PCR And Sequencing Technique Detection Of Gardnerella Spp Associated With Urinary Tract Vaginosis

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KEYWORDS
Gardnerella spp., 16SrRNA gene, DNA sequencing, Bacterial vaginosis

ABSTRACT
Introduction: Gardnerella is a bacteria originally described in bacterial vaginosis, which can colonize the urinary tract and is often associated with vaginosis when present as a single-species flora. Commonly found in vaginal tracts, and it is also associated genital tract problems.
Methods: The current study was to identify the genetic variation of 16SrRNA gene in Gardnerella spp. which isolated from urine samples. In this study, 100 samples of urine from of women suffering from Urinary tract problems collected between August to September 2023 in Thi-Qar province of Iraq.
Results: The PCR technique results noted that (90%) of urine samples had Gardnerella sp. isolates by 16SrRNA gene. The bacterial samples recorded in NCBI (LC774556, LC774557, LC774558, LC774559, LC774560 LC7745561, LC774562 and LC774563) a 278bp genetic fragment that partially encompassed a coding region of the 16SrRNA gene was enlarged in this study. The phylogenetic tree was built in order to evaluate the precise phylogenetic distances, in addition to other related bacterial genomes. The present results exhibited that Gardnerella spp. sequences showed around 99% homology. Genetic variations of 16SrRNA gene were identified, the major variations were variably distributed in the (LC774556, LC774557) of the studied samples; while the variation in (G176A) was identified only in LC774556 sample. Also, the deletion mutation (A116 del) was noticed in all studied samples, while (T129 del) was showed in (LC774556, LC774558, LC774560) samples.
Conclusion: The infection with Gardnerella vaginalis. may be related with Bacterial vaginosis tract; Also, the DNA sequencing used to detect the genetic variation of Gardnerella vaginalis.

1. Introduction

Urinary tract is a common vaginal condition in women of reproductive age. significantly more, prevalent in women compared to men, with a ratio of roughly 8:1 [1]. Furthermore, around 50-60% of adult women will encounter at least one UTI during their lifetime [2]. Moreover, (UTI) can have harmful effects on an individual's quality of life and may lead to the development of depression [3].

The pathogenic gut bacteria can induce UTI through retrograde infection when they colonise the vaginal introitus or periurethra [4]. Because of the relationship between the stomach and bladder, vaginal microbiome, namely Gardnerella vaginalis, is often not considered as a potential cause of UTIs, even if it can indeed cause of infection. The clinical significance of G. vaginalis has been underestimated until now, mainly due to its infrequent detection in traditional urine culture [5]. The G. vaginalis is a type of rod-shaped bacterium that may survive with or without oxygen. It is classified as Gram-variable, meaning its staining characteristics can vary. G. vaginalis is commonly seen in bacterial vaginosis, a condition in which the usual balance of bacteria in the vagina is disrupted. This condition is often caused by the presence of several bacteria, particularly those that thrive in oxygen-deprived environments. [3].

G. vaginalis is commonly found in vaginal tracts, but it is also associated with bacterial vaginosis and genital tract infections in men [6], impaired semen quality [7], acute hip arthritis [8], vertebral osteomyelitis, and bacteraemia [9].

A complex bladder microbiome exists in addition to classical bacteria, as revealed by 16SrRNA gene amplification and sequencing and the enhanced quantitative urine culture (EQUC) technique [10]; additionally, Gardnerella, a commen pathogen of BV, triggers causes of UTI [11]. The sole Gardnerella species known to exist is G. vaginalis, which has been found to be the predominant habitant of the vaginal tract in women who have been diagnosed with bacterial vaginosis (BV) [12].

G. vaginalis is a significant contributor to the development of abnormal vaginal microbiota. However, there is limited knowledge regarding the genetics, physiology, and diversity of G. vaginalis

strains found in women with vaginosis. Analysis of the G. vaginalis genome suggests that its pathogenic capabilities may be underestimated [13]. In healthy human vaginal microbes in reproductive age is usually predominated by Lactobacillus types. which products, such as hydrogen peroxide (H2O2), bacteriocin, and lactic acid are believed to play an important role in maintenance of the normal vaginal flora by inhibiting invade by other pathogens [27]. A Sexually active women are more likely to develop bacterial vaginosis (BV_; the symptoms of BV were a white-grey color vaginal discharge which may have a "fishy" odor, abnormal discharge which may be more noticeable during menstruation and after sex and vulval or vaginal irritation or soreness [28]. The recent search in genetics have showed new hight light on the genetic taxonomic diversity and heterogeneity within the genus of Gardnerella. The sequence analysis of whole genome were carried out in 81 Gardnerella strains by Vaneechoutte and colleagues in 2019 [29]. Found at least 14 groups distinct enough to be classified as separate species, within the taxon formerly known as G. vaginalis [29]. It was an important breakthrough in the field that resulted in an emended description of G. vaginalis and, for the first time, the inclusion more species of Gardnerella, namely G. swidsinskii, and G. leopoldii, G. piotii, [29]. The objective of this investigation was to identify the genetic variation of 16SrRNA gene in Gardnerella spp. by DNA sequencing technique form women suffering from bacterial vaginosis.

2. Methodology

Sample Collection

One hundured of urine samples from women had no take any antimicrobial agents, and which were obtained using transurethral catheters. Upon retrieval, the samples were promptly dispatched to the laboratory for storage and refrigeration with boric acid, during the specified time from August and September 2023 in Thi-Qar province of Iraq.

DNA Extraction

Twent five ml of urine samples, at 5000 g rpm were centrifuged for 30 minutes, then, the sediment of urine were harvested and treated for DNA extraction by the Mag-MAX™ microbiome ultra nucleic acid Isolation Kit (ThermoFisher Scientific,Waltham, USA) rendering to the manufacturer's instructions.

The reaction mixture had one microliter of each forward and reverse primer, 5µl of extracted DNA and 12µl of Master mix and completed the total volum to 20µl with nuclease free water. The primer was used to *16SrRNA* gene of *Gardnerella* species as detection primer registered in Table (1).

Table (1): Primer sequences of *16SrRNA* gene [14]

No.	Gene	Primer Sequences (5´- 3´)	Size Product
1	*16SrRNA*	F: GGTAGACAGGACCGATGAAG R: GAACAGTCAATCCCACGACC	278bp

The thermocycling protocol for the PCR amplification of *16SrRNA* gene was described in table (2).

Table (2):The *16SrRNA* gene program

Step	Temperature, °C	Time	Cycle
Initial dena.	95	5min	1
Den.	95	30sec	30
An.	60	30sec	
ExT.	72	30sec	
Finnal ext.	72	5min	1

Sequencing

Sanger type of sequencing method was performed using the ABI3730XL type automated DNA

sequencing by Macrogen of 90 PCR product samples forward)for the *16SrRNA* gene, and their genotypes were revealed by the Geneious software version 10.2 after alignment with reference sequences in the ncbi Gene Bank. The *Gardnerella* spp. gene variants were annotated using SnapGene Viewer ver. 4.0.4 (www.snapgene.com/).

Phylogenetic tree

The phylogenetic tree study was conducted using the NCBI-BLAS alignment identification and neighbour distance phylogenetic tree analysis (Mega,11), as well as the Multiple sequence alignment analysis based on ClustalW alignment analysis.

3. Results and discussion

The molecular diagnosis of *Gardnerella* spp. in urine samples by *16SrRNA* gene recorded that 90% (90/100) of UTI diagnosis as *Gardnerella* spp. According to PCR assay of *16SrRNA* gene, the bands shows in the following Fig. (1) for determination of the size of this gene which at 278bp.

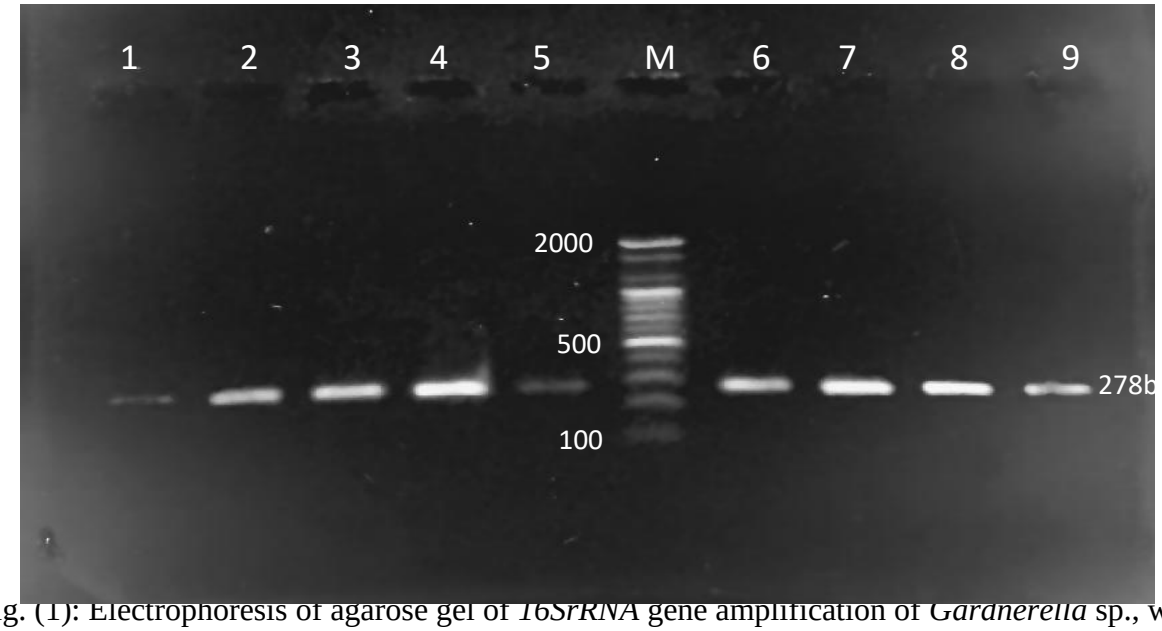

Fig. (1): Electrophoresis of agarose gel of *16SrRNA* gene amplification of *Gardnerella* sp., wherever M: ladder, 1-9: positive results

The amplified products were validated for their identification by conducting an NCBI BLASTn analysis on the sequencing reactions. The NCBI BLASTn engine revealed a significant sequence similarity between the sequenced samples and *Gardnerella* spp. sequences, specifically in the 278bp PCR amplicons of the currently targeted 16SrRNA sequences.

The NCBI BLASTn engine detected approximately 99% similarity with the intended target, which only partially overlapped with the coding region of the *16SrRNA* gene sequences. Through a comparison of the observed DNA sequences of the now examined samples with the obtained DNA sequences (GenBank acc. no. L08167), the specific characteristics of these sequences were emphasized within the amplified sequences (Table 3).

Table (3): Shows the positions and lengths of the 278 PCR amplicons utilized to amplify a part of the *16SrRNA* gene from *Gardnerella* spp. genomic DNA sequences. (GenBank acc. L08167).

Amplicon	Reference locus sequences (5′ - 3′	length
***16SrRNA* gene**	*GGTAGACAGGACCGATGAAGGACGTGACGGGCTGCGATATGCCTCGGGGAGCTG CCGAGTGGGCTTTGATCCGAGGATTTCCGAATGGGGAGACCCGGCCACTGTTATG GGTGGTACACCACAGTTTTTGTGGGGGGTACGCAGGGAAGTGAAACATCTC AGTACCTGCAGGAAAGGATACTCCGTGAGTAGTGGCGAGCGAAAGCGGATCAGGC TAAACCGAGTACGTGTGATAACCGTCAGGTGTTGCGTATTCGGGGTCGTGGGATT GACTGTTC**	278bp

***** Forward primer sequences;

** reverse primer sequences

The alignment analysis of the 278bp sequence samples identified 3 nucleic acid changes when compared to the corresponding *Gardnerella* spp. reference sequences . The sequences were produced by matching our samples with the most relevant sequences included in the NCBI database (GenBank acc.no. L08167).

The sequences of *16SrRNA* gene of of *Gardnerella vaginalis* in studied samples were submitted in the Gene Bank with accession number as: LC774556, LC774557, LC774558, LC774559, LC774560, LC774561, LC774562, and LC7745563.

A phylogenetic tree was constructed using the analyzed *16SrRNA* sequences from the bacterial samples under investigation. Among the deposited DNA sequences, the phylogenetic tree included the samples that were examined (LC774559, LC7745563, LC774561, LC774560, LC774557, LC774558, LC774556, and LC774562). Consistently matched with its closely associated sequences using the Tamura-Nei mode. The current tree has a total of 30 aligned nucleic acid sequences. The tree contained *Gardnerella* spp. which was the sole nucleic acid sequences present in the tree. The genetic sequences of *Gardnerella* spp. were analysed, and the resulting *16SrRNA* sequences were grouped into multiple neighbouring phylogenetic branches, indicating a high level of diversity within this organism .

The results of phylogenetic tree recorded the study isolate (LC774562) were closely relation with other isolates CP033836.1.422564, CP033836.1.541338, LT629773, LT629773.1.1229096, LT629773.1.1105956, AP012332, CP083173, those isolates found in separated branch. While, the detected phylogenetic distances amongst other studied samples ((LC774559, LC7745563, LC774561, LC774560, LC774557, LC774558, LC774556) that presented in other branch, and related incorporated organisms (10.6767), Provide a distinct indicator of the consecutive similarity of the integrated bacterial sequences, as shown in (Fig. 2).

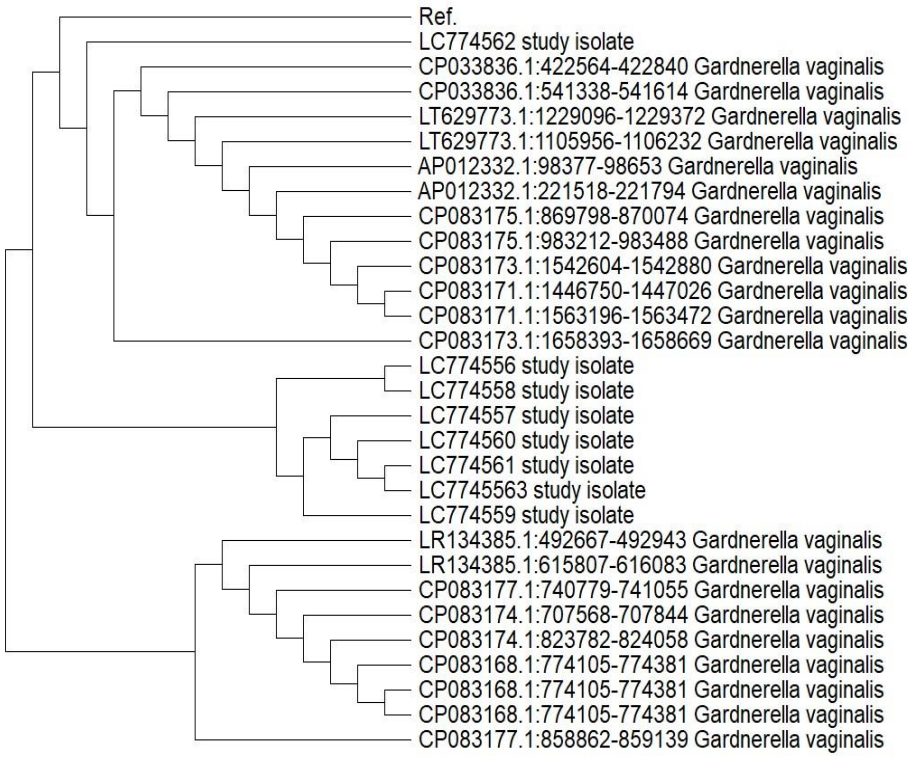

Fig. (2): The phylogenetic tree was constructed using the Neighbor-Joining method, as described by [15]. The tree that provides the best possible outcome is displayed. (next to the branches). The evolutionary distances were calculated using the Maximum Composite Likelihood technique [16], and are expressed in terms of the number of base substitutions per site. There were 30 nucleotide sequences included in this analysis. The codon positions considered were the first, second, third, and noncoding positions. All instances of unclear places were eliminated for each pair of sequences using the paired deletion option. The completed dataset consisted of a total of 278 locations. The evolutionary studies were performed using MEGA11 [17].

Table (4): Average estimates of genetic distances at DNA level.

	Ref.	LC774556	LC774557	LC774558	LC774559	LC774561	LC774562	LC774563
LC774556	1.1385424415							
LC774557	0.2051237503	0.1203119382						
LC774558	0.0495427493	0.3815495459	0.1106029948					
LC774559	0.0000000000	1.1498237033	0.2051237503	0.0497492410				
LC774560	0.0000000000	1.1498237033	0.2051237503	0.0497492410	0.0000000000			
LC774561	0.0000000000	1.1610090352	0.2063976510	0.0499574709	0.0000000000	0.0000000000		
LC774562	0.0000000000	1.1498237033	0.2051237503	0.0497492410	0.0000000000	0.0000000000	0.0000000000	
LC774563	0.0036533232	1.1717391445	0.2142727367	0.0544356048	0.0036533232	0.0036667188	0.0036533232	0.0036533232

Discussion

The present results of PCR technique recorded that 90% (90/100) of urine samples diagnosis as *Gardnerella* spp. Because of the prevalent using of DNA sequencing, as 16SrRNA gene sequencing had fundamental role in the properate identification of *Gardnerella* isolates.

The 16SrRNA sequencing is crucial for identifying bacteria that exhibits atypical phenotypic profiles, uncommon bacteria, bacteria that grow slowly, bacteria that cannot be cultured, and illnesses that do not show up in culture tests. The study offered valuable insights into the causes of infectious

diseases, aiding clinicians in prescribing appropriate antibiotics and identifying the optimal duration of therapy and infection control measures [18].

According to Bunyan et al. [19], the results of PCR detection verified that, of 100 high vaginal swab samples, 67 (89.33%) came from miscarriage women and 72 (96.00%) from women who did not have miscarriage vaginosis.

The study conducted by Srinivasan et al. [20] utilized PCR to target the variable region of the 16SrRNA genes for species-level identification. The purpose of this investigation was to examine the relationships between the presence of specific bacterial species and the clinical diagnostic criteria of bacterial vaginosis.

The present investigation was to ascertain the genetic variations of 16SrRNA gene in 8 samples of *Gardnerella* spp. that were taken from urine samples of women suffering from UTI disease. The 16SrRNA gene sequences of 11 clinical isolates of *Gardnerella* spp. genotype 1 exhibit complete sequence similarity (100%) with the previously documented *16SrRNA* gene sequence (ATCC 14019, genotype 1). The *16SrRNA* gene sequences of *Gardnerella* spp. genotype 2 closely resemble those of *G. vaginalis* strain 409-05 (genotype 2), which was obtained from a woman without symptoms of bacterial vaginosis and had a Nugent score of 9 [13]. Also, Degaim *et al*., [21] recorded that the using of *16SrRNA* gene to detect the different pathogenic bacteria as *S. aureus*. Nevertheless, the similarity in genetic sequence (genotype 2) between *Gardnerella* spp. isolates and the commensal isolate 409-05 does not indicate that genotype 2 can be considered a reliable indicator for asymptomatic BV [22].

Subsequently, the identified nucleoids variations of *16SrRNA* gene were pragmatic in the (LC774556 and LC774557) of studied samples such as: (A139T, G144A, G162A), while the variation in (G176A) was detected only in LC774556 ssssssample. Likewise the deletion mutation (A116 del) was detected in all studied samples, while (T129 del) was showed in (LC774556, LC774557, LC774558, LC774560) samples. The study conducted by Srinivasan *et al*., [23] utilized *16SrRNA* genes to investigate the connections between bacterial vaginosis and bacterial morphotypes in infected women.

Within the National Centre for Biotechnology Information, one of those positive samples was recoded [24,25,30,31]. The phylogenetic tree the most relative sequences to investigated samples (LC774559, LC7745563, LC774561, LC774560, LC774557, LC774558, LC774556) were found in separated branch belonged to *Gardnerella* spp., while local isolate (LC774562) was positioned in other branch of this tree.

Base on the analysis of the specific *16SrRNA* gene sequencing by NCBI-BLASTn, which can identify among different species of *Gardnerella* spp. The similarity of the investigated specimens (L08167) to other strains was measured by NCBI-BLASTn analysis of the *16SrRNA* gene, there recorded in genbank are in concordance of 100% homology with *Gardnerella* spp. isolated from vaginal secretion while the homology with other strains was 99.64% (CP033836 LR134385, LT629773.1, AP012332.1, CP083177.1, CP083175.1,CP083174.1, CP083173.1, CP083171.1).

The present findings align with the results obtained by Abed and Kandala [26], who reported an 99% identification rate of the bacterial vaginosis-associated bacteria (*Gardnerella* spp.) in pregnant women through sequencing. This identification was achieved by comparing the bacterial sequence with the established strains of *Gardnerella* spp. in the Gene Bank database.

4. Conclusion and future scope

The urinary pathogenic potential of the individual species may be different, DNA sequencing used to detect the genetic variation of Gardnerella spp. with genetic variation.

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