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Bioinformatics-Based Investigation of Vaginal Microbiota in Women Experiencing Recurrent Aborted Women

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Abstract:

Recurrent pregnancy loss (RPL) is one of the most difficult problems in reproductive health since effective treatments are rarely accessible and its causes are frequently unclear.To identify the independent culture vaginal microbiota of recurrent aborted women by sequencing *16S ribosomal RNA* .Vagina specimens were collected from recurrent abortion women . Multiplex PCR technique was performed based *16S RNA* gene to identify the uncultured bacteria. The DNA sequencing method was performed using Sanger's method and phylogeny for mutation analysis by using NCBI BLAST analysis. *Megasphera, Atopobium*, *Mobiluncus*, *Erysipelothrix*, *Catibacterium*, *and Bifdobacterium* were diagnosed . This alignment study revealed the *16S ribosomal RNA* gene's substitution mutations and nucleotide alignment similarities across isolates . Phylogenetic tree analysis of local isolates related to NCBI-BLAST showed genetic relationship with some variation or subsituation of nitrogen bases of target nucleotides. The maternal vaginal microbiome has an important role in the recurrent pregnant loss etiology.

Keywords: Bioinformatics ; Vaginal Microbiota ; Recurrent Aborted Women; Multiplex PCR technique

Introduction

The majority of lower vaginal tract infections in the general population are caused by bacterial vaginosis (BV), affecting 30% of women (1A 60% loss rate is thought to occur either before to or shortly after implantation in humans (2). The majority of pregnancy failures after implantation happen too soon to be noticed, The majority, however, present as clinical miscarriages. The risks of these pregnancies include preterm birth, placental abruption, and stillbirth, even though the majority of women who experience recurrent miscarriages will go on to give birth to live babies (3) . The relationship between alterations in vaginal microecology throughout pregnancy and the success of the pregnancy is attracting more attention from researchers (4) and are doing extensive research on vaginal microecology. It is possible to identify non-cultivable bacteria in the vaginal microbiome using molecular techniques, such as A. vaginae, which is hard to cultivate (5). Molecular identification methods that target genes (mostly the *16S rRNA* sequence) of

while more expensive than the gold standard, some bacterial genera or species allow for fuller characterization of the vaginal flora. Microbiomes can now be studied with higher levels of sensitivity and accuracy than traditional culture-based assays thanks to methods that extract bacterial DNA, amplify it via polymerase chain reaction, and identify target genes, frequently using the bacterial *16S rRNA* gene . These techniques might explain whether bacteria play a harmful or protective function in health and illness. (6). One or more highly variable areas are amplified and then sequenced for the *16S rRNA* gene, and the data acquired enables the taxonomic makeup and diversity. This technique reveals the presence of microorganisms and the proportion of the overall population that can be ascribed to a particular taxon (i.e., phyla or genera) inside a sample (7).The present study aimed to identify the independent culture vaginal microbiota of recurrent aborted women by sequencing *16S ribosomal RNA* gene and detect the genetic variations between the diagnosed local isolates in compared with world strains recorded in Gene bank.

Materials and Methods

Collection of samples

A total of 120 swabs (high vaginal) were collected from three group of women(30 recurrent abortion women , 30 pregnant woman and 60 healthy women whom clinically diagnosed by the Gynecologist .The studied groups aged from (16 - 46) years old attended the women's and children's Teaching hospital in AL-Diwaniyah city during the period from November , 2024 to April, 2025. The swabs placed in Amies transport media then transported to the laboratory. Each specimen had two duplicate swabs ;one it used for wet ,stained preparation ; and the other was used for a PCR test that would directly detect the presence of bacteria that cause vaginitis.

Multiplex Polymerase Chain Reaction

Multiplex PCR technique was performed based *16S ribosomal RNA* gene. The PCR primers for *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 (Iraq) (table 1).

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

Primer	Sequence (5'-3')		Product Size bp	Genbank
Megasphaera sp.	F	GACGATCAGTAGCCGGTCTG	400	LN998020.1
	R	ATTCCGCTTTCCTCTCCGAC		
Atopobium sp.	F	GGCACGCTTAACACATGCAA	480	AJ581675.1
	R	CCTACGTATTACCGCGGCTG		
Mobiluncus sp.	F	CACTGGGACTGAGATACGGC	520	OX458307.1
	R	GCATCCCCACACCTAGTTCC		
Erysipelothrix sp.	F	GGAGGCAGCAGTAGGGAATT	600	OQ355811.1

	R	CCACATAGTCCAGCGCTTGT		
Cutibacterium sp.	F	TTCGATGCAACGCGAAGAAC	310	LC414574.1
	R	ACAGGCTCGCAACTCTTTGT		
Bifidobacterium sp.	F	GCAGCAGTGGGGAATATTGC	720	MW750419.1
	R	ACATCTCACGACACGAGCTG		

Using the PrestoTM Mini gDNA Bacteria Kit and following the manufacturer's instructions, bacterial isolates' genomic DNA was extracted. Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer, USA, was used to measure the DNA content (ng/L) and assess the purity of the RNA at absorbance (260/280 nm), and it was used to examine the extracted total DNA. Using the GoTaq ®Green PCR master kit, the PCR master mix reactions were made in accordance with manufacturer's instructions. The PCR tubes were then all transferred to an Exispin vortex centrifuge and spun at 3000 rpm for 3 minutes before being put into a BioRad-USA T100 PCR Thermocycler. Optimase ProtocolWriterTM online program was used to generate the multiplex PCR thermocycler conditions protocol for each gene, which was then carried out using standard PCR thermocycler settings (table 2).

Table (2) PCR Thermocycler Conditions

Multiplex PCR step	Temp.	Time	repeat
Initial Denaturation	95°C	5min	1
Denaturation	95°C	30sec.	35 cycle
Annealing	58°C	30sec	
Extension	72°C	1 min	
Final extension	72°C	5min	1
Hold	4°C	Stop	-

The agarose gel electrophoresis technique was used to evaluate the mPCR results.With the use of a UV Transilluminator, PCR products were seen .

DNA sequencing method

Sanger's approach for identifying genetic relationships and analyzing genetic variation (substation mutations) based on the *16S ribosomal RNA* gene in bacterial isolates was used to execute the DNA sequencing procedure.The multiplex PCR products were sent to the Korean company Macrogen for DNA sequencing using an Applied Biosystems machine. The DNA sequence was analyzed using Molecular Evolutionary Genetics study version 6.0 (Mega 6.0), which is based on sequence-based ClustalW alignment analysis, and multiple sequence alignment research of the genes. The evolutionary distances were calculated using the phylogenetic tree UPGMA algorithm and the Maximum Composite Likelihood approach. the identification of homologous sequences and analysis of mutations using NCBI BLAST. The genes were finally uploaded to the NCBI-Genbank database in order to get Genbank accession numbers.

Statistical analysis :

Data were collected, compiled, analyzed, and presented using Microsoft Office Excel 2010 and the Statistical Package for Social Sciences (SPSS) version 25. The chi-square test was used to determine if any two category variables were related. P-values under 0.05 and 0.01, respectively, were considered to be at the highly significant level of significance (8).

Results

The resalts of multiplex PCR based on amplification of the *16S rRNA* gene of *Megasphera, Atopobium, Mobiluncus, Erysipelothrix, Catibacterium, and Bidifidobacterium* in vaginal swabs . Gave positive results with percent 178 (33.0 %), while the negative was362(67.0%). (Figure 1)

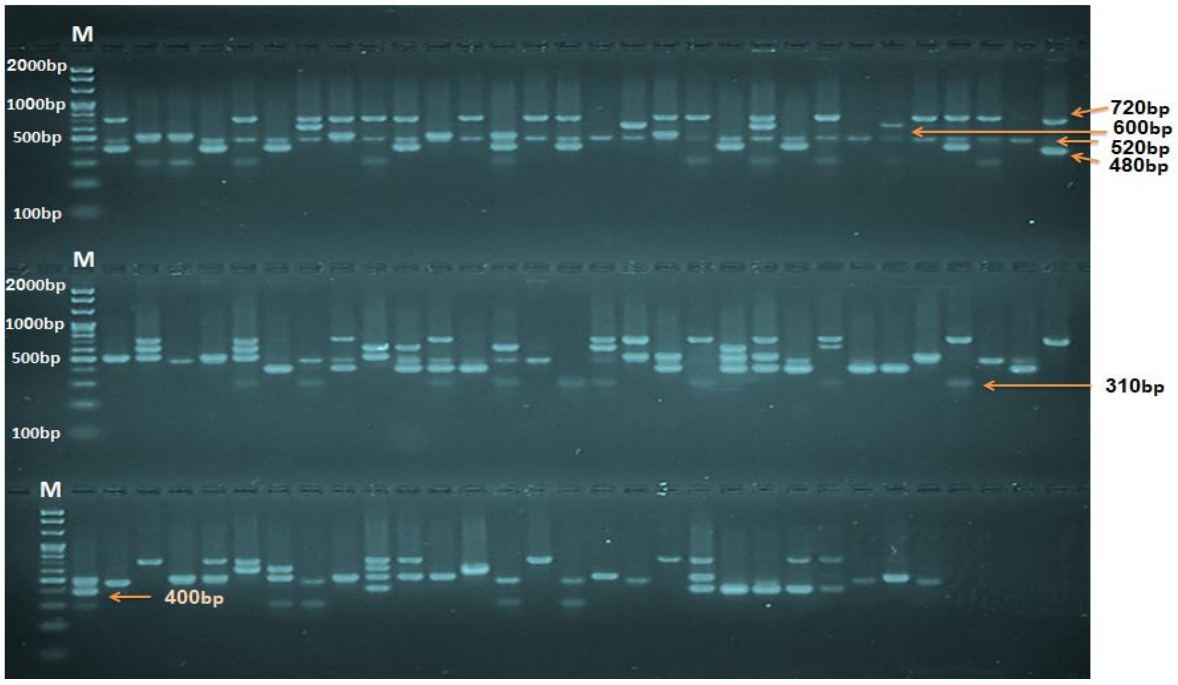

Figure (1): Agarose gel electrophoresis image that showed Multiplex PCR product analysis based on *16S ribosomal RNA* gene for detection Bacterial vaginosis bacterium. M (Marker ladder 2000-100bp). The lanes for *Catibacterium, Megasphera , Atopobium, Mobiluncus , Erysipelothrix* and *Bidifidobacterium* were showed positive at 310bp, 400bp , 480bp , 520bp, 600bp and 720bp PCR product size respectively.

The phylogenetic tree was created using MEGA 6.0's Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree). The nearby isolated Nos.38,58,36 of *Megasphaera* sp. IQ.abortion, IQ.pregnant, and IQ. Heathy isolates showed genetic related to NCBI-BLAST *Megasphaera massiliensis* strain NBRC (CP084019.1). at total genetic changes (0.0060-0.0010%) .*Atopobium* sp. F0209 isolate (EU592966.1) at total genetic changes (0.04-0.01**).***Mobiluncus porci* strain RF-GAM-744-WT-7 (MN537552.1) at total genetic changes (0.0100-0.0020%) **;***Erysipelothrix* sp. 'B 2344/87' isolate (EF050041.1) showed genetic changes at level (0.0120-0.0020%) ;*Bifidobacterium* sp. strain MRG-SR-352 (MW036293.1). at total genetic changes (0.0050-0.001) *Biifidobacterium* sp. strain MRG-SR-352 (MW036293.1) at total genetic changes (0.0050-0.0010%).

The total DNA homology identity percent of six uncultured bacterial isolates in the present study in recurrent abortion women was 98.27% with a mutation rate 1.73% and a mutation numbers (42) that recorded with accession no.in world GeneBank (Table 1) ,where the *Atopobium* sp. gave a low identity (97.93%) with a high genetic variation (2.07%) in a rate of 8 mutations . While , the *Cutibacterium* sp. gave a high identity (98.43%) with a low genetic variations (1.57%) in a rate of 3 mutations.

Figure (2) shows the NCBI -BLAST Homology sequence identity analysis between the 3 study groups . The results revealed that percent of identity were varied according to causative agents and cases of study groups, where ranged from 97.92 to 99.47 %.The high percent was recorded in a healthy group while the lowest in pregnant group . While the bacteria , *Cutibacterium* sp. gave a high percent of identity (99.47%) and the lowest was 97.92%

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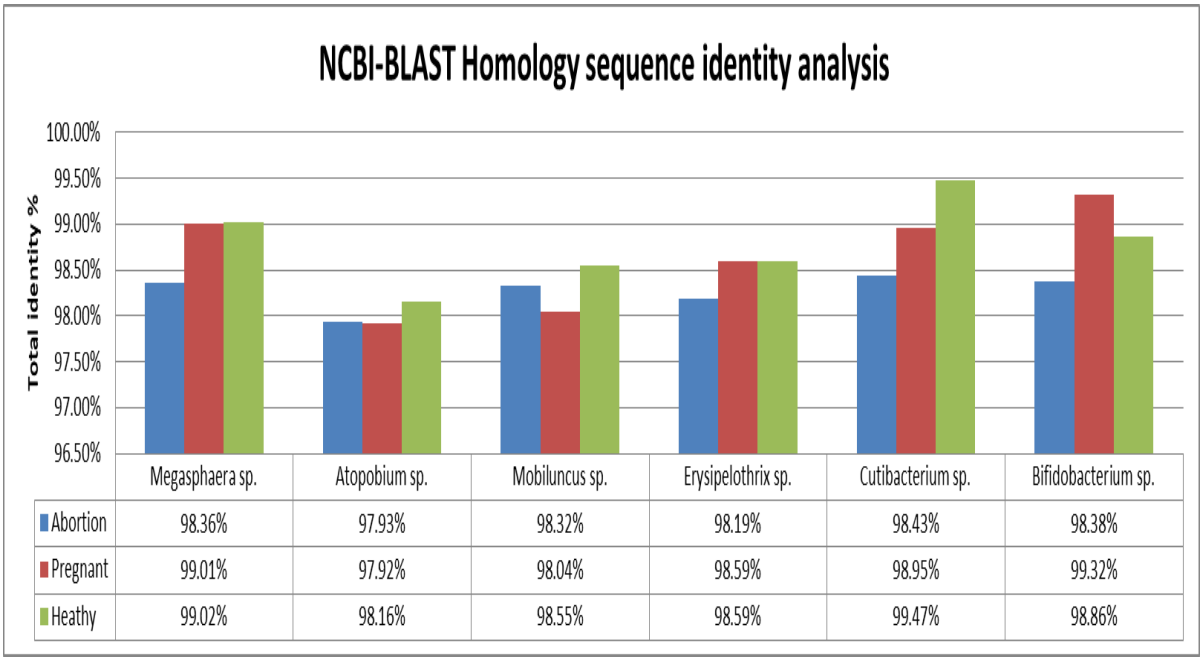
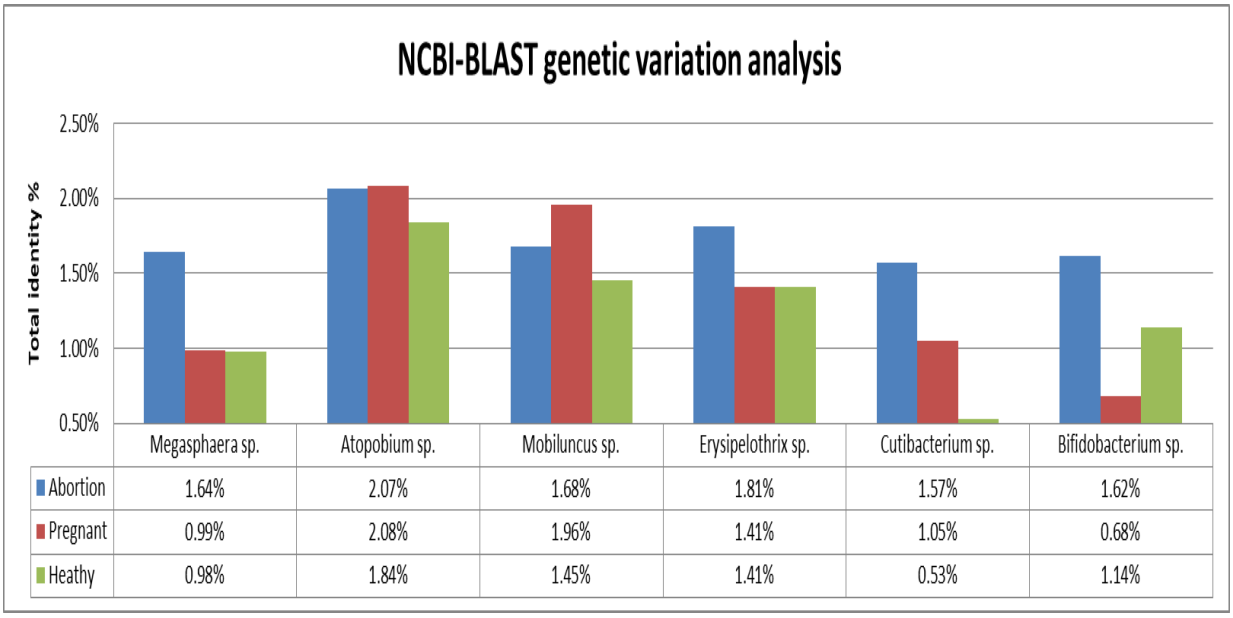


	Megasphaera sp.	Atopobium sp.	Mobiluncus sp.	Erysipelothrix sp.	Cutibacterium sp.	Bifidobacterium sp.
Abortion	98.36%	97.93%	98.32%	98.19%	98.43%	98.38%
Pregnant	99.01%	97.92%	98.04%	98.59%	98.95%	99.32%
Heathy	99.02%	98.16%	98.55%	98.59%	99.47%	98.86%

Figure (2) The NCBI-BLAST Homology sequence identity analysis between abortion, pregnant, and healthy bacterial vaginosis isolate

The results revealed that percent of genetic variation analysis were varied according to causative agents and cases of study groups, where that percent was high in the *Atopobium* sp. 2.08 % in a pregnant in compared with the lowest one (0.53%) for the bacteria , *Cutibacterium* sp. in healthy group (Fig 3).



	Megasphaera sp.	Atopobium sp.	Mobiluncus sp.	Erysipelothrix sp.	Cutibacterium sp.	Bifidobacterium sp.
Abortion	1.64%	2.07%	1.68%	1.81%	1.57%	1.62%
Pregnant	0.99%	2.08%	1.96%	1.41%	1.05%	0.68%
Heathy	0.98%	1.84%	1.45%	1.41%	0.53%	1.14%

Figure (3) genetic variation analysis between recurrent abortion ,pregnant and healthy bacteria vaginosis isolatie

Discussion

First and foremost, choosing the right primer combinations and paying attention to the hypervariable parts of the bacterial *16S rRNA* gene may make a difference in the outcomes (9). According to the results of the present investigation, universal primers may effectively gather microbial diversity for both short and long read sequences(10) . The findings of this study demonstrated that all bacterial groups that have been described in associated research may be consistently identified using these primer combinations. However, additional comparison research with primers amplifying other hypervariable areas of the bacterial *16S rRNA* gene would be required. The issue of choosing the right primer combinations for vaginal microbiome research has drawn a lot of interest.

. There hasn't been an ideal option discovered yet, thus further investigation is required to choose the ideal, universal primers for researching the vaginal microbiota. (11).

Given the increasing significance of species-level categorization, selecting databases and techniques for comparing and categorizing sequences to the appropriate taxonomic levels is an essential methodological problem. The most recent local research by Mahdey *et al*. (2012)

. showed a significant risk of pregnancy when *Megasphera* and *Atobopium* are infected. *Atopobium* produces lactic acid as one of its main metabolic products, which is interesting. However, Dipeptidyl peptidase activity is also known to exist in several species.

, which allows them to significantly increase their ammonium production in different settings (13). BV bacteria, such as *G. vaginalis*, may use the latter as a substrate (14). Because *Atopobium* and *Mobiluncus* spp. are resistant to metronidazole, it is difficult to successfully treat infections with these organisms, which explains their crucial involvement in the development of chronic and recurrent BV (15). Gram-stained rod anaerobic bacteria are *Mobiluncus* spp. Additionally, such bacteria have a connection to BV (16).

Using PCR, these bacterial species were identified in women with BV at 84.5% and in women with no BV infection at 38%. Furthermore, *M curtisii* was only revealed to be present in BV-affected women at 65.3%.

The main mutation of local isolates showed many mutation either by substitution or insertion. This research used the sequencing approach to show the qualitative makeup of the vaginal microbiota in pregnant women. Walther-Antonio *et al*. (17) studied the vaginal microbiome of pregnant women. was

given to the variation in the bacterial vaginal composition in agreement with previously published research, which highlight the fact that the genus Lactobacillus predominates in the physiological vaginal microbiota during prenancy(18). The predominance of this genus in pregnant women is linked to higher estrogen production. Glycogen builds up as a result and is converted to lactic acid, which encourages the growth of *lactobacilli* (19). It is important to remember that not all species of the genus Lactobacillus are created equal. This is not a rule, however. *Atopobium* sp., within this genus, was prevalent in our investigation in relation to the studied group, whereas others appeared in lesser proportions. Despite the fact that it is still unclear what exactly these species do in the vagina during pregnancy, several studies have shown that they may play a dominant role during the transitional period between "normal" and "abnormal" microbiota or when *Lactobacilli* dominance is being restored following antibiotic therapy. This is also most probable because the genome also encodes for the pore-forming toxin inerolysin, which is linked to *Gardnerella* spp. vaginolysin. Microorganisms have clonal variations that, in certain circumstances, support vaginal health and, in other circumstances, are linked to dysbiosis and illness (20). In other instances, the investigation also discovered a high concentration of bacteria belonging to the species *Bifidobacterium*. Progesterone, the main prenatal hormone, which promotes a rise in the relative abundance of *Bifidobacterium* and reaches its maximum concentrations in the last trimester of pregnancy, is most likely the cause. study conducted by the group of Nuriel-Ohayon *et al*. These results are confirmed in pregnant women, a mouse model, and in vitro settings (21). *Bifidobacterium* species have a significant impact on both the fetus and the mother during pregnancy (increasing insulin sensitivity and the immune system). They are the predominant kind in the digestive system of healthy infants and are transferred to the baby after normal delivery and nursing (22). Early in infancy, *Bifidobacteria* play an essential role for the immune system's development, and they also create lactic acid, which may break down the oligosaccharides found in human milk. Infants with illness states have lower concentrations of these microorganisms (23). Therefore, it may be inferred that the rise in *Bifidobacterium* bacteria during pregnancy is related to the body's natural preparation for giving birth. *Bifidobacterium* is thought to be a part of the bacterial microbiota in nature. Additionally, several bacteria that are also found in BV, such as *Megasphaera* or *Atopobium*, have the capacity to create lactic acid, suggesting that species other than lactobacilli may be able to shield a person from the spread of pathogenic bacteria (19). The current research successfully analyzed six local isolates using *16S rRNA* gene sequencing from each group that had recurrent abortion loss, pregnant women, and six individuals from the health group, demonstrating the genetic variety brought on by mutations. A mutation is a change in the nucleotide sequence that may result in the failure of certain biological processes or the creation of completely new ones. Both spontaneous mutations and mutations brought on by exposure to certain chemicals are possible. The bacterial make-up of the human vaginal microbiome in various women groups has been identified using *16S rRNA* sequencing. The adoption of culture independent approaches to describe bacterial diversity based on the categorization of partial *16S rRNA* gene sequences (24,25) has significantly improved our knowledge of the human vaginal microbiome. Results Sequencing and bioinformatics studies revealed that samples were 97%–99% aligned. It is important to note that our bioinformatics analysis had first revealed any mutations that were found. But the majority of studies that have been released to far have reported by (26). Patients with RPL were found to have sequence variations. Novel mutations in women who have repeated miscarriages are a useful tool for identifying the genetic roots of complicated reproductive diseases. These results supported the notion that examining a carefully chosen set of genes might be a potent strategy for determining the molecular causes of complicated disorders. The bioinformatics analysis presented here is an effective method for locating mutations (possibly having moderate or strong functional effects) linked to the etiology of RPL, and some of these variants (and genes), after being definitively functionally validated, will be used as RPL biomarkers (27). The *16S rRNA* gene of the uncultured regional isolates of vaginal microbiota underwent DNA sequencing for phylogenetic tree analysis in RPL, where it was compared to global isolates from NCBI-Genbank before the current isolates were included into the database. The discovery of specific vaginal bacteria

linked to an increased risk of miscarriage in the phylogenetic-tree analysis of the *16S rRNA* gene should enhance early-pregnancy screening programs and encourage early interventions to minimize early pregnancy loss (28).

Conclusion:

This study concludes that the vaginal microbiome plays a significant role in the etiology of recurrent pregnancy loss (RPL). Through 16S rRNA gene sequencing, several bacterial genera—including *Megasphaera, Atopobium, Mobiluncus, Erysipelothrix, Cutibacterium,* and *Bifidobacterium*—were identified in women with a history of recurrent abortion. Phylogenetic and mutation analyses revealed genetic variations in these local isolates compared to global strains, with mutation rates ranging up to 2.08%. These findings suggest that specific compositional and genetic changes in the vaginal microbiota may contribute to pregnancy loss, highlighting the potential for microbiome profiling to serve as a biomarker for RPL risk and informing future therapeutic strategies.

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