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Molecular Detection and Genotypic Study of Eggerthella -Like Uncultured Bacterium by 16SrRNA Gene that Isolated from Bacterial Vaginosis Women with and without-Miscarriage in AL-Hillah City.

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Abstract

The present study was conducted to determine Eggerthella spp by culture independent method from both bacterial vaginosis(BV) women without and without miscarriage . Another aim was to sequence the 16SrRNA gene for phylogenetic study of the local isolates of Eggerthella spp in comparison to world Eggerthella spp isolates in NCBI Gen bank and lastly deposition of the current isolates in Gen bank. Hundred and fifty (150) high vaginal swabs from BV women with and without miscarriage were collected from the Hospital for Maternity and children and private clinics of Babylon city , where Seventy five samples(75) were taken from married BV women without miscarriage and Seventy five samples (75) from BV women with miscarriage . The patient's age (15– 45) years. The samples was taken by disposable swabs, 16SrRNA gene detection by polymerase chain reaction technique. Results revealed that from a total 150 swabs, 31(41.33%) and 18(24.00%) of Eggerthella spp obtained by PCR from BV women with and without -miscarriage respectively. Phylogenetic study of 16SrRNA gene has shown that local isolates Eggerthella spp (NO.1 and NO.2) have shared a higher homology with other isolates of Eggerthella spp available in the GenBank. Sequence similarity was (98.51% and 98.31%) for isolates (No.1 and No.2) respectively.

Keywords*:* Eggerthella -like uncultured bacterium, Bacterial vaginosis(BV), 16SrRNA gene ,Culture independent Technique ,DNA sequencing, Miscarriage

Introduction

Bacterial vaginosis is a vaginal normal flora disturbance, where the typically plentiful hydrogen peroxide producing *lactobacillus* are scarce and increased the growth of other anaerobe bacteria as *Gardnerella vaginalis , Atopobium vaginae, Bacteroides spp, Mobiluncus spp. and Prevotella spp* [1].Clinically a vaginal discharge and a rotten fish vaginal odour are a typical symptoms , although some

BV women remain asymptomatic [2]. Bacterial vaginosis is a main causes of vaginal complaints in childbirth age women (pregnant and non-pregnant) [3]. BV is correlated with adverse pregnancy outcomes like preterm labour , miscarriage and increasing the risk for infections that transmitted sexually such as human immunodeficiency virus (HIV) [4].

Bacterial vaginosis (BV) represents a reduction in *Lactobacilli* and acquisition of a diverse group of anaerobic and facultative bacteria . It can be diagnosed by various tests ranging from clinical indicators depended on the presence of vaginal discharge , high pH, fishy odour, and clue cells as determined by microscopy and molecular methods . Molecular technology is objective and capable of detecting fastidious bacteria, allows quantitation for more convenient and accurate testing for BV and is suitable for selfcollecting vaginal swabs [5] [6].

Employment of *16Sr RNA* gene sequencing has had a major impact on the field of vaginal microbiota. This culture-independent ways demonstrated the complexity of the vaginal microbiota and detected clusters of bacteria that associated with genital health or inflammation[7] [8] [9]. Additionally , further advantages conferred by 16SrRNA gene include its universality , the presence of multiple gene copies which makes it an abundant and easily detectable target, its highly conserved regions which enables the construction of a widerange universal polymerase chain reaction (PCR) primers, and the presence of highly variable regions for the identifying individual species [10][11][12][13]. Therefor this study conduct to detection fastidious ,unculturable bacteria that cause BV and had a serious adverse effect on pregnant outcome . Also this study aim to determine the sequence homology and phylogenetic tree between local isolates and isolates that deposed in NCBI GenBank.

Materials and Methods

Sample Collection

One hundred and fifty(150) high vaginal swabs samples were collected which diagnosed as bacterial vaginosis by the physician (seventy five from BV women with miscarriage and seventy five from BV women without- miscarriage) were recovered All samples or individual were attended to Maternity and Pediatrics Hospital and outpatient clinics of Gynecology in Al-Hillah city/ Iraq, during the duration from (November 2018 to June 2019). After taking the permission from the patients for examination and sampling, three cotton swab of high vaginal discharge obtained from each woman by brushing a swab across the vaginal wall.

DNA Extraction

G-Spin^{TM} Total DNA extraction kit (iNtRON/ Korea) was using for extracting DNA from all frozen high vaginal swabs according to manufacture instructions**.** Nanodrop spectrophotometer was used for checked concentration and purity of DNA that extracted from high vaginal swab which checked and measured by reading the absorbance at (260 /280 nm).

Primer Design and Uniplex PCR

Molecular detection was conducted by uniplex PCR with a primer as illustrated by [14] and imported from Macrogen Company as shown in the table (1).

Table 1: Primer for amplification of *16S rRNA* gene of *Eggerthella spp*.[14]

Bacterium	**Primer Sequence (5' --- 3') CR product (b**		**GenBank code**
Eggerthella spp	AACCTCGAGCCGGGTTCC CGGCACGGAAGATGTAATC	236	AY738656

PCR Reaction Mixture

The reaction of PCR for detection *16SrRNA* gene was done in a volume 20 µl, which included : Maxime

PCR Pre mix kit (Bioneer, Korea), 5 µl (20 ng/µL) of sampling DNA, 1 µL (10pm/µl) of each forward and reverse primers and the volume of the mixture was complete by using nuclease free water.

PCR Thermocycler Program

PCR conditions were used to detect *16SrRNA gene* of *Eggerthella spp* are present in table (2). After that agarose gel electrophoresis confirmed the PCR amplicon**.**

Table 2. Amplifying Conditions of *16 SrRNA* gene [14].

Steps	**Temperature**	Time	**Number of cycles**
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec.	38 cycle
Annealing	55 °C	30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1

DNA Sequencing

DNA sequencing of the amplicon was carried out by Macrogen Company in Korea by using the AB DNA sequencing system. phylogenetic study was proposed dependent on the identify alignment on the NCBIBlast and neighbor distance. phylogenetic study and sequence alignment analysis based on ClustalW alignment analysis.

Statistical Analysis

Statistical software package SPSS 23 was used to analyzed the result. Pearson Chi-square test and odds ratio with (95%) confidence was used to determine the statistical difference between groups.

Results and Discussion

Molecular Detection of *Eggerthella-spp*.

Vaginal microbiome dysbiosis due to anaerobic bacteria overgrowth result in bacterial vaginosis which is correlated with increased the genital mucosa inflammation. Furthermore, BV increases susceptibility to sexual transmitted infections (STIs) and is related with adverse outcomes of pregnancy [15]. A variety of microorganisms are responsible for BV, One of which is anaerobic bacteria that considered as vaginal normal flora and presents in great numbers because of reduce in the growth of *Lactobacillus spp* resulting in vaginal infections, which are considered as a common cause of miscarriage [16]**.** The current results showed that the number of *Eggerthella-spp* was (32.66%) the distribution of this microorganism in vaginosis women with miscarriage was (41.33%). While there distribution in vaginosis women without miscarriage was (24.00%) as showed in table (3) and figure (1). Statically there was no significant difference observed with a P value ($p \leq 0.005$). Amplification of *16SrRNA* gene of *Eggerthella-spp* by PCR to confirm the existence of *16S rRNA* gene that appeared in molecular weight 236 bp as conducted by [17] figure(2) was exclusively used to proceed for the sequencing analysis. Five species of bacteria (*A. vaginae, Eggerthella-like , G. vaginalis, , Leptotrichia spp* and *Megasphera ph. 1)* were identified in majority of women with bacterial vaginosis and can therefore be regarded as bacterial indicators of this disease . [18].Our results detected *Eggerthella spp* from both vaginosis miscarriage and non-miscarriage similar to study conducted by [19] who detected *Eggerthella* sp. type 1 *16SrRNA* gene sequence was in high percentage from BV women against women without BV.

Table 3. PCR detection percentage of *Eggerthella-spp*.

Types of samples	Positive (%)	Negative (%)
Vaginal swab from miscarriage women	31(41.33%)	44(58.66%)
aginal swab from non-miscarriage wom	18(24.00%)	57(76.00%)
Total	49(32.66%)	101(67.33%)
P –value	0.063	

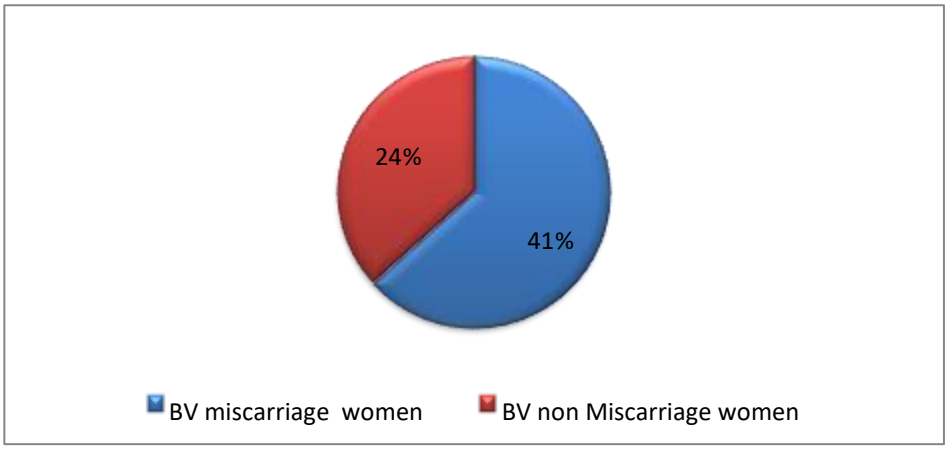

Figure 1. Distribution Of *Eggerthella-spp*. isolates in BV miscarriage and non-miscarriage women

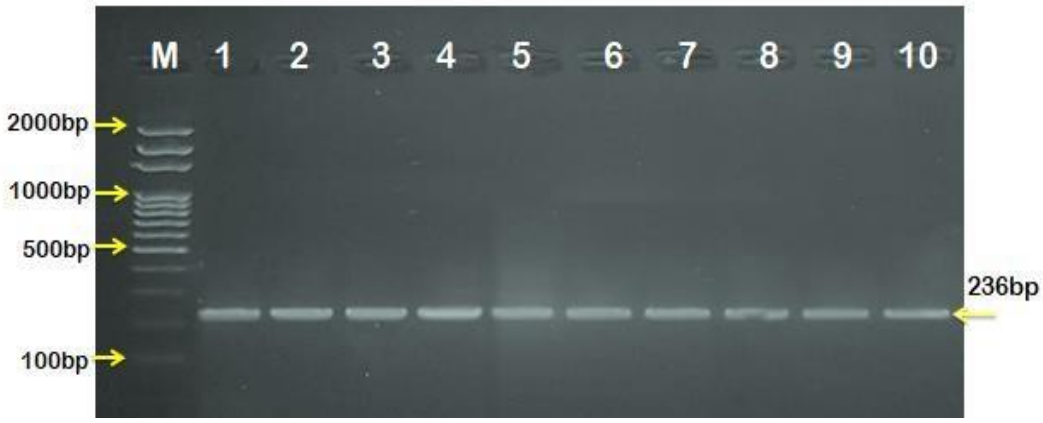

Figure 2. PCR amplicon Analysis For *16S rRNA* Gene In *Eggerthella-spp* by Agarose Gel Electrophoresis. M (Marker Ladder 2000-100bp). Positive *Leptotrichia spp*. sample shown at Lane (1-10) with 230bp Product Size.[17].

Phylogenetic Analysis

DNA sequencing was carried out in order to phylogenetic confirmative of *Eggerthella spp* based on *16S rRNA* gene detection . Two isolates(one from vaginosis miscarriage women and one from vaginosis non miscarriage women), were sent for sequencing after that submission in NCBI-GenBank database to get accession number codes (MN165522 and MN165523) frequently**.** phylogenic study of *16SrRNA gene* of *Eggerthella spp* isolated from miscarriage women with BV and BV women without miscarriage illustrated that the local *Eggerthella spp* isolates (No.1) and (No.2) were genetically related to NCBI-Blast uncultured *Eggerthella sp.* clone 123-f2 68 isolate (AY738656.1) at sequence homology identity (98.51% and 98.31%) for isolates (No.1 and No.2) whereas other NCBI-Blast *Eggerthella spp* showed differences out of the tree at a genetic variation (0.005-0.025%) as shown in figure (3,4) and table (4). The Nucleotide variations Substitution analysis between local *Eggerthella sp* isolates *16S rRNA* gene and NCBI BLAST *Eggerthella sp* isolates were showed highly transitional substitutions between (C) nucleotide that substituted by (T) nucleotide at (29.38)% from total nucleotides. Whereas highly nucleotide variations Substitution at transversionsal substitutions were showed at (*1.34*%) between (G) nucleotide that substituted by (T) and (C) nucleotide. As showed in table (5).

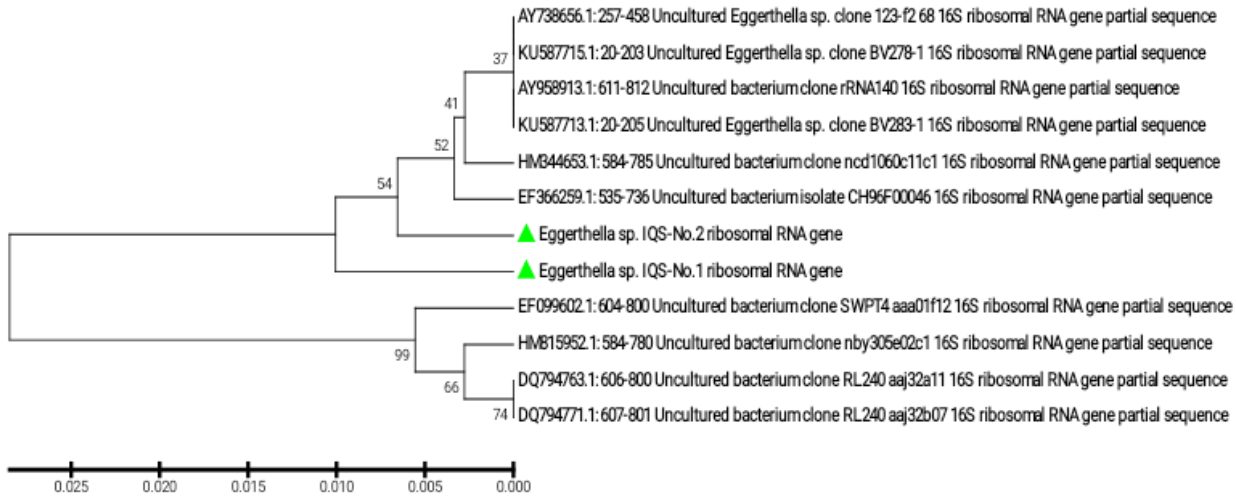

Figure 3. Phylogenetic Tree Analysis depend on Partial *16SrRNA* Gene Sequence of *Eggerthella spp.*
(IQB.E.No.1 and IQB.E.No.2) Local Isolates. Phylogenetic Tree was Conducted Using (MEGA 6.0 Version) . In a Total Genetic alteration (0.005-0.025%).

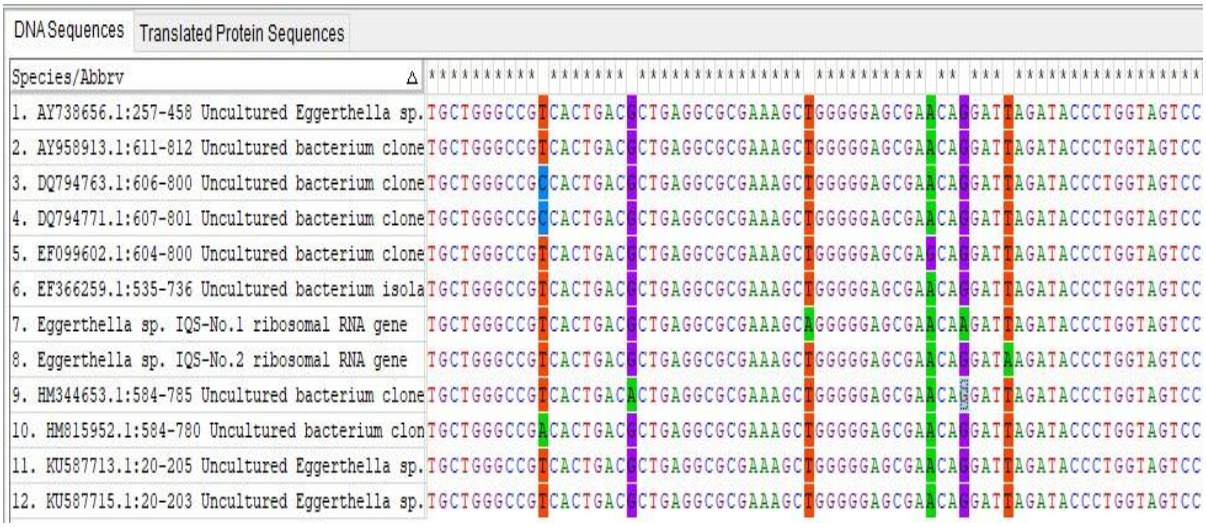

Figure 4. Partial Sequence Alignment Analysis of *16SrRNA* Gene of Local *Eggerthella spp*. (IQB.E.No.1 and IQB.E.No.2) With Gene Bank *Eggerthella spp*. Isolates *16SrRNA*. Partial Sequence Alignment Analysis was Conducted by Clustalw Alignment Tool In (MEGA 6.0 Version). Which Showed The sequence Alignment identity As (*) With Different *Eggerthella spp*.

Table 4. Sequence Homology (%) between *Eggerthella spp*.(IQB.E.No.1) and (IQB.E.No.2) *16S rRNA* gene local isolates and Gen bank deposited *Eggerthella spp*. Isolates.

Local isolates dentity Isolates oAccession Numbe NCBI BLAST	**Country**	**Homology (%)**
late of *Eggerthella s*cultured *Eggerthe* AY738656.1 No. 1 *p.* clone 123-f2 68	USA	98.51%
late of *Eggerthella s*cultured *Eggerthe* AY738656.1 No.2 *p.* clone 123-f2 68	USA	98.31%

Table 5. Nucleotide Variations Substitution Analysis Between Local *Eggerthella spp* Isolates *16SrRNA* Gene and NCBI *Eggerthella spp* Isolates

	6
	8
	7
	2

The table showed the probability of substitution from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are showed in bold and those of transversionsal substitutions are showed in *italics*. Evolutionary analyses were conducted in MEGA6.

Conclusions

Culture independent detection of unculturable , fastidious bacteria associated with bacterial vaginosis would support the early diagnosis in pregnancy and promote early curative to decrease the complication of pregnancy like miscarriage and preterm delivery.

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