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Molecular Detection and Genotyping of Gardnerella Vaginalis, 16S rRNA Gene from Bacterial Vaginosis Miscarriage Women in AL-hillah City

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Abstract--- *This study was aimed to determine the Gardnerella vaginalis in vaginosis women without miscarriage and vaginosis women with miscarriage. Also other aim, the DNA sequencing was performed for phylogenetic tree analysis of 16SrRNA gene in local Gardnerella vaginalis isolates in comparison with NCBI-Genbank global Atopobium vaginae isolates and finally submission of the present isolates in NCBI-Genbank database. One hundred fifty (150) high vaginal swabs were collected from women with vaginosis (Seventy five samples were taken from married vaginosis women without miscarriage and Seventy five samples from vaginosis women with miscarriage) from Babylon city hospital and private clinics. The age of patient (15– 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. Gardnerella vaginalis was isolated on Columbia agar supplemented with 5% fresh blood with addition of antibiotics, the study confirmed that 15 (20.00%)and 23(30.66%)of Gardnerella vaginalis out of 150 swabs isolated from miscarriage and non-miscarriage vaginosis women respectively. According to molecular detection of the 16S rRNA gene, the study revealed that 67(89.33%)and 72(96.00%)of Gardnerella vaginalis out of 150 swabs obtained from miscarriage and non-miscarriage vaginosis women respectively. BLAST analysis showed that the 16S rRNA gene shared more than 98- 99% sequence compatibility with the sequences of Gardnerella vaginalis. Furthermore, the phylogenetic tree analysis of the 16S rRNA gene indicated that local Gardnerella vaginalis (NO.1 and NO.2) isolates shared higher homology with other Gardnerella vaginalis isolates available in the GenBank. The homology of the nucleotides was (98.50%) respectively.*

Keywords--- *Bacterial Vaginosis, Molecular Identification, Gardnerella Vaginalis, 16 s rRNA Gene Sequence, Miscarriage.*

I. INTRODUCTION

Bacterial Vaginosis is a dysbiotic condition of the vaginal cavity that has deleterious effects during pregnancy [1]. Many novel, fastidious and uncultivated bacterial species are related with Bacterial vaginosis that is one of the most common genital infections among women in the childbearing age. These are called bacterial vaginosis associated bacteria (BVAB), present in trace amount and have a significant role in the infection. Biofilm forming by these bacteria led to increase chance of bacterial vaginosis incidence due to increase bacterial resistant[2]. A healthy vaginal flora, dominated by *Lactobacillus* species, has an important role in the protection against genital infections,

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which are considered as a common cause of miscarriage [3][2].Clinically a profuse vaginal discharge and a rotten fish vaginal odour are characteristic symptoms, although some women with BV remain asymptomatic [**4]**

Bacterial vaginosis 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 [5].

Gardnerella vaginalis is a facultative anaerobic Gram-variable rod that is involved together with many other bacteria mostly anaerobic in bacterial vaginosis in some women as a result of a disruption in the normal vaginal microflora [6].

Gardnerella vaginalis is strongly associated with bacterial vaginosis (BV) and is one of the most frequently isolated bacteria from women with symptoms of BV [7] [8]. Abundance of *G. vaginalis* in vaginal samples has also been associated with preterm labor [9].

An influential factor in the pathogenicity of *Gardnerella vaginalis* is its adhesive ability, which enables bacteria to attach to the epithelium of the vaginal mucosa, colonize it and form a biofilm [10]

Biofilm formation is key for the development of disease since it confers heightened antibiotic tolerance and resistance to host immune defenses making diseases chronic and/or relapsing [11][12]

Other virulence factors produced by *G. vaginalis* are cytolysins that cause cell death by activating the protein kinase pathway in human epithelial cells. Among the best-studied cytolysins is vaginolysin, a member of the cholesterol-dependent family of pore-forming toxins that lyses human red blood cells and vaginal epithelial cells [13].

II. MATERIALS AND METHODS

Sample Collection

The total number of samples were collected (150) high vaginal swabs samples from bacterial vaginosis women (seventy five vaginosis women with miscarriage and seventy five vaginosis women without miscarriage) were recovered All samples or individual were admitted to out-patient clinics of Gynecology and Babylon Maternity and Pediatrics Hospital in Al-Hilla city/ Iraq. 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 (Amsel's 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.

Bacterial Isolation

Gardenerella vaginalis grow on Columbia agar supplemented with 5% fresh blood with addition of Naldixic acid, Gentamycin and Nystatin and the colonies tend to be smooth, small colonies(hear pin) circular entire, glistening, and opaque colonies β- hemolysis surrounded with double hemolysis zone the addition of Antibiotics

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were allowed to selective isolation of *G. vaginalis* that are described by [14]. The isolates were showed to be catalase negative, oxidase variable, coagulase negative, and mannitol fermentation negative. Also, all isolates were urease negative, methyl red and citrate utilization test positive Also the isolates were confirmed by Vitek 2 system**.**

III. MOLECULAR DIAGNOSIS

DNA Extraction

Kit (G-Spin^{TM} Total DNA extraction kit (iNtRON/ Korea)) was used in DNA extraction from all frozen high vaginal swabs as well as grown bacterial colonies.

Detection of Specific Gene Markers By PCR

The primer was used to 16SrRNA gene a detection primer listed in Table (1) according to Fredricks *et al*. [15]. and PCR conditions were used to detect 16SrRNA gene of *G. vaginalis* are present in table (2).

PCR Reaction Mixture

Reaction mixture contained 1µl of forward primer, 1µl of reverse primer, 5µl of extracted DNA and 13µl of nuclease free water. These PCR Master mix reaction component placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer and tracking dye).

Successful PCR amplification was confirmed by gel electrophoresis on 1.5% agarose gels for 50 min at 70 V.

Table 1: Primer for Amplification of *16S rRNA* Gene of *Gardnerella Vaginalis*

Bacterium	**Primer Sequence (5' -------- 3')**		**PCR product size**	**GenBank code**
Gardnerella vaginalis	F	GGGCGGGCTAGAGTGCA	210	AY738695
	R	GAACCCGTGGAATGGGCC		

Table 2: Amplification Conditions of *16 SrRNA* Genes[15]

Steps	Temperature	Time	No. of cycles
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	38 cycle
Annealing	62	30sec	
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

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DNA Sequencing

The amplicon 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.

IV. RESULTS AND DISCUSSION

Cultural and Molecular Detection of Isolates

The isolation and identification of *Gardnerella vaginalis* isolates creates by cultural and biochemical characteristics and also confirmed by Vitek 2 system out of 150 high vaginal swab 15 (20.00%)and 23(30.66%) isolated from miscarriage and non-miscarriage vaginosis women respectively.

Our result showed *G. vaginalis* isolated were higher than the rates of result conducted in Basrah by Mahdi and Al-Hamdani[16] who found the rates of isolated *G. vaginalis* were 6.2%, 7.6% among women with and without habitual abortion respectively.

Whereas Jabuk [17] was found that there was no *G. vaginalis* in Babylon city on BV patient's. this variation may be due to the Geographic distribution and to type of sample and the Antibiotic's uptake.

The risk to get *G. vaginalis* resulted from their ability to biofilm formation and may be establish chronic persistent infection [18][19].

Study proposed by Bunyan *et al*.[20]found that the isolates of *G vaginalis* are capable to adhere to epithelial cells of vagina and causes infections.

While the result of PCR detection confirmed that out of 150 high vaginal swab samples 67(89.33%)and 72(96.00%)of *Gardnerella vaginalis* obtained from miscarriage and non-miscarriage vaginosis women respectively as show in table (3) and figure (1) according to molecular detection by Bunyan et al [21].

Table 3: Cultural and PCR Detection Percentage of *Gardnerella Vaginalis*

Types of samples	**Culture Number**		**PCR Number**	
	Positive (%)	**Negative (%)**	**Positive (%)**	**Negative (%)**
Vaginal swab from BV women with miscarriage	15 (20.00%)	60(80.00)%	67(89.33%)	8(10.66)%
Vaginal swab from BV women without-miscarriage	23(30.66%)	52(69.33)%	72(96.00%)	3(4.00)%
Total	38(25.33)%	112(74.66)%	139(92.66)%	11(7.3)%

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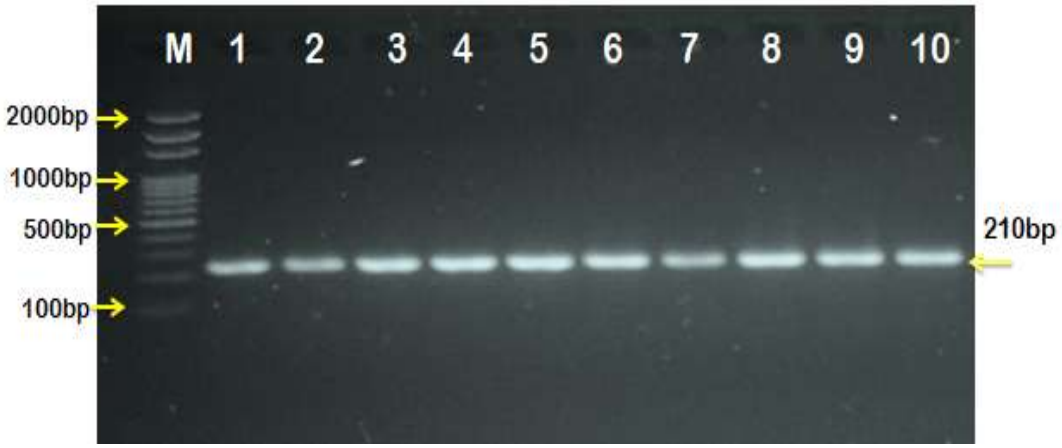

Figure 1: Agarose Gel Electrophoresis Image That Showed PCR Product Analysis For *16S rRNA* Gene In *Gardnerella Vaginalis*. M (Marker Ladder 2000-100bp). Lane (1-10) Some Positive *Gardnerella Vaginalis*. Samples at 210bp Product Size.[21]

The present study agree with study conducted by Srinivasan *et al.* [22] who used *16SrRNA* genes to examine associations between bacterial vaginosis and bacterial morphotypes in infected women. The *16S rRNA* genes used in PCR targeting the variable region of the gene with species-level identification used for investigated the associations between the presence of individual bacterial species and clinical diagnostic characteristics of BV by Srinivasan *et al*.[23]

The current study was in agreement with study that conducted by Mahdey and Abed [24]who found *Gardenrella vaginalis* comprised high percentage (66.67%) from total vaginosis women. Lactobacilli depletion, combined with the presence of either *G. vaginalis* or *A. vaginae*, is also a highly accurate predictor of BV [25].

Also our result similar to study done in Iraq by Bunyan *et al*. [9]who showed that *Gardnerella vaginalis* was isolated from preterm labor in percentage (20%).

An imbalance in the normal vaginal bacteria is therefore known as bacterial vaginosis. Chorioamnionitis is the main cause of preterm delivery, and various previous reports have stated that chorioamnionitis occurs against a background of BV, When BV causes vaginitis or cervicitis and then progresses to inflammation of all fetal membranes, it can in turn cause premature rupture of membranes and labor [26] [27]

Phylogenetic Study Analysis

As the widespread use of PCR and DNA sequencing, *16S rDNA* sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. For bacterial identification,*16SrDNA* sequencing is important in case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections. It provided insights into an etiology of infectious disease, so helps clinicians to prescribe antibiotics and in determining the duration of treatment and infection control procedures [28].

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Two isolates (one isolate from BV women without miscarriage *G. vaginalis*. (IQB.G.No.1) and the other isolates from vaginosis women with miscarriage *G. vaginalis*. (IQB.G.No.2), sent for sequencing after that submission in NCBI-GenBank database to get accession number codes (MN165532 and MN165533) frequently.

DNA sequencing method was performed at the beginning to confirm the diagnosis of PCR and determined the nucleotide sequences and closed relationship with others world strains by study the genetic variation between local and word isolates that recorded in GenBank by used phylogenetic tree method that based on the presence of genetic variation.as show in figure (2).

Figure 2: Multiple Sequence Alignment Analysis of *16SrRNA* Gene Partial Sequence For Local *G. vaginalis*. (IQB.G.No.1 and IQB.G.No.2) With NCBI-Blast of *G. vaginalis*. Isolates*16SrRNA* 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 (*)and Differences In 16SrRNA Gene Nucleotide Sequences

Phylogenetic tree analysis was indicated that the Local *G. vaginalis* isolate (No.1 and No.2) were showed genetically closed related to NCBI-Blast *Gardnerella vaginalis* strain DNF00536 (KU726661.1) at sequence homology identity (98.50%) for both isolates whereas other NCBI-Blast *Gardnerella vaginalis* showed differences out of the tree at total genetic change (0.5-1.5%) as show in figure 3 and table 4.

The current results similar with results conducted by Abed and Kandala [29]who found that the results of sequencing of BV associated bacteria *G. vaginalis* isolated from pregnant women was identified in percentage of 87.5% by comparing its sequence with the standard strains of *G. vaginalis* in Gene Bank.

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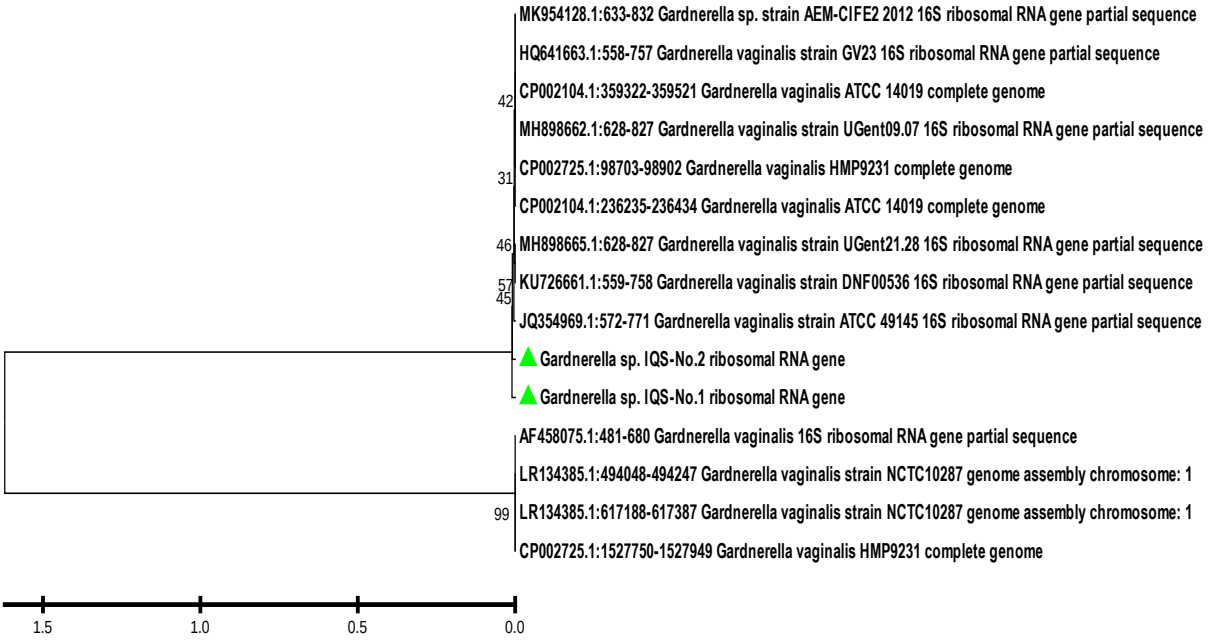

Figure 3: Phylogenetic Tree Analysis Based on *16SrRNA* Gene Partial Sequence That Used For Genetic Relationship Analysis of Local *Gardnerella spp*. (IQB.G.No.1 and IQB.G.No.2). The Phylogenetic` Tree was Constructed Using Unweighted Pair Group Method With Arithmetic Mean (UPGMA Tree) In (MEGA 6.0 Version) At Total Genetic Change (0.5-1.5%).

Table 4: The NCBI-BLAST Homology Sequence identity (%) between local *Gardnerella spp*. (IQB.G.No.1 and IQB.G.No.2) *16S rRNA* gene isolates and NCBI-BLAST submitted *Gardnerella spp.* 16S rRNA gene Isolates

Local isolates		**NCBI-BLAST Homology Sequence identity**		
	NCBI BLAST identity isolates	**Accession number**	**Country**	**Identity (%)**
Gardnerella sp. isolate No.2	*Gardnerella vaginalis* strain DNF00536	KU726661.1	USA	98.50%
Gardnerella sp. isolate No.2	*Gardnerella vaginalis* strain DNF00536	KU726661.1	USA	98.50%

The Nucleotide variations Substitution analysis between local *G. vaginalis* isolates *16SrRNA* gene and NCBI BLAST *G. vaginalis* isolates were showed highly transitional substitutions between (C) nucleotide that substituted by (T/U) nucleotide at (**32.60**)% from total nucleotides. Whereas highly nucleotide variations Substitution at transversionsal substitutions were showed at (*6.26*%) between (G) nucleotide that substituted by (T) and (C) nucleotide. As showed in table (5).

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Table 5: Nucleotide Variations Substitution Analysis between Local *Gardnerella spp* Isolates *16S rRNA* Gene and NCBI *Gardnerella spp* Isolates

	A	**T/U**	**C**	**G**
A	-	*4.07*	*4.74*	**0.00**
T/U	*4.64*	-	**32.60**	*6.26*
C	*4.64*	**27.97**	-	*6.26*
G	**0.00**	*4.07*	*4.74*	-

V. CONCLUSIONS

The identification of *Gardnerella vaginalis* 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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