ID: JCDR/2012/4115:2306

Original Article

Microbiology Section

Effect of pH on the Adherence, Surface Hydrophobicity and the Biofilm Formation of *Gardnerella Vaginalis*

UDAYALAXMI, GOPALKRISHNA BHAT, SUBBANNAYYA KOTIGADDE, SHASHIDHAR KOTIAN

ABSTRACT

Background: Bacterial vaginosis is a common cause of the abnormal vaginal discharge in women of the reproductive age group. Although bacterial vaginosis is prevalent, not much progress has been made in identifying the factors which are responsible for and those which are associated with bacterial vaginosis and its pathophysiology. Here, we would like to evaluate the effect of the change in the pH of the vagina on the virulence factors of *Gardnerella vaginalis,* the main organism which has been indicated in this vaginal condition.

Objective: The objective of the present study was to observe the effect of pH on the adherence, surface hydrophobicity and the biofilm formation of 10 strains of *Gardnerella vaginalis* which were isolated from cases of bacterial vaginosis.

Result: We found that the adherence to the vaginal epithelial cells and the surface hydrophobicity was maximum at a lower pH (pH- 3,4,5) and minimum at a higher pH (pH- 6,7), but the pH did not have a significant effect on the biofilm formation.

Conclusion: An increase in the pH of the vagina which is observed during bacterial vaginosis, probably occurs much later during the disease. During the early part of the disease, the bacteria adhere to the vaginal epithelium, multiply in large numbers and form a thick biofilm which is not affected by the rise in pH, which occurs probably due to the metabolic activities of this large bacterial population. This also explains the fact that all women with bacterial vaginosis do not have an elevated vaginal pH.

Key Words: Adherence, Bacterial vaginosis, Biofilm, Gardnerella vaginalis, pH, Surface Hydrophobicity

INTRODUCTION

Bacterial vaginosis represents a unique and complex change in the flora of the vagina, which is characterized by a reduction in the prevalence and the number of lactobacilli and an increase in the concentration of *Gardnerella vaginalis* and the resident anaerobic bacteria [1,2]. *Gardnerella vaginalis, Bacteroides* spp, *Peptostreptococcus* spp, *Mobiluncus* spp. *Prevotella* spp. *Porphyromonas* spp., *Atopobium vaginae and Mycoplasma hominis* are the microorganisms which are usually associated with cases of bacterial vaginosis [1,2]. *G.vaginalis* is considered as an indicator organism for bacterial vaginosis because of its regular association with it [3,4]. In a study, most of the isolates of *G.vaginalis* which were isolated from cases of bacterial vaginosis showed good adherence to the vaginal epithelial cells, they formed a biofilm, they showed good surface hydrophobicity and they produced phospholipase C and protease enzymes [5]. Another study confirmed a greater virulence potential of *Gardnerella vaginalis* which was related to that of other bacterial vaginosis associated anaerobes [6].

Although bacterial vaginosis is prevalent, not much progress has been made in identifying the factors which are responsible for and are associated with bacterial vaginosis and its pathophysiology [7]. The normal vaginal ecosystem in mature women is maintained by lactobacilli that secrete lactic acid and hydrogen peroxide. The resulting pH < 4.5 is thought to limit the overgrowth of opportunistic microbes [7, 8]. The lactobacilli maintain their dominance through a combination of acidity, hydrogen peroxide, lactocins, and other bacteriocins to inhibit the growth of other bacteria [8]. In the present study, we intended to learn the effect of pH on the adherence, surface hydrophobicity and the biofilm formation of *G.vaginalis.*

MATERIALS AND METHODS

Vaginal swabs were collected from 10 women who were known cases of bacterial vaginosis. The swabs were inoculated onto human blood bilayer agar with Tween 80 and a *G.vaginalis* selective supplement and the agar plates were incubated at 37ºC for 2 days [9]. *G.vaginalis* was isolated and identified on the basis of the colony morphology, gram staining and standard biochemical reactions [9]. This study had the approval of the institutional ethics committee.

Bacterial strains: This study was conducted on 10 vaginal isolates of *G.vaginalis* which were isolated from cases of bacterial vaginosis. A control strain, *G.vaginalis* ATCC14018 was included in every assay.

Effect of pH on the adherence of G.vaginalis to the vaginal epithelial cells: The adherence of *G.vaginalis* to the vaginal epithelial cells was studied by performing an adherence assay, as has been described previously [10]. Vaginal swabs were collected from healthy volunteer women. The vaginal discharge was eluted into 2ml of 0.85% sterile saline. The vaginal epithelial cells were washed in sterile saline 3 times until the vaginal epithelial cells were washed free of the adherent bacteria. Finally, the cells were suspended in citrate phosphate buffered saline of 5 different pH values (pH 3–pH 7).

The vaginal isolates of *G.vaginalis* were inoculated on human blood agar plates and these were incubated at 37ºC for 48 hours in a candle jar [9]. The colonies were harvested from 3 human blood agar plates by using 0.15 M sterile saline and they were centrifuged at 2500 rpm for 15 minutes. The pellet was resuspended in 0.15 M

sterile saline. This process was repeated 2 times and the pellet was then suspended in citrate phosphate buffered saline of 5 different pH values (pH 3–pH 7). The concentration of the bacterial cells was adjusted to give an OD_{540} of 0.1.

1ml of the standard bacterial suspension was mixed with an equal volume of the standard vaginal epithelial cell suspension and this was incubated at 37ºC in a shaker water bath at a speed of 35 rotations per minute for 45 minutes. The epithelial cells were washed free of the non-adherent bacteria by passing them through a membrane filter of pore size, 8 µm. The membrane filter was carefully removed and inverted over a slide, air dried, alcohol fixed and gram stained. The average number of bacteria which were adherent to 30 cells was counted. We considered an average of > 10 adherent bacilli /cell as moderate adherence [10].

Effect of pH on the surface hydrophobicity of G.vaginalis: A standard bacterial suspension was prepared in citrate phosphate buffered saline of pH 3–7, as has been described above. The concentration of the bacterial cells was adjusted to an OD_{600} of 0.3 (OD initial). To 3ml of the bacterial suspension, 300 µl of ρ-Xylene was added and this was vortexed for 1 minute. The suspension was allowed to stand at 25ºC for 30 minutes. The aqueous and the hydrocarbon phases got separated. The OD_{600} of the aqueous phase was determined (OD final) [10,11]. The hydrophobicity index was calculated by using the following formula [10].

$$\text{Hydrophobicity index} = \frac{\text{OD initial} - \text{OD final}}{\text{OD initial}} \times 100$$

Surface hydrophobicity index ≤ 20 was graded as low and >20 as high [10, 11].

Effect of pH on the biofilm formation of G.vaginalis: A standard bacterial suspension was prepared in citrate phosphate buffered saline of pH 3–7, as has been described above. Then, 200 µl of the suspension was dispensed into microtitre plate wells. The plate was incubated at 37ºC for 18 hours. The microtitre plate wells were washed with 0.85% sterile saline and 100 µl of Bouin's fixative was added. The plate was incubated at room temperature for 10 minutes. The contents were aspirated and washed in 0.85% saline, 20 µl of 1% crystal violet was added to this and it was kept for 1 minute. The plate was vigorously washed with water and air dried. The absorbance was read at 490 nm by using an ELISA reader. All the tests were performed in duplicate. The biofilm formation was graded as, OD of < 0.1 – weak or non-biofilm producers; OD of 0.1–0.2 – moderate and an OD of > 0.2 – high. Sterile saline which was incorporated into one of the wells, served as a blank. *P. aeruginosa* ATCC 27853 was used as a positive control [12,13].

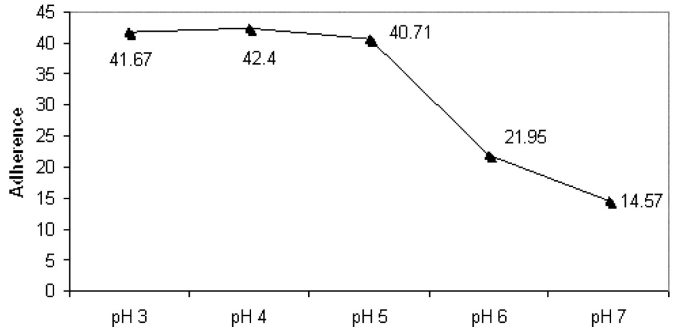
Statistical analysis: The statistical analysis was done by the Kruskal Wallis test. We used SPSS, version 11.5 software for the statistical analysis.

RESULTS AND DISCUSSION

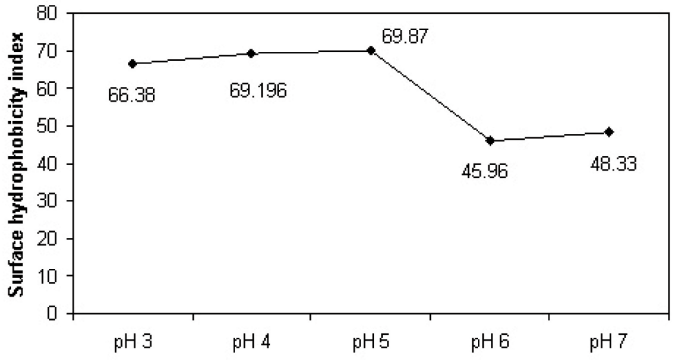
Effect of pH on the adherence of G. vaginalis to the vaginal epithelial cells: The presence of clue cells in the vaginal discharge of the cases of bacterial vaginosis indicated the role of the adherence which was exhibited by *G.vaginalis* in the pathogenesis of bacterial vaginosis. In the present study, the rate of the adherence differed in the different vaginal isolates. All the isolates showed good adherence at an optimum pH of 5. We found that the average adherence was better at a lower pH (pH- 3,4,5) than at a higher pH (pH- 6,7) $P < 0.001$ [Table/Fig-1]. Adherence is not always a property of pathogens, because some studies showed that lactobacilli which are the predominant normal flora in normal women, also exhibited good adherence. The vaginal epithelial cells and the bacteria have a net negative charge that creates an electrostatic repulsive force which is reduced at a lowered pH [14]. It is known that the optimum adherence of *G.vaginalis* to the vaginal epithelial cells is at pH-5.4 and that of lactobacilli is at pH-4.4. At a higher pH (4.8 and 5.4), the lactobacilli do not compete for the binding sites which are occupied by *G.vaginalis* [15].

Effect of pH on the Surface hydrophobicity index: The surface hydrophobicity and adherence are directly proportional to each other. All the isolates showed a good surface hydrophobicity at an optimum pH of 5. We found that the average surface hydrophobicity index increased at a lower pH (pH -3, 4, 5) and that it decreased at higher pH (pH- 6, 7) $P < 0.05$ [Table/Fig-2].

Effect of pH on the biofilm formation: A recent study which investigated the composition and the spatial organization of the bacteria which were associated with the vaginal epithelium in biopsy specimens by using a broad range of fluorescent bacterial group-specific rRNA-targeted oligonucleotide probes, concluded that even though bacterial vaginosis was associated with a variety of bacterial groups, only *G.vaginalis* developed a characteristic adherent biofilm that was specific for bacterial vaginosis [16]. A biofilm which was comprised of confluent *G.vaginalis,* with other bacterial groups being incorporated in the adherent layer was a prominent feature of bacterial vaginosis [15]. In the present study, all the isolates were found to be good biofilm producers at the pH range which was under study. So, a change in the pH did not affect the biofilm formation $p = 0.1$ [Table/Fig-3]. Another study showed that the biofilm type of growth protected G.vaginalis from the lactic acid and the hydrogen peroxide which were produced by lactobacilli [17]. Thus, the biofilm formation is a protective virulence factor of *G.vaginalis.*



[Table/Fig-1]: Effect of pH on adherence (average numbers of bacteria adherent/ vaginal epithelial cell) of *G.vaginalis* to vaginal epithelial cells (A mean of adherence assays performed on 10 isolates) $P < 0.001$



[Table/Fig-2]: Effect of pH on surface hydrophobicity of *G.vaginalis* (Mean of surface hydrophobicity indices of 10 vaginal isolates) $P < 0.05$

Strain No.	[a] Biofilm formation at various pH				
	pH 3	pH 4	pH 5	pH 6	pH 7
1	0.13	0.14	0.17	0.11	0.12
2	0.20	0.22	0.19	0.14	0.16
3	0.19	0.15	0.19	0.13	0.21
4	0.21	0.22	0.23	0.18	0.26
5	0.15	0.20	0.23	0.20	0.21
6	0.13	0.15	0.16	0.12	0.11
7	0.20	0.32	0.30	0.26	0.18
8	0.18	0.20	0.19	0.12	0.19
9	0.15	0.17	0.19	0.16	0.13
10	0.38	0.35	0.24	0.17	0.14
Biofilm (Mean OD_{490} ± SD)	0.19 ± 0.07	0.21 ± 0.07	0.21 ± 0.04	0.16 ± 0.05	0.17 ± 0.05

[Table/Fig.3]: Effect of pH on biofilm formation by 10 G.vaginalis isolates
[a] The ability to produce biofilm is not affected by the change in pH $P = 0.1$.

CONCLUSION

An increase in the pH of the vagina which is observed during bacterial vaginosis probably occurs much later during the disease. During the early part of the disease, the bacteria adhere to the vaginal epithelium, multiply in large numbers and form a thick biofilm which is not affected by the rise in pH, which occurs probably due to the metabolic activities of this large bacterial population. This also explains the fact that all women with bacterial vaginosis do not have an elevated vaginal pH.

ACKNOWLEDGEMENT

The authors are deeply indebted to Manipal University for providing the necessary infrastructure which was required for this study.

REFERENCES

[1] Rein MF. Vulvovaginitis and cervicitis. In *Principles and practice of infectious diseases*. Mandell GL, Bennett JE and Dolin R (eds.). Churchill Livingstone: Harcourt, *Inc. United States of America.* 2000;1218–34.
[2] Sobel JD. Bacterial vaginosis. *Annu Rev Med.* 2000;51:349-56.
[3] Menard JP, Fenollar F, Henry M, Bretelle F, Raoult D. Molecular quantification of the *Gardnerella vaginalis* and the *Atopobium vaginae* loads to predict bacterial vaginosis. *Clin Infect Dis.* 2008;47:33-43.
[4] Demba E, Morison L, Maartem Schim VDL, Awasana AA, Goodling E, Bailey R, et al. Bacterial vaginosis, the vaginal flora patterns and the vaginal hygiene practices in patients who presented with the vaginal discharge syndrome in Gambia, west Africa. *BMC Infect Dis*. 2005;5:12-24.
[5] Udayalaxmi J, Bhat GK, Kotigadde S. The biotypes and the virulence factors of *Gardnerella vaginalis* which was isolated from cases of bacterial vaginosis. *Indian J Med Microbiol*. 2011; 29: 165-68.
[6] Patterson JL, Stull-Lane A, Girerd PH, Jefferson KK. Analysis of the adherence, biofilm formation and the cytotoxicity suggests the greater virulence potential of Gardnerella vaginalis which is relative to that of other bacterial-vaginosis-associated anaerobes. *Microbiol.* 2010;156:392-99.
[7] Hay P. Life in the littoral zone: lactobacilli losing the plot. *Sex Transm Infect.* 2005;81:100-02.
[8] Aroutcheva A, Gariti D, Simon M, Shott S, Faro J, Simoes JA, et al. The defence factors of vaginal lactobacilli. *Am J Obstet Gynaecol*. 2001;185:375-79.
[9] Udayalaxmi, Bhat G, Kotigadde S, Shenoy S. Comparison of the methods of the diagnosis of bacterial vaginosis. *JCDR.* 2011; 5: 498-501.
[10] Pascual LM, Daniele MB, Ruiz F, Giordano W, Pajaro C, Barberis L. *Lactobacillus rhamnosus* L60, a potential probiotic which was isolated from the human vagina. *J Gen Appl Microbiol*. 2008;54:141-48.
[11] Kaushik JK, Kumar A, Duary RK, Mohanty AK, Grover S, Batish VK. The functional and the probiotic attributes of an indigenous isolate of *Lactobacillus plantarum. PLoS One.* 2009; e8099.
[12] Mathur T, Singhal S, Khan S, Upadhayay DJ, Fatma T, Rattan A. Detection of the biofilm formation among the clinical isolates of *Staphylococci*: An evaluation of three different screening methods. *Indian J Med Microbiol.* 2006;24:25-29.
[13] Suman E, Singh S, Kotian S. *Pseudomonas aeruginosa* biofilms in hospital water systems and the effect of a sub-inhibitory concentration of chlorine. *J Hosp Infect.* 2008;70:199-201.
[14] Sobel JD, Schneider J, Kaye D, Levision ME. Adherence of bacteria to the vaginal epithelial cells at various times of the menstrual cycle. *Infect Immun.* 1981;32:194-97.
[15] Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA. Acid production by the vaginal flora in vitro is consistent with the rate and the extent of the vaginal acidification. *Infect Immun*. 1999; 67:5170-75.
[16] Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S, Hale LP, et al. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol.* 2005;106:1013-23.
[17] Jennifer LP, Philippe HG, Nicole WK, Kimberly KJ. Effect of the biofilm phenotype on the resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. *Am J Obstet Gynaecol.* 2007;197:170-77.

AUTHOR(S):
1. Dr. Udayalaxmi
2. Dr. Gopalkrishna Bhat
3. Dr. Subbannayya Kotigadde
4. Dr. Shashidhar Kotian

PARTICULARS OF CONTRIBUTORS:
1. Selection Grade Lecturer, Department of Microbiology, Kasturba Medical College, Manipal University, Mangalore, India.
2. Associate Professor, Department of Microbiology, Kasturba Medical College, Manipal University, Mangalore, India.
3. Professor, Department of Microbiology, KVG Medical College, Sullia, India.
4. Department of Community Medicine, Kasturba Medical College, Manipal University, Mangalore, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. J. Udayalaxmi
Selection Grade lecturer, Department of Microbiology,
Kasturba Medical College, Mangalore-575001, India.
E-mail: udayalaxmi68@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS:
None.

Date of Submission: **Feb 09, 2012**
Date of Peer Review: **Apr 17, 2012**
Date of Acceptance: **Jun 30, 2012**
Date of Publishing: **Aug 10, 2012**