collected from healthy asymptomatic women in the reproductive age group, and the vaginal discharge was eluted into 2 ml of 0.85 per cent sterile saline. The adherence of the isolates to these vaginal epithelial cells was determined[12]. An average of >10 adherent bacilli/cell was considered as good degree of adherence[7,12]. Haemagglutination assay was performed according to Scott *et al*[13]. The suspension giving a carpet of erythrocytes over the bottom of the well was considered as positive. The same test was done using 2 per cent human, sheep and chick red blood cells (RBCs)[13]. Biofilm formation in microtiter plates was determined as done in previous studies[14,15]. The biofilm formation was graded as, optical density (OD) <0.1 as weak or non-biofilm producers, OD 0.1-0.2 as moderate and an OD >0.2 as good biofilm producer. *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control[14,15]. For protease production *G. vaginalis* isolates were inoculated into skim milk agar with 5 per cent horse serum and incubated in the candle jar (5-10% CO_2) at 37°C for 48 h. Clear zone around the growth indicated protease enzyme activity[16]. Brain heart infusion (BHI) agar supplemented with 1 per cent gelatin egg yolk was used for phospholipase production. *G. vaginalis* isolates were inoculated and incubated in the candle jar (5-10% CO_2) at 37°C for 48 h. Pearly layer was seen around growth in lipase-positive organism. *P. aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (25923) were used as

negative reference strains[17]. Surface hydrophobicity of the isolates was determined as given in the previous studies[6,18]. Surface hydrophobicity index ≤20 was graded as low and >20 as high. Biotyping was done using three tests - hippurate hydrolysis, phospholipase and O-Nitrophenyl β-D-galactopyranoside (ONPG) as described previously[7,19].

Statistical analysis: Statistical analysis was done using SPSS version 16 (IBM, Chicago, USA). The various biotypes and virulence factors of *G. vaginalis* from women with or without BV were compared using Chi-square test.

Results & Discussion

A total of 176 isolates of *G. vaginalis* were obtained from 811 women with abnormal vaginal discharge. According to Nugent's criteria, vaginal samples were classified into three groups. Group A consisted of 75 women with Nugent score 4-6 (not consistent with BV by Amsel's criteria). These were considered as non-BV cases. Group B included 89 women (Nugent score ≥7 and BV case by Amsel). These were considered as BV cases. Group C included 12 women with Nugent score 0-3 (non-BV case by Amsel) having other vaginal infections such as vulvovaginitis (n=6), cervicitis (n=4) and HIV reactive (n=2). These were also considered as non-BV cases. There were 23 women with *Candida* in group A, 49 in group B, and eight in group C. As group C has only 12 women, all comparisons were made between groups A (non-BV) and B (BV). Table I shows the virulence factors expressed by the three groups.

A study proposed a conceptual model to explain the pathogenesis of BV[20]. In our study most isolates of *G. vaginalis* obtained from BV cases exhibited various virulence factors such as adherence to vaginal epithelial cells, biofilm formation, phospholipase C production, protease production, surface hydrophobicity and haemagglutination rather than the non-BV cases (Table I).

According to a previous study, *G. vaginalis* was the first isolate to adhere to the vaginal epithelial cells causing the scaffolding to which other organism adhere[21].

Table I. Virulence factors expressed by *Gardnerella vaginalis* isolated from the genital tract of women with and without bacterial vaginosis (BV)

Virulence factors	Number of isolates tested	Number tested in each group	Results	Group A n (%)	Group B n (%)	Group C n (%)	*P* (A vs. B)
Adherence to vaginal epithelial cells	141	A - 60 B - 72 C - 9	Good	15 (25)	60 (83.3)	5 (55.5)	<0.001
			Poor	45 (75)	12 (16.6)	4 (44.4)	
Biofilm formation	133	A - 49 B - 74 C - 10	Moderate or good	9 (18.4)	58 (78.4)	6 (60)	<0.001
			Poor	40 (81.6)	16 (21.6)	4 (40)	
Phospholipase C production	145	A - 60 B - 76 C - 9	Positive	21 (35)	63 (82.9)	5 (55.5)	<0.001
			Negative	39 (65)	13 (17.1)	4 (44.4)	
Protease production	145	A - 60 B - 76 C - 9	Positive	22 (36.6)	51 (67.1)	3 (33.3)	<0.01
			Negative	38 (3.33)	25 (32.9)	6 (66.6)	
Surface hydrophobicity	145	A - 60 B - 75 C - 10	High	25 (41.7)	58 (77.3)	5 (50.0)	<0.01
			Low	35 (58.3)	17 (22.67)	5 (50.0)	
Haemagglutination with human RBC	141	A - 59 B - 73 C - 9	Positive	6 (10.1)	45 (61.64)	4 (44.4)	<0.001
			Negative	53 (89.8)	28 (38.35)	5 (55.5)	
Haemagglutination with sheep RBC	141	A - 59 B - 73 C - 9	Positive	9 (15.2)	34 (46.5)	2 (22.2)	<0.01
			Negative	50 (84.7)	39 (53.4)	7 (77.7)	
Haemagglutination with chick RBC	141	A - 59 B - 73 C - 9	Positive	3 (5.1)	27 (36.9)	-	<0.001
			Negative	57 (94.9)	46 (63.0)	9 (100)	

RBC, red blood cell

However, another study reported that adherence of *G. vaginalis* was inhibited by the lactobacilli and adherent lactobacilli were displaced by *G. vaginalis*[22]. Other groups studied the influence of biofilm formation by *G. vaginalis* and other anaerobes from the time of their initial adhesion until biofilm formation in BV cases. It was reported that the synergistic interaction occurred between *G. vaginalis* and anaerobes from commensal status till biofilm formation[23,24]. *G. vaginalis* was shown to have the highest virulence potential due to higher initial adhesion, cytotoxicity and the ability to form a biofilm[25].

Past studies have reported difference in genotype and *in vitro* expression of virulence factors between BV and non-BV isolates of *G. vaginalis*[26,27]. The BV-associated isolate encoded a different variant of a biofilm-associated protein gene and demonstrated greater adherence, aggregation and biofilm formation than that isolated from healthy vagina[26]. In our study also BV-associated isolates showed better adherence, biofilm formation, haemagglutination, phospholipase and protease production in comparison to non-BV isolates. In our study, maximum number of isolates belonged to biotypes 6, 2 and 3. Biotype 3 was more associated with non-BV rather than BV (Table II); biotypes 6, 2 and 1 were more associated with cases of BV. Maximum virulence factors were expressed by biotypes 6, 2 and 1 (Table III).

Our earlier study showed no relationship between biotypes and virulence factors or biotypes from BV and non-BV cases[7]. Another study showed that phospholipase-producing biotypes 1, 2, 3 and 4 were frequently associated with cases of BV rather than non-BV and also the patient acquired a different biotype after treatment[27]. In a longitudinal study on biotypes of *G. vaginalis* biotypes 2, 3 and 7 were more frequently isolated from cases of BV and biotype 7 was more isolated from non-BV cases[27]. In our study biotypes 6, 2 and 1 showed association with various virulence factors.

In conclusion, majority of *G. vaginalis* isolates from cases of BV exhibited more number of virulence factors than those from healthy women. Biotype 3 was more prevalent in non-BV cases while biotypes 6, 2 and 1 were associated with cases of BV and expressed maximum virulence factors.

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Table II. Different biotypes of *G. vaginalis* isolated from the genital tract of women with and without bacterial vaginosis (BV, n=143)

Biotypes	Group A n (%)	Group B n (%)	Group C n (%)	Total
1	3 (2)	12 (8)*	1 (0.6)	16 (11.1)
2	8 (5.5)	22 (15.3)*	2 (1.3)	32 (22.3)*
3	16 (11.1)*	3 (2)	3 (2)	22 (15.3)*
4	8 (5.5)	3 (2)	1 (0.6)	12 (8.3)
5	1 (0.6)	6 (4.1)	0	7 (4.8)
6	8 (5.5)	23 (16)*	2 (1.3)	33 (23)*
7	4 (2.7)	1 (0.6)	0	5 (3.5)
8	10 (7)	6 (4.1)	0	16 (11.1)
Total				143

*P<0.001, showing association between biotype and respective group

Table III. Association of 143 biotyped isolates with virulence factors of *G. vaginalis* isolated from the genital tract of women with and without bacterial vaginosis (BV)

Biotypes	Good adherence n (%)	Biofilm producers n (%)	Good surface hydrophobicity n (%)	Agglutination of human RBC n (%)	Phospholipase producers n (%)	Protease producers n (%)
1	11 (7.6)	7 (4.8)	13 (9)	6 (4.1)	16 (11.1)	8 (5.5)
2	20 (13.9)	17 (11.8)	19 (13.2)	10 (6.9)	32 (22.3)	21 (14.6)
3	7 (4.8)	4 (2.7)	4 (2.7)	5 (3.5)	0	7 (4.8)
4	7 (4.8)	1 (0.6)	7 (4.8)	5 (3.5)	0	5 (3.5)
5	5 (3.5)	4 (2.7)	5 (3.5)	4 (2.7)	7 (4.8)	4 (2.7)
6	17 (11.8)	19 (13.2)	20 (13.9)	18 (12.5)	33 (23)	18 (12.5)
7	1 (0.6)	1 (0.6)	0	0	0	1 (0.6)
8	8 (5.5)	4 (2.7)	10 (6.9)	3 (2)	0	6 (4.1)
P	0.084	0.033	0.003	0.139	<0.001	0.446

RBC, red blood cell

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***Conflicts of Interest*:** None.

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