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PREVALENCE OF BIOFILM FORMATION IN BACTERIAL PATHOGENS IN VAGINAL WOMEN INFECTIONS

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Abstract

Background and aims: Bacterial vaginosis (BV) is a vaginal microbiome dysbiosis that is reported to be the most common gynecological condition in women of reproductive age. **Methods:** A total 120 swab samples were collected from patients with age 18 –50 years, during the period from 7th November, 2021 to 28th February, 2022 that admitted to Al-Zahra Teaching Hospital, Al Kut Hospital, , and private clinics in Wasit province. A total 70 bacterial isolates were detected by culture characterization on the different media, biochemical investigation, analytical profile index 20 and Vitek2 automated system and investigation about biofilm production using microtitre plate (MTP). **Results:** There were 70 bacterial-isolated samples. Results of this study has been showed that the high rate of bacterial biofilm formation was in recorded as 17.1%, 14.3% and 12.9% in *Escherichia coli, klebsiella pneumoniae* and *staphylococcus epidermidis* respectively, as they form strong biofilm.biofilm formation was more prevalent in gram-negative bacteria (73%), *Klebsiella pneumoniae* (37.41%), *Escherichia coli* (28.78%) *Staphylococcus* spp. (23.57%) was the only gram-positive isolate formed strong biofilm. **Conclusion:** In Wasit province, there is a development in bacterial production of biofilm. This may have a harmful impact on Iraqi women and should be seen as a concerning health situation.

Keywords: Bacterial vaginosis (BV), Biofilm production, Microtitre plate (MTP)

Introduction

Bacterial vaginosis (BV) is a vaginal microbiome dysbiosis that is reported to be the most common gynecological condition in women of reproductive age (1). As well as BV has been recognized as one of the most perplexing mysteries in medicine and there is a large research gap around BV (2). Understanding the disease of BV is critical for motivating research towards improved diagnosis and treatment of BV and its negative effects (3).There has been a significant rise in clinician and patient awareness of bacterial vaginosis as a common cause of vaginal discharge over the last decade. There has also been a lot of speculation about the involvement of BV in upper genital tract infections including pelvic inflammatory disease and premature labor and delivery (4).

In terms of aetiology and pathophysiology it is perplexing bacterial vaginosis is the most significant reason of vaginal discharge affecting 20-25% of the general population and up to 50% of women attending sexually transmitted diseases clinics (5) .

Regardless of the fact that studies have demonstrated that clinicians who make empirical diagnosis without the use of laboratory testing commonly misdiagnose the aetiology of a vaginal infection empirical diagnoses remain the standard in many practices. The most common signs of BV include odor and vaginal discharge. Pruritus is frequently not apparent (6). In addition; the bacterial vaginosis could be defined clinically or microbiologically. When three of the following four criteria are achieved the clinical or Amsel criteria allow for the diagnosis of BV (7).

(1) Vaginal pH greater than 4-5.
(2) Positive "whiff" test when vaginal fluid is combined with 10% KOH.
(3) Presence of clue cells contaminated squamous epithelial cells.
(4) Presence of a homogenous vaginal discharge microbiologically the BV syndrome is defined by a shift in vaginal microbes away from a lactobacillus-dominated flora to a vaginal environment dominated by anaerobes and facultative anaerobes such as Gardnerella, mycoplasmas, Prevotella/Porphyromonas, Mobiluncus and diminished numbers of lactobacilli, particularly those that produce hydrogen peroxide (8,9).

Currently, BV treatment is based on eradicating anaerobic/facultatively anaerobic organisms with metronidazole or clindamycin. The recommended oral metronidazole dosage is 500 mg twice daily. Although, the initial 2 mg dosage may improve compliance, seven days regimen is more effective especially when cure rates at three weeks following therapy are compared. In addition, metronidazole is available in topical forms with cure rates comparable to oral (10). Although topical therapies avoid unpleasant systemic side effects there are growing theoretical worries that management of anaerobes in the lower genital tract may be insufficient to prevent upper tract difficulties associated with BV (11). Moreover, symptoms include vaginal odour and a grey-whitish discharge, Itching, irritation, burning, bleeding, soreness, pain during sexual intercourse, stomach cramps, and peeling skin around the vulva are among symptoms that women may experience (12). According to studies, symptoms may be absent in up to 83% of women (13). The most painful symptom, the malodor, is frequently characterised as fishy; nevertheless, some women report smelling 'piquant' or'musky,' as well as like 'a dead thing' or 'trash.' The milky discharge is thin and uniform (11).

Material and method

Study design

A total 120 swab samples were collected by gynecologist from patients with age 18 –50 years, during the period from 7th November, 2021 to 28th February, 2022 that admitted to Al-Zahra Teaching Hospital, Al Kut Hospital, and private clinics in Wasit province. Bacterial isolates were detected by culture characterization on the different media, biochemical investigation, analytical profile index 20 and Vitek2 automated system.

Biofilm microtitre plate

Microtitre plates were infected with 250 µl of broth culture per well for each bacterial strain. As a control, sterile nutrient broths were infected. Plates were covered and incubated for 24 hrs. At 37°C, the bacterial culture broth was withdrawn from each well after incubation. To eliminate germs that were not adherent to the wells, each well was washed three times with 300 mL of sterile

PBS and violently shaken. Biofilms adhered to the wells were fixed for 15 mins. in 250 liters of 96% ethanol each well. The ethanol was then taken from the plate, and it was allowed to dry. After staining each well for five min, 0.2 ml of crystal violet solution (2% *w/v*), and the excess was rinsed away with water. According to (14) the quantitative assessment of biofilm generation was accomplished by adding 200 µl of 33% glacial acetic acid (v/v) each well, incubating for 15 mins.and measuring absorbance at 570 mm using a Versa Max Plate Reader. Tahmourespour and Kermanshahi's approach was used to reproduce all experiments three times under the identical experimental circumstances. The strains were categorized as follows based on their non-adherent status: Individual bacterial strains were injected into microtitre plates with of broth culture each well as a control, sterile nutrient broths were infected. Plates were covered and incubated for 24 hrs. at 37 °C. The bacterial culture broth was withdrawn from each well after incubation. To eliminate germs that were not adherent to the wells, each well was washed three times with 300 ml of sterile PBS and violently shaken. Biofilms adhered to the wells were fixed for 15 mins. in 250 liters of 96% ethanol each well. The ethanol was then taken from the plate, and it was allowed to dry. The surplus crystal violet solution (2% w/v) was rinsed away with molecular-grade water after each well was stained for five min. The quantitative assessment of biofilm generation was accomplished by adding of 33% glacial acetic acid (*v/v*) each well, incubating for 15 min, and measuring absorbance at 570 mm using a Versa Max Plate Reader (14). Tahmourespour and Kermanshahi's approach was used to reproduce all experiments three times under the identical experimental circumstances. The strains were categorized as follows based on their non-adherent status. The microorganism was found if the optical densities were less than or equal to 0.120. They were classed as weakly adherent if their optical densities were greater than 0.120 but less than 0.240 if the optical densities in either medium surpassed a certain threshold, the strain was categorized as severe 0.240.

Statistical analysis

Data were entered and analyzed using Statistical Package for Social Sciences (SPSS) software program version 26. All categorical variables were presented by frequency and percentages. Association between categorical variables was assessed by the Chi-Square test or Fisher's Exact Test (if > 20% of expected cell counts is less than 5 accordingly. *P*-value was equal to or less than 0.05 as significant (15).

Results:

Sample collection:A total 120 swab samples were collected from patients with age 18 –50 years, during the period from 7th November, 2021 to 28th February, 2022 that admitted to Al-Zahra Teaching Hospital, Al Kut Hospital, , and private clinics in Wasit province. A total 70 bacterial isolates were detected by culture characterization on the different media, biochemical investigation, analytical profile index 20 and Vitek2 automated system and investigation about biofilm production using microtitre plate (MTP).

Table (1): Distribution of bacterial isolates according to genus species

Bacterial Isolate	Frequency	Percent	t-test	P-Value

Enterococcus faecalis	5	4.2	23.095	0.0001
Enterobacter cloacae	4	3.3		
Pseudomonas aeruginosa	6	5.0		
Escherichia coli	12	10.0		
Staphylococcus hominis	2	1.7		
Staphylococcus warneri	1	0.8		
Streptococcus thoraltensis	4	3.3		
Acinetobacter baumannii	4	3.3		
Klebsiella pneumoniae	10	8.3		
Staphylococcus epidermidis	8	6.7		
Staphylococcus haemolyticus	6	5.0		
Streptococcus agalactiae	3	2.5		
Enterococcus faecium	3	2.5		
Pseudomonas putida	1	0.8		
No Growth	51	42.5		

Results of in the current study has been showed that the high rate of bacterial was in *pseudomonas aeruginosa* (5.0%), *Escherichia coli* (10.0%), *klebsiella pneumoniae* (8.3%) and *staphylococcus epidermidis* (6.7%) in all groups of pregnant women. In addition the positive outcomes were indicated in 57.5%. The high incidence and risk of developing UTI in the course of pregnancy were in HVS was related to abnormal anatomical and physiological changes that occur during this period (16). Furthermore, previous history of UTI, increased age, multiparity, sexual activity, history of catheterization, immunodeficiency and lower socioeconomic status are identified as factors likely to increase risk of UTI during pregnancy (17). Current study outcomes were as the same to(18) who recorded that overall prevalence of bacterial isolates in HVS was that the predominant bacterial isolates were *E. coli* 17 (33.3%) followed by coagulase-negative staphylococci 15 (30.0%) and Staphylococcus spp. 14 (27.5%). While positivity was recorded in 15.5% (50/323) isolates. In addition (19) reported that bacteria are the most common agents causing UTI including *Escherichia coli, Proteus mirabilis, Klebsiella* spp., *Pseudomonas aeruginosa, Enterobacter spp., Enterococci, Citrobacter, Staphylococcus aureus*, coagulase-negative staphylococci, Streptococcus spp. and others. Gram-negative bacteria are the major isolates causing UTI in which the predominant isolates are Escherichia coli accounting majority (80–90%) of infection (20).In the present study The data were incompatible with(21) who showed that HVS in pregnancy may be caused by symptomatic or asymptomatic Bactria are *E. coli, k. pneumoniae, P.aeruginosa, S. aureus* and *Streptococcus* spp. which occurs in 2–11% of

pregnancies worldwide and is a major predisposition to the development of acute pyelonephritis in 20–50% of untreated cases. Furthermore, untreated UTI in pregnancy is associated with a 50% increase in the risk of maternal complications of pregnancy which raise extent of preterm labor, prematurity and low birth weight resulting in high perinatal morbidity and mortality (22). Early diagnosis and clinical management reduce the incidence of these complications (23). Nevertheless, in developing countries including Ethiopia, urine culture screening is not routinely done as part of antenatal care and treatment is empirical which may lead to emergence and spread of antimicrobial-resistant strains which is a leading cause of treatment failure in UTI.

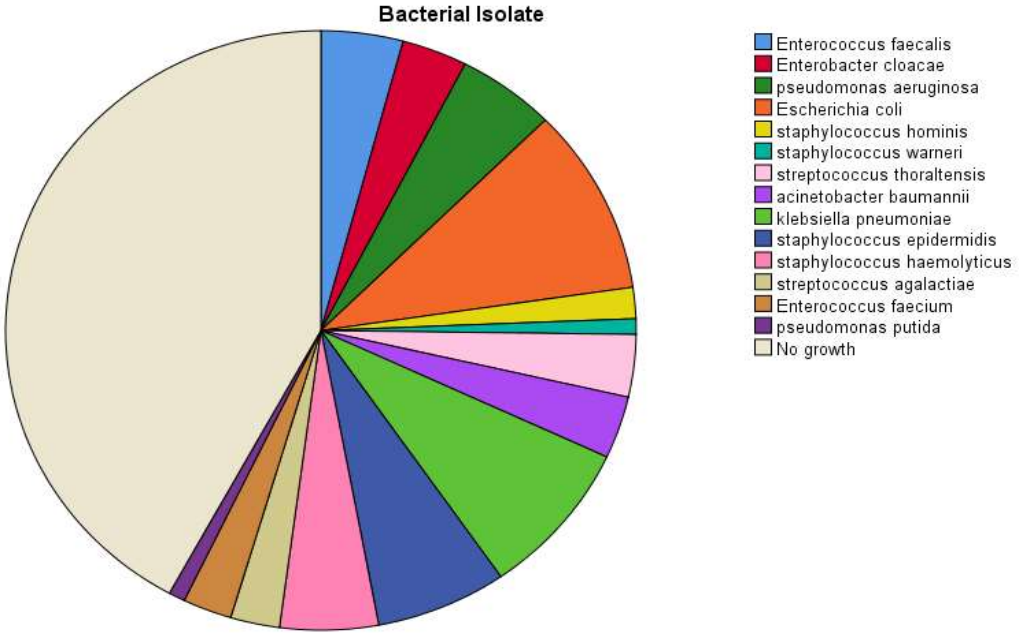

Figure 1: Distribution of bacterial isolates according to genus species and types of bacterial isolate

Table 2: MTP Method that used for biofilm production and percent of each type

Biofilm	Frequency	Percent
Non	12	17.1
Weak	12	17.1
Moderate	15	21.4
Strong	31	44.3

In this study strong biofilm formation was more dominant (44.3%) than non-producers (17.1%), weak (17.1%) and moderate (21.4%) in all patients.The bacterial vaginitis recommended treatments are wide range, by the guidelines from the CDC and the American College of Obstetricians and Gynecologists (ACOG) (24). However, the recommended treatments are still unsatisfactory, and high recurrence rates and resistance rates are frequent because the pathogenesis

process involved is not yet well understood. Biofilms are protective for the bacteria residing within as they can trap antibiotics before they reach their target, protect bacteria from the effects of the host immune system, and keep bacteria in a metabolically quiescent state induced by nutrient limitation inside the biofilm. The central role of bacterial vaginitis is attributed to its higher virulence potential and ability to form biofilms that are stronger than those formed by other bacterial vaginitis -associated bacteria (25). Thus, bacteria remains the primary pathogen of interest to study bacterial vaginitis occurrence and recurrence. Efforts have been made to explore which microorganisms participate in the formation of a polymicrobial bacterial vaginitis biofilm. Current data were in agreement with(26) study tested the ability of 10 clinical isolates of *G. vaginalis* to form biofilms; most of them were noted to be weak or moderate producers of biofilms in vitro, moreover, the MIC of some antibiotic was higher too for biofilm-forming isolates (0.099 ± 0.041 μg/mL vs. 23.7 ± 9.49 μg/mL;) the resistance rate was 27.3%, and the MBEC of clindamycin was 28.4 ± 6.50 μg/mL. Additionally in many studies have demonstrated that there was strong biofilm formation in vitro in bacterial species (27,28), although the data regarding the effects of pH and medium composition are controversial (29) found that a low pH induced strong biofilm formation (30) found that bacteria produced a greater amount of biofilm according to pH especially in 4.2. On the other hand (31). Results showed that the combined rate of biofilm formation in isolates was 87.9%, 26.3%, 26%, and 47.1% of isolates were able to create strong, moderate and weak biofilms, respectively. The distribution of UTI in male and female were 45% and 79.5%, respectively.

Table 3: MTP Method that used for biofilm production and percent of each type

Bacterial Isolate * Biofilm Crosstabulation						**Chi-Square**	**P-Value**
Bacterial Isolate	**Biofilm**				**Total**		
	Non	**Weak**	**Moderate**	**Strong**			
Enterococcus faecalis	0	1	0	4	**5 (7.1%)**	**63.039**	**0.009**
Enterobacter cloacae	1	0	1	2	**4 (5.7%)**		
pseudomonas aeruginosa	4	1	0	1	**6 (8.5%)**		
Escherichia coli	1	1	3	7	**12 (17.1%)**		
staphylococcus hominis	0	1	0	1	**2 (2.9%)**		
staphylococcus warneri	0	0	0	1	**1 (1.4%)**		
streptococcus thoraltensis	1	3	0	0	**4 (5.7%)**		

acinetobacter baumannii	2	0	0	2	**4 (5.7%)**		
klebsiella pneumoniae	0	0	4	6	**10 (14.3%)**		
staphylococcus epidermidis	1	5	2	1	**9 (12.9%)**		
staphylococcus haemolyticus	0	0	4	2	**6 (8.6%)**		
streptococcus agalactiae	1	0	0	2	**3 (4.3%)**		
Enterococcus faecium	1	0	1	1	**3 (4.3%)**		
pseudomonas putida	0	0	0	1	**1 (1.4%)**		
Total	**12 (17.1%)**	**12 (17.1%)**	**15 (21.4%)**	**31 (44.3%)**	**70 (100%)**		

Discussion

Results of this study has been showed that the high rate of bacterial biofilm formation was in recorded as 17.1%, 14.3% and 12.9% in *Escherichia coli, klebsiella pneumoniae and staphylococcus epidermidis* respectively, as they form strong biofilm.High vaginal swab bacteria in pregnant women contribute about 25% of all infections and are among the most frequent clinical bacterial infections. Pregnancy changes in women that include anatomical, physiological and hormones, current data were in agreement with(32)as the proportion of biofilm formation was more prevalent in gram-negative bacteria (73%), *Klebsiella pneumoniae* 52(37.41%), *Escherichia coli* 40(28.78%) and *Proteus mirabilis* 7(5.04%). *Staphylococcus spp* 33(23.57%) was the only gram-positive isolate formed strong biofilm. All cultures demonstrated resistance to more than one drug. Other studies showed that management of strong biofilm formation in HVS isolates has been largely empirical without the use of a urine culture and susceptibility testing to guide therapy. This practice is a risk for development of antimicrobial resistance among uropathogens. Else-where, antimicrobial resistance is a major health problem in the treatment of UTI caused by *Escherichia coli Klebsiella pneumonia*, the dominant uropathogens in pregnant women causing difficulties in treatment due to strong biofilm formation (33-35). At hospitals, it was found that 96% of pregnant women with UTI were treated empirically with 18% having extended spectrum ß-lactamases (ESBL) and 36% with multidrug resistant *Escherichia coli* strains (36).

Conclusions

According to the findings of the current study, *E. coli, K. pneumoniae,* and *S. epidermidis* were the most frequent bacteria capable of generating biofilm in women's Wasit hospitals. Also, there

was a substantial proportion of bacterial resistance to the most commonly used antibiotics; particularly, *E. coli* was the most common bacterium in biofilm development.

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