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ORIGINAL RESEARCH ARTICLE

Molecular Diagnosis of *Lactobacillus spp.* Isolated from Females Infected with Bacterial Vaginosis

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ABSTRACT

Background: Indigenous *lactobacilli* in the vaginal microbiota play a crucial role in maintaining women's health. It can contribute to microbial homeostasis by producing lactic acid, hydrogen peroxide, and a range of antimicrobials, which inhibit the growth of pathogens. This dynamic interplay helps preserve the vaginal ecosystem and prevent infections. Bacterial vaginosis (BV) is a clinical condition characterized by decreased microbiota and an increased number of aerobic and anaerobic pathogenic microorganisms, such as *Gardnerella spp., genital mycoplasmas,* and *Candida spp.*

Objectives: This study aimed to isolate, purify, identify, and diagnose the vaginal Lactobacilli from females who are in vaginitis dysbiosis in Baghdad city, using molecular techniques

Methods: Of forty-two adult females suffering from bacterial vaginitis, only 12 isolates of the purified Lactobacillus spp. were identified on a molecular level in this study.

Results: The results of molecular diagnosis using 16S rRNA revealed that seven isolates were diagnosed as Lactobacillus crispatus, while the other five isolates were Lactobacillus gasseri. The diagnosis protocol included application of a specific forward and reverse 16S rRNA primers with an amplicon size of 154bp *(for L. crispatus)* and 322bp *(for L. gasseri).*

Conclusion: The polymerase chain reaction (PCR) method stands out as the most efficient technique for characterizing the vaginal microbiome's composition. The predominant *Lactobacillus spp.* among BV-infected women are *L. crispatus,* followed by *L. gasseri.*

Keywords: Bacterial Vaginitis; *Lactobacillus crispatus*; *Lactobacillus gasseri*; Molecular Diagnosis; Probiotic bacteria.

1. INTRODUCTION

The human body is a complex, interconnected organism composed of many systems, including microorganisms such as those in the vagina. The vagina comprises a complex microecosystem that hosts billions of microorganisms, which are crucial for maintaining the health of the female reproductive tract. These microorganisms play a significant role in minimizing the risk of gynecological infections [1]. *Lactobacillus* is a Gram-positive, facultative anaerobic or microaerophilic rod-shaped bacterium. They are a major group of lactic acid bacteria (LAB), named as such because most members convert lactose and other sugars to lactic acid [2,3]. Lactobacillus

belongs to the family *Lactobacillaceae* and consists of 170 species and 17 subspecies [4]. The traditional phenotypic methods that were available, and which are still very important in classifications, are: morphology, mode of glucose fermentation, growth at certain temperatures (10°C and 45°C), and range of sugar utilization, These and other characteristics have not been useful for discriminating the closely related bacteria in the ecological niche of the normal human vagina, which mainly belong to the *Lactobacillus* [5,6]

A healthy human vagina is dominated by Lactobacilli, which play an important role in protecting the host from urogenital infections, such as those caused by urogenital tract infections. Additionally, it is widely recognized that the microbial balance between Lactobacilli as the dominating flora (microbiota) and other, mainly gram-negative anaerobes can be upset and frequently result in the syndrome of bacterial vaginitis [1,7].

The identification of *Lactobacillus* as probiotic bacterial isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) [8]. In general, about 17 phenotypic tests are required to identify a *Lactobacillus* isolation accurately to the species level [9]. Moreover, the derivation of simple yet rapid identification methods is required to deal with the large numbers of *Lactobacillus* isolates obtained during microbial ecological studies of ecosystems.

The application of *16S rRNA* gene sequences for the investigation of bacterial phylogeny and taxonomy has emerged as the predominant housekeeping genetic marker; this preference can be attributed to several key factors. These causes incorporate: its widespread presence in nearly all bacteria, where it often exists as part of a multigene family, or operons [10]. The role of the *16S rRNA* gene has not changed throughout history, suggesting that random variations in the sequence offer a more precise indication of evolutionary time; thus, the *16S rRNA* gene is sufficiently large for informatics applications [11]. Due to such complexity, this study aimed to isolate, purify, identify, and diagnose the vaginal Lactobacilli from females who are in vaginitis dysbiosis, in Baghdad city, using molecular techniques.

2. MATERIALS AND METHODS

2.1 Specimen collection and isolation of vaginal lactobacilli

Vaginal specimens were obtained from forty-two women between the ages of 14 and 50 years, with BV ecosystems in the National Centre for Educational Laboratories in Medical City, Baghdad. Lateral vaginal walls were swabbed with sterile cotton-tipped applicators. The swabbing was supervised by a gynecologist's consultation, then the swabs were rolled onto a slide for Gram stain of vaginal smear and then streaked on de Man-Rogosa Sharpe agar (MRS agar, HiMedia, India), incubated anaerobically at 37°C for 48 h using an anaerobic jar. The individual colonies were re-streaked twice on an MRS agar plate for pure culture. Identification of Lactobacillus species was performed by phenotypic criteria: Gram-positive rod-shaped cell morphology, a negative catalase activity, endospore stain, and motility test [12,13]

2.2 Molecular analysis:

2.2.1. Primer's preparation:

The primers (BioCorp) that are used in PCR amplification were diluted by adding nuclease-free water according to the manufacturer's instructions. 754 µL of free nuclease water was added to the Forward primer to get 100 pmol/µL to prepare stock primer. Then, 10 µL of the previous solution was diluted by adding 90 µL of free nuclease water to get a final volume of 10 pmol/µL as a working primer.

For the Reverse primer, 588 µL of free nuclease water was added to the primer to get 100 pmol/µL. Then, 10 µL of the previous solution was diluted by adding 90 µL of free nuclease water to get a final volume of 10 pmol/µL. This primer was prepared for *L. gasseri.*

For *L. crispatus,* 750 µL of free nuclease water was added to the Forward primer to get 100 pmol/µL. Then, 10 µL of the previous solution was diluted by adding 90µL of free nuclease water to get a final volume of 10 pmol/µL.

For the Reverse primer, 800µL of free nuclease water was added to the primer to get 100 pmol/µL. Then, 10 µL of the previous solution was diluted by adding 90 µL of free nuclease water to get a final volume of 10 pmol/µL. After primer preparation, it is ready for the preparation of the PCR mixture [10].

2.2.2. DNA extraction:

The boiling method described by [14]. was applied for DNA extraction. Briefly, this method included transferring a pure isolate to Eppendrof's tubes that were previously containing 500µL of free nuclease water. These tubes were then boiled in a water bath for 10 min. After centrifugation (13000 rpm) for 5 min, the supernatant was used as template DNA for the PCR procedure.

2.2.3 Preparation of PCR mixture:

The primers 10 pmol/µL were used, and the reaction mixture was prepared according to the procedure suggested by the manufacturer. Seven microliters of the DNA template were used, which was mixed with a PCR mixture composed of 12.5 µL of Green Master Mix, 2.5 µL from each primer forward and reverse, and 3 µL of nuclease-free water to get a final volume of 25 µL.

2.2.4 PCR amplification procedure:

The PCR programs applied in this study were based on the method described by [14]. The PCR reaction conditions were as follows: predenatured at 95°c for 10 minutes, melted at 95°c for 30 seconds; optimized annealing at 51,53,50,52°c for 30 seconds; extension at 72°c for 30 seconds; 40 cycles; a final extension at 72°c for 8 minutes. PCR primers are shown in (Table 1); the PCR products were visualized after electrophoresis in 0.8% agarose gels and staining with ethidium bromide, as seen in (Figures 1 and 2).

Table 1: PCR primers and running programs.

Species	primers	Sequences	Initial denaturation	cycle NO.	denaturation	annealing	Extension	Final Extension
L. crispatus	452F	5′-GATAGAGGTAGTAACTGGCCTTTA-3′	95°c/10 min	40	95°c/30sec	52°c/30sec	72/30sec	72/8 min
	1023 R	5′-CTTTGTATCTCTACAAATGGCACTA-3′	95°c/10 min	40	95°c/30sec			
L. gasseri	*L.gassF*	5′-AGCGAGCTTGCCTAGATGAATTTG-3′	95°c/10 min	40	95°c/30sec	52°c/30sec	72/30sec	72/8 min
	L.gassR	5′-TCTTTTAAACTCTAGACATGCGTC-3′	95°c/10 min	40	95°c/30sec			

3. RESULTS

3.1 Morphological and Biochemical Identification of Lactobacilli spp.

According to morphological and biochemical characteristics, forty-two isolates were obtained and described as *Lactobacillus* spp.; all these isolates were collected from females aged between 14 and 50 years.

These isolates have demonstrated the same morphological and biochemical characteristics. However, the colonial characteristics of Lactobacilli spp. are observed on MRS agar. The colonies showed a typical morphological characteristic, which is: small colonies without pigment, white or cream color, and round. This description agrees with that reported in [15].

The microscopical features of *Lactobacilli spp*. isolates are Gram-positive, with size variations ranging from short to elongated, large rod-shaped forms, and non-sporing bacilli. They frequently occur alone, in pairs, or in chains of different lengths [16].

The biochemical characteristics, including the catalase test, endospore stain method, and motility test, were negative for all isolates; these characteristics classify the isolates of the study as the genus of *Lactobacillus* spp. [16].

3.2 Molecular identification:

The purified forty-two Lactobacillus spp. isolates were considered for molecular diagnosis. The primers 452F, 1023R, *L.gassF*, and *L.gassR* were used as the identification primers. Some optimization on the thermal cycling program was done; this optimization included the application of 52ºC as annealing temperature. The optimization in the annealing temperature allowed a better resolution for molecular diagnosis compared to the other temperatures (50 ºC, 51 ºC, 53 ºC), which gave somewhat less obvious resolution (Figure 1, 2). The molecular diagnosis result of the vaginal Lactobacilli isolates included *L. crispatus* (7/ 42) and *L. gasseri* (5/42) in percentages of 17%, 12% respectively, while the other 20 isolates showed a negative result for *Lactobacillus* spp. (Table 2)

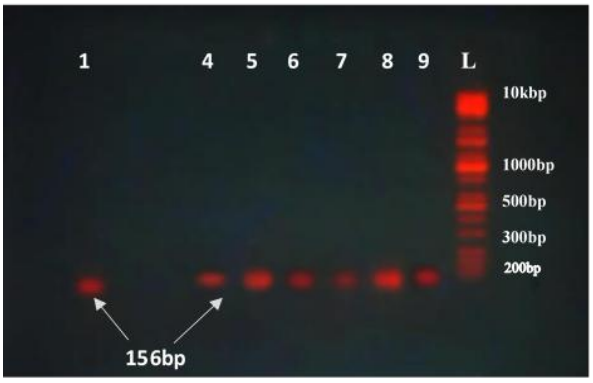

Figure 1. Agarose gel electrophoresis (1 % agarose, supplied with ethidium bromide at 75V) for the 16S rRNA gene for the detection of *L. crispatus* (amplified size 154bp as compared with 10kbp DNA ladder (L)), using template DNA prepared by the boiling method. Lines 1, 4, 5, 6, 7, 8, 9 represent positive results. 10kbp.

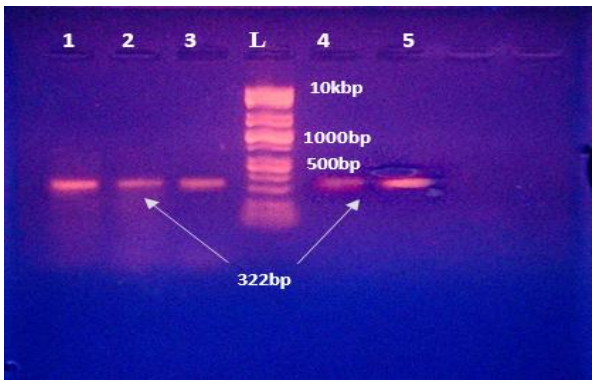

Figure 2. Agarose gel electrophoresis (1 % agarose, supplied with ethidium bromide at 75V) for the 16S rRNA gene for the detection of *L. gasseri* (amplified size 322bp as compared with 10kbp DNA ladder (L)) using template DNA prepared by the boiling method, Lines 1, 2, 3, 4, 5 represent positive results.

Table 2*:* PCR primers and running programs

Lactobacillus spp.	***+ result/ total***	***Lactobacillus spp. %***
L. crispatus	*7/ 42*	*17%*
L. gasseri	*5/ 42*	*12%*
Total	*12/ 42*	*29%*

4. DISCUSSION

This study highlights the distribution of vaginal *Lactobacillus* spp., particularly *L. crispatus* and *L. gasseri*, based on morphological and molecular findings. These results provide insight into their role in maintaining vaginal microbiota and their potential association with dysbiosis. [17] studied 98 healthy, pregnant Japanese women and found that *L. crispatus* was 61 %, and *L. gasseri* was 34 %. [18] have mentioned the presence of *L. crispatus* in 48% and *L. gasseri* in 30% of 23 Swedish women. These studies dealt with the *16S rDNA* analysis, which confirms that the DNA studies have found that the most prevalent species of vaginal Lactobacilli in women were homologous to the type strains of *L. crispatus* and *L. gasseri*, which support the findings of this study. The difference in percentage might be related to the healthy, non-BV-infected females; furthermore, it might be attributed to geographical distribution, technique used, and/or socioeconomic situation of the studied case. On the other hand, [19] study has reported that the usual vaginal Lactobacilli percentages are *L. crispatus* and constitute a percentage of (22%), and *L. gasseri* (10%), [20] showed that the only species found in the human vagina was *L. crispatus* (34%). Likewise, the taxonomically identified to species level *16S rRNA* gene, were 8 isolates belonging to *L. crispatus*, 6 isolates to *L. gasseri*.

The isolation of *L. crispatus* has been strongly associated with a normal vaginal microbiota and absence of vaginal dysbiosis; also, the presence of *L. crispatus* promotes stability of the vaginal microbiota, which might be a reason for the low rate of its presence in BV-infected women. A precise diagnosis is considered very critical in the development of suitable bacterial replacement therapy for the treatment of vaginosis.

5. CONCLUSION

The polymerase chain reaction (PCR) method stands out as the most efficient technique for characterizing the vaginal microbiome's composition. The predominant Lactobacillus spp. among BV-infected women are *L. crispatus*, followed by *L. gasseri*. Furthermore, the purification and isolation of the BV probiotic bacteria present significant challenges due to vaginal dysbiosis. Additional work is required in this field to plot a complete phylogenetic map for the distribution of these bacteria in Baghdad and other cities in Iraq.

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CONFLICTS OF INTEREST

There were no conflicts of interest in this study.

AUTHOR CONTRIBUTIONS

The Author creates all paper elements.

DATA AVAILABILITY STATEMENT

Data Availability Statement: All data have been gathered in the biology department laboratory.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval for the study was secured from the Clinical Laboratory/Biology Department at the College of Science, Mustansiriyah University

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