



ISSN: 0067-2904

Molecular and Bacteriological Detection of Some Bacterial Vaginosis Associate Bacteria in Women

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Abstract

Bacterial vaginosis (BV) is one of the most common genital infections among women in the childbearing age. Many novel, fastidious and uncultivated bacterial species are related with BV. These are called bacterial vaginosis associated bacteria (BVAB), present in trace amount and have a significant role in the infection. A total of 80 vaginal swabs were obtained from 80 pregnant and non-pregnant women. Samples were collected from different hospitals in Baghdad city and Al-Kut city.

Clinically, 60 sample among 80 were gave positive results depending on Nugent score and Amsel criteria ,the Bacteriological test showed the percentages of gram negative bacteria (*E.coli* ,*K.pneumoniae*, *P.mirabilis*, *Ps.aeruginosa* and *A.baumannii* were) were (38.3 ,28.3, 26.6,20,16.6,15)% respectively, while, the percentages of gram positive bacteria (*Lactobacillus* spp., *S. epidermidis* , *S. aureus*, *S. agalactiae* and *S. faecalis* were) (100, 46.6, 38.3,18.3 ,11.6)% respectively.

The results of molecular detection revealed that the specific primers amplified in 48 sample of vaginal swabs and produced a single band with a molecular weight of 210 bp for *G. vaginalis* gene, and 9 samples of vaginal swabs for *Leptotrichia/Sneathia* gene and produced a single band with a molecular weight of 320bp, While negative results for other bacteria such as *Atopobiumvaginae* and Bacterial Vagionosis Associated Bacteria 1,2,3 (BVAB1, BVAB2 and BVAB3) .

The results of *Lactobacillus*Spp., showed that among 60 samples of *Lactobacillus*Spp., 28 isolates (46.66%) were *L. acidophilus* and the PCR product appeared as a single DNA band with a molecular weight of 192bp,while 32 isolates (53.33%) were *L. fermentum*,the PCR product appeared as single DNA band with a molecular weight of 600 bp. Sequencing of 16s rRNA of *G.vaginalis*and *L.fermentum*was agreed with PCR identification give similar identity in percentages (87.5 and 100)% respectively with the standard strains in NCBI web site. While for *Leptotrichia* spp. it differs and related to modified nucleic acid bacteria in comparison with NCBI web site and hence is considered as a novel BV associated bacteria in vagina.

Keywords: Bacterial vaginosis, Molecular detection, PCR-technique.

التشخيص الجزيئي والبكتريولوجي لبعض أنواع البكتريا المرتبطة بالتهابات المهبل عند النساء

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الخلاصة

بعد التهاب المهبل البكتيري من أكثر الأمراض التناسلية شيوعا ويصيب النساء في سن البلوغ. بعض أنواع البكتريا التي تتصف بكونها نادرة، شرهة و غير قابلة للزرع لها علاقة بهذا المرض، هذه البكتريا تسمى (البكتريا المرتبطة بالتهاب المهبل)، حيث توجد باعداد قليلة وتلعب دورا مهما في الإصابة.

تم الحصول على 80 عينة سريريته من 80 امرأة. جمعت العينات من مستشفى اليرموك في مدينة بغداد ومستشفى الزهراء والكوت في مدينة الكوت . ثمانون امرأة خضعت لهذه الدراسة، ستون منها تم اختيارها كحالة مرضية اعتمادا على اختبار (Amsel criteria ,Nugent Score). اظهرت نتائج التشخيص البكتريولوجي للمسحات المهبلية تواجد انواع مختلفة من البكتريا السالبة لصبغة كرام (*K.pneumoniae* , *P.mirabilis*, *P.aeruginosa* and *Acinetobacter spp.*) بنسب (28,3، 26,6، 20 ، 16,6 ، 15) % على التوالي اما البكتريا الموجبة لصبغة كرام فكانت (*S. epidermidis* , *Lactobacillus spp* , *S. aureus* , *Streptococcus agalactiae* and *Enterococcus faecalis*) بنسب (100، 46,6 ، 38,3 ، 18,3 ، 11,6) % على التوالي. اظهرت نتائج التشخيص الجزيئي باستخدام تقنية PCR للكشف عن ستة انواع من البكتريا النادرة و الغير مستزرعة احتواء 48 عينة على حزمة مفردة بحجم جزيئي (210) زوج قاعدي تابعة الى بكتريا *Gardnerella vaginalis*، بينما 9 عينات تحتوي على بكتريا *Leptotrichia* اذ اظهرت حزمة مفردة بحجم جزيئي (320) زوج قاعدي. بينما اظهرت النتائج عدم تواجد باقي الانواع المدروسة *Atopobium vaginae* والانواع المرتبطة بالمهبل *Bacteria 1,2,3 BVAB1, BVAB2 and BVAB3*). و اظهرت نتائج بكتريا *Lactobacillus spp* تواجد بكتريا *Lactobacillus acidophilus* في 28 عينة اذ اعطت حزمة مفردة بحجم 192 زوج قاعدي. بينما 32 عينة اعطت نتيجة ايجابية لبكتريا *Lactobacillus fermentum* بظهور حزمة مفردة بحجم 600 زوج قاعدي. تم مقارنة نتائج النتائج التابع للجين المتخصص لبكتريا *Gardnerella vaginalis* , *Leptotrichia spp* و *L.fermentum* بواسطة قاعدة بيانات المركز القومي للمعلومات الحيوي (NCBI) وقد اشارت النتائج بأن *Gardnerella vaginalis* , *L.fermentum* لديها نسبة تماثل (92 و 87,5) % مقارنة بالسلاسل المعزولة عالميا، بينما *Leptotrichia spp* لا تتماثل وعدت نوع جديد من الانواع المعزولة من المهبل .

Introduction

Vaginal infection is one of the most common gynecological infections. Bacterial vaginosis is a significant public health problem because of the majority of cases throughout the world causing medical, social and economic consequences and most prevalence vaginal infections affecting women in the reproductive age[1,2]. When the condition of vaginal flora disturbance , lactobacilli are scarce and other bacteria are overly abundant such as *Mycoplasma hominis*, *Gardnerellavaginalis*, *Ureaplasmaurealyticum* and anaerobes such as *Prevotella*, *Mobiluncus* and *Bacteroides* [3-7].

Bacterial vaginosis is diagnosed by two gold standards Amsel's clinical criteria and the laboratory-based Nugent Gram staining evaluation.

The diagnosis of vaginal flora based on conventional culture-dependent methods and biochemical identification methods [8]. These morphological and biochemical assessments failed to clearly reveal the microbiological variations among BV individuals alone. In addition, numerous vaginal bacteria have been considered as novel, fastidious bacteria (of which some remain uncultivated) involving *Megasphaeraspp.*, *Gardnerellavaginalis*, *Atopobiumvaginae*, *Dialisterspp.*, *Leptotrichia/Sneathia*, an *Egg ertella*-like bacterium and three novel bacteria in the order *Clostridiales* which are: Bacterial Vaginosis Associated Bacterium 1,2,3 (BVAB-1) (BVAB-2) and (BVAB-3) respectively[2,9]. These are considered as excellent diagnostic indicators of BV either alone or in combination because they may have important functional roles despite their low abundances, [9,10].

These microorganisms are strict anaerobes and are either hard to recover or unrecoverable by conventional culture methods [11].

Other study have used broad-range 16S rRNA gene PCR, a cultivation-independent method, to characterize the community of vaginal bacteria. This molecular study have discovered a large number of novel, fastidious uncultivated bacterial species [9].

The aims of this study to detect the presence of some BV related bacteria by molecular and bacteriological methods.

Materials and Methods

Collection of samples

In this study, eighty samples of vaginal swabs were collected from women who were in the reproductive age ranging from 16 to 45 and 10 samples from healthy women as control group.

The patients had symptoms of abnormal vaginal discharge, odor and itching or burning. These women were attended hospitals of Baghdad (Al-Yarmouk teaching hospital) and Al-Kut (Al-Zahraa teaching hospital and Al-Kut hospital) during period from February 2014 to February 2015. The sampling was carried out by specialized clinicians and all subjects underwent speculum examination.

Clinical diagnosing

Two criteria Nugant's test, and Amsel's criteria were used to diagnosis bacterial vaginosis associated bacteria (BVAB), in women which present in trace amount and have a BV infected according to methods described by [12,13].

Bacterial Isolation, identification and Biochemical Identification

A total of 60 clinical specimens of vaginal swabs were subjected to morphological, cultural characterization, microscopic examination and biochemical tests according to Bergys Manual of Systematic Bacteriology [14] and confirmed the identifying of bacteria by API System (Bio Merieux /France).

Molecular Analysis of BV Associated Bacteria using PCR Technique

DNA extraction

DNA was extracted from all frozen swabs as well as isolated bacteria by using Exi-prip Genomic DNA kit (Bioneer/ Korea). The protocol was designed for extraction genomic DNA of gram-negative and gram-positive bacteria using Exi-prep fully automated nucleic acid extractor.

PCR Analysis

In order to detect the BV associated bacteria by using the primer specific for 16S rRNA according to [9]. PCR was used as a diagnostic technique. The reaction mixtures was described in Table-1.

Table 1- The mixtures adopted in PCR analysis for all primers

PCR reaction components	Final Concentration	Volume for (1) tube(μl)
Deionized water	-----	8.5
Green Master Mix (2X)	1x	12.5
F- Primer	10picomol	1
R- Primer	10picomol	1
Template DNA	60ng/μl	2
Total reaction volume		25

❖ Molecular Detection of BV Associated Bacteria Genes by 16S rRNA

The set of PCR reactions was performed for diagnosing BV associated bacteria by using the primer specific for 16S rRNA according to [9]. The reaction mixture was described in Table (2-7), conditions were described in Tables (2-11). The gene was detected by PCR with primers, (Table 2-10).

Table 2- Oligonucleotide primers sequences and PCR products for detection of BV associated bacteria [9]

Primer	Sequence(5' ----- 3')	PCR Product(bp)
Atop-442F	5-GCAGGGACGAGGCCGCAA-3	623
Atop-1017R	5-GTGTTTCCACTGCTTCACCTAA-3	
BVAB1-1019F	5-GTATATTTTCTACGGAACACAGG-3	261
BVAB1-1280R	5-TTTGCTCCGGATCGCTCCTT-3	
BVAB2-619F	5-TTAACCTTGGGGTTCATTACAA-3	405
BVAB2-1024R	5-AATTCAGTCTCCTGAATCGTCAGA-3	
BVAB3-999F	5-CTTGAACGATGTAGAGATACATAA-3	278
BVAB3-1278R	5-TGCTTCGCCTCGCGACGTC-3	
<i>G.vag</i> 644F	5-GGGCGGGCTAGAGTGCA-3	210
<i>G.vag</i> 851R	5-GAACCCGTGGAATGGGCC-3	
Lepto-395F	5_CAATTCTGTGTGTGTGAAGAAG-3	320
Lepto-646R	5_ACAGTTTTGTAGGCAAGCCTAT-3	

Table 3- The program of all BV associated bacteriaprimer used in PCR analysis [9]

Steps	Temperature	Time
Initial Denaturation	94°C	5 min
Denaturation	94°C	1 min
Annealing	(50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70)°C	1min
Extension	72°C	1min
Final extension	72°C	7 min

No. of cycles = 40 cycle

Detection of three types of BV associated bacteria by automated sequencing

According to the results of PCR product, several DNA samples from BV associated bacteria isolates and *Lactobacillus* Spp. Were subjected to sequencing by NICEM Company / USA using AB13730XL APPLIED BIOSYSTEM machine, which gave the identity of the genes comprised with the original genes in Gene bank by blast program which is available at the national center biotechnology information (NCBI).

Results and Discussion

Clinical Diagnosis of BV

The results of the present study revealed that sixty samples out of eighty samples taken from women patient gave positive results according to Amsel's criteria and Nugant's criteria. Patients completed necessary questionnaires regarding symptoms, age, use of contraceptives or condom use, pregnancy, demographic data, reproductive and sexual health history including vaginal discharge. All these data will be mentioned to explain the relationship between these factors and BV.

Amsel's Clinical Criteria

Amsel's clinical criteria was considered as one of the most standard method for BV diagnosis [13]. Amsel's Criteria revealed that all sixty samples of women patients gave positive results. While all control samples (10 samples) were negative for Amsel's Clinical Criteria.

Nugent's clinical criteria

Nugent clinical criteria is one of the golden standards for diagnoses BV; it is regarded as a more objective scoring system for BV diagnosis based on observed morphotypes [12,13].Nugent score revealed that out of sixty samples from women patients only 45 samples were classified as BV, whereas 15 patients were classified as intermediate according to Nugent's scoring system Table- 5. While all control samples (10 samples) were classified as normal.

Table 4- The number of Bacteria counted by Nugent's Score

Score	<i>Lactobacillus</i> Morphotype /Vision field	<i>Gardnerella</i> Morphotype /Vision field
Normal	15- 28	<1
Intermediate	2-4	1-4
BV	1-2	12-20

Identification of Bacterial Isolates

All bacterial isolates were characterized according to Bergey's Manual of Systematic Bacteriology [14] ,as well as, other characters reported by [15-18] . Cultural, morphological and biochemical characteristics for each bacterial genus in addition to the use of APi 20 system identification kit, revealed the appearance of different types of bacteria, including gram-positive and gram-negative bacteria in 60 samples of vaginal swabs as showed in Table-5 which described that *Lactococcus* spp had the highest isolation ratio 100%,while others microorganisms showed variety in their percentages of distribution in gram positive bacteria , *S. epidermidis* *S. aureus* , *Streptococcus agalactiae* and *Enterococcus faecalis* in percentages (46.6,38.3,18.3 ,11.6)% respectively .Whereas the percentages of distribution for gram negative bacteria *E.coli*, *P.mirabilis*,*K.pneumoniae*,*P.aeruginosa*, and *A.baumannii*, were (38.3,26.6, 28.3,20,16.6,) %respectively comparison with the result of Bacterial distribution in control samples (ten) were as follows: *Lactobacillus* spp. were 100%, *E.coli* 85%, *S.epidermidis* 75%,and *S.aureus* 25%.

The percentage of *Lactobacillus* spp. as the predominant in vaginal flora, is an indicator of healthy vagina but its presence in low amounts with a high proportion of other pathogens is pathogenic. They play an important role in protecting against pathogens invasion or overgrowth by production of

hydrogen peroxide, bacteriocins, and lactic acid [19]. *The Enterococcus faecalis* was considered as one of the common pathogens in the genital tract and urinary tract [15].

The *Streptococcus agalactiae* (GBS) is one of the most important pathogens of GTI infections including bleeding and rupture membrane after birth [20]. The present study revealed that, *S. epidermidis* considered as one of the most common microorganisms in infections, especially those associated with the presence of foreign bodies like IUD [21]. It was observed that *S. aureus* was highly associated with abortion [22].

Hirabayashi and Okada [23] confirmed the ability of *E.coli* isolation from patients in genital clinics and births in large proportion. Lafta, [24] who found the percentage of *K. pneumoniae* that isolated from urinary tract infection reached to 50 % of *Klebsiella* spp., this confirms the fact that *K. pneumoniae* play important role in causing UTI and it may reach genital tract.

Table 5- The different types of bacteria isolated from BV

The bacteria	Percentage%		
Gram positive bacteria		Gram negative bacteria	
<i>Lactobacillus</i> spp.	100	<i>E.coli</i>	38.3
<i>S. epidermidis</i>	46.6	<i>K.pneumoniae</i>	28.3
<i>S. aureus</i>	38.3	<i>P.mirabilis</i>	26.6
<i>Streptococcus agalactiae.</i>	18.3	<i>Pseudomonas aeruginosa</i>	20
<i>Enterococcus faecalis.</i>	11.6	<i>A.baumannii</i>	16.6

DNA Extraction

The genomic DNA was extracted efficiently from all 60 vaginal swabs samples by using the Exiprep™ 16 plus Bacteria Genomic DNA kit (Bioneer / Korea). The purity and concentration data of total DNA extracted from all samples were described in Table-6.

Table 6- The purity and concentration data of total DNA extracted from all samples

Isolates of bacteria	A260/A280 ratio	Yield : ng/ml
Gram positive bacteria	1.8- 2	22.8- 488
Gram negative bacteria	1.7- 2	30.9- 584

Molecular Analysis of genomic DNA by using PCR technique

Molecular Diagnosis For Detection Of 16S rRNA Gene For G.vaginalis

In this study, the results of molecular detection for *16s rRNA* gene showed that 48 out of 60 samples gave positive results to *G.vaginalis* with a specific primer; the PCR product appeared as a single DNA band with a molecular base of 210 bp Figure-1, and the optimum annealing temperature for the detection of *G.vaginalis* gene was 67°C for 1 min.

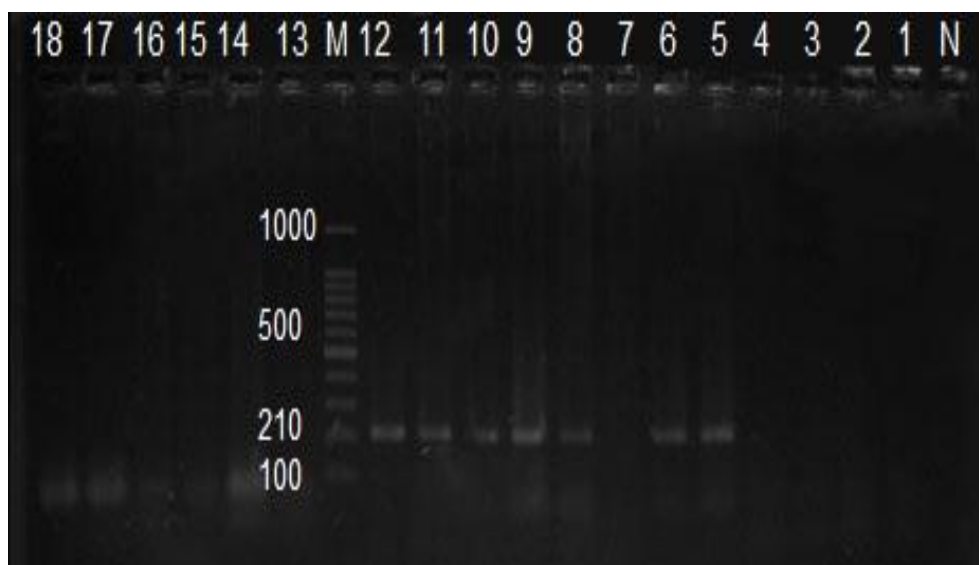


Figure 1- Agarose gel electrophoresis of PCR product using specific primer for detected *G. vaginalis* gene products (210) bp, Electrophoresis was performed on 2% agarose gel (1.5hr, 5V/cm, 1XTris-borate buffer) and visualized under U.V. light after staining with ethidium bromide. N: negative control. M: 100-1500 bp DNA marker Lanes (1,2,3,4,7,13,14,15,16,17,18) negative results. Lanes (5,6,8,9,10,11,12) positive results.

According to the study by [5], who's reported the incidence of *Gardnerella vaginalis* was 31.1%.while the study that was conducted in India by [25] reported the incidence of this infection in women was 18% in different ages ranging (15 - 49) years old.

Molecular Diagnosis For Detection Of 16S rRNA Gene For *Leptotrichia/Sneathia*

The results as showed in Figure-2 revealed among 60 samples, only 9 samples had *Leptotrichia/Sneathia* gene. The PCR product was 320 bp in size, which is the same size obtained by [9] when they used the same primer. The results of the optimum annealing temperature was 58°C for 1 min, ().

Leptotrichia/Sneathia. established considerable relations with increasing sexual exposure. These conclusions suggest that *Leptotrichia/Sneathia* spp. might be just epidemiologically related with BV, or sexually transmitted “passengers” but not involved in BV development. A similar scenario applies to some *Ureaplasma* and *Mycoplasmaspp.*[26,27].



Figure 2- Agarose gel electrophoresis of PCR product using specific primer for detected *Leptotrichia/Sneathia* gene product (320)bp. Electrophoresis was performed on 2% agarose gel (1.5hr, 5V/cm, 0.5XTris-borate buffer) and visualized under U.V. light after staining with ethidium bromide. Lanes (1,5,6,8,28,30,34,53,56) positive N: negative control. M: 100-1500 bp DNA marker.

Molecular Diagnostic For Detection of 16S rRNA Gene For *Atopobiumvaginae*.

The 16s rRNA gene was amplified using the PCR technique for the detection of *A.vaginae*. The results showed that all 60 samples were negative for *A.vaginae*, These results suggest that PCR detection of selected key novel vaginal bacteria may be useful for the microbiological diagnosis or confirmation of BV [9].

Molecular Diagnostic For Detection of 16S rRNA Gene For BVAB(BVAB1, BVAB2and BVAB3)Using PCR Technique.

The 16s rRNA gene was amplified using the PCR technique for the detection of BVAB1 BVAB2 and BVAB3. The results showed that all 60 samples were negative for BVAB1; BVAB2 and BVAB3 . BVAB1,BVAB2 and BVAB3were fastidious vaginal bacterium and it is considered as an excellent marker of BV, either alone or in combination with each other in the order *Clostridiales* (BVAB1 ,BVAB2 and BVAB3), and with *Leptotrichia/Sneathia*, *Atopobiumvaginae*. These results suggest that PCR detection of selected key novel vaginal bacteria may be useful for the microbiological diagnosis or confirmation of BV [9].

Molecular diagnostic for detection of 16S rRNA gene for *Lactobacillus* spp

The 16s rRNA gene was amplified using the PCR technique for detection of *Lactobacillus* spp. (*L. acidophilus*, *L. fermentum* , *L. gasseri* , *L. jensseni*, and *L.iners*) . The results showed that among the 60 samples of *Lactobacillus* spp, 28 isolates (46.66%) were *Lactobacillus acidophilus* Figure-3, the PCR product as a single DNA band with a molecular weight of 192 bp , while 32 isolates (53.33%) were *Lactobacillus fermentum* Figure-4. The PCR product appeared as single DNA band with a

molecular weight of 600 bp .Whereas the PCR product appeared negative result for the other three species of *lactobacillus* primers used in this study.

The results revealed that the optimum annealing temperature was 61°C for 1 min. Generally, *Lactobacillus acidophilus* is considered to comprise most of the healthy vaginal *Lactobacillus* flora.

Generally, The present study revealed that different type of *Lactobacillu* species included *L.acidophilus* and *L.fermentum* present in healthy vagina of women in Iraq differ from other counters and this result are in agreement with other study showing a predominance of species within the *L. acidophilus* complex and the fact that only one or a few *Lactobacillus* species colonize the healthy human vagina. [28].Other study by [29] showed the predominant species isolated from 80 Indian women were *L. reuteri* present in 26 (32.5%) women, *L. fermentum* in 20 (25%), and *L. salivarius* in 13 (16.25%) women.

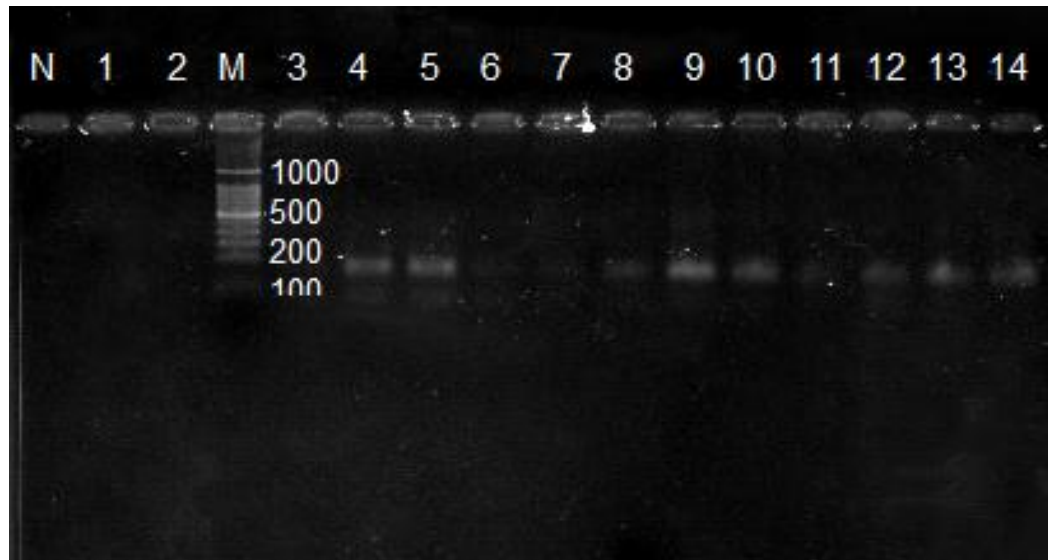


Figure 3- gel electrophoresis of PCR product using specific primer for detected *lactobacillus acidophilus* gene product (192) bp .Electrophoresis was performed on 2% agarose gel (1.5hr, 5V/cm, 1XTris-borate buffer) and visualized under U.V. light after staining with ethidium bromide. N: negative control. M: 100bp DNA marker Lanes (4-14) isolates positive results. Lanes (1,2,3) isolates negative results. M: 1 Kb ladder, N: negative control.

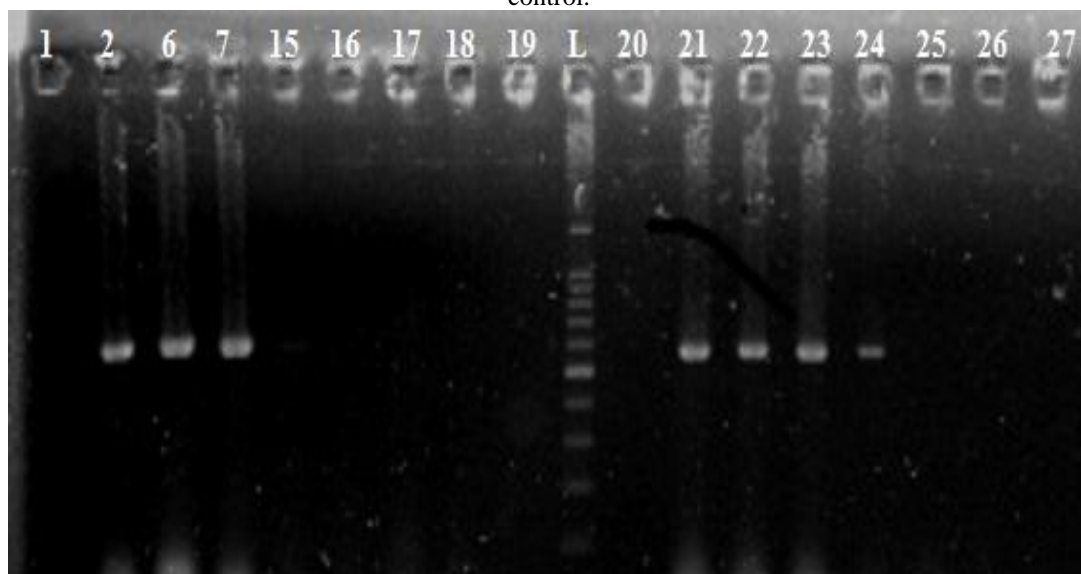


Figure 4- Agarose gel electrophoresis of PCR product using specific primer for detected *lactobacillus fermentum* gene product (600) bp .Electrophoresis was performed on 2% agarose (1.5hr, 5V/cm, 0.5XTris-borate buffer) and visualized under U.V. light after staining with ethidium bromide.N: negative control. M: 100bp DNA marker Lanes (4-14) isolates positive results. Lanes (1,2) isolates negative results.

Relationship among Bacterial Vaginosis associated Bacteria

The results of this study showed that nine patients were positive for *Leptotrichia Sneathia.*, which classified as BV using Nugent score and gave a positive result using Amesl's criteria, while 39 patients that were positive for *G. vaginalis* and were classified as BV using Nugent score and gave a positive result using Amsel's criteria and 9 patients were classified as intermediate using Nugent score and gave positive result using Amsel's criteria. The use of 16S rRNA gene sequence-based analyses have frequently been used for the study of the vaginal microbiota, thus revealing the presence of many many aerobic and anaerobic species not previously detected by culture. Synergy between *G. vaginalis* and *Atopobium vaginae* has been proposed as they are often discovered together in BV. BVAB2, BVAB3, *Megasphaera* type I, *Leptotrichia Sneathia.*, *Sneathia spp.*, and *G. vaginalis* established considerable relations with increasing sexual exposure, while *A. vaginae* and *G. vaginalis* were frequently detected in women with no sexual experience. The *Leptotrichia Sneathia.* might be just epidemiologically related with BV, or sexually transmitted "passengers" but not involved in BV development. A study by [27] showed that six of the eight BV- CO were rare or absent in sexually-inactive women which are: *Sneathia spp.*, *Megasphaera* type I, *Leptotrichia Sneathia.*, BVAB1, BVAB2 and BVAB3. While *A. vaginae* and *G. vaginalis* were the most common BV-COs in sexually-inexperienced women. However, the clinical consequences of all these genera in the vaginal ecosystem remain unknown [30].

Results of sequencing

The results of sequencing of specific gene to detect BV associated bacteria *G. Vaginalis* solateas shown in Figure-5 revealed that the species was identified in percentage of 87.5% by comparing its sequence with that in the database in gene bank by Blast program with gaps in percentage (1%). While other sample of BV associated bacteria isolate was *Leptotrichia/Sneathia* as shown in Figure-6 revealed that the species was belonged to Modified microbial nucleic acid bacteria that is not related to *Leptotrichia Sneathia.*, and this is considered as a novel BV associated bacteria in vagina. The results of sequencing of *L.fermentumas* revealed that the species was identified in percentage 92% by comparing its sequence with that in the database in gene bank by Blast program with no gaps (0%).

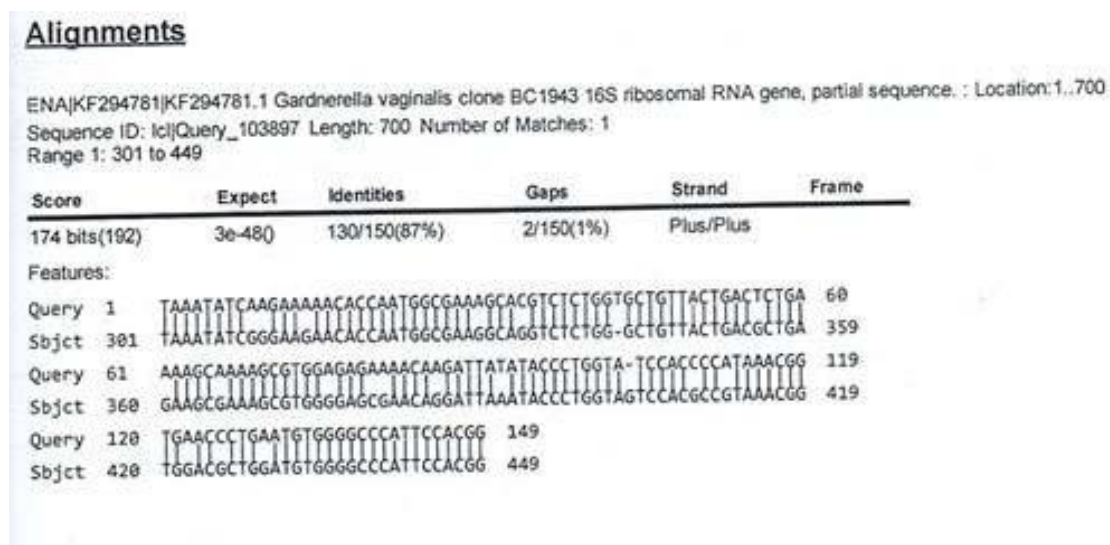


Figure 6- Local pairwise alignment of BV associated bacteria *G. vaginalis* isolate.

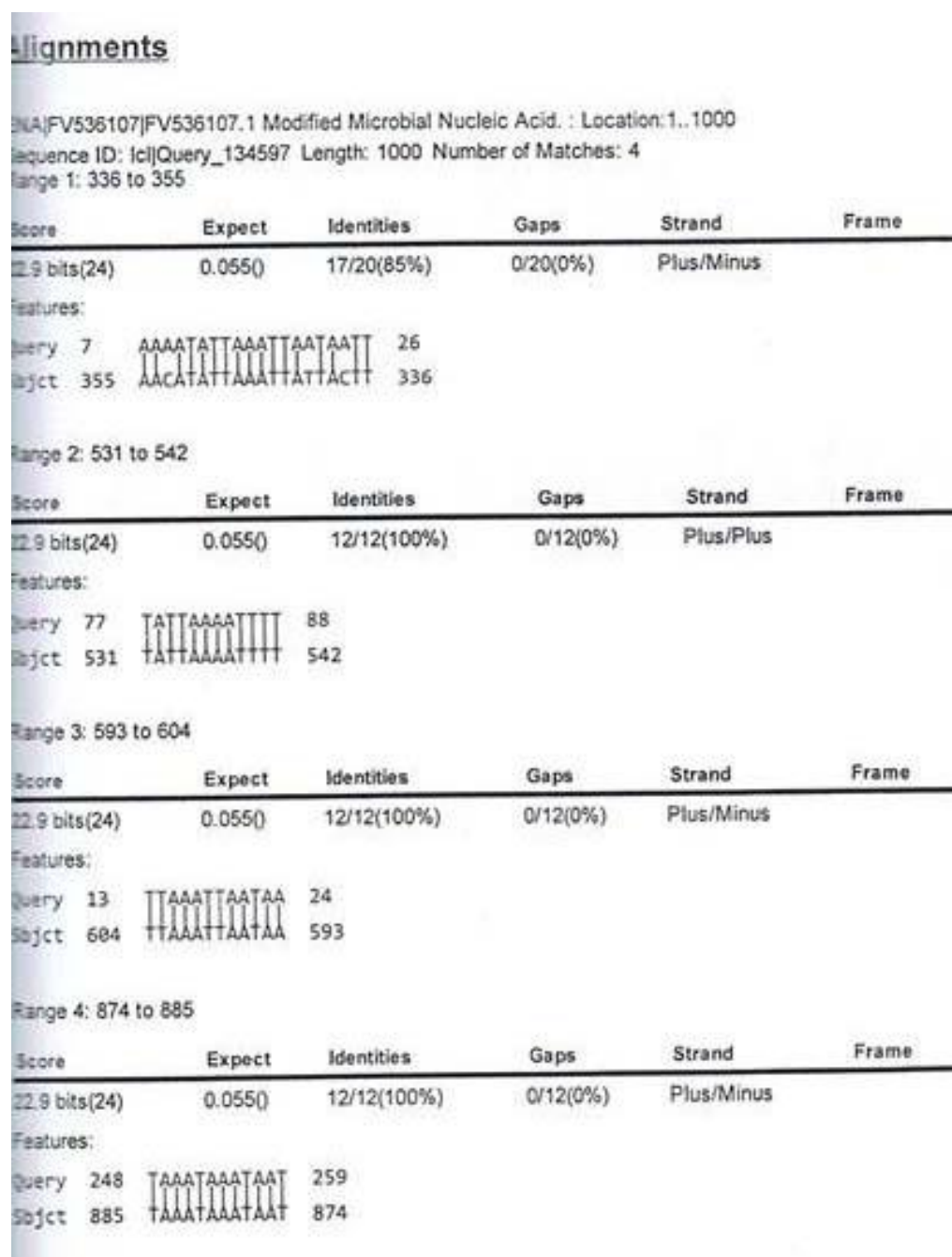


Figure 6- Local pairwise (alignment) of BV associated bacteria *Leptotrichia spp.* isolate which gave negative result (modified nucleic acid bacteria).

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