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Biofilm formation by *Streptococcus agalactiae* is affected by pH changes *in vitro*

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Abstract

Background: The capacity of *Streptococcus agalactiae* to cause infections is related to their ability to produce biofilms which allow bacteria to adhere to tissues causing pathogenesis and promote their resistance to antibiotics resulting in prolonged infections. Therefore, the aim of current study was to study the ability of these bacteria to form biofilms and the effects of pH changes on this ability.

Materials and Methods: A total of 25 confirmed isolates of *Streptococcus agalactiae* were collected in this study. The isolates were obtained by vaginal swaps from pregnant women who were complaining from abnormal vaginal discharge. Biofilm was detected by semi- quantitative microtiter plate test (biofilm assay) using TSB supplemented with 1% glucose.

Results: In this study, all strains of GBS were biofilm former and 52% of them were strong biofilm formers while 48% were moderate biofilms formers. Also, results showed that the ability of the studies isolates to form biofilms was weak at low pH and enhanced at high pH. Therefore, high pH plays a critical role in the pathogenesis of chronic vaginal bacterial infections.

Key Words: Streptococcus agalactiae, pH, biofilm, vaginal infection.

1. INTRODUCTION

Group B streptococcus (GBS) or *Streptococcus agalactiae* is a Gram-positive encapsulated bacterium exhibiting β-hemolysis on blood agar. The organism lives commensally in the gastrointestinal and genitourinary tracts of up to 30% of healthy adults. In addition, GBS is facultative anaerobic, catalase-negative and gram-positive cocci. Their metabolism is mainly fermentative and lactic acid is the predominant end product [1].

Colonization and persistence of bacteria in different host niches is dependent on the adherence capacity of GBS to host cells and tissues. This adhesion facilitates bacterial cells aggregation and formation of sessile communities known as biofilms. Bacterial biofilms represent well-known virulence factors with a vital role in persistence and chronic infections [2]. In the host environment, bacteria are often protected from the immune system by building sessile colonies embedded in an extracellular matrix of polysaccharides representing the biofilm. For GBS the bacterial capsule and type IIa pili have been demonstrated to play an important role in biofilm formation [2]. Biofilm formation is a complex aggregation of microorganisms growing on a solid surface. Biofilms are usually found on solid substance submerged in or exposed to some aqueous solution [3].

Biofilms allow long-term bacterial persistence and protect bacteria from recognition by immune system and controlling the expression of bacterial surface-associated structures, such as pili and the capsule, which are both involved in promoting bacterial biofilm formation [4].

A decrease in the number of normal flora lowers the concentrations of H_2O_2 and lactic acid and results in proliferation, and subsequent biofilm formation, of pathogenic bacteria. In addition, biofilms formation is poor in acidic environment while an increase in pH enhances bacterial bacterial ability to form biofilms [5].

Taken together, the aim of current study was to investigate the *in vitro* effects of pH changes on biofilms formation in *Streptococcus agalactiae* bacterial strains.

2. MATERIAL AND METHOD

Bacterial samples

All samples were obtained by vaginal swaps from pregnant women performed by a gynecologist who admitted them to Al-Hilla Surgical Teaching Hospital and Babylon Maternity and Paediatrics Hospital during the period from February to November 2017. however, out of 300 samples collected , only twenty five isolates of *Strep. agalactiae* were obtained.

Bacterial identification

The samples were processed on blood and Edward modified medium agars, incubated at 37 ºC. The identification of gram positive bacteria was performed by standard biochemical methods using Catalase, CAMP and Oxidase tests.

Detection of biofilm formation

Tissue culture plate method (TCP) assay (also called semi quantitative microtiter plate test (biofilm assay) described by [6] was considered as a standard test for detection of biofilm formation as follow:

1- Isolates from fresh agar plates were inoculated in TSB containing 1% glucose and incubated aerobically for 72 hrs at 37°C and then diluted 1:100 with TSB.
2- Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates wells were filled with 150 µl of the diluted cultures and only broth served as control to check non-specific binding of media. Each isolate was inoculated in triplicate.
3- The tissue culture plates were incubated for 24 hrs at 37°C. After incubation, content of each well was gently removed by tapping the plates. The wells were washed four times with phosphate buffer saline (pH 7.2) to remove free-floating bacteria.
4- Biofilms formed by adherent sessile organisms in plate were fixed by placing in oven at 37°C for 30 minutes.
5- All wells stained with crystal violet (0.1 % v/v). Excess stain was rinsed-off by thorough washing with deionized water and plates were kept for drying.
6- 150 µl of acetone/ethanol (20:80, v/v) mixture was added to dissolve bounded crystal violet. The optical density (O.D.) at 360 nm was recorded and the results were interpreted according to [7] (Table 1).

Table 1 Classification of bacterial adherence and biofilm formationby TCB method

Mean of O.D. value at 630 nm	Adherence	Biofilm formation
< 0.120	Non	Non
0.120 – 0.240	Moderately	Moderate
> 0.240	Strong	High

Effect of pH changes on biofilm formation

TSB was prepared and pH was adjusted to 4,5,6 using HCl or NaOH. These pH values were used to reproduce the vaginal pH in normal conditions (pH 4) and in case of infection (pH 5 ,6) [8].

3. RESULTS AND DISCUSSION

Results of current study revealed that all strains of GBS were biofilm former, however, 52% of isolates were strong biofilm former while 48% were moderate biofilm former with 1% glucose concentration. Results showed that all isolates were able to produce biofilm in different quantities which provided strong evidence that GBS isolates could be the main pathogens in vaginal infections. Also, results showed that all GBS isolates have high capacity to grow as biofilms (Table 2).

Table 2 Capacity of biofilm production by GBS (n= 25)

Capacity of Biofilm production	No. of GBS isolates (%)
Strong	13 (52)
moderate	12 (48)
Weak	0 (0)
Total	25 (100)

This assay was repeated as triplicate to increase its accuracy. According to the mean OD value at 630 nm, the results were interpreted as none, moderate and high biofilm former when the means of OD value were <0.120, 0.120-0.240, and >0.240, respectively. *In addition, the results revealed that all GBS isolates were biofilm former high and moderate mode were account for (52%,48%) respectively while there is no isolates that express non biofilm formation ,as shown in table(2*) (هذا الكلام مكرر ومن الافضل حذفه). The crystal violet microtiter plate test was a simple and rapid method to quantify biofilm formation of different bacterial strains.

Crystal violet is a basic dye known to bind negatively-charged molecules on cells surfaces, to nucleic acids and to polysaccharide, therefore, it gives an overall measure of whole biofilm. It has been used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of Gram positive bacteria [9].

In present study results showed that all isolates were able to produce biofilm in different quantities which gave strong evidence that GBS isolates could be the main colonizer in vaginal infection (هذا الكلام مكرر ايضا). Also results showed that all GBS isolates have high capacity to grow as biofilm.

Effect of pH changes on biofilm formation

Fluctuations in pH values (from 4 to 6) had their effects on bacterial biofilms formation. The results of current study showed that the isolates of interest formed biofilms at pH 6 and pH 5 but not at pH 4 (Table 3).

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Table 3 Effects of pH value on biofilm formation by *Strep. agalactiae* (n=25).

pH value	Biofilm formation by GBS		
	Strong *No. (%)	Moderate No. (%)	Weak No. (%)
4	0 (0.0%)	0 (0.0%)	25 (100%)
5	8 (32%)	10 (40%)	7 (28%)
6	13 (52%)	12 (48%)	0 (0.0%)

* Number of GBS bacterial isolates.

This result is in agreement with results reported by previous studies [8,10, 11] in which biofilm production was investigated under neutral and acidic pH conditions. They found that larger amounts of biofilm were formed at pH 6.5 compared to pH 4.2. Probably it could be due to poor bacterial growth at low pH. In contrast, another study [12] found that low pH could induce biofilm formation .

In the present study there was no biofilm formation at pH 4, maybe there is an association between vaginal pH and the presence of pathogens as reported by [13]. However, patients who are carrying potentially pathogenic bacteria (including GBS) had a conditions of altered microbiota such as bacterial vaginosis (BV) [14]. In the latter , vaginal pH is increased because Lactobacillus spp. are replaced by anaerobic bacteria, perhaps, enabling the colonization of GBS [15].

The use of TSB media with pH (5 to 6) had changed the ability of bacteria to produce biofilm with an increase in the percentage from 32% to 52%. As a result, we could conclude thatbiofilm formation by bacteria is pH-dependent and the increase in pH of media resulted in increased ability of bacteria to produce biofilm. Thus, a rise in vaginal pH may contribute to extended survival of colonizing GBS. These results were similar to those reported by [10] where biofilm formation by GBS in TSB varied with pH and the amounts of cells attached were increased with the increase of pH. Moreover, it was suggested that increasing vaginal pH could be a risk factor for vaginal infection as it may influence both GBS survival and biofilm production [8].

Healthy women normally show in vaginal fluid (Gram-stain smears) a prevalence of lactobacilli microflora composed of Lactobacillus spp. The latter support and maintain a vigorous ecosystem by producing lactic acid, hydrogen peroxide, and bacteriocins. These products have antibacterial properties against vaginal pathogens. Some Lactobacillus spp. also produce, *in vitro*, thick protective biofilm that protects them from harmful microbial proliferation [16].

Lactic acid is thought to play a critical and complex role in maintaining healthy vaginal environment [17]. It is thought that one important function of lactic acid is inhibiting the growth of potentially pathogenic organism. Instillation of probiotic lactobacilli has potentially significant impact on the health of women and, therefore, it is important to understand how the vaginal microbes change and adapt to the presence of these strain [18]

4. CONCLUSION

Streptococcus agalactiae have the ability to produce biofilms and this ability was weak at low pH value and moderate to strong at high pH values. Also, it seemed that high pH could be a risk factor that promotes bacterial vaginal infections.

Ethical Clearance: It was obtained from Ethics Committee at Al-Hilla Surgical Teaching Hospital and Babylon Maternity and Paediatrics Hospital.

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Conflict of Interest: None to declare.

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