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Evaluation of Different Detection Methods of Biofilm Formation in the Clinical Isolated from Pregnant and Non- Pregnant Women with Genital Tract Infection

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ABSTRACT

This study was carried out during the period (March to July 2012). A total of two hundred and fifty high vaginal swabs were collected from (100 pregnant and 150 non- pregnant) women patients with genital tract infection at the age ranged between (18- 55) years, who attended the gynecology clinics and obstetrics department of Maternity Teaching Hospital in Erbil city. Vaginal swab samples were collected and direct examined for pH measurement, microscopic Gram stain examination and culture techniques. Isolated microorganisms were identified using microscopical, morphological, biochemical tests, analytic profile index system and Vitek 2 compact system. The results showed that positive vaginal cultures were detected in 233 (93.2%) women patients, among pregnant were 95 (95%), while among non- pregnant were 138 (92%). The total number of bacteria isolates obtained from women patients were (191) isolates. These isolates were distributed between Gram- positive bacteria 118 (61.8%) and Gram- negative bacteria 73 (38.2%). All Gram-positive and Gram- negative bacterial isolates (191) were screened for biofilm production as one of the virulence factors by using two different methods (tube method and tissue culture plate method) and the results showed that the tissue culture plate method was the most sensitive method for detection of biofilm production. The results by tube method were 94 (49.2%) as non or weak and 97 (50.8%) as strong and moderate biofilm producers, while by tissue culture plate method using ELISA (Enzyme- linked immuno sorbent assay) system were 68 (35.6%) as non or weak and 123 (64.4%) as strong and moderate biofilm producers.

Keywords: Genital Tract Infection, Bacterial pathogens, Biofilm formation

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INTRODUCTION

Vaginitis is a common infection in females [1]. Vaginitis is an inflammation of the vaginal mucosa, whose incidence appears to be increasing, as estimated that 75% of women will experience at least one episode of vaginitis[2]. Biofilm are a group of microorganisms attached to a surface and covered by an exopolysaccharide matrix. Various changes occur during their transition from plank tonic to a surface attached community [3]. According to a publication by the National Institutes of Health, more than 60% of all infections are caused by biofilms [4]. Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents[5]. Bacterial vaginosis is considered a common vaginal disorder in women of reproductive age [6]. The bacteriological agents associated with vaginitis include a wide variety of bacteria that are dominated by overgrowth and marked by deficiency of hydrogen peroxide producing Lactobacilli[7]. The most bacterial agents causing vaginitis include *Staphylococcus aureus, Escherichia coli,* Group B Streptococci (GBS), *Listeria monocytogenes, Klebsiella pneumoniae, Acinetobacter* spp., *Neisseria gonorrhoeae* and in addition to Chlamydia[8]. Enterobacteriaceae the opportunistic pathogens most commonly include *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp. and *Serratia* spp. they are normal human gastrointestinal flora. There are several virulence factors, including endotoxins, capsules, adhesion proteins, and resistance to multiple antimicrobial agents[9]. The role of the biofilms in chronic diseases is increasingly recognized [10]. The frequency of bacterial infectious disease experts at the centers for disease control and prevention estimate that 65% of them produce biofilms[11]. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold [12]. Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*[13]. Glycocalyx is refer to all polysaccharide- containing substance found external to the cell wall, from the thickest capsules to the thinnest slime layers. All bacteria have at least a thin slime layer. A slime layer is less tightly bound to the cell wall and is usually thinner than a capsule. Slime layers allow bacteria to adhere to objects in their environments[14]. The slime is made up of bacteria in stationary phase, hydrophilic polysaccharides (glycocalyx) and minerals such as calcium which are essential to the structural integrity of the biofilm[15]. It is advantageous for bacteria to form stable communities of protection

rather than live as free planktonic cells[16]. Figure 1 demonstrate the biofilm formation by bacteria [17]. Adherence is a property common to many pathogenic microorganisms (Gram-positive and Gram-negative bacteria) and viruses by attaching to host structures[18]. After the attachment to a surface, the bacteria increase synthesis of exopolysaccharide and increase antibiotic resistance. They also develop an increased resistance to UV light and increased genetic exchange[19]. In patients with bacterial vaginosis, dense pathogenic biofilms cover the epithelial surface. Such biofilms resist host defences and exogenous antimicrobials, allowing the development of infections [20].

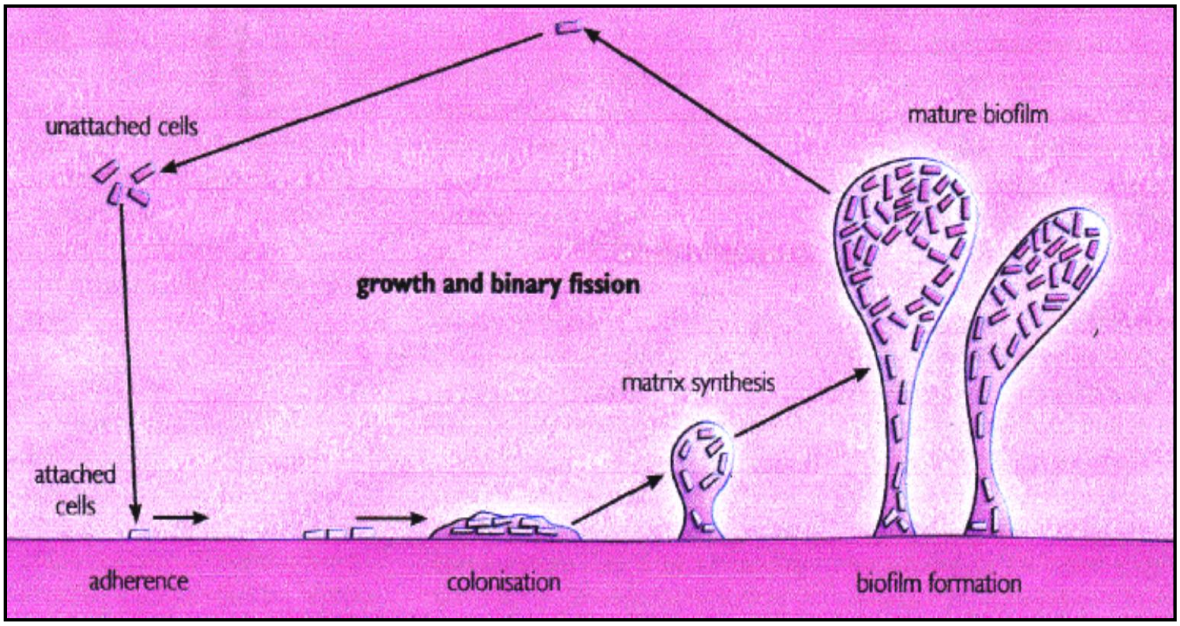


Figure 1: Biofilm formation (Cooper and Okhiria, 2006)

MATERIALS AND METHOD

Samples collection

High vaginal swabs were collected from two hundred and fifty (250) women patients with vaginal symptoms who attended the gynecology clinics and obstetrics department of Maternity Teaching Hospital in Erbil city during the period from March to July 2012. All vaginal swabs were taken from married women patients, of these 100 swabs from pregnant and 150 were from non-pregnant women. The age of these patients ranged between (18- 55) years. High vaginal swabs were taken from women patients suffering with abnormal vaginal discharge, itching, burning and lower abdominal pain. The samples were taken from each women patient (by doctors) using sterile swabs stick and speculum. Vaginal swab for each patient were transported to the laboratory by inoculating the swab into a sterile tube containing 3 ml of normal saline. The samples were examined by staining with Gram stain and pH measurement were performed.

Isolation of microorganisms

For isolation of microorganisms, the specimen of vaginal swab was directly inoculated on culture media: Blood agar, MacConkey agar and thioglycolate broth were incubated aerobically at 37˚C for 24-48 hours, and Chocolate agar plates were incubated micro-aerophilically at 37˚C for 24-48 hours. Microaerophillic incubation was in a candle jar supplied 5-10% Co2 [21].

Identification of microorganisms

Pure colonies of isolated microorganisms were identified using morphological, biochemical tests including API system[9]. Species identification and antibiograms for pathogens were performed using Vitek 2 compact system[6].

Assay of pH factor:

The pH was measured with pH indicator paper or (Uripath 3 strip) held with forceps and dipped into the saline preparation in the tube which contain vaginal swab[22].

Biofilm detection was done by the following methods

All bacterial isolates were tested by the following two methods for detection of biofilm formation (tube method and tissue culture plate method)

Tube method

Tube method was performed by a loopful of the isolate from agar plate was inoculated into a glass test tube containing 5ml of trypticase soy broth (TSB) and incubated overnight at 37˚C. Each tube was decanted, washed with phosphate buffer saline (pH 7.2), stained with 0.25% safranin, and then gently rotated to ensure uniform staining and the contents were gently decanted. The tube were then placed upside- down to drain. The color of the inner surfaces of the tubes was observed. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. The absence of a film or the presence of a ring at the liquid-air interface was interrupted as a negative result (–). Based on biofilm production, the positive results were recorded as strong (+++), moderate (++) and week (+) [23].

Tissue culture plate method

A loopful of test organism from overnight culture on nutrient agar was inoculated into 10ml Trypticase soy broth with 1% glucose. The broth was incubated at 37˚C for 24 hours. The culture was further diluted 1:100 with fresh medium. 96 wells flat bottom tissue culture plates were filled with 0.2ml of diluted cultures individually. Only sterile broth was served as blank. The culture plates were incubated at 37˚C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 0.2ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and bottoms of the wells were

fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA auto reader at wave length 570nm. Experiment was performed in triplicate and repeated thrice. Average of OD values of sterile medium were calculated and subtracted from all test values[24]. The results were interpretated according to that described by Bose *et al* [3]as shows in table (6), the data obtained was used to classify the OD value less than 0.120 was considered as non- biofilm producers, 0.120- 0.240 as moderate biofilm producers, and more than 0.240 as strong biofilm producers.

RESULTS AND DISCUSSION

A total of two hundred and fifty (250) high vaginal swabs were collected from women patients attending Maternity Teaching Hospital in Erbil city suspected of having vaginitis (We exclude the patients who are unmarried). The results showed that among 250 high vaginal swabs only 233 (93.2%) showed culture positive, 138 (92%) were non- pregnant and 95 (95%) pregnant, while 17 (6.8%) samples showed culture negative, 12 (8%) were non- pregnant and 5 (5%) pregnant. The statistical analysis showed no significant differences of infection among non- pregnant and pregnant as shown in table (1). Statistical difference were determined by Chi- square (X^2) test. Probability value (P-value) less than (< 0.05) was considered as statistically significant (*), while P-value more than (> 0.05) was considered as statistically not significant. The results of this study indicated that high rate of vaginitis detected in pregnant and non- pregnant women with symptoms were 233 (93.2%), similar results recorded by Masood *et al*., [25] from Pakistan reported that (86.6%) and . While in a study done in Nigeria by Isibor *et al*.[26], their results showed that (74.6%) of samples had positive culture. However, lower percentage of infections were reported by other investigators, Jarjees [27] from Erbil (Iraq) reported the rate was (68.3%) and Al- Muk and Hasony [28] from Basrah (Iraq) (67.6%), Abdul Razzak *et al*., [29] from Babylon (Iraq) (64.3%), Khan *et al*.,[30] from Pakistan (64%). The results also showed that the percentage of positive culture in pregnant women 95 (95%) were higher than non- pregnant women 138 (92%), but statistically not significant difference. Our results seem to agree with finding by Jarjees [27] from Erbil (Iraq) who reported in pregnant (71%) and in non- pregnant (67%), and agree with Isibor *et al*.,[26] from Nigeria who found that positive culture among pregnant women (53.6%) was higher than non-pregnant women (46.4%). The high incidence of infection in pregnant women could be attributed to hormonal changes [31]. The differences between our results and others might due to sample size and our target populations were selected by physician only women patients with symptoms of

vaginitis like abnormal vaginal discharge, itching, burning and lower abdominal pain. Table (2) represented the both Gram- positive and Gram- negative bacteria were involved as causative agents of the infection. The incidence of Gram- positive bacteria 118 (61.8%) was relatively higher than Gram- negative bacteria 73 (38.2%). This result is in agreement with Al- Muk and Hasony [28] from Basrah (Iraq), they reported in pregnant women the rate of Gram- positive bacteria (31%) higher than the Gram- negative bacteria (13.5%), and agree with Saini *et al*., [32] from India, he reported the Gram positive bacteria (52.2%) higher compared to Gram- negative bacteria (47.4%). Our results disagree with Jarjees [27] in Erbil (Iraq) her result showed that the isolated Gram-negative bacteria (80.44%) were more than Gram-positive bacteria (19.53%). The presence of Gram positive in vaginal samples may be due to that Staphylococci are normal flora of intestine and vagina [33], also Staphylococci whose natural habitatis the intestinal tract of humans can cause infection by the ascending route [34]. The most common isolated microorganisms from the vaginal women with vaginitis was *Escherichia coli* 42 (57.5%), also the frequency of *Escherichia coli* in non- pregnant 30 (56.6%) higher compared to pregnant 12 (60%) are showed in table (3). These results were agreement with those reported in our country such as by Jarjees [27] from Erbil (Iraq) in non-pregnant (53.2%) and in pregnant (48.6%). The low frequency of infection noticed by *Pseudomonas aeruginosa* was 2 (2.7%) and *Pseudomonas luteola* was 1 (1.4%). Similar findings were obtained by Al- Jammaly and Abdulla [35] from Mosul (Iraq) (1.9%) and Mumtaz *et al*., [36] from Pakistan (1.8%), they reported that the incidence of infection with *Pseudomonas aeruginosa*. The highest percent of the isolates belonged to Gram-positive bacteria were *Staphylococcus haemolyticus* 19 (16.1%), *Staphylococcus auricularis* 19 (16.1%), followed by *Staphylococcus aureus* 13 (11%), *Staphylococcus saprophyticus* 8 (6.8%) and *Staphylococcus epidermidis* 5 (4.23%) are represented in table (4). Similar finding have been reported by Alim *et al*., [37] from Kabul (Afghanistan) (10.28) and Adegoke and Okoh [38] from South Africa (12.5%), they reported the rate of *Staphylococcus aureus* isolated in vaginitis infection. Agree with Al- Musawi *et al*.,[2] from Al- Diwaniya (Iraq), who reported a prevalence of isolated *Staphylococcus aureus* was (5.6%) and *Staphylococcus saprophyticus* was (4.8%). Also agree with Arianpour *et al*., [39] from Iran reported *Staphylococcus saprophyticus* constitute (5.8%). The percentages of *Staphylococcus* spp. (*S. aureus, S. epidermidis, S. saprophyticus*) in patients with vaginitis may reach to 62% [40]. The coagulase negative Staphylococci are normal human flora and sometimes cause infection in immunocopromised patients[34]. In our result the prevalence of *Staphylococcus aureus* among non-pregnant women with vaginitis was 8 (11.1%) and pregnant was 5 (10.9%). Respectively lower than those recorded by Jarjees [27] from Erbil (Iraq) in non- pregnant (12.9%) and in pregnant were

(14.9%). The involvement of *S. aureus* in vaginal discharge or infection of the female genital tract has been an issue in the microbiology of vaginal infections. The presence of *S. aureus* along side with other bacteria such as *Escherichia coli, Enterococcus faecalis, Klebsiella* spp., *Proteus* spp. and *Pseudomonas aeruginosa* may be attributed to immune status of the individual, lack of personal hygiene regarding the proximity of the vagina to the urethral tract from where these bacterial species may readily gain access into the vagina. Faecal organisms from the anus could also be introduced into the vagina during cleaning by using the ascending route (back to front) instead of the descending route front to back [26]. The results showed that the relation between the pH and the highest percentage of infection occurred at pH 7 was 4 (100%) in pregnant and non-pregnant women, followed by pH 6 was 140 (92.1%) distribution among non- pregnant was 87 (88.8%) and among pregnant was 53 (98.1%) as in table (5) and figure (2). This result was higher with other studies who depended on Amsel's clinical criteria which have considered vaginal pH ≥ 4.5 as one of four criteria to diagnosis BV that obtained by Al- Musawi *et al*., [2] from Al- Diwaniya (Iraq) who reported the prevalence of vaginitis infections was (53%) at pH > 4.5. But disagreement with Al- Muk and Hasony [29] from Basrah (Iraq) who reported at pH > 4.5 the rate of infections in pregnant was (10.2%). The role of Lactobacilli in preserving the vaginal health as being the predominant vaginal flora and mentioned that when Lactobacilli decreased due to many causes like douching and use of antibiotics; other pathogenic organisms would dominate [7]. If the number of Lactobacilli falls, the resulting increase in pH favors an overgrowth of anaerobic and facultative bacteria which can develop into vaginitis. The biofilm found in bacterial vaginosis is highly organized. Clue cells are epithelial cells whose surfaces are heavily coated with bacteria. In contrast to bacterial vaginosis, adherent biofilms were not observed on the epithelium of most healthy controls[41]. We evaluated (191) isolates bacteria by two screening methods tube method (TM) and tissue culture plate method (TCP) by using ELISA for their ability to form biofilms. Among (191) isolates as seen in figure (3 and 4), TCP method detected 123 (64.4%) as (strong and moderate) biofilm producers, while in TM detected 97 (50.8%) as (strong and moderate) biofilm producres. Similar findings were in agreement with our results reported by Mathur *et al*., [24] from India showed that out of 152 isolates tested, the percentage of biofilm producers identified by TCP method was (53.8%) as (strong and moderate) and by TM showed (41.4%) isolates were picked up as (strong and moderate) biofilm producers, and agree with Bose *et al*., [3] from India who reported (54.19%) isolates as (strong and moderate) in TCP method biofilm producers, while in tube method (42.46%) isolates were found as strong and moderate biofilm producers, and agree with Hassan *et al*., from Pakistan, who reported among (110) isolates, TCP method detected (63.7%) as

(strong and moderate) biofilm producers and by TM the number of biofilm producers were (52%) as (strong and moderate). The ability to adhere to materials and to form biofilm is an important feature in the pathogenesis of clinical isolates [41]. A dense bacterial biofilm was attached to at least 50% of the intact epithelial surface in 90% of the biopsy specimens from subjects with bacterial vaginosis. Bacteria were nearly homogeneously composed of stacked short rods with almost no free spaces between single cells and the epithelial surface[41]. In the present part of the study. Generally we compare between two methods TM and TCP method for biofilm formation as in table (7) out of 118 Gram- positive bacteria isolates, the percentage of biofilm producers by TCP method were 95 (80.5%) higher than in TM were 78 (66.1%) biofilm producers as (strong and moderate). While out of 73 Gram- negative bacteria isolates in TCP method were 28 (38.4%) isolates produce biofilm and in TM were 19 (26%) biofilm producers as (strong and moderate). Other study have Similar rate obtained by Zubair *et al*., [43] from India reported that the rate for biofilm formation by Gram- negative bacteria were about (59.4%). The detection of biofilm formation by TCP method more accurate than the TM, because in TCP method showed the biofilm formation (as strong and moderate) higher in all isolates bacteria (Gram- positive and Gram-negative) compared to TM. In tube method, it was hard to differentiate between moderate, weak and non-biofilm producers due to the changeability in the results detected by different observers. In accordance with the preceding studies, TM cannot be suggested as general screening test to identify biofilm producing isolates [24]. We comparison of two method TM and TCP and each method studied separately to detected the ability of all isolated bacterial vaginosis for biofilm formation biofilm the results presented in table (8). Among *Staphylococcus* spp. in TCP method, the most common species which produce biofilm *Staphylococcus aureus* were 12 (92.3%) isolates as (strong and moderate) biofilm formed. *Staphylococcus epidermidis* were 4 (80%) as (strong and moderate) produced biofilm. Our result was higher than different results have been obtained by Bendouah *et al.,* [44] from Canada biofilm producer among *Staphylococcus aureus* were (80%). Khan *et al.,*[22] from India who reported that *Staphylococcus aureus* were (64.89%) biofilm formed by TCP method. Knobloch *et al*.,[45] who reported that out of 128 isolates of *S. aureus* by TCP method which detected (57.1%) as biofilm producer. In another study by Ruzicka *et al.,*[46] noted that out of 147 isolates of *S. epidermidis* by TM detected biofilm formation were (53.7%). But vary small number have been obtained by Hassan *et al*., [5] from Pakistan, who reported biofilm producer by TCP method for *S. aureus* (11.4%) and *S. epidermidis* (37.1%).

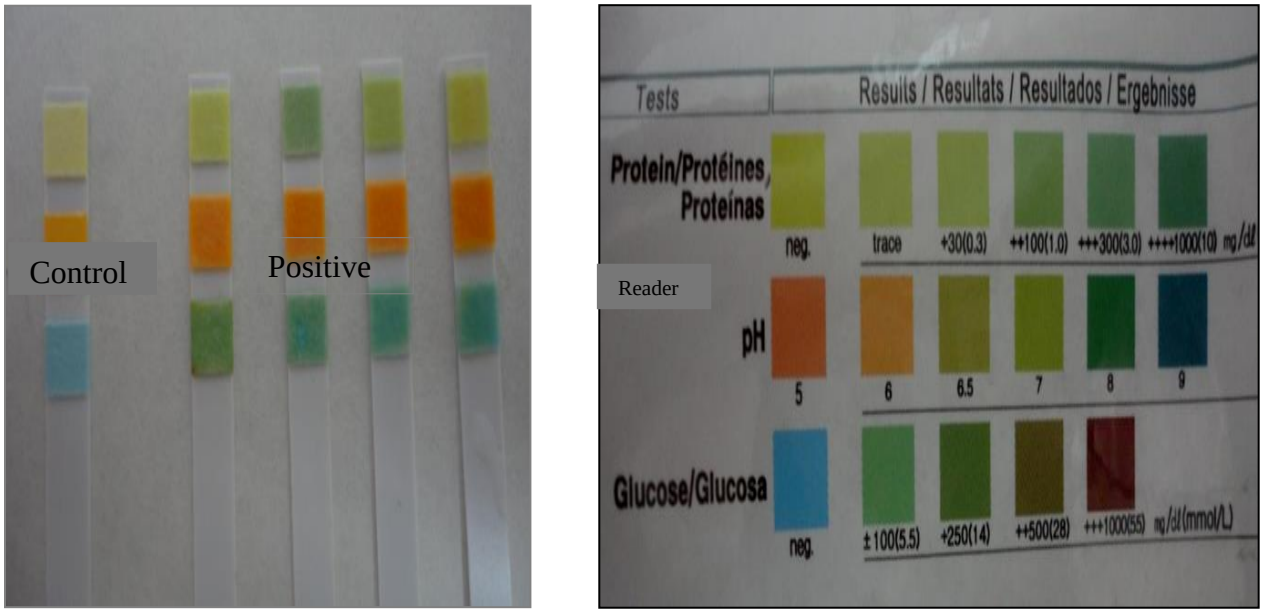
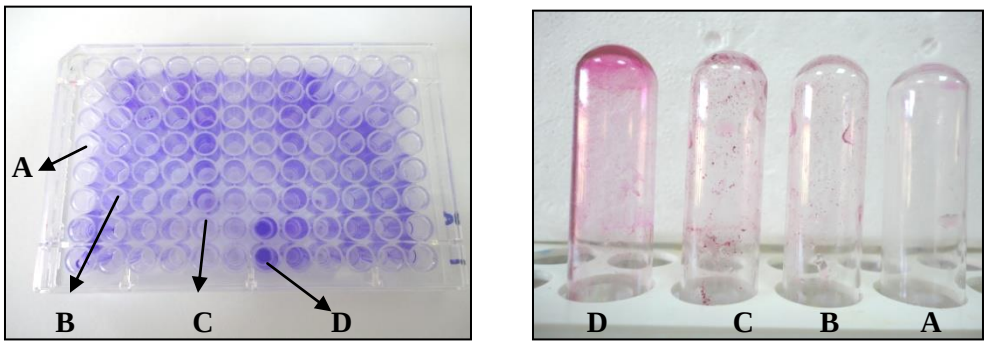


Figure 2: Assay of pH for vaginal discharge by Uripath3 strip



Tissue culture plate method (TCPM) **tube method (TM)**

Figure 3: Detection of biofilm producer by TCPM and TM: (A) Control, (B) Non or weak biofilm producer, (C) Moderate biofilm producer, (D) High

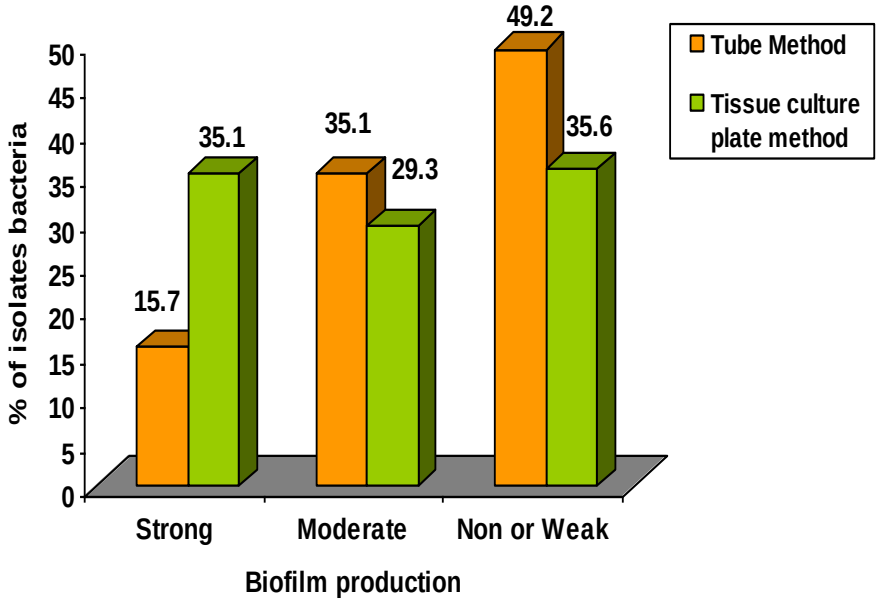


Figure 4: The percentage of biofilm formation by tube method and tissue culture plate method

Table 1: Distribution of women patients with vaginitis in relation with pregnant and non-pregnant

Culture results	Non- pregnant		Pregnant		Total	
	No.	%	No.	%	No.	%
Positive culture	138	92 %	95	95 %	233	93.2 %
Negative culture	12	8 %	5	5 %	17	6.8 %
Total	150	60%	100	40 %	250	100 %
Chi- square (x^2)			0.85 N.S.			

Note: N.S. = No Significant

Table 2: Distribution of Gram- positive and Gram- negative bacteria in relation to pregnant and non- pregnant women with vaginitis

Bacterial vaginosis	Non- pregnant		Pregnant		Total	
	No.	%	No.	%	No.	%
Gram- positive bacteria	72	57.6 %	46	69.7 %	118	61.8 %
Gram- negative bacteria	53	42.4 %	20	30.3 %	73	38.2 %
Total	125	65.4 %	66	34.6 %	191	100 %
Chi- square (x^2)	2.68 N.S.					

Note: N.S. = No Significant

Table 3: Distribution of Gram- negative bacteria in vaginitis in relation to pregnant and non-pregnant women

Isolated Gram- negative bacteria	Non- pregnant		Pregnant		Total	
	No.	%	No.	%	No.	%
Escherichia coli	30	56.6 %	12	60 %	42	57.5 %
Klebsiella pneumoniae	12	22 .6%	2	10 %	14	19.1 %
Proteus mirabilis	3	5.6 %	2	10 %	5	6.8 %
Pseudomonas aeruginosa	2	3.8 %	0	0 %	2	2.7 %
Pseudomonas luteola	1	1.9 %	0	0 %	1	1.4 %
Serratia fonticola	1	1.9 %	1	5 %	2	2.7 %
Serratia plymuthica	1	1.9 %	0	0 %	1	1.4 %
Enterobacter aerogenes	0	0 %	1	5 %	1	1.4 %
Acinetobacter lwoffii	1	1.9 %	0	0 %	1	1.4 %
Raoultella ornithinolytica	1	1.9 %	0	0 %	1	1.4 %
Pantoea agglomerans	0	0 %	1	5 %	1	1.4 %
Sphingomonas paucimobilis	0	0 %	1	5 %	1	1.4 %
Ewingella americana	1	1.9 %	0	0 %	1	1.4 %
Total	53	72.6 %	20	27.4 %	73	100 %

Table 4: Distribution of Gram- positive bacteria in vaginitis in relation to pregnant and non-pregnant women

Isolated Gram- positive bacteria	Non- pregnant		Pregnant		Total	
	No.	%	No.	%	No.	%
Staphylococcus aureus	8	11.1 %	5	10.9 %	13	11 %
Staphylococcus lentus	1	1.4 %	2	4.3 %	3	2.54 %
Staphylococcus epidermidis	5	6.9 %	0	0 %	5	4.23 %
Staphylococcus hominis	1	1.4 %	2	4.3 %	3	2.54 %
Staphylococcus intermedius	1	1.4 %	0	0%	1	0.85 %
Staphylococcus haemolyticus	15	20.8 %	4	8.7 %	19	16.1 %
Staphylococcus auricularis	5	6.9 %	14	30.4 %	19	16.1 %
Staphylococcus saprophyticus	2	2.8 %	6	13 %	8	6.8 %
Staphylococcus capitis	1	1.4 %	1	2.2 %	2	1.7 %
Staphylococcus sciuri	1	1.4 %	1	2.2 %	2	1.7 %
Staphylococcus warneri	1	1.4 %	0	0 %	1	0.85 %
Staphylococcus cohnii	1	1.4 %	0	0 %	1	0.85 %
Kocuria kristinae	6	8.3 %	1	2.2 %	7	5.9 %
Kocuria rosea	0	0%	1	2.2 %	1	0.85 %
Kocuria varians	3	4.2 %	1	2.2 %	4	3.4 %
Enterococcus faecalis	12	16.6 %	7	15.2 %	19	16.1 %
Enterococcus casseliflavus	1	1.4 %	0	0 %	1	0.85 %
Enterococcus gallinarum	1	1.4 %	0	0 %	1	0.85 %
Enterococcus faecium	3	4.2 %	0	0 %	3	2.54 %
Dermacoccus nishinomiyaensis /Kytococcus sedentarius	1	1.4 %	0	0 %	1	0.85 %
Alloiococcus otitis	1	1.4 %	0	0%	1	0.85 %
Micrococcus luteus/ lylae	0	0%	1	2.2 %	1	0.85 %

Lactococcus garvieae	1	1.4 %	0	0%	1	0.85 %
Granulicatella adiacens	1	1.4 %	0	0 %	1	0.85 %
Total	72	61 %	46	39 %	118	100 %

Table 5: The relation between pH in pregnant and non- pregnant women and positive culture of vaginal swabs

pH	Non- pregnant		Pregnant		Total	
	No. & % of samples	No. & % of positive culture	No. & % of samples	No. & % of positive culture	No. & %of samples	No. & % of positive culture
pH 5	49&32.7%	48&98 %	45&45%	41&91.1 %	94&37.6%	89&94.7%
pH 6	98& 65.3%	87&88.8%	54&54%	53&98.1%	152&60.8%	140&92.1%
pH 7	3& 2%	3&100 %	1&1%	1&100%	4&1.6%	4&100%
Total	150& 60%	138&92%	100&40%	95&95%	250&100%	233&93.2%
Chi-square (x^2)	1.93 N.S.					

Table 6: Classification of results for detection of biofilm formation based on OD values by TCP method

Mean OD value	Adherence	Biofilm Formation
< 0.120	Non	Non / weak
0.120- 0.240	Moderate	Moderate
> 0.240	Strong	Strong

Table 7: Relation of biofilm formation by tube method and tissue culture plate method for Gram positive and Gram negative bacterial vaginosis

Biofilm formation		Total No. of isolated bacteria (191)				Chi-square (X^2)
		Total No. of isolated Gram- positive bacteria (118)		Total No. of isolated Gram-negative bacteria (73)		
		No.	%	No.	%	
Tube method	Non/Weak	40	33.9%	54	74%	36.04*
	Moderate	48	40.7%	19	26%	
	Strong	30	25.4%	0	0%	
Tissue culture plate method	Non/Weak	23	19.5%	45	61.6%	38.57**
	Moderate	38	32.2%	18	24.7%	
	Strong	57	48.3%	10	13.7%	

* Significant (P<0.05) for Tube method

** Significant(P<0.05) for Tissue culture plate method

Table 8 Frequency of biofilm formation by tube method and tissue culture plate method in bacterial vaginosis

Bacterial isolated	Total No. of isolated	Biofilm formation					
		Tube method (TM)			Tissue culture plate method (TCPM)		
		Non /Weak	Moderate	Strong	Non /Weak	Moderate	Strong
Staphylococcus aureus	13	3 (23.1%)	3 (23.1%)	7 (53.8%)	1 (7.7%)	2 (15.4%)	10 (76.9%)
Staphylococcus lentus	3	0	2 (66.7%)	1 (33.3%)	0	2 (66.7%)	1 (33.3%)
Staphylococcus epidermidis	5	1 (20%)	2 (40%)	2 (40%)	1 (20%)	1 (20%)	3 (60%)
Staphylococcus hominis	3	0	3 (100%)	0	0	2 (66.7%)	1 (33.3%)
Staphylococcus intermedius	1	1 (100%)	0	0	0	1 (100%)	0
Staphylococcus haemolyticus	19	8 (42.1%)	8 (42.1%)	3 (15.8%)	3 (15.8%)	7 (36.8%)	9 (47.4%)
Staphylococcus auricularis	19	7 (36.84%)	10 (52.63%)	2(10.53%)	4 (21.1%)	8 (42.1%)	7 (36.8%)
Staphylococcus saprophyticus	8	1 (12.5%)	6 (75%)	1 (12.5%)	2 (25%)	0	6 (75%)
Staphylococcus capitis	2	1 (50%)	0	1 (50%)	1(50%)	0	1 (50%)
Staphylococcus sciuri	2	0	1 (50%)	1 (50%)	0	0	2 (100%)
Staphylococcus warneri	1	0	1 (100%)	0	0	0	1 (100%)
Staphylococcus cohnii	1	1 (100%)	0	0	0	1 (100%)	0
Kocuria ristinae	7	3 (42.8%)	2 (28.6%)	2 (28.6%)	4 (57.2%)	0	3 (42.8%)
Kocuria rosea	1	0	0	1 (100%)	0	0	1(100%)
Kocuria varians	4	3 (75%)	0	1 (25%)	2 (50%)	1 (25%)	1 (25%)
Enterococcus faecalis	19	5 (26.3%)	6 (31.6%)	8 (42.1%)	2 (10.5%)	8 (42.1%)	9 (47.4%)
Enterococcus casseliflavus	1	1 (100%)	0	0	1(100%)	0	0
Enterococcus gallinarum	1	1 (100%)	0	0	1 (100%)	0	0
Enterococcus faecium	3	2 (66.7%)	1 (33.3%)	0	0	2 (66.7%)	1 (33.33%)
Dermacoccus nishinomiyaensis	1	1 (100%)	0	0	0	1(100%)	0
Alloiococcus otitis	1	1 (100%)	0	0	1 (100%)	0	0
Micrococcus luteus	1	0	1 (100%)	0	0	1 (100%)	0
Lactococcus garvieae	1	0	1 (100%)	0	0	1 (100%)	0
Granulicatella adiacens	1	0	1 (100%)	0	0	0	1 (100%)
Escherichia coli	42	36 (85.7%)	6 (14.3%)	0	28 (66.7%)	10 (23.8%)	4 (9.5%)
Klebsiella pneumoniae	14	10 (71.4%)	4 (28.6%)	0	10 (71.4%)	4 (28.6%)	0
Proteus mirabilis	5	3 (60%)	2 (40%)	0	3 (60%)	1 (20%)	1 (20%)
Pseudomonas aeruginosa	2	0	2 (100%)	0	0	1 (50%)	1 (50%)

Pseudomonas luteola	1	1 (100%)	0	0	1(100%)	0	0
Serratia fonticola	2	0	2 (100%)	0	0	1 (50%)	1 (50%)
Serratia plymuthica	1	0	1 (100%)	0	0	0	1 (100%)
Enterobacter aerogenes	1	1(100%)	0	0	1(100%)	0	0
Acinetobacter lwoffii	1	0	1 (100%)	0	0	0	1 (100%)
Raoultella ornithinolytica	1	1 (100%)	0	0	1 (100%)	0	0
Pantoea agglomerans	1	1 (100%)	0	0	1 (100%)	0	0
Sphingomonas paucimobilis	1	0	1 (100%)	0	0	0	1 (100%)
Ewingella Americana	1	1(100%)	0	0	0	1 (100%)	0
Total	191	94 (49.2%)	67 (35.1%)	30 (10.7%)	68 (35.6%)	56 (29.3%)	67 (35.1%)

CONCLUSION

It has been suggested that the ability to form biofilms on surface greatly contributes to the virulence of *Staphylococcus epidermidis*. This ability depends on the production of polysaccaharide intracellular adhesion molecules, encoded by the intracellular adhesion (ica) locus including the icaA gene, icaB gene, icaC gene and icaD gene [23]. The present study noticed that for biofilm production by TCP method strong and moderate) by bacteria *Escherichia coli* was 14 (33.3%), *Klebsiella pneumoniae* was 4 (28.6%). Similar finding have been obtained by Hemachandran *et al*., [42] from India who reported that the ability to adherent *Escherichia coli* biofilm producer (43%) and the ability of *Klebsiella* spp. (31%), and agree with Hassan *et al*., [5] from Pakistan, who reported in TCP method biofilm producer by bacteria *Escherichia coli* was (27.1%) and *Klebsiella pneumoniae* (15.7%).

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