Isolation and Identification of pathogenic bacteria from women with vaginal infections

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Abstract

The bacterial biota of the human vaginal canal can have a significant impact on women and their newborns' health. Changes in the vaginal microbiota have been linked to a variety of negative health consequences, including preterm delivery, pelvic inflammatory illness, and HIV infection. New insights into bacterial diversity in this essential niche have been revealed by cultivation-independent molecular approaches, particularly in women with the common disease bacterial vaginosis (BV). Lactobacilli colonize healthy women the most, while other bacteria may be present as well. The microbiology of BV is extremely diverse. Bacteremia, urinary tract infections, intra-abdominal infections, and endocarditis are all caused by Enterococcus faecalis.

Keywords: Bacterial vaginosis, Biofilm Formation, E. faecalis, Quorum Sensing, Salicylic acid and Benzoyl peroxide.

Introduction

The vaginal environment is unusual in terms of bacterial colonization. It changes considerably over one's life as a result of developmental and hormonal changes. It is lined by stratified squamous epithelium, which regresses after maternal estrogen wears off. The vaginal flora in children is made up of skin commensals and stomach microorganisms. When a woman approaches menarche, lactobacilli take over the flora and her pH drops to around 4 (Hay, 2002).
Biofilm is a bacterial population embedded in polysaccharide intercellular adhesion (PIA), which helps bacteria stick to biological and non-biological surfaces, survive for long periods of time, cause chronic and periodic infection, and increase antibiotic resistance (Zeena, 2018). Attachment, Maturation, and Dispersion are the three steps of biofilm formation. To create a biofilm, bacteria must be close enough to the surface and stick to it. Once attached, bacteria adhere to the surface due to weak Vander wall forces between bacterial cells and the surface (Mohammed, 2021).

Biofilms are a complex structure that clings uniformly to surfaces in the presence of water. Microorganisms encase themselves in a protective mucilaginous coat to produce them. Bacteria, fungi, yeasts, and protozoa are the most common organisms that produce them. They can be found in live organisms on both hard and soft surfaces (Raghad, 2021). As a result, they fall under the category of "viscoelastic" biofilms, which have both liquid and solid qualities (similar to slug slime) (Donlan, 2002). Biofilms include dental plaque and the slimy covering that covers tanks and pipes. Van Leeuwenhoek discovered microbial biofilms by observing microorganisms with his rudimentary microscopes on tooth surfaces. Following that, several scientists investigated biofilms, most notably Heukelekian and Heller in 1940 and Zobell in 1943, but comprehensive evaluation of biofilms had to wait until the introduction of the electron microscope, which allowed highly-resolution photo microscopy at far higher amplifications than light microscopy (Kreth et al., 2008).

Common Genital Infections

Vaginitis is one of the most common medical disorders among women, and acottect diagnosis is necessary for effective treatment and recurrence prevention (Kent, 1991). According to Kuar et al., (2008), 25-50% of those infected with the virus develop a variety of clinical symptoms, ranging from asymptomatic to severe inflammatory symptoms. Infected females get vulvovaginitis, cervicitis, and urethritis as symptoms of infection. According to many research, there is no link between the use of various contraceptive techniques and recurrent vaginal infections (Valiani et al., 2011; Akbarzadeh et al., 2010).

The most frequent illness in women of reproductive age is bacterial vaginosis (Gingelmaier and Verner et al., 2016). Bacterial vaginosis is the invasion of the vaginal cavity by
aerobic bacteria. It occurs when the vaginal ecology is disrupted, with a significant loss of typical bacterial flora (Lactobacilli) and an increase in anaerobic microorganisms (Nobel et al 2004; Demoba et al 2005; Lakshmi et al 2012).

The species Enterococcus faecalis was the most frequently related with the presence of all three indications of bacterial vaginosis, including pH>4, altered color of vaginal discharge, and a positive amino smelling test (Bubu et al 2017; Romanik et al 2007). E. faecalis was previously classified as part of the Streptococcus genus and was known as Streptococcus faecalis until 1984.

The quorum sensing (QS) system is a rapidly evolving topic in which we are gradually learning more about how bacteria interact and regulate their activities in bacterial sociality. QS-related autoinducers may be entrenched in the crosstalk between host and parasite microorganisms, in addition to collectively influencing bacterial behavior (Liang, 2021). QS is a cell-to-cell communication system that allows bacteria to sense their surroundings and regulate their density and behavior, allowing them to exist in multicellular environments. They use their community to manage self-competition and communicate with their host collectively (Bassler and Losick, 2006).

In 1970, Kenneth Nealson, Terry Platt, and J. Woodland Hastings discovered quorum sensing when they saw "conditioning" of the media in which they had grown the bioluminescent marine bacteria Aliivibrio fischeri (Nealson et al., 1970). (von Bodman and colleagues, 2003).

Materials and Methods

In this prospective study, 80 married women of reproductive age were included, with ages ranging from (15-60) years. Swabs from patients in general hospitals were obtained and transferred to the laboratory in septic conditions. The swabs were inoculated on nutritional agar, blood agar, mannitol salt agar, and MacConkey agar, and incubated at 37°C for 24 hours. The colony morphology was determined based on the size, height, and form of the colonies (Verner et al., 2016; Mancuso et al., 2015) and the presence of biochemical tests. It should be
mentioned that all patients in this study (vaginal infections) were selected depending on their history and clinical examination by specialist doctors. Samples that taken from vagina women were collected from deep parts of the vagina by a cotton sterile media swab. Then all samples were transported to a microbiological laboratory for isolation, identification of bacteria and for further investigations (Tahrir, 2020). All cultures media (Nutrient agar, Mannitol salt agar, Blood agar, MacConkey agar, Brain heart infusion broth, and Lauryl broth) were prepared according to the instructions of the companies and autoclaved at 121°C for 15 min (Zeena, 2018).

The ability of bacteria to produce biofilms was tested using the Congo Red Agar (CRA) method. Sucrose 50g/L and congo red 0.8g/L dye were added to Brain Heart Infusion agar to make the medium for this assay. The bacteria were streaked on congo red agar medium and incubated at 37 degrees Celsius for 24 hours (Raghad, 2020).

Results

Eighty samples were collected from vaginal infections women during November 2020 to March 2021. All samples were cultured on nutrient agar, blood base agar, mannitol salt agar, and MacConcy agar medium with duplicate then sub-culture on nutrient agar medium. Out of 80 samples, 32 (40%) were Gram positive (pure culture), whereas 48 (60%) were Gram negative (pure culture). BV was discovered in 80 women between the ages of (15 -60) (89 %). As indicated in Table (1), statistical analysis revealed a significant difference in the purity of bacterial isolates from vaginal infections.

Table (1): The number and percentage of bacterial isolates from vaginal infections

<table>
<thead>
<tr>
<th>Sex</th>
<th>Total No. of samples</th>
<th>Gram-positive</th>
<th>Gram-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>80</td>
<td>32(40%)</td>
<td>48(60%)</td>
</tr>
</tbody>
</table>

Morphological, Physiological and Biochemical tests of *E. Faecalis*, *E. faecium*, and *E. coli* isolates
Summary of the main characteristics of the isolates of *E. faecalis*, *E. faecium*, and *E. coli* species are shown in (Table 2) as revealed by Gram staining, growth and biochemical tests.

**Table (2):** Results of morphological, physiological and biochemical tests of *E. faecalis, E. faecium*, and *E. coli* spp isolates.

<table>
<thead>
<tr>
<th>Pattern of pathogen</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Coagulase</th>
<th>Mannitol</th>
<th>NaCl</th>
<th>Starch</th>
<th>Gelatin</th>
<th>Citrate</th>
<th>Gram Staining</th>
<th>Hemolysis</th>
<th>Casain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>variable</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>-</td>
<td>-</td>
<td>variable</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>variable</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

**Effect of Salicylic acid and Benzoyl Peroxide on biofilm formation of *E. faecalis* and *E. coli***

The antibacterial activity of SA and BP were examined against isolates of *E. faecalis* and *E. coli*. MIC assay results indicated that SA and BP concentrations 50 mM inhibited bacterial growth and biofilm of *E. coli* in all tubes when viewed with naked eye and spectrophotometer reader while 100Mm of SA and BP inhibited bacterial growth and biofilm of *E. faecalis* (Figure 1, 2, 3, and 4). The medicinal properties of salicylic acid have been known since ancient times. There are studies on the inhibition of planktonic cell growth with SA demonstrated significant antimicrobial properties (P value<0.01).
**Figure (1):** Effect of different concentration of SA and BP on growth of *E. faecalis*

**Figure (2):** Effect of different concentration of SA and BP on growth of *E.coli*
Figure (3): Effect of different concentration of SA and BP on biofilm production of *E. faecalis*.

Figure (4): Effect of different concentration of SA and BP on biofilm production of *E. coli*.

**Identification of Biofilm forming pathogenic bacteria**

A- **Tube method**
Tube method was used as a qualitative assay for detection the biofilm forming bacteria isolated from vaginal infection. The results showed that 10 (12.5%) isolates were strong biofilm former, 20 (25%) moderate, while 50(62.5%) isolates were weak or non-biofilm forming, as shown in figure (5).

**Figure (5):** Biofilm formation of pathogenic bacteria by using Tube method (TM) 1: Indicates to non-biofilm forming, 2: moderate biofilm forming, 3: strong biofilm forming of pathogenic bacteria isolated from vaginal infection

### B- Congo Red Agar method

Congo red agar method used for detection the biofilm forming bacteria. The results showed that 15 (12.5%) isolates were strong biofilm by giving black colonies, 20 (25%) moderate that give pink color colonies and 45(56.25%) weak or non-biofilm that give red color as shown in **table (3) and figure(6).**

**Table (3):** Grading of biofilm formation by different methods

<table>
<thead>
<tr>
<th>Biofilm formation</th>
<th>CRA</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>15 (18.75%)</td>
<td>10 (12.5%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>20 (25%)</td>
<td>20 (25%)</td>
</tr>
<tr>
<td>Non-biofilm</td>
<td>45 (56.25%)</td>
<td>50 (62.5%)</td>
</tr>
</tbody>
</table>
Figure (6): Strong biofilm formation of *E. Faecalis* by using Congo red agar method (CRA).

**Molecular identification**

**A- DNA extraction of *E. faecalis***

Complete genomic DNA was extracted according to the kit technique. Electrophoresis on a 1% agarose gel revealed the presence of DNA. Photo seen under UV gel document, figure (7).
Figure (7): Genomic DNA of *E. faecalis*

**Detection of luxS gene by PCR technique of *E. faecalis***

PCR was used to amplify the *luxS* gene of *E. faecalis* from the extracted DNA. By comparing the band of each amplified *luxS* gene to a typical molecular DNA ladder (2000bp), the band of each amplified *luxS* gene was described in 203 bp (Figure 8).

**Figure (8):** Patterns of 1% of agarose gel electrophoresis reveal DNA bands of *E. faecalis* 1,2,3,4,5, DNA bands, L DNA ladder. Figure on the left shown DNA ladder bands used in the study from Bioneer company in Korea.

**Discussion**
Between November 2020 and March 2021, 80 swabs from women with vaginal infections were collected for the study. They care for children and mothers at Abi Alkhasib General Hospital and Basrah Hospital in Iraq. This quantity of isolates was satisfied due to the funding support and the short study time.

*Enterococcus faecalis* which were identified depending on biochemical characteristics, Sujata et al. (2016) found that *E. faecalis* accounts for 20% of vaginal infection, while Ghasemie et al. (2016) found that 8.14 % of women with vaginal discharge had *Enterococci*. *E. faecalis* accounted for 89.95% of the isolates, and *Enterococci* were found in the human intestine and can be transmitted to the vagina in low-income communities socioeconomic status (Ghasemi et al., 2016).

According to Jacobsen et al., (2008), bacterial vaginosis is the most common type of infection with bacterial vaginosis, the highest rate of infection was seen among women aged 30-40 years (8.8%), while the lowest rate was seen among women aged 10-20 and 50-60 years (1.3%), while the highest rate of infection was seen among women aged 31-40 years , and the rate of infection was low below 20 years, which could be due to high sexual exposure prevalence of bacterial vaginosis and its relationship to non-pregnant women's risk factors.

*Enterococcus* species was diagnosed on nutrient agar, mannitol salt agar and blood base agar, based on the appearance, where it was appear as fermentation process. The color of the medium changed from red to yellow. It found negative results for both catalase and oxidase tests, indicating that the bacteria do not have the enzyme cytochrome oxidase, which is a feature of *E. faecalis*, *E. faecium*. All the *E. coli* isolates were found to produce bright pink colonies on MacConkey agar is a selective and differential culture medium for bacteria. It is designed to selectively isolate Gram-negative bacteria and differentiate them based on lactose fermentation. Lactose fermenters turn red or pink on McConkey agar, and non-fermenters do not change color. The media inhibits growth of Gram-positive organisms with crystal violet and bile salts, allowing for the selection and isolation of gram-negative bacteria (Gupta et al., 2013).

Two approaches were employed to detect biofilm-forming bacteria among pathogenic bacteria isolated from vaginal infection in the current study. The Congo red agar method and
the tube method are both qualitative approaches. The present investigation found that 35 of
the vaginal isolates were positive "strong slime producers" toward CRA, which is a simple,
quick, and reliable laboratory approach for determining potential (Jain and Agarwal, 2009).
According to Kaiser et al., (2012), the CRA has a number of advantages over other approaches,
including the fact that it is inexpensive, rapid, and easy to apply, even in small laboratories. To
induce bacteria to make LPS, sugar was added to the medium, and Congo red was used to look
for exopolysaccharide in bacteria (Freeman et al., 1989).

When viewed with the naked eye and spectrophotometer reader, SA and BP at
concentrations of 50mM inhibited bacterial growth and biofilm of E. coli in all tubes, while
100mM of SA and BP inhibited bacterial growth and biofilm of E. faecalis when compared to
control, whereas Lattab et al., (2017) indicated that SA concentrations of 100 mM inhibited
strains growth in all tubes because of SA potential (Chow et al.,2011; Rosenberg et al.,2008).
Furthermore, SA has been shown to limit bacterial aggregation and surface attachment by
inhibiting the formation of teichoic acid and slime-associated proteins in wild and
polysaccharide adhesions deficient bacteria (Muller et al., 1998).

According to Landini et al., (2010), the SA medicinal qualities have been reduced of
planktonic cell growth has been shown to have strong antibacterial effects and biofilm
inhibition is that the SA influences quorum sensing (QS), also known as cell to cell
communication, which is necessary for individual cell development into mature biofilms (Davies
et al., 1998).

The antibacterial activity of benzyol peroxide was investigated in this study, and it was
found to suppress microbial growth and biofilm formation. When compared to tetracycline and
minocycline, BP demonstrated equal activity, giving it a viable option for cost-effective
treatment (Bojar et al., 1995).

The LuxS gene specific for E.faecalis was used to identify a quorum sensing gene. The
LuxS gene was found in all E.faecalis isolates in the current study. This suggests that the isolates
can detect and respond to cell population density through gene regulation using quorum
sensing or quorum signaling (Shao et al., 2012).
Conclusion

We can conclude from this research that Gram-negative and Gram-positive bacteria were isolated from vaginal infections women. In addition, there is a high rate of vaginal infections at the age group 21-35 years. Furthermore Some bacterial isolates have the ability to form biofilm. *E. faecalis* was regarded as a significant biofilm formation. We can also conclude that SA and BP inhibit bacterial growth depending on concentrations, and we can also retain that SA and BP decreased biofilm formation. Lastly, Homolog of luxS which encodes the protein responsible for AI-2 production has been found in a *E. faecalis*,AI-2 plays a key role in *E. faecalis* biofilm development.

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