RESEARCH ARTICLE

Molecular Identification and Genotyping of *Atopobium Vaginae*, 16S rRNA Gene from Bacterial Vaginosis Miscarriage Women in Al-Hillah City

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ABSTRACT

This 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 global *A. vaginae* isolates and finally submission of the present isolates in NCBI-Genbank database.

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 15 to 45 years. The sample was collected by disposable swabs, genomic DNA was extracted from these swabs. 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.

Keywords: Bacterial vaginosis, Molecular identification, *Atopobium vaginae*, 16 s rRNA gene sequence , miscarriage

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INTRODUCTION

Bacterial vaginosis (BV) is an ecological imbalance of the vaginal microbiota affecting mostly women of reproductive age group (pregnant and non-pregnant)and it is the most prevalent cause of vaginal discharge and malodour[1,2].

Bacterial Vaginosis (BV) develops when the normally predominant peroxide producing *lactobacillus species* in the vagina are replaced by mixed predominantly anaerobic flora consisting of *Gardnerella vaginalis, Mycoplasma hominis, Mobiluncus species, Bacteroides species,Prevotela species, Peptostreptococcus species, Fusobacterium species and Porphyromonas species*.[3] However, BV is associated with adverse pregnancy outcomes such as preterm birth as well as miscarriage and increased risk for acquiring sexually transmitted infections such as HIV.[4] Bacterial vaginosis can be diagnosed using several tests ranging from clinical indicators depended on the presence of vaginal discharge and odor, elevated pH, and clue cells as determined by microscopy and molecular assays. Molecular technologies are objective and able to detect fastidious bacteria, enable quantitation allow for more convenient and accurate testing for BV and are ideal for self-collected vaginal swabs.[5,6] Cultivation-independent (molecular) analyses, most notably sequencing of 16S rRNA genes polymerase chain reaction (PCR)-amplified from microbial community DNA, offer a more systematic approach to detecting microbes in natural habitats.[7]

Fredricks et al, the first group to use molecular biology techniques to characterize vaginal flora, suggested the involvement of new bacterial species in BV.[8] Preliminary studies have shown that *A. vaginae* is associated with BV.[8,9]

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A molecular tool that based on the quantification of *A. vaginae and G. vaginalis* is both highly sensitive and specific for the diagnosis of BV.[10]

Atopobium vaginae is a newly discovered bacterium frequently found in women with BV.[11] *A vaginae* is an anaerobic Gram-positive bacterium recognized as a causative agent of bacterial vaginosis and associated with preterm delivery.[12] 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. Genetic differences within *Atopobium* species may explain why single species can be associated with both health and disease.[13] Similar to *G. vaginalis*, the presence of the organism at a high concentration is highly sensitive and specific for the diagnosis of BV.[14,15] In addition, the presence of *G. vaginalis* and *A. vaginae* together is associated with disease recurrence and associated with late miscarriage and prematurity.[15-17]

A. vaginae had a fermentative type of metabolism with a large amount of volatile amines formation (methylamine, dimethylamine, trimethylamine, etc.) and short chain volatile fatty acids that can explain unpleasant odor of vaginal discharge caused by anaerobic microorganisms.[18]

The anaerobic respiration of the bacteria of the genus *Atopobium* involves the glycolytic pathway which is carried out through numerous enzymes. In this way, the enzyme D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acts as a pathogenicity factor blocking the complement system (C5a and C1q) function.[19]

Subjects and Study Design

Sample Collection

This study involved (150) samples were collected from women with bacterial Vaginosis (seventy five from vaginosis women with miscarriage and seventy five from vaginosis women without miscarriage), all subjects were within 15–45 years of age. the sample include high vaginal swabs from married female were admitted to the out-patient clinics of Gynecology Babylon Maternity and Pediatrics Hospital during the period from (November 2018 to June 2019). Three cotton swabs of vaginal discharge were obtained from each woman by brushing a swab across the vaginal wall.

The first swab was used for clinical diagnosis (nugent criteria), while the second swab was immersed in plain tube-containing 5ml of Brain heart infusion broth supplemented with 15% glycerol and frozen immediately at –20°C to be used for molecular diagnosis. The third swab was placed into Aims' transport media to be used for the bacteriological diagnosis .

Clinical Diagnosing

Nugent's criteria test was used to diagnosis Bacterial vaginosis according to methods described by.[20]

Bacterial Isolation

A. vaginae was isolated by inoculation on Columbia blood agar supplemented with 2 mg/mL Amphotericin B, 30 mg/mL Nalidixic acid and 4mg/mL Colistin in an anaerobic chamber (Gas Pack) at 37˚C for 48–72 hours. The grown colonies were grey-white in color and non-hemolytic, catalase-negative. In microscopic examination, *A. vaginae* appear as gram-positive, small cocci, and arranged in single cells, in pairs or short chains.

Bacterial DNA Extraction

Total DNA from samples, all frozen high vaginal swabs as well as grown bacterial colonies, were extracted by using G-Spin™ Total DNA extraction kit (iNtRON/ Korea) and done according to company instruction. The concentration and purity of the isolated DNA samples were measured by the Nano Drop spectrophotometer before the performance of PCR. The extracted DNA then stored at –20°C until PCR assay.

Polymerase Chain Reaction

The PCR assay was performed for detection *A. vaginae* by using the primer specific for 16S rRNA according to the study[21] and provided from Macrogen Company as following Table.1. PCR master mix for gene was prepared by using Maxime PCR PreMix kit (Bioneer, Korea) and this master mix done

Table 1: primer for amplification of 16s rRNA gene of *Atopobium vaginae*.

Bacterium	*Primer Sequence (5'-------- 3')*		*PCR product size*	*GenBank code*
Atopobium sp	F	GCAGGGACGAGGCCGCAA	558	AY738658
	R	GTGTTTCCACTGCTTCACCTAA		

Table 2: Contents of the **PCR** reaction mixture with their volumes

PCR master mix	*Volume*
Genomic DNA 5-50ng	5µL
Primary primers forward (10pmol)	1µL
Primary primers reverse (10pmol)	1µL
PCR water	13 µL
Total	20µL

Table 3: Amplification conditions of 16 sRNA genes[21].

Steps	*Temperature*	*Time*	*No. of cycles*
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	
Annealing	55	30sec	38 cycle
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	–

according to company instructions as showed in Table 2. PCR thermocycler program were done according to Ranjit et al.[2] by using convential PCR thermocycler system as was listed in Table 3. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator.

DNA Sequencing Method

The DNA sequencing method was performed for phylogenetic tree analysis of the 16S rRNA gene in the local *A. vaginae* isolates and compared with NCBI-Genbank global *A. vaginae* isolates and final submission of the present isolates in NCBI-Genbank database. The amplified 16S rRNA gene was sent to Macrogen Company in Korea for performing the DNA sequencing by using the AB DNA sequencing system. The phylogenetic tree analysis was performed based on the NCBI-Blast alignment identification and neighbor distance phylogenetic tree analysis (Mega version 6) and multiple sequence alignment analysis based ClustalW alignment analysis and the evolutionary distances were computed using the maximum composite likelihood method by phylogenetic tree UPGMA method.

RESULTS

Clinical Diagnosis of Bacterial Vaginosis

Nugent's Clinical Criteria

The results of the present study revealed that out of 75 samples from Vaginosis women vaginosis miscarriage women only 65 diagnosis as BV and 10 diagnosis as intermediate according to Nugent scoring system while out of 75 samples from vaginosis women without miscarriage only 58 miscarriage women patients samples were classified as BV, whereas 17 patients were classified as intermediate according to Nugent's scoring system as showed in Table 4.

Culture and molecular analysis

The results of this study showed that the number of *A. vaginae* isolated on culture media from the vaginosis women with miscarriage was 9 (12.00%) and 5 (6.66%) from vaginosis women without miscarriage. The results of PCR technique show that *A. vaginae* detected in highest percentage 116 (77.33%) positive samples for 16S rRNA gene of *A. vaginae*, divided into 69 (92%)vaginosis miscarriage women, and 47 (62.66%)vaginosis women without miscarriage out of total 150 swabs as showed in Table 5.

Amplification of 16srRNA gene of *A. vaginae* by PCR to confirm the presence of 16S rRNA gene in that appeared in molecular weight 558 bp was exclusively used to proceed for the sequencing analysis.

Two isolates, sent for sequencing were after that submission in NCBI-GenBank database to get accession number codes (MN165520 and MN165521) frequently. DNA sequencing method was performed for phylogenetic confirmative of *A. vaginae* on 16S rRNA gene detection as show in Figures 2 and 3 and Table 6.

DISSCATION

Vaginosis is a dysbiotic condition of the vaginal cavity that has deleterious effects during pregnancy.[24] Different

Table 4: The result of Nugent criteria

Nugent score	*Number*	*Result of smear*
0–3	Excluded	NO BV
4–6	27	Intermediate BV
7–10	123	BV
	Total number =150 sample	

Table 5: Percentage of positive sample in both culture test and PCR

Types of samples	*Culture Number*		*PCR Number*	
	Positive (%)	*Negative (%)*	*Positive (%)*	*Negative (%)*
Vaginal swab from miscarriage women	9(12.00%)	66(88.00)	69(92.00)	6(8.00)
Vaginal swab from non-miscarriage women	5(6.66%)	70(93.33)	47(62.66)	28(37.33)
Total	14(9.33%)	136(90.66)	116(77.33)	34(22.66)

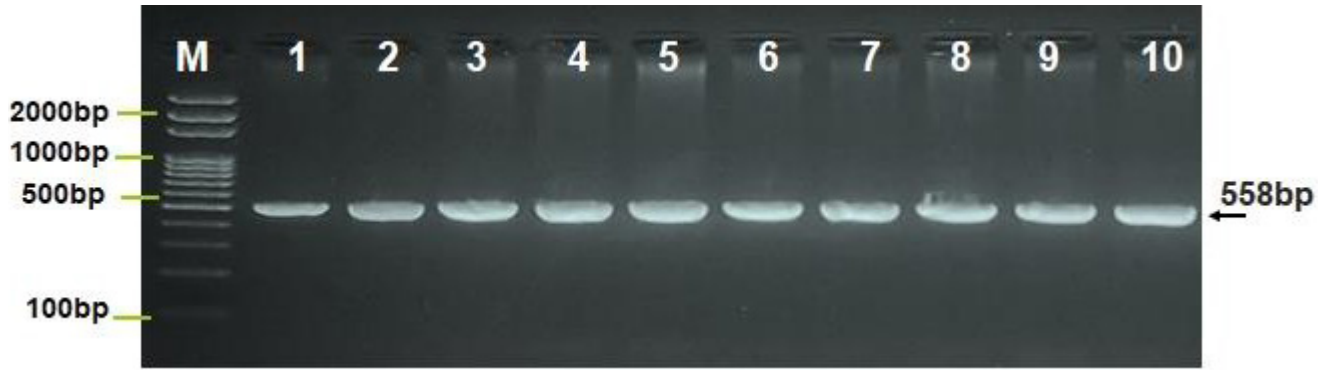

Figure 1: Agarose gel electrophoresis image that showed PCR product analysis for 16s rRNA gene in Atopobium sp . M (Marker ladder 2000-100bp). Lane (1-10) some Positive Atopobium sp. samples at 558bp product size.

Figure 2: Multiple sequence alignment analysis of 16s rRNA gene partial sequence for local *Atopobium sp* from vaginosis miscarriage and vaginosis without miscarriage women isolates (No.1 and No.2) with NCBI-Blast of *Atopobium sp.* isolates 16s r RNA gene (NCBI-BLAST online). The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) with different Atopobium sp. isolates

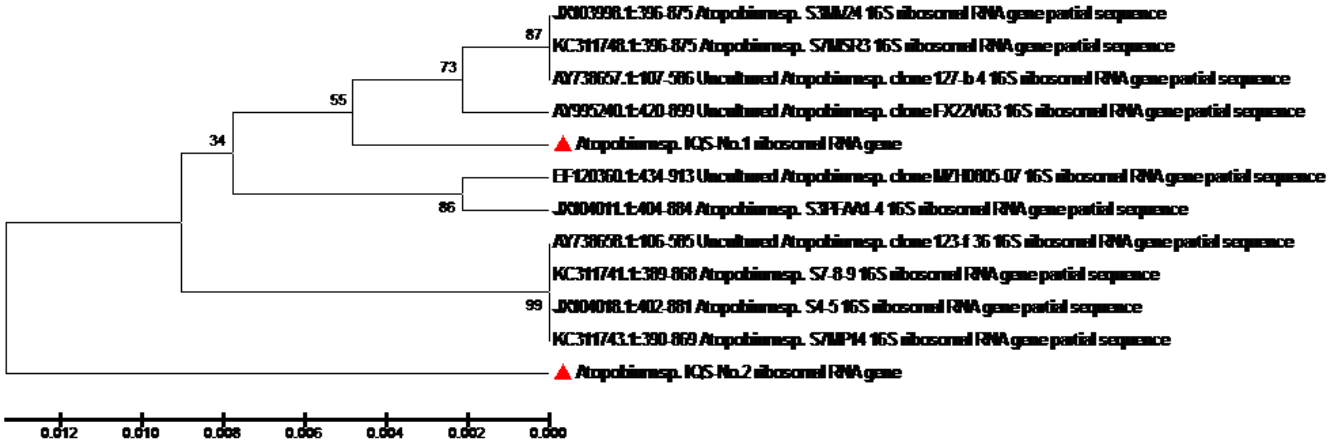

Figure 3: Phylogenetic tree analysis based on 16S ribosomal RNA gene partial sequence that used for genetic relationship analysis of local *Atopobium sp* vaginosis miscarriage and vaginosis without miscarriage women isolates . (No.1 and No.2). The phylogenetic` tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). at total genetic change (0.002-0.012%)

Table 6: Tthe NCBI-BLAST homology sequence identity (%) between local *Atopobium sp.* 16S rRNA gene isolates and NCBI-BLAST submitted *Atopobium sp.* 16S rRNA gene Isolates

Local isolate	*NCBI BLAST identity isolate*	*NCBI-BLAST Homology Sequence identity*		
		Accession number	*Country*	*Identity (%)*
Atopobium sp. isolate No.1	Uncultured *Atopobium* sp. clone FX22W63	AY995240.1		
USA	99.17%			
Atopobium sp. isolate No.2	Atopobium sp. S7MP14	KC311743.1	USA	98.75%

micro-organisms causes BVs, one of them were anaerobes that considered as vaginal flora and there presents in large number due to decrease in Lactobacilli growth lead to causing vaginal infections, which are considered as a common cause of miscarriage.[25] Women diagnosis as BV by Nugent scoring system . The BV subjects were selected dependent on the basis of microscopic examination of high vaginal swabs smeared on microscope slides, gram-stained, and scored by the Nugent method for further diagnosis using traditional and molecular methods. The Nugent score examination results showed large gram positive rods (*L. species*) and gram variable rods (*G. species*) and curved rods like *(M. species*). However , BV can be asymptomatic in about 50% of women and for that reason microbiological method such as Nugent´s scoring system required a restricted skill in the using of microscopy to examining the smear and differentiation between bacterial species ,so this way remain underutilized in routine diagnosis of BV.[14] Interestingly, the major metabolic products of *Atopobium* include lactic acid, In addition to producing lactic acid, however, some species are also known to have dipeptidyl peptidase activity and produce significant amounts of ammonium in other environments,[26] the latter potentially acting as a substrate for BV microorganisms, including *G. vaginalis*.[27] While the commensal relationship between the ammonium-

producing *Prevotella bivia* and the utilization of ammonium by *G. vaginalis* has been demonstrated previously it is also possible that other associations exist. However, it remains to be determined whether *Atopobium* species, *Atopobium*-related species, and other species detected here are involved directly in BV or simply react opportunistically when vaginal conditions change.[27]

Atopobium sp was isolated for the first time in Iraq by microbiological culture media, its an aerobic gram-positive, rod shaped **.** *A. vagina* is considered to be specific for BV.[8, 28] this type of bacteria found in healthy women but in low frequency,[29,30] otherwise in other studies it consider a cause of miscarriage.[31,32]

Our molecular tool is reliable and efficient, allowing precise identification and quantification of several microorganisms involved in vaginal flora anomalies.[20] The molecular tool is definitely more powerful than the Nugent score for identifying BV.[10,20]

In this investigation, the isolation rate of *A. vaginae* in women with BV was 62.66% from vaginosis women without miscarriage . This result agreement study conducted by Cartwright C. et al.,[33] who found association of *A. vaginae* with bacterial vaginosis when detected *A. vaginae* in 66% (25/38) of bacterial vaginosis cases and in 10% (4/44) samples with intermediate vaginal flora. It was not detected in asymptomatic women.Burton *et al*. used a different set of A. vaginae-specific PCR primers to detect *A. vaginae* in 50% of Canadian BV patients.[34] Marrazo *et al* reported the sensitivity and specificity of *A. vaginae* for BV to be 96 and 80%, respectively.[35] This bacterium was detected by PCR in 96% of patients with BV.[36]

Molecular analysis of 16 s rRNA gene by PCR was highly sensitive in detection uncultured microorgnism in our study *Atopobium* species was discovered in high percent in both vaginosis miscarriage women and vaginosis non miscarriage women . This result in agreement with result proposed Mahdey and Abd who detected *Atobopium* sp in high load and came in the second rank and accounted for 67 (69.79%) and also found this microorganism On the other hand, women with *Atobopium* sp. infection have a chance of getting multiple miscarriage of approximately 3 times than those without such infection.[37] Our result was disagreement with study conducted by Abed and Kandala **w**ho found that all sixty vaginosis women was negative for detection of 16S rRNA gene for *A. vaginae*.[38]

The association between *A. vaginae* and BV is now established. Our study demonstrates an association between *A. vaginae* and miscarriage , but it remains unclear whether *A. vaginae* is simply a marker of other vaginal anomalies in a complex polymicrobial condition or if it plays a direct pathogenic role alone or as part of a microbial imbalance. The transmissibility of BV suggests that it results from external contamination rather than an internal disequilibrium.[16,65,39]

The inability to prevent recurrences reflects our lack of knowledge about its origins. The initial event that shifts anaerobic predominance remains enigmatic. Recurrences have been associated with same-sex partners, whether exclusive or not. Reduced rates, on the other hand, are associated with condom use and estrogen-containing oral contraceptives.[16,35,38]

In the phylogenetic-tree analysis of 16S rRNA gene, *A. vaginae* isolated from vaginosis women with miscarriage and vaginosis women without miscarriage . the phylogenetic tree analysis was indicated that the local *Atopobium sp* isolate No.1were show genetic closed related to NCBI-Blast *Uncultured Atopobium sp. clone FX22W63* and local *Atopobium sp* isolate No.2 were show closed related to NCBI-Blast *Atopobium sp. S7MP14* at sequence homology identity (99.17% and 98.75%) respectively whereas other NCBI-Blast Atopobium sp showed differences out of the tree at total genetic change (0.002-0.012%).

CONCLUSIONS

The identification of selected vaginal bacteria associated with an increased risk of miscarriage could support screening programs early in pregnancy and promote early therapies to reduce early pregnancy loss.

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