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Molecular Diagnosis of Vaginal Microbiota Associated with Spontaneous Abortion in Women

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Abstract

Spontaneous abortion (SA) is a sever disease in which a women losses her foetus usually before 24 weeks of the pregnancy. About 10% -50% of pregnancy cases are run out with SA for reasons related with women's age and health. This condition occurrence may increases if the patient has a history of bacterial vaginosis. This study aimed at the characterization and isolation of vaginal microbiota generally and highlight on commensal bacteria becoming opportunistic when circumstances are favourable. From October to December 2022, about 100 samples were collected, 50 specimens from women with SA and 50 specimens from healthy pregnant outcomes (control). The high vaginal swabs samples were collected from Babylon Teaching Hospital for Maternity and Children and Imam Sadiq Teaching Hospital in Babylon, Iraq. To culture the samples, they were transported by media swab to laboratory. The culture of bacteria was done on 4 types of agar media: UTI chromogenic agar, MRS agar, MacConkey agar and blood agar. The first identification of bacteria was based on phenotypic traits of colonies, using manual biochemical tests and gram stain. Finally, the diagnosis was confirmed genetically by extracting bacterial genomic DNA for 20 of bacterial isolates under study and using PCR technique for 16S rRNA Loci gene and sequencing. The current study results showed difference in bacterial genera in women with SA compared with healthy women. It was also noted that embroilment of *Enterococcus faecalis* occurred in most cases of SA with an estimated percentage of 56% (28/50), thus defeating *Escherichia coli* by 32% (16/50) and 4% (2/50) for *Klebsiella pneumonia* and 4% (2/50) for *Enterococcus gallinarum.* In this study, some very rare bacteria species were identified including *Acinetobacter junii* at 2% (1/50) and *Corynebacterium coyleae* at 2% (1/50). While the percentage of bacteria associated with healthy women was: 30% (15/50) for *E. faecalis*, 26% (13/50) for *E. coli*, 18% (9/50) for *K. pneumonia*, 24% (12/50) for *Staphylococcus epidermidis*, and 2% (1/50) for *Metabacillus niabensis* (which was diagnosed for the first time in Iraq as well as the rest of the world in a clinical sample).

Keywords: Spontaneous abortion, Vaginal microbiota, Bacterial vaginosis, 16S rRNA gene sequencing.

التشخيص الجزيئي للأحياء المجهرية المهبلية التي ترتبط مع الإجهاض التلقائي عند النساء

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الخلاصة

الإجهاض التلقائي (SA) هو مرض خطير حيث تفقد المرأة جنينها عادة قبل الاسبوع الرابع والعشرين من الحمل . تقريبا 10% _50% من حالات الحمل تنتهي بإجهاض تلقائي لأسباب تتعلق بعمر المرأة وصحتها. حدوث هذه الحالة ربما يزداد اذا كانت المريضة مشخصة مسبقا بالتهاب المهبل البكتيري. هدفت هذه الدراسة الى توصيف وعزل البكتريا المهبلية بصورة عامة وتسليط الضوء على البكتريا المتعايشة عندما تصبح انتهازية حين تتهيأ لها الظروف. خلال الفترة من تشرين الاول الى كانون الاول 2022 تم جمع حوالي 100 عينة 50, عينة

من نساء لديهن اجهاض تلقائي و 50 عينة نساء لديهن نتائج صحية للحمل (مجموعة السيطرة) . تم جمع العينات من مستشفى بابل التعليمي للولادة والأطفال ومستشفى الامام الصادق التعليمي في بابل / العراق. كانت العينات عبارة عن مسحات مهبلية عالية (HVS), تم نقل المسحات المهبلية العالية بواسطة وسائط المسحة الى المختبر للزراعة بعد ذلك. بعد زراعة البكتيريا على 4 أنواع من وسائط أغار وهي أغار التهاب القناة البولية الكروموجيني ، MRS اغار، اغار ماكونكي و اغار الدم اعتمد التحديد الأول للبكتيريا على السمات المظهرية للمستعمرات باستخدام الاختبارات البيو كيميائية اليدوية وصبغة غرام. في النهاية، تم تأكيد التشخيص وراثيًا عن طريق استخراج DNA الجينوم البكتيري لـ 20 عزلة بكتيرية قيد الدراسة وتقنية polymerase chain reactionللجين 16S rRNA Loci وتسلسلها. اظهرت نتائج الدراسة الحالية الاختلاف في الأجناس البكتيرية لدى النساء المصابات بـ SA مقارنة بالنساء السليمات ، وقد لوحظ أن الإصابة ب *Enterococcus faecalis* حدثت في معظم حالات SA بنسبة تقديرية بلغت56٪ (50/28) ، وبذلك تتغلب على *Escherichia coli* *بنسبة* 32٪ (50/16) و 4٪ (50/2) *Klebsiella pneumonia*و 4٪ (50/2) لـ *Enterococcus gallinarum*. في هذه الدراسة، تم التعرف على أنواع بكتيريا نادرة ، بما في ذلك *Acinetobacter junii* بنسبة 2٪ (50/1) و *Corynebacterium coyleae* بنسبة 2٪ (50/1) ، بينما كانت النسبة المئوية للبكتيريا المصاحبة للمرأة السليمة: 30٪ (50/15) *Enterococcus faecalis* ، 26٪ (50/13) *Escherichia coli*، 18٪ (50/9) *Klebsilla pneumonia*، 24٪ (50/12) *Staphylococcus epidermidis*، و 2٪ (50/1) *Metabacillus niabensis*الذي تم تشخيصه لأول مرة في العراق وكذلك في بقية العالم في عينة سريرية.

1. Introduction

One of the most difficult issues in the branch of fertility and obstetrics is spontaneous abortion (SA). It is one of the most serious problems associated with infertility, with a possibility of adverse health effect. SA is a severe disease characterized by multiple pregnancy losses [1, 2]. The causes of SA are unknown, making diagnosis and treatment difficult. Abortions leave many childbearing couples unable to have babies of their own, a situation that has detrimental effects on their own households and society as a whole [3]. SA is influenced by a variety of factors including chromosomes, hereditary factors, dissection of the endocrine glands, anomalies of the placenta, an infection, and the immune system, thrombosis, and the environment, to name a few [4]. Vaginal flora serves as the body's first line of defense against invading harmful germs and aids in maintaining the dynamic balance and mutual restriction of dependency [5]. Role of the vaginal microbiome is another unidentified aspect of SA. In many studies, the researchers have compared the prevalent bacterial species to bacterial isolates from healthy patients to learn how each of them altered the environment in the vagina. A healthy vaginal microbiota influences immunological responses to the invading pathogens and boosts host immunity by producing substances including bacteriocins, hydrogen peroxide, and biosurfactants [6]. Women may become particularly susceptible to infection if there is a difference in the vaginal microbiota's makeup and activity. In fact, the presence of Atopobium vaginae increases the production of inflammatory cytokines such as tumor necrosis factor, interleukin (IL)-1, IL-6, and IL-8, all of which may affect the performance of the innate mucosal barrier [7].

The vaginal flora of a woman evolves throughout the course of her lifetime and may altered its components by age, pregnancy, hormone-driven instability, sexual relationship, usage of probiotics and antibiotics, and other medications, which might result in an imbalance [8]. The microenvironment of a healthy vaginal flora is predominated by *Lactobacillus* species, specifically *Lactobacillus jensenii*, *Lactobacillus crispatus, Lactobacillus iners* and *Lactobacillus gasseri*. Although the last two types of *Lactobacilli* are considered causative agents of primary infection complications after probiotic treatments due to their opportunistic susceptibility, especially in immunocompetent people. A healthy female genital tract contains a microbiome populated by lactic acid and hydrogen peroxide producing bacteria that try to maintain a low pH to protect against infections, despite all of these communities of microbes are dominated by *Lactobacillus* and are relatively simple when compared to the gut microbiome [8, 9].

Bacterial vaginosis (BV), which is characterized microbiologically by an excess of anaerobic bacteria and/or a decrease in the quantity of Lactobacillus species, may result from a change in the bacterial composition of the vagina [9]. Nugent scoring system was used to determine if there was an increase or a reduction in the amount of *Lactobacillus* spp. and Lactobacillus morphotype [10]. BV, also known as vaginal dysbiosis, is a common vaginal condition characterized by abnormal alteration in the vaginal microbiome (VMB) and an increased incidence of anaerobic bacteria [11]. Recent studies have related vaginal dysbacteriosis or the vaginal microbiome (VMB) to gynecological cancer, gestational diabetes, complications during pregnancy, and preterm birth [12]. As BV is linked to negative the outcome of reproductive health like pelvic inflammation disease, SA, and period delivery, it is a significant health concerns affecting women of fertility age, their children, and associates [11]. Age, socioeconomic status, using antibiotics, sexual activity, and ethnicity have all been identified as risk factors in the pathogenicity of BV. Numerous bacterial species have been isolated and described according to culture techniques, by using high vaginal swab (HVS), a medical procedure performed in obstetrics and gynecology to test vaginal discharge for the presence of BV.
Whilemolecular techniques have shown the limitations of culture by demonstrating that the va gina was an intricate ecosystem with a variety of uncultured or hard-to-identify bacteria [2, 11, 13]. Understanding this vaginal condition and the host-microbiota interactions requires knowledge of vaginal microbiota [8]. This study aimed at the characterization and isolation of vaginal microbiota generally and highlight on commensal bacteria that becomes opportunistic when circumstances are favourable.

2. Material and Methods

2.1 Study Sitting and Duration

This study was conducted between October to December 2022 in the College of Science for Women at the University of Babylon / Iraq.

2.2 Patients and Specimen's Collection

The present study was done on 100 (HVS) from women (50 patients with SA and 50 with a healthy pregnancy outcome). The patients and controls were all between the ages of 17 and 45 years. These specimens were taken from women who attended the Babylon Teaching Hospital for Maternity and Children and the Imam Sadiq Teaching Hospital in Babylon, Iraq, by a specialist physician.
Patients were the women who had SA from the first weeks of pregnancy until the sixth month, when an ultrasound scan confirmed the death of the fetus. Using a speculum and HVS,

samples were taken from the vagina in the vicinity of the cervix without interfering with the external reproductive system or urine. Women who had an induced abortion or an ectopic pregnancy were excluded from this study. HVS was kept at 4°C until it was time to cultivate it, at which point it was delivered to the lab to be cultured on several types of agars.
Controls included the women who completed their pregnancies by natural delivery or caesarean section.

2.3 Bacterial Isolation and Identification

The HVS were cultured on different agar media, including urinary tract infection chromogenic agar (UTIC) (Condalab, Spain), deMan, Rogosa, Sharpe (MRS) (Hi media, India), blood and chocolate agar (Hi media, India), MacConkey agar (Hi media, India) and Mannitole salt agar (Hi media, India).

All the above media were prepared according to the instructions supplied by the manufacturing companies. The media inoculated with specimens were incubated aerobically and anaerobically at 37 °C for 24 hours. The primary diagnosis of bacterial isolates depended on the morphological characters of colonies like hemolysis presence, margin shape, size, color, etc. Microscopic examination by using Gram stain in addition to the classical biochemical tests like catalase and oxidase tests and mannitole salt fermentation test.

2.4 Genetic Study

2.4.1 Genomic DNA Extraction and PCR Amplification

A single colony of 20 types of bacteria under interest isolates (*E.faecalis, E.coli, K.pneumonia, S.epidermidis, E.gallinarum, A.junii C. coyleae* and *M.niabensis*) was inoculated in nutrient agar (Hi media, India) and incubated at 37°C for 24 hours to obtained new and fresh colony.
Bacterial genomic DNA was extracted according to the guidelines provided by the manufactured scientific institutions (Favrogene Kit). Primers were dissolved and prepared according to the manufacturer's instructions (Biotech Corp., Pingtung, Taiwan) [14]. This primer is a universal primer designed to target specific region in this gene and to amplify 938 bp of 16S rRNA gene for all types of bacteria. The forward primer sequence, 357F (5-AGAGTTTGATCCTGGCTCAG -3) and Reverse primer sequence 806R (CCGTCAATTCCTTTGAGTTT). AccuPower PCR premix was used to perform the PCR reaction (Bioneer, Daejeon, South Korea). One U of Taq DNA polymerase, 250 µM of dNTPs, 10 µM of Tris-HCl (pH 9.0), 30 µM of KCl, and 1.5 µM of $MgCl_2$ were all in every 20 µl of the PCR premix. Fifty ng of genomic DNA and ten pmol of each primer were added to the reaction mixture [15]. The thermal cycler for PCR was used to carry out the following program. The first phase in the amplification process was the initial denaturation, which took place at 94 °C for 5 min. Next, there were 30 denaturation cycles, annealing, and elongation at 61 °C for 1 min and at 72 °C, followed by a final extension at 72 °C for 10 min (18), 1% (w/v) agarose gel melted in TBE buffer (1 X), ethidium bromide pre-staining (0.5 g/ml), and samples electrophoresis the for 45 minutes at 100 volts were applied to test for amplification. By selecting a 938-bp ladder from the 1–19 16S rRNA PCR output as a molecular size marker (Bioneer, South Korea), the accuracy and presence of a single, pure, and distinct band in each of the PCR-fixed bands was verified to be successfully exposed to sequencing [14, 15].

2.4.2 DNA Amplicon Sequencing for PCR

The clarified PCR was done for 20 bacterial isolates (amplicons were commercially sequenced from the termini, forward, and reverse, as per instructions from the sequencing company (Macrogen Inc., Geumchen, Seoul, South Korea). Only translucent chromatographs from ABI sequence databases were further investigated to ensure that the annotations and

discrepancies were not the result of PCR or artifact sequencing. Virtual positions and other details of the PCR pieces acquired were determined by comparing the samples' observed nucleic acid sequences to the bacterial database conserved standard sequences [15, 16].

2.4.3 Analysis of Sequencing Data

The sequencing outcomes of the PCR products of various samples were modified, coordinated, and assessed with "Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA)". Each sequenced sample's PCR amplicons was numbered, as were the alterations that were found and where in the referring genome they were found [15].

2.4.4 Design of a Comprehensive Phylogenetic Tree

A specific and comprehensive tree was built in this study using the neighbor-joining Mega-6 procedure as described by the NCBI-BLAST, server was used to compare the detected variations to their neighbors' homologous reference sequences. After that, a fully inclusive tree was created using the neighbor-joining strategy and aligned with the observed version of the *E. faecalis* strain as well as the reference strain KP317676.1 [14].

3. Results

3.1 Bacterial Isolation and Identification

The primary diagnosis of bacterial isolates which depended on the color changes in UTIC agar in addition to microscopic and morphological characterization, was supported by a genetic study using 16S rRNA PCR and sequencing. The current study results showed a difference in bacterial genera in women with SA compared with healthy women, and it was noted that embroilment of *Enterococcus faecalis* occurred in most cases of SA with an estimated percentage of 56% (28/50), thus defeating *Escherichia coli by* 32% (16/50) and 4% (2/50) for *Klebsiella pneumonia* and 4% (2/50) for *Enterococcus gallinarum.* In this study, very rare bacteria species were identified, including *Acinetobacter junii* at 2% (1/50) and *Corynebacterium coyleae* at 2% (1/50). While the percentage of bacteria associated with healthy women was: 30% (15/50) for *E. faecalis*, 26% (13/50) for *E. coli,* 18% (9/50) for *K. pneumonia, 24*% (12/50) for *Staphylococcus epidermidis*, and 2% (1/50) for *Metabacillus niabensis* (Figure 1).

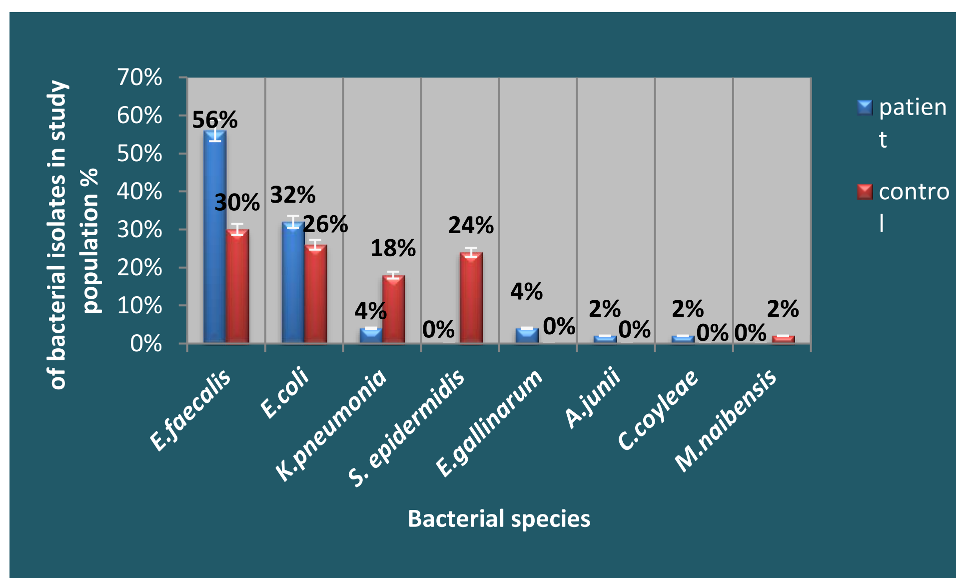

Figure.1: Type and percentage of bacterial isolates in SA and control.

3.2 Genotyping Assay

3.2.1 PCR Amplification of 16S rRNA Gene and Sequencing

The local sample was included by using a locus-specific primer (16S rRNA) to generate amplicons of approximately 938 base pairs in length. The 938 bp region of the 16S rRNA gene was amplified by PCR using the isolated bacterial genomic DNA as a template. Each ribosomal amplicon was checked to make sure it had distinct bands before it was sent for sequencing. Prior to sending these ribosomal amplicons for sequencing, it was ensured that every amplified amplicon displayed clear, distinct and crisp bands, (Figure 2).

Figure 2: Illustration gel electrophoresis profile of PCR products (938bp) for 20 type of bacteria.

By using NCBI BLAST, the sequencing reactions demonstrated and confirmed the amplified products' identification. The sequenced samples and *E . faecalis* (The accession number is NZ_KB944666.1), *E. coli (The accession number is NC_002695.2)*, *C. coyleae* (accession number NZ_FNRU01000002.1) , *A. junii* (The accession number NZ_CP059558.1), *E. gallinarum* (accession number NZ_KZ846567.1), *K. pneumonia* (accession number NC_016845.1) , *M. niabensis* (accession number MW559669.1) , and *S. epidermidis* (accession number NZ_CP035288.1) sequences displayed complete sequences of similarities, according to the NCBI BLAST search engine with regard to the 938-bp PCR amplicons of the ribosomal gene. The confirmed identification of the amplified products was shown by the sequencing reactions using NCBI BLAST. Regarding 938-bp PCR amplicons of the ribosomal gene, the sequenced samples and their targets showed complete sequences of similarities, according to analysis by the NCBI BLASTN search engine [15].

Nucleotide composition analysis was compared to those derived using the more standard alignment-based method. The 16-S ribosomal RNA (rRNA) region was covered by approximately 97% of the predicted target, according to the NCBI BLASTN engine. Through contrasting the returned DNA sequences with the recognized DNA sequences of the samples being tested (Gene Bank accession number KP317676.1), as shown in Figure 3, it was possible to determine the precise positions and other details of the retrieved PCR fragments.

3.2.2 DNA Sequencing for Enterococcus faecalis

The sequencing result of *E. faecalis,* which has 99.35% compatibility with the subject of *E. faecalis* in NCBI under accession number NZ_KB944666.1, showed 1 transition (A/G) and 2 transversions (G/T and T/G) when compared Query with Subject, as shown in Figure 3. Query represents DNA of the samples, while Subject represents DNA of the NCBI database.

Figure 3: Alignment analysis of *E. faecalis* with gene bank at NC

The other bacterial isolates were also compared to subject of NCBI in the same method.

3.2.3 DNA Sequencing for Metabacillus niabensis

The sequencing results of *M. niabensis,* which has 89% compatibility with the subject of *M. niabensis* in NCBI under accession number MW559669.1, showed that there, were a large number of transitions and transversions when Query was compared with Subject, Figure 4.

Figure 4: Alignment analysis of M. niabensis with gene bank at NCBI

3.2.4 A Phylogenetic Tree Based on the Sequence of 16S rRNA

In the current investigation, 16S rRNA PCR amplicons was used to construct an accurate phylogenetic tree. The S1–S20 samples and additional related DNA sequences were used to construct this phylogenetic tree. Several species were included in this tree. Close relatives of those species are also shown in Figure 5.

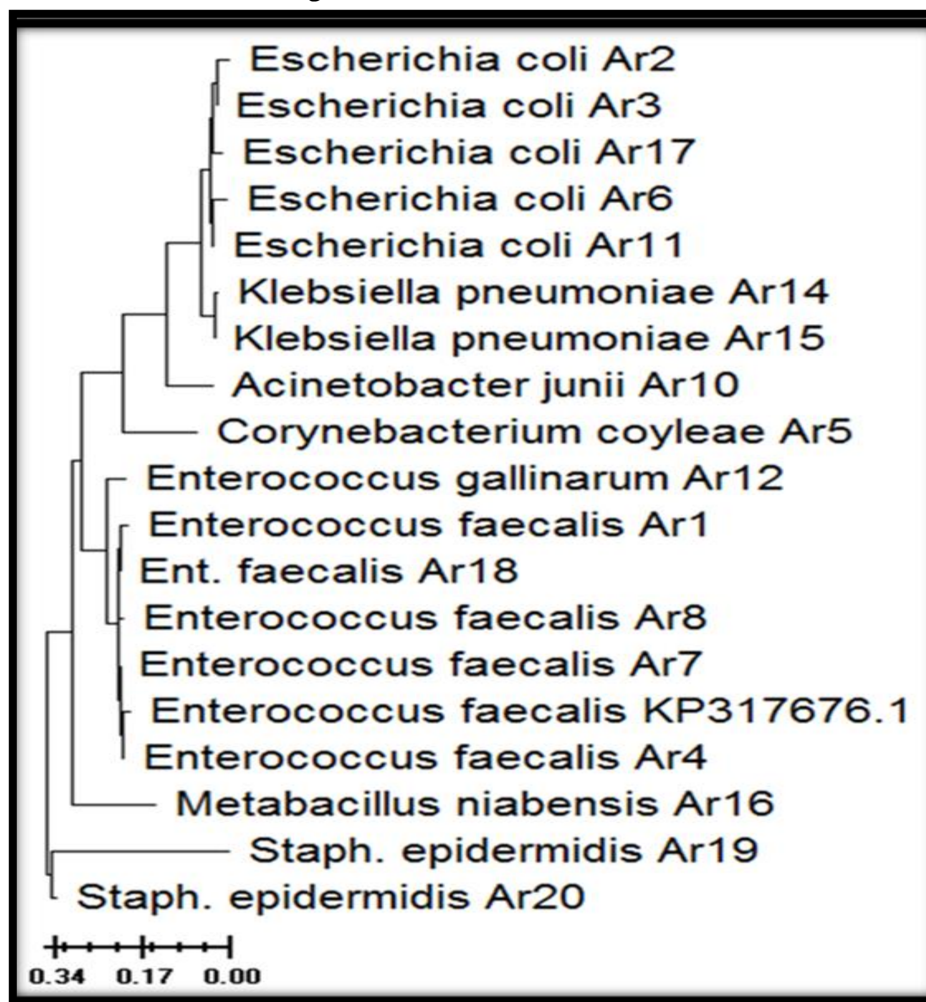

Figure 5: Construction Phylogeny tree of bacterial species and alignment with reference strain based on Neighbor-Joining tree Mega 6 software

This 16S rRNA-based comprehensive tree has provided an extremely inclusive tool about the high ability of such genetic fragments to efficiently identify these bacterial samples using the genetic fragments. Interestingly, this phylogenetic analysis observed the highest species detection specificity with regard to 16S rRNA-based PCR. These phylogenetic protocols in turn give a further indication of the power of currently utilized 16S rRNA specific primers to discriminate among the currently investigated strains.

4. Discussion

The essential initial step in the effective establishment of infection is frequently bacterial adhesion to the surfaces of the host cells. Bacterial pathology is caused by pathogenic bacteria that may penetrate the tissue after adherence and access target cells [16]. Additionally, cellular invasion is regarded as a key virulence component since it gives pathogens a means of circumventing immune system of the host, causing tissue harm. Fimbriae of various species are known to play an important role in bacterial adhesion to cellular surfaces due to their ability to detect a variety of membrane cell receptors such as integrins, cadherins,

selectins, and carcinoembryonic antigen-related adhesion molecules, all of which are involved in mediating bacterial invasion [17]. This is normally true, but when pathogenic bacteria are present in the vagina of pregnant women, particularly in the second trimester, it causes severe inflammation results in the pregnancy sac to burst and break before any discomfort or bleeding occurs. SA has been most frequently linked to bacterial infections such as Enterococcus faecalis, Escherichia coli, and Klebsiella pneumonia. Early pregnant women who have BV are more likely to have an abortion [17, 18]. In most cases, bacteria are not present in amniotic fluid. Yet, there are a variety of ways that bacteria may enter the amniotic cavity, with an ascending pathway from the lower genital tract being the most common. A local inflammatory response may be triggered by bacteria in the amniotic cavity that can be found using molecular microbiologic techniques or culturing. This condition is known as intra-amniotic infection. There is strong evidence connecting intra amniotic infection to unfavorable pregnancy complications, including spontaneous preterm labor and delivery, the onset of acute histologic chorioamnionitis and funisitis, and a fetal inflammatory response [19].

Enterococcus faecalis was found to be superior to all other species of identified bacteria in this research, and according to the molecular results from a recent study .Although *Enterococcus faecalis* is a common opportunistic pathogen [20], but because of the increase in resistance to antibiotics, the vital role of enterococci as an infection-causing agent has grown in some strains as well as their known pathogenic potential .*E. faecalis* can persist in critical settings with insufficient nutrition provided and an elevated pH level ranging up to 11.5, as it has demonstrated good adaptation to such ecosystems with rich nutrients, low levels of oxygen and a complex habitat. It is necessary for enterococci to have the capacity to colonize the host's tissues, overcome non-specific and immunological defense mechanisms and induce pathogenic effects [21]. Adherence tests have demonstrated that enterococci can colonize host tissues by attaching to intestinal and urinary tract epithelial cells, as well as heart cells, via adhesins that are produced on the bacterial surface [21, 22]. *E. faecalis* is the most frequently isolated pathogen in cases of aerobic vaginosis (32%) [22]. The pathogenic effects of aerobic microorganisms such as *E. faecalis* have been shown to cause spontaneous abortion [22, 23, 24].
Escherichia coli was the other bacterial species isolated from SA women. *E. coli* is one of the primary etiological agents in instances of aerobic vaginitis. It has been demonstrated that due to the physical proximity of the anorectic/vaginal region, some enterobacteria can operate as uropathogens and can be related to cases of bacterial vaginosis [25]. The presence of greater *E. coli* colonization in the vaginal introitus (>105 CFU/mL) in women with a history of UTI emphasizes the significance of the vaginal milieu in the pathophysiology of recurrent UTI [26]. BV during pregnancy can exacerbate. A local study by al Juber and Hammoudi on bacterial vaginosis in pregnant women found that 14.8% of *K. pneumonia* and *Klebsiella spp.,* implicated in both AV and BV [27]. BV and AV are vaginal dysbioses characterized by a reduction in lactobacilli and are associated with chorioamnionitis, preterm delivery, spontaneous abortion and low birth weight [13,28].

Similar studies have shown that pregnant women with gestational diabetes have a higher rate of infection than pregnant women without the condition. When bacteria were isolated from healthy women and from women who had genital tract infections, *Enterococcus gallinarum* represented the most common 23 (28.39%) of the bacteria. The potential for pathogenic bacterial infection was examined which highlighted how vaginal infections affect preterm birth, the rupture of the vaginal membranes and placental infections [29, 30].

Corynebacterium coyleae, it is a part of the symbiotic microbiota of the urinary tract, mucous membranes, genital system, skin, etc., was another bacterium that was identified and was thought to represent commensal strains as it is uncommon to find them connected with newborn infections [31, 32]. This species' potential for causing infection has not been completely identified. Using blood cultures from six patients who experienced episodes of fever of unknown origin, Funke *et al*. made the initial discovery in 1997. One of the patients had the virus that causes HIV infections. However, there is still a need for more clinical research. The underlying issue in the other five individuals, however, required prior surgical intervention [32]. Also, individual cases of each confirmed sepsis, probable sepsis and soft tissue infection, a suspected post-transfusion bacteremia, neonatal bacteremia, injuries from burns, samples of pleural fluid, abscess formation, and ulceration have all been treated with *C. coyleae*. Regarding its formal pathogen status, current debate continues. Non spore forming, non-acid fast, straight to slightly curved gram-positive rods, frequently with tapered ends, occasionally club shaped or ellipsoidal, organized in angular or palisade formations. It is yet unknown how this specific. *Corynebacterium* species contributes to the pathogenesis of human diseases and what role it plays in infection [32, 33].

The distinction between colonization and an infectious state is manifestly lacking. *Corynebacterium coyleae* is an infrequently isolated species with scant published data from clinically important samples. However it should still be considered a pathogen that can cause complex urinary tract infections, mainly in women [33].

In this study, *Acinetobacter junii* was isolated from SA women. It is also considered a rare species and thought to be an uncommon type of human-infected bacteria, was isolated from South African women in a similar study. Gram-stained smear analysis under a microscope uncovered tiny gram-negative cocci. Its colonies are non-hemolytic on sheep blood agar. The isolate tested positive for catalase but tested negative for cytochrome oxidase and indole. *Acinetobacter* is a common bacterium that exists everywhere in the environment. It can be separated from a variety of sources such as food, water, waste and soil. *Acinetobacter*, which is often non-pathogenic, can lead to life-threatening infections in severely ill patients [34]. It occasionally makes up a minor portion of the human skin flora in about 25% of healthy individuals. In retrospective research, digestive tract colonization was observed in both newborns and adults (77% of 73 patients). *Acinetobacter* is a member of the microflora of the oral cavity, upper respiratory system, and genitalia. Rectal colonization of *Acinetobacter* carries the danger of the "translocation" phenomenon (transfer from the gut to form infected areas in the lungs or other organs) [35]. *Acinetobacter* colonization is a type of gram-negative bacteria. Some strains have a polysaccharide capsule which typically indicates a high level of virulence. The thick polysaccharide and polypeptide capsule shields bacteria from the immune system of the host cell [34, 35]. Also, it allows bacteria to adhere to surfaces, including those on the human body and inanimate objects. Moreover, the polysaccharide capsule prevents bacterial dehydration. *Acinetobacter* produces non-pigmented colonies that, when produced by an encapsulated strain, are also mucoid.

In a study that is closely related to this one, *A. junii* was found in samples of amniotic fluid when there was no intra-amniotic inflammation, and it was the second-most common microorganism found (n = 14) out of a total of 79 microorganisms [36].

Staphylococcus epidermidis, a common commensal bacterium of human skin and mucosa, is also diagnosed in healthy pregnant women. Although *S. epidermidis* was once thought to be nonpathogenic, it is now understood to be an important opportunistic pathogen. It maintains things in balance, promotes the skin's immune system, and uses colonization resistance to stop

opportunistic microorganisms from creating disease [37]. According to some researchers one of the most significant species in this group has been identified as *Staphylococcus epidermidis* (*S. epidermidis*). It is a gram-positive, facultatively anaerobic, non-spore-forming, non-motile, catalase-positive, and coagulase-negative bacteria that causes many nosocomial and hospital infections. The accessory gene regulator (Agr) types I, II, and III provide virulence traits to the *S. epidermidis* strains isolated from clinical infections in humans [38].

This research diagnosed *Metabacillus niabensis* for the first time in Iraq as well as the rest of the world in clinical samples, specifically in the vagina. The isolated species of the genus *Metabacillu*s used in this study can help guide future research on this genus and improve its taxonomic diversity [39].

When cotton waste composts were used to cultivate mushrooms, *Metabacillus fastidiosus*, the type species of the genus *Metabacillus*, was discovered for the first time by Patel and Gupta who named the type strain of *Metabacillus niabensis*. *M. niabensis* is a gram negative, aerobic, motile bacterium that can grow between 15 and 40°C. The main fatty acid identified in *Metabacillus niabensis* is 12-methyl-tetradecanoic acid, and this bacterium is catalase, β-galactosidase and oxidase positive [40]. *Bacillus* systematics study using molecular approaches resulted in significant developments in the taxonomy of the genus. Numerous species have lately been reclassified, entering new genera, leaving nearly exclusively species from the *Bacillus cereus* group in the genus *Bacillus.* These species are so phylogenetically related that only sequencing of the 16S rRNA gene will differentiate them.

Bacillus species and nearby genera have a cell wall typical of gram-positive bacteria while exhibiting gram-positive (in early cultures), gram-variable or gram-negative staining. *Bacillus* and similar genera can survive in various conditions for extended periods of time because they can generate spores which make them resistant to high and low temperatures as well as typical-sanitizers.

Given the similarity of physical, biochemical, and genetic patterns among close-related species, identifying *Bacillus* and related taxa using traditional methods is particularly hard. The identification of new species is made possible by polyphasic strategy that combines phenotypic and genotypic methodologies for a more accurate assessment of this group's taxonomic and phylogenetic relations. Several identification systems contain databases (DB) focused on clinically significant bacteria, which are more restrictive than drug-related microbiota. These species frequently originate from their surroundings, and because of the variety in their physiologies and dietary requirements, their metabolic patterns are not always included in the DB [39, 40].

Several studies show that molecular methods like the sequencing of housekeeping genes must be applied in an attempt to identify species of bacteria isolated from productive sites. The ribosomal RNA 16S subunit, encoded by the rrs gene, is currently extensively employed in bacterial identification. Yet, the 16S rRNA gene sequences of several *Bacillus* species are remarkably similar. Furthermore, the 16S rRNA gene in bacteria can exist in several copies. Depending on the replicated copy, this gene can produce various identifications, leading to an unclear and imprecise analysis. Extra genes, such as rpoB (which encodes the beta subunit of RNA polymerase), gyrA (which encodes the alpha subunit of DNA gyrase), and gyrB (which encodes the beta component of DNA gyrase), are required for the separation of these species, must be sequenced [41].

5. Conclusions and Recommendations

The molecular identification of bacterial isolates and 16S rRNA gene sequencing detected the high percentage of *Enterococcus faecalis* in SA. *Enterococcus faecalis* strains possess an arsenal of virulence factors that are located in virulence genes and contribute to their ability to cause disease. The presented tree has added an accurate and inclusive phylogenetic distribution and positioning of the studied S1-S20 that originated from the differences in the nucleic acid substitutions within the same detected species.

Real-Time PCR vaginal microbiota detection must be introduced to find microorganisms that are slow to grow, difficult to cultivate or are hard to detect.

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