Molecular Detection of Bacterial vaginosis Isolated from Preterm Labor Patients in Wasit City, Iraq

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KEYWORDS

Bacterial vaginosis, Preterm birth, Gardnerella vaginalis, Megasphaera, Atopobium vaginae

ABSTRACT

The aim of this study was to evaluate the role of bacterial vaginosis in preterm labor. There is growing evidence that infections, particularly those that spread from the lower genital tract, might trigger preterm labor. One of the main causes of newborn morbidity and mortality is preterm birth. This is a cross-sectional study which was carried out in the labor facilities in local hospital in Wasit. A total 90 swab samples were collected by gynecologist from patients in Preterm and Full-term labor Bacteriological diagnosis was done using Molecular quantification methods have been reported recently, but the specific risk factors they might identify remain unclear. We carried our study on 90 pregnant women, divided into two groups. First group delivered preterm and other group at full term. There are 38 out of 45 women who had no bacterial infection in the group of full-term pregnancy compared to 19 out of 45 in the preterm group , so the incidence of BV was significantly more in preterm group. The commonest isolated pathogen was G. vaginalis, followed by Megasphaera and Atopobium vaginalis. More than one-third (37.8%) of patients with preterm delivery have Gardnerella infection when compared to only 6.7% of those who delivered at term , Around one-quarter (26.7%) of patients who delivered preterm were diagnosed with Megasphaera. Only 4.4% of full-term patients had positive tests for this infection and 22.2% of patients with preterm labour compared to 4.4% of full-term pregnancies diagnosed with Atopobium infection.

It was concluded that bacterial vaginosis is considered as one of the most common vaginal infection in pregnancy and it have role in preterm labor.

1. Introduction

Preterm delivery is defined as delivery before 37 weeks of gestation or 259 days after the last menstrual period (LMP). It is one of the main reasons for infant mortality in the world, accounting for 35% of infant deaths annually (1). Preterm delivery is still responsible for 70% of neurological complications, disabilities, and deaths, which impose a substantial economic healthcare burden. Several methods are available for diagnosing, including investigation of cervical dilation, assessment of cervical activity with a dynamometer, performing sonography to determine gestational age, evaluation of cervical length using abdominal or vaginal ultrasound, and evaluation of vaginal fibronectin levels and salivary estriol (2). A full-term pregnancy is one that lasts between 39 weeks and 40 weeks and 6 days. This is the ideal time for a baby to be born, as it gives the baby's lungs, liver, and brain time to fully develop. Babies born full term are less likely to have health problems than babies born preterm or late term (3).

Bacterial vaginosis (BV), or vaginal dysbiosis, is one of the most common vaginal conditions associated with aberrant changes in the vaginal microbiome (VMB). BV is the most prevalent vaginal infection in women of reproductive age, with estimated occurrences ranging from 5% to 70% (4). Bacterial vaginosis is characterized by a reduction of the resident lactic-acid producing Lactobacillus spp. and an overgrowth of anaerobic bacteria (5). such as Gardnerella vaginalis, Atopobium vaginae, Ureaplasma urealyticum, Mycoplasma hominis, Prevotella, Peptoniphilus, Megasphaera, Mobiluncus, and several fastidious and uncultured bacteria, including BV-associated bacteria (BVAB-1 to 3) (6). The most common bacteria isolated from BV is Gardnerella vaginalis that grows under appropriate microaerophilic condition and the anaerobic bacterium Atopobium vaginae.A hallmark of BV is the presence of a highly structured polymicrobial biofilm on the vaginal epithelium and made up of bacterial cells packed inside a network of polysaccharide fibrils, mainly populated by Gardnerella vaginalis with strong adhesion to vaginal epithelium. A species-specific PCR assay for the detection of Bacterial vaginosis targeting the 16S rRNA gene was arranged (7).

BV is the most common cause amongst all the known causes of vaginal discharge and is characterized

by a malodorous, vaginal discharge, in women of childbearing age, presence of clue cells in wet mount, and fishy odor (positive KOH amine test). It may be diagnosed both clinically and microbiologically. Some recent studies imply that it is sexually transmitted, with more pathogenic strains of Gardnerella vaginalis being identified and recovered in the male partners of females diagnosed with bacterial vaginosis (8).

Various factors that might contribute to the development of BV include frequent baths, douching, smoking, engaging in multiple sexual partners, using over-the-counter intravaginal hygiene products, experiencing severe stress, and increased frequency of sexual intercourse. BV might also be more prevalent among women who do not frequently change their underwear (4). In clinical settings, BV is typically diagnosed using Amsel criteria (three of the following four criteria should be present: clue cells on wet mount microscopy; a 'fishy' odor after adding 10% KOH to vaginal secretions; vaginal pH.4.5; and thin, homogenous vaginal discharge (9).

BV is associated with adverse obstetric and gynaecologic outcomes including infertility, preterm birth, premature labour, post-partum endometritis, pelvic inflammatory disease, miscarriage, preterm prelabour rupture of membranes, chorioamnionitis, intrauterine infection, and an increased risk of acquiring human immunodeficiency virus (HIV) and other sexually transmitted infections STIs (12). In the last decade, phylogenetic analyses of vaginal samples (mostly bacterial 16S ribosomal RNA gene sequencing) have shown that bacterial communities in the vagina are more complex than previously thought. The first study using molecular methods to characterize the VMB was published in 2002 (11) it is now clear that there are different subtypes of BV that can be stratified using molecular approaches, and some subtypes of BV may be more tightly linked to preterm birth than others (26). Multiplex PCR polymerase chain reaction refers to the use two or more primer sets designed for amplification of different targets are included in the same PCR reaction. Using this technique, more than one target sequence in a clinical specimen can be amplified in a single tube.

2. Methodology

Subjects of study

This is a cross-sectional study which was carried out in the labor facilities in local hospital in Wasit. A total 90 swab samples were collected by gynecologist, from patients in Preterm and Full-term labor , during the period from 31th July to 31th of December of 2023 that admitted to Al-Zahra Teaching Hospital, Al Kut Hospital, Fairooz Hospital and private clinics in Wasit province. Bacterial isolates were detected by Multiplex PCR (MPCR.

Inclusion Criteria

- Inclusion criteria according to definition of Preterm labor are delivering a baby prior to the end of the 37th week of pregnancy, which is the primary cause of newborn mortality.
- Inclusion criteria according to definition of full term (control) Babies born between 39 weeks and 40 weeks and 6 days.

Exclusion Criteria

- Exclusion criteria (abortion case, IUD-intra uterine death, still death) abortion is described as a consequence, rather than an act or a choice. It is the "death of the fetus, sometimes with passage of products of conception (fetus and placenta), before 20 weeks gestation.

Specimens Collection

Vaginal swab: Collect vaginal fluid sample using the swab by contacting the swab to the lower third of the vaginal wall and rotating the swab for 10 to 30 seconds to absorb fluid. Immediately place the swab into the transport tube contains 1 ml tryptone water media prepared by add 15 g to 1 liter of distilled water. Mix well, distribute into final containers and sterilize by autoclaving at 121°C for 15

minutes. Store prepared media below 8°C and carefully break the swab shaft against the side of the tube. Swabs were preprocessed and stored at −80 °C within an hour of collection (31).

Molecular Analyses

Multiplex PCR technique was performed bacterial vaginosis (*Gardnerella vaginalis , Megasphaera sp., and Atopobium vaginalis*) based on 16S ribosomal RNA gene were designed in This study using NCBI Genbank database and primer 3 plus. These primers were provided by Scientific Reserecher. Co.Ltd in Iraq as following table. Bacterial genomic DNA was extracted bacterial isolates by using (**Presto™ Mini gDNA Bacteria Kit**) as and done according to company instructions.

Table 1: The 16S ribosomal RNA gene PCR primers with their nucleotide sequence and product size

Primer	**Sequence (5'-3')**		**Product Size**	**Genbank**
G. vaginalis 16S rRNA gene	F	ACTGAGATACGGCCCAGACT	653bp	ON878197.1
	R	CCAGGTAAGGTTCTTCGCGT		
Megasphaera sp. 16S rRNA gene	F	GGACGAACAGGACATCGGTT	510bp	LN998020.1
	R	CCACATACTCCACCGCTTGT		
A. vaginalis 16S rRNA gene	F	ATAAAGTGGCGAACGGCTGA	390bp	AJ585206.2
	R	GCTTCTTCTGCAGGTACCGT		

Table (2): Standard multiplex PCR master mix protocol: Table (3): Multiplex PCR Thermocycler Conditions

Statistical analysis

PCR step	Temp.	Time	repeat
Initial Denaturation	95°C	3min	1
Denaturation	95°C	30sec.	34 cycle
Annealing	58°C	30sec	
Extension	72°C	1 min	
Final extension	72°C	5min	1
Hold	4°C	Stop	-

Data were entered in Excel and then transformed into the software program Statistical Package for Social Sciences (SPSS) version 26. Descriptive statistics was used to describe both categorical and numerical variables. Frequencies and percentages were used for categorical variables. Normality tests were conducted. To assess the association between categorical variables, the Chi-square test was used while the independent samples t-test was used to assess the presence of differences between sample means. Fisher's Exact Test was used instead of Chi-square when more than 20% of cells have expected values less than 5 and considering a P-value less than 0.05 as significant (15).

3. Result and Discussion

The results of this study were dependent on the analysis of collected data related to 90 female patients at the time of their delivery. The sample consisted of 40 patients who gave birth at term (control) and the remaining 40 were preterm.

The sociodemographic features of the study sample are demonstrated in Table 4. This table shows that a higher percentage of the participating women (32.2%) were belonging to the age group 17- 23 years old. Those between 24 and 30 years were with a slightly lower percentage (30%). Only 11 women (12.2%) were above 37 years old.

More than half of the sample (56.6%) mentioned living in the AL-Hay district followed by 17.7% who lived in the AL-Kut district. The majority of the sample (87.8%) was housewives and didn't work outside their homes.

Table 4: Frequency distribution of socio-demographic features of the study sample (n=90)

Socio-demographic features		**Frequency**	**Percentage**
Age (Years)	17-23	29	32.2
	24 - 30	27	30.0
	31 - 37	23	25.6
	38-44	11	12.2
Residency	AL-Hay	51	56.6
	AL-Fajer	9	10.0
	AL-Kut	16	17.7
	AL-Moafaqia	7	7.7
	Nasiriya	5	5.5
	Baghdad	2	2.2
Occupation	Governmental job	11	12.2
	Housewife	79	87.8

Molecular analysis

Amplification of 16srRNA gene of (*G. vaginalis* , *Megasphaera sp.*, and *A. vaginalis*) by m PCR to confirm the presence of 16S rRNA gene in that appeared in molecular weight at 653bp, 510bp , and 390bp PCR product size respectively. The results of PCR technique show that there are 38 out of 45 women who had no bacterial infection in the group of full-term pregnancy compared to 19 out of 45 in the preterm group, so the incidence of BV was significantly more in preterm group. The commonest isolated pathogen was *G. vaginalis*, followed by *Megasphaera* and *Atopobium Vaginalis*. More than one-third (37.8%) of patients with preterm delivery have Gardnerella infection when compared to only 6.7% of those who delivered at term , Around one-quarter (26.7%) of patients who delivered preterm were diagnosed with *Megasphaera*. Only 4.4% of full-term patients had positive tests for this infection and 22.2% of patients with preterm labour compared to 4.4% of full-term pregnancies diagnosed with *Atopobium* infection.

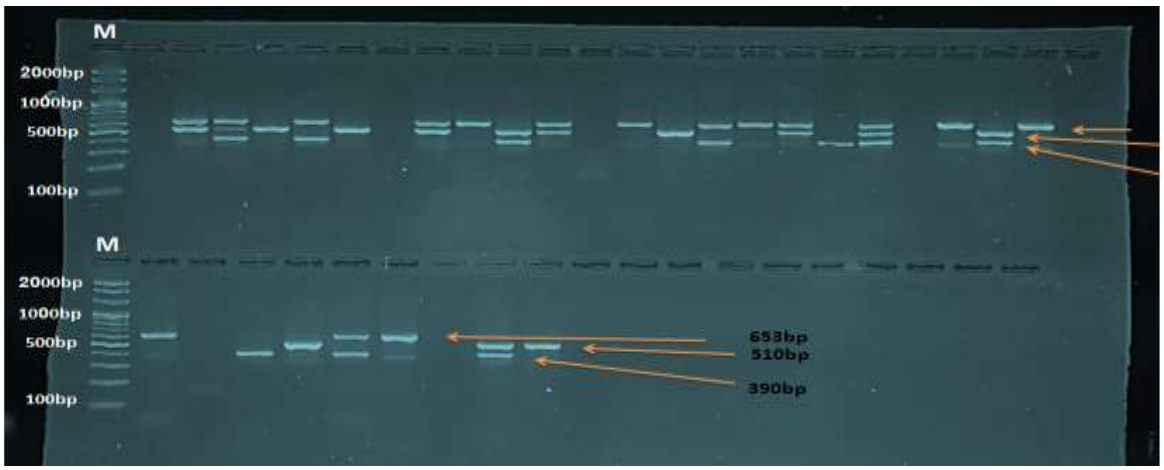

Figure (1): Agarose gel electrophoresis image that showed Multiplex PCR product analysis based on 16S ribosomal RNA gene for detection Bacterial vaginosis bacterium from patients samples. M (Marker ladder 2000-100bp). The samples lanes showed some positive samples for Gardnerella , Megasphaera, and Atopobium were showed positive at 653bp, 510bp , and 390bp PCR product size respectively.

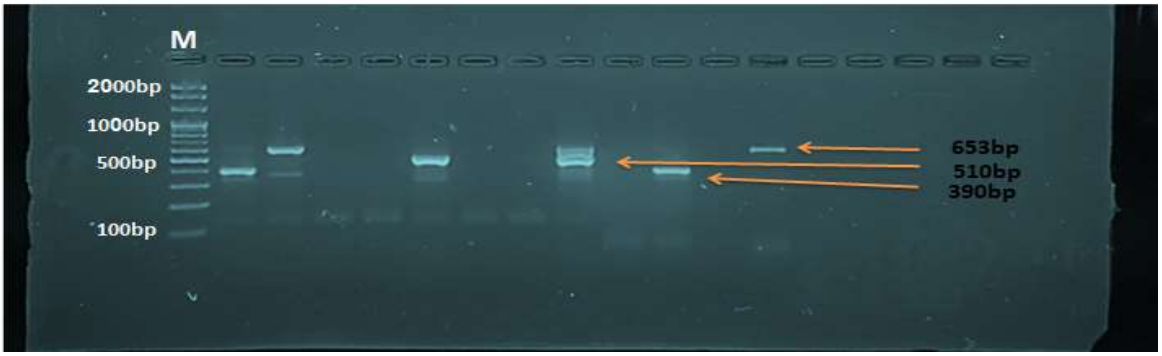

Figure (2): Agarose gel electrophoresis image that showed Multiplex PCR product analysis based on 16S ribosomal RNA gene for detection Bacterial vaginosis bacterium from control samples. M (Marker ladder 2000-100bp). The samples lanes showed some positive samples for Gardnerella , Megasphaera, and Atopobium were showed positive at 653bp, 510bp , and 390bp PCR product size respectively.

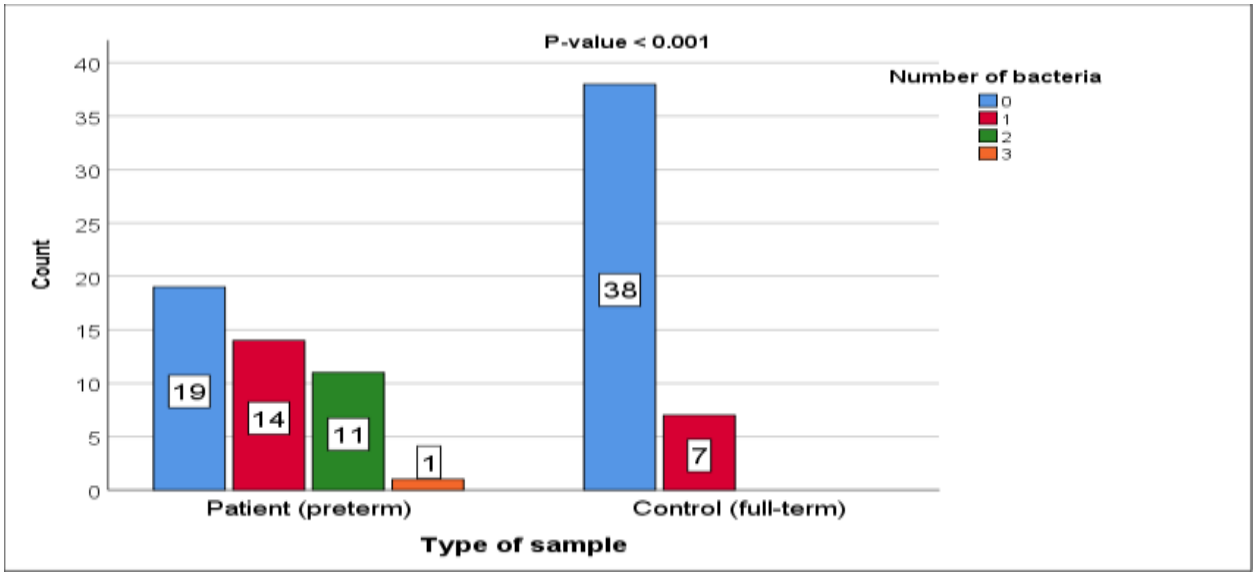

Figure 3: The distribution of number of bacterial infections among the study groups.

In Figure 3, there was a statistical association between the number of bacteria present in preterm-delivered patients and full-term patients (P-value <0.05). There are 38 out of 45 women who had no bacterial infection in the group of full-term pregnancy compared to 19 out of 45 in the preterm group. No one in the full-term group had two or three bacteria while 12 out of 45 preterm women had more than one type of selected bacteria in their examination

1. *Gardnerella Vaginalis*

Table 5 shows that there was a statistically significant association between the sample type (time of delivery) and the presence of *Gardnerella* infection among the study participants (P-value <0.05). More than one-third (37.8%) of patients with preterm delivery have Gardnerella infection when compared to only 6.7% of those who delivered at term. Most of the sample (77.8%) didn't have any Gardnerella infection.

Table (5): The association between the time of delivery and the presence of *Gardnerella Vaginalis* infection among 90 study samples

G. Vaginalis	**Type of sample**		**Total**	**P-value**
	Patient (preterm)	**Control (full-term)**		
Negative	28 62.2%	42 93.3%	70 77.8%	<0.001
Positive	17 37.8%	3 6.7%	20 22.2%	
Total	45 100%	45 100%	90 100%	

The most prevalent infectious condition affecting the female genital tract in women of childbearing age is vaginitis. *G. vaginosis* is an anaerobic facultative organism and is the most common cause of bacterial vaginosis (14).

A growing number of studies have reported that *Gardnerella* clades, genomic species, or amplicon sequence variants were differently associated with specific pathogen phenotypes BV and PTB (15).

G. *vaginalis* is the most prevalent vaginal infection in women of reproductive age, with estimated occurrences ranging from 5% to 70%. The prevalence of G. *vaginalis* among women fluctuates from 20% to 60% across various countries. In the United it is rates are lowest in Australia, New Zealand, and Western Europe. it's exhibits a higher prevalence among black women compared to white women. In addition, it is more frequently encountered in women with multiple sexual partners. *Gardnerella* has consistently been identified as a significant pathogen in BV, indicating a notable prevalence of *Gardnerella* in these populations (12).

study was carried out in Suliamania teaching and Azadi teaching hospitals to assess the bacterial vaginosis rate of *G. vaginalis* among women with preterm labor. For each patient, a special high vaginal swab (HVS) was tested for color, pH, whiff test, and clue cells, in addition to the cultivation of HVS

and urine for detecting other bacterial isolates. The results revealed an overall rate of infection of 76.67%, 33.33% for *G. vaginalis*. A total of women in their reproductive years suffered from preterm labor from 24 to 37 weeks of pregnancy. The relationship between clinical history and type of microorganism distribution was significant P<0.05, via the history of vaginal discharge and rupture of membrane (16).

GV was isolated from 16 out of 24 (66.7%) in the BV positive group. There was a significantly higher preterm birth rate, below 34 weeks (22.7% vs. 6.2%, p = 0.019) in women with BV (17).

Gardnerella vaginalis has been suggested as the principal cause of bacterial vaginosis although the isolation rate of this organism from patients of bacterial vaginosis is variable 6-94%, due to broad diversity in selection of patient's material, methods, and criteria for establishment of diagnosis (12).

Laboratory evaluation and clinical assessment were performed for 100 female patients. Bacteriological diagnosis were done using direct Gram staining for clue cells, bacterial culture and amsel's criteria, scoring was done by Nugent score system. We carried our study on 100 pregnant women, divided into two groups. First group delivered preterm and other group at full term. 33 patients out of 50 pts with preterm group had BV (66%) while 24 patients out of 50 patients had BV (48%) in full term group, so the incidence of BV was significantly more in preterm group. The commonest isolated pathogen was *G. vaginalis*, followed by beta hemolytic *streptococci* and *staph aureus*. There was a significantly higher preterm birth rate, below 34 weeks (P-value <0.05). It was concluded that bacterial vaginosis is considered as one of the most common vaginal infection in pregnancy and it have role in preterm labor (19).

A study admitted to Babylon Maternity and Al-Hilla Teaching aims to isolate and identify G. *vaginalis* from preterm labor, clinical sample were collected from preterm labor patients with (bacterial vaginosis. These high vaginal samples were subjected to different methods of identification of *G. vaginalis.* Results It was found that 30 (20%) isolates were recovered dependent on direct extraction to high vaginal swab on molecular level. Depending on molecular identification, it was found that (53%) percentage bacterial vaginitis , Then these bacteria were diagnosed as *G. vaginalis* after amplification depending on 16SRNA(28).

2. *Megasphaera* sp.

In Table 6, the statistical analysis found a significant association between patients' age of delivery and the occurrence of *Megasphaera* infection with a P-value =0.004. Around one-quarter (26.7%) of patients who delivered preterm were diagnosed with *Megasphaera*. Only 4.4% of full-term patients had positive tests for this infection. The majority 84.4% of the total sample had negative tests for *Megasphaera* infection. Only 15.6% of the total sample were diagnosed with this infection.

Table 6: Association between the study sample and the presence of *Megsphaera i*nfection among the study sample

Megasphaera	**Type of sample**		**Total**	**P-value**
	Patient (preterm)	Control (full-term)		
Negative	33 73.3%	43 95.6%	76 84.4%	0.004

Positive	12 26.7%	2 4.4%	14 15.6%	
Total	45 100%	45 100%	90 100%	

Vaginal carriage of *Megasphaera* is strongly associated with BV, and pregnant women with BV have an elevated risk for spontaneous preterm birth, BV has long been linked to elevated risk for preterm birth, more recent vaginal microbiome studies have identified higher MP1 carriage in women who go on to deliver preterm and potentially contribute to PPROM and/or spontaneous preterm birth (26).

Megasphaera type 1 was shown to be useful for the molecular diagnosis of BV and has been included as a target in commercially available nucleic acid amplification tests for the diagnosis of BV. Furthermore, pregnant women with a prior history of preterm delivery and increasing levels of *Megasphaera* type 1 through mid-pregnancy were more likely to experience spontaneous preterm delivery (25). An association between *Megasphaera* species and spontaneous preterm birth was also noted in a case-control study of mostly African American women (21). A recent study showed that women with pelvic inflammatory disease were more likely to test positive for *Megasphaera* species among other anaerobes (23).

Vaginal *Megasphaera* species have also been shown to be associated with increased risk for HIV acquisition and among a group of mostly South African women, *Megasphaera* type 2 was associated with increased risk but not *Megasphaera* type 1 (24, 25).

Vaginal swabs were collected during all three trimesters from 38 pregnant Indian women who delivered spontaneous term (n=20) and preterm (n=18) neonates. Paired-end sequencing of V3-V4 region of 16S rRNA gene was performed using the metagenomic DNA isolated from vaginal swabs (n=115). Abundance of Megasphaera sp. (PTB: 1.45%, TB: 0.0007%) is significantly (p- value < 0.05) higher in 1st trimester of preterm delivering mothers No particular species of Megasphaera sp. was found to be significantly different between TB and PTB samples (27).

Samples collected as part of the Vaginal Human Microbiome Project (VaHMP). Briefly, mid-vaginal wall swab samples were collected and DNA was extracted from the swabs, DNA samples were randomized to avoid batch effects and the V1-V3 region of the 16S rRNA gene was amplified using polymerase chain reaction (PCR). MP1 is highly prevalent in the VaHMP cohort, colonizing 33.2% of women in the study. These findings suggest that this highly prevalent organism colonizes the vaginal environment and remains present and transcriptionally active during pregnancy. This capability combined with the ability of MP1 to maintain colonization during pregnancy suggests that this organism is a candidate for future studies investigating the proposed model where ascending infection of vaginal organisms contributes to in preterm labor and/or birth (26).

3. *Atopobium Vaginalis*

Table 7 also shows a significant statistical association (P-value =0.013) between the patients' group and the presence of *Atopobium* infection among the study sample.

There were 22.2% of patients with preterm labour compared to 4.4% of full-term pregnancies diagnosed with *Atopobium* infection. Infection with *Atopobium* was present in 86.7% of the total sample. A total 90 women included, 36 delivered before 37 weeks of gestation (40%). Molecular methods were used to prospectively quantify *Atopobium vaginae* in vaginal fluid samples from women admitted for spontaneous preterm labor with intact membranes.

Table 7: Association of sample groups and the presence of *Atopobium* infection

Atopobium	Type of sample		Total	P-value
	Patient (preterm)	Control (full-term)		
Negative	35 77.8%	43 95.6%	78 86.7%	0.013
Positive	10 22.2%	2 4.4%	12 13.3%	
Total	45 100%	45 100%	90 100%	

Atopobium vaginae are a newly discovered bacterium frequently found in women with BV. *A vaginae* is an anaerobic bacterium recognized as a causative agent of bacterial vaginosis and associated with preterm delivery. Although vaginal microbial communities of some healthy women have high proportions of *A. vaginae*, the genus *Atopobium* is more commonly associated with bacterial vaginosis, a syndrome associated with an increased risk of adverse pregnancy outcomes and the transmission of sexually transmitted diseases (28) .

The primary outcome measure was the relationship between bacterial concentration at admission and preterm delivery, before 37 weeks of gestation. Molecular quantification detected high concentrations of *A vaginae* (10(6)/mL or more: 25.0% in the preterm group and 9.3% in the term group, P=0.04) more often in women with preterm deliveries compared with term deliveries (29).

We demonstrated that the microbiome of participants across PTB and term gestational delivery groups differed significantly. The PTB group was significantly belonging to Atopobium vaginae, Peptostreptococcus anaerobius, Gardnerella vaginalis, Prevotella bivia, Mycoplasma hominis, Parvimonas spp., Aerococcus spp., and Prevotella. A. vaginae emerged as the most predominant microbiome among other significant PTB-associated taxa (meanAtopobium = 0.446, PAtopobium = 0.001, qAtopobium , 0.05, permutation test) We further examined the proportion of vagitypes associated with PTB across all participants who delivered preterm. The proportion and prevalence of A. vaginae remained significantly high in all PTB participants (30).

the study included 813 pregnancies, vaginal samples were PCR analyzed and 793 Nugent scores were available. High vaginal loads of either or both of A. vaginae and G. vaginalis were associated with preterm birth (hazard ratio [HR], 3.9; 95% confidence interval {CI}, 1.1–14.1; P = .031). A high vaginal load of A. vaginae was significantly associated with shortened time to delivery and therefore pregnancy length. After multivariate analysis, A. vaginae levels ≥108 copies/mL remained significantly associated with delivery before 22 weeks of gestation (adjusted HR, 4.7; 95% CI, .2–17.6; P = .014) (31).

Study was aimed to determine the Atopobium vaginae associated bacterial vaginosis (BV) in vaginosis women and women with miscarriage. Also other aim, the deoxyribonucleic acid (DNA) sequencing was performed for phylogenetic tree analysis of 16S rRNA gene in local A. vaginae isolates in comparison with NCBI-Genbank globa. One hundred fifty high vaginal swabs were collected from women with vaginosis (75 samples were taken from married vaginosis women without miscarriage and 75 samples from vaginosis women with miscarriage) from Babylon city hospital and private clinics. The age of patient was 15 to 45 years. The sample was collected by disposable swabs, genomic DNA was extracted from these swabs and 16S rRNA gene detection by polymerase chain reaction technique. *A. vaginae* was isolated on Columbia blood agar supplemented with antibiotic for the first

time in Iraq, the study confirmed that 9 (12.00%) and 5 (6.66%) of *A. vaginae* out of 150 swabs isolated from miscarriage and non-miscarriage vaginosis women respectively. According to the detection of the 16S rRNA gene, the study revealed that 69 (92.00%) and 47 (62.66%) of *A. vaginae* out of 150 swabs obtained from miscarriage and non-miscarriage vaginosis women respectively. Basic Local Alignment Search Tool (BLAST) analysis showed that the 16S rRNA gene shared more than 98–99% sequence compatibility with the sequences of *A. vaginae*. Furthermore, the phylogenetic tree analysis of the 16S rRNA gene indicated that local *A. vaginae* (NO.1 and NO. 2) isolates shared higher homology with other *A. vaginae* isolates available in the GenBank. The homology of the nucleotides was between (99.17 and 98.75%) respectively (28).

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