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Investigating Bacterial Vaginal Discharge Etiology in Pregnant Women by Microscopic Examination and PCR

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Authors
Bahare Ghanbari, *PhD* [1,2]
Majid Akbari, *PhD* [1,2]
Nazila Najdi, *PhD* [3]
Mohammad Arjomandzadegan, *PhD* [2,1]
Azam Ahmadi, *PhD* [2*]

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[1]Department of Microbiology, School of Medicine, Arak University of Medical Sciences, Arak, Iran.
[2]Infectious Diseases Research Center IDRC), Arak University of Medical) Sciences, Arak, Iran.
[3]Department of Obstetric and Gynecology, School of Medicine, Taleghani Hospital, Arak University of ,Medical Science , Arak, Iran.

** Correspondence*
Infectious Research Center, Arak University of Medical Sciences, Arak, Iran
Email: akbari@arakmu.ac.ir

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ABSTRACT

Backgrounds: Abnormal vaginal discharge is a common problem among pregnant women. The most common cause of these discharges is bacterial vaginosis (BV), which has numerous complications and causes problems for pregnant mothers and their fetuses. The purpose of this study was to determine the BV frequency among pregnant women referring to a gynecology clinic in Arak city using Amsel and Nugent criteria, Alberta guideline, and PCR.
Materials & Methods: This descriptive study was performed on 70 vaginal samples of pregnant women in Arak to investigate the most common causes of vaginal discharge according to Amsel and Nugent criteria and polymerase chain reaction (PCR) method using specific primers targeted towards three bacteria: *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus curtisii.* Data were analyzed using SPSS software and Chi-square test.
Findings: In this study, ten (14.28%) out of 70 pregnant women had positive bacterial vaginosis according to Amsel criteria. According to Nugent criteria and Alberta guideline, three (4.29%) cases were diagnosed with definite BV, 20 (32.26%) cases with intermediate BV with clue cells, 42 (67.74%) cases with intermediate BV without clue cells, and finally five (4.29%) cases with negative BV. Also, according to PCR, the frequency of *G. vaginalis*, *M. curtisii,* and *A. vaginae* in vaginal samples was 71.42% (50 cases), 64.28% (45 cases), and 30% (21 cases), respectively.
Conclusion: According to the obtained results, the prevalence of definite bacterial vaginosis was lower than that of vaginitis, and most patients suffered from nonspecific vaginitis.

Keywords: Vaginal Discharge, Vaginosis, Bacterial, Pregnant Women, Polymerase Chain Reaction.

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Introduction

Abnormal vaginal discharge is a common problem among pregnant women. The most common cause of these vaginal discharges is bacterial vaginosis (BV) [1]. The causes of abnormal vaginal discharges could be divided into three main groups: infectious, non-infectious, and chronic vaginitis [2]. Infectious vaginitis is the most prevalent type of vaginitis that most women suffer from. Bacterial vaginosis (BV) is the most common cause of infectious vaginitis [2, 3]. Since the publication of the first microbiological study of the human vagina in 1892 by Albert Doderlein, the vaginal microbiota of healthy fertile women has been considered to contain mainly Gram-positive bacilli of the genus *Lactobacillus* [4]. Healthy vaginal mucosa in women of childbearing age is composed of a non-keratinized squamous epithelium consisting of about 28 cell layers covered by a mucosal layer that is constantly lubricated by cervicovaginal fluid [5]. The apical vaginal epithelium is composed of dead cornified cells that are non-infectious and therefore act as a shield against pathogens [6]. However, these protective layers are constantly at risk and could eventually be disrupted, allowing pathogens to invade and cause diseases [7]. Occasionally, these infections are caused by a variety of interactions between pathogens in the vaginal environment, such as bacterial vaginosis (BV) [5].

Bacterial vaginosis is caused by the accumulation of mixed bacteria such as *Gardnerella vaginalis*, *Atopobium vaginae*, and *Mobiluncus* spp. When these bacteria overgrow in the vaginal ecosystem, they could product virulence factors such as cytolysins, sialidase (sialidase levels are higher in women with bacterial vaginosis) [8], prolidase, and pili and biofilm, which result in BV. The main symptom of bacterial vaginosis is an increase in homogeneous gray-white vaginal discharge with an odor like fish amine without inflammation [9, 10]. The most important risk factors associated with BV include: education, age, race and ethnicity, low socio-economic status, vaginal douching, smoking, etc. Bacterial vaginosis (BV) is a polymicrobial disorder caused by changes in the vaginal ecosystem, a reduction in peroxidase-producing lactobacilli (as the normal vaginal flora), and their replacement by anaerobic bacteria, including *G. vaginalis*, *A. vaginae*, *Mobiluncus*, *Prevotella*, *Megasphaera*, *Leptotrichia*, *Essensia*, *Dialister*, *Bacteroides*, *Peptostreptococcus*, *Clostridium*, *Vionella*, *Mycoplasma* spp., and BVAB (BV associated bacterium) 1, 2, and 3 [11, 12]. Bacterial vaginosis causes many complications and problems for pregnant women, including increased risk of preterm delivery, premature rupture of membranes, chorioamnionitis, amniotic fluid infection, postpartum endometriosis, urinary tract infection, pelvic inflammatory disease, postpartum complications, miscarriage, etc [13, 14]. Bacterial vaginosis is diagnosed based on Amsel and Nugent criteria [1] and molecular methods [9]. Amsel diagnostic criteria include four cases, if three of which are confirmed in the sample, the sample is considered as BV positive. These criteria include abnormal vaginal discharge, pH greater than 4.5, the presence of clue cells in the vaginal discharge slide, and a positive whiff test [8]. To confirm the results of Amsel test as a primary screening method, Nugent test is performed. In the Nugent scoring criteria, a score of 0-3 is considered as a healthy sample, a score of 4-6 as an intermediate flora, and a score of 7-10 as bacterial vaginosis. In the case of scores 4 to 6, if no clue cells are observed in the Gram-stained smear, this indicates altered vaginal flora, and the results are indeterminate for bacterial vaginosis, but if clue cells are observed, they suggest the transition of vaginal flora towards bacterial vaginosis, and it is recommended to repeat the vaginal smear test [15-20].

Many studies have used molecular methods such as PCR to identify the causes of bacterial vaginosis [21-23]. Genetic analysis using PCR in various studies has shown that *G. vaginalis*, *A. vaginae*, and *Mobiluncus* spp. are the most prevalent bacteria in vaginal discharges.

Backgrounds:

Objectives: The aim of this study was to investigate the BV frequency among pregnant women referring to a gynecology and obstetrics clinic in Arak city based on Amsel and Nugent criteria and PCR method using specific primers for these bacteria.

Materials and Methods

Sample size and collection: This descriptive study was performed on 70 vaginal discharge samples collected from pregnant women during their first visit to a gynecologist in Arak in order to investigate the frequency of BV based on Amsel and Nugent criteria and PCR method using specific primers for *G. vaginalis*, *A. vaginae*, and *Mobiluncus* spp bacteria.

Inclusion criteria included pregnant women with vaginal discharge. All women participating in the study filled out the informed consent form. Exclusion criteria included: non-pregnant women, pregnant women without vaginal discharge, and pregnant women who did not fill out the informed consent form.

Samples were collected in the gynecologist's office using three sterile cotton swabs and a disposable speculum. After examining the quality of vaginal discharge by the physician, the first swab was used for Gram staining smear to examine clue cells and bacteria, and the second swab was used for pH determination and whiff test. Vaginal pH was determined using litmus paper, and any color change on the paper was reported. Whiff test was also performed by adding 10% potassium hydroxide to a clean glass slide and reporting any amine odor. The third swap was also placed in 2 mL of PBS (phosphate buffered saline) solution and transferred to the laboratory for PCR.

Amsel test: Amsel test includes four items, if three of which are confirmed in the sample, the sample is considered as BV positive. These criteria include abnormal vaginal discharge, pH greater than 4.5, the presence of clue cells in the vaginal discharge slide, and a positive whiff test [8].

Nugent test: In the slide prepared from vaginal discharge, Nugent scoring criteria were examined. According to the Nugent criteria, a

Table 1) Nugent scoring criteria applied in this study to evaluate bacterial vaginosis in vaginal discharge samples [26]

Lactobacilli Morfotype (Gram-Positive Bacilli)	Score	Gardnerella (Gram-Negative Coccobacilli)	Score	Mobiluncus (Curved Gram-Variable Rods)	Score	Total
30 or more	0	0	0	0	0	0
5-30	1	<1	1	<1	1	3
1-4	2	1-4	2	1-4	1	5
<1	3	5-30	3	5-30	2	8
0	4	30 or more	4	30 or more	2	10

Table 2) Alberta guideline for microscopic cellular and bacterial analysis of vaginal smears for BV [27]

Nugent Score (N-Score)[a]	Yes	Yes
Microscopic cellular components[b]	Adult women (>13 - ≤ 55 yrs)	Post-menopausal women (> 55 yrs)
Nugent Score (N-Score)[c]	Yes	Yes
Clue cells [d]	Reporting the presence of clue cells	Reporting the presence of clue cells
Polymorphonuclear cells (PMNs) [d]	Moderate (3+) or heavy (4+ amounts)	Moderate (3+) or heavy (4+ amounts)
Epithelial Cells [e]	No	No
Non-sufficient quantity of vaginal sample	Reporting the insufficiency of sample for assessing vaginitis. Requiring immediate recollection	Reporting the insufficiency of sample for assessing vaginitis. Requiring immediate recollection

a: See Table 1 for Nugent scoring criteria (N-score).
b: Post-menopausal women (>55 yrs.) should have an additional comment added to all vaginal smear reports: "Results may not be reliable in post-menopausal women. Correlate with the clinical picture."
c: The presence of clue cells is looked for and reported if the N-score is ≥4. If the N-score is indeterminate (i.e., 4-6), then additional fields should be examined for clue cells before reporting.
d: If the N-score is indicative of BV (i.e., 7-10), then clue cells are only reported if found as part of the routine microscopic examination.
e; The presence of 3+ to 4+ PMNS is reported.

Table 3) PCR primer sequences used in this study

Primers	Length	Sequences, 5'→ 3'	Annealing Temperature
Atopobium vaginae	248		63.5
Forward		CTGGGGGCTCAACCCCTA	
Reverse		TGCGGCACGGAAAGTATAATCT	
Mobiluncus Curtisii	361		61.5
Forward		GAGGAACACCGATGGCGAAG	
Reverse		AGCTGACGACAACCATGCAC	
Gardnerella vaginalis	465		63.5
Forward		TTGGTGGAGGGTTCGATTCTG	
Reverse		TTGGTGGAGGGTTCGATTCTG	

score (NS) between 7-10 was considered as positive for bacterial vaginosis, a score between 4-6 as intermediate bacterial vaginosis, and a score lower than 3 as negative and healthy in terms of bacterial vaginosis [24, 25].

Patients participating in this study were categorized into three groups according to the Nugent criteria and Alberta guideline, including healthy patients with negative BV (NS: 0-3), patients with intermediate BV (NS: 4-6), and patients with positive BV (NS: 7-10).

PCR and DNA extraction: DNA extraction from clinical specimens was performed using the YTA Genomic DNA Extraction Mini Kit (for blood cells/cultured YT9040; Yekta tajhiz Company, Tehran, Iran) according to the manufacturer's instructions. The concentration and purity of the extracted DNA samples were determined using a NanoDrop spectrophotometer and gel electrophoresis, respectively. Specific primers related to *A. vaginae, M. curtisii,* and *G. vaginalis* were designed in this study.

Findings

In this study, 70 vaginal samples were collected from pregnant women referring to a gynecology and obstetrics clinic with complains of vaginal discharge. The age of the study participants was between 13 and 55 years. Bacterial vaginosis was positive in ten (29.14%) cases based on the Amsel criteria as shown in Table 4.

Table 4) Amsel test results regarding bacterial vaginosis in vaginal discharge samples

Amsel Test	Frequency (%)
Positive	10 (14.29)
Negative	60 (85.71)
Total	70 (100)

In the Nugent test, Gram-stained vaginal smears were analyzed to identify three bacteria, *Lactobacillus* spp., *G. vaginalis*, and *Mobiluncus* spp.

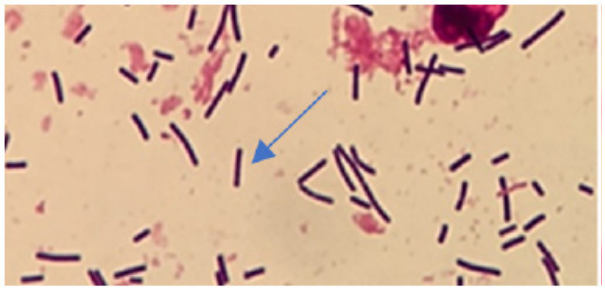

Figure 1. *Lactobacillus* spp

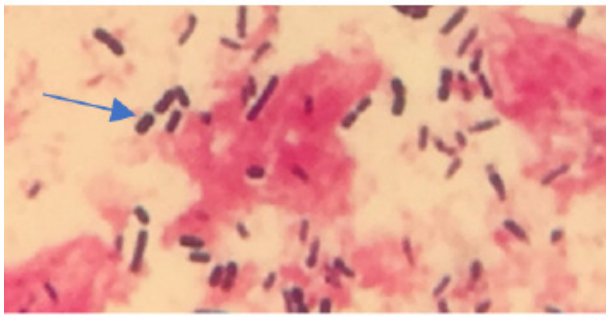

Figure 2. *Gardnerella vaginalis*

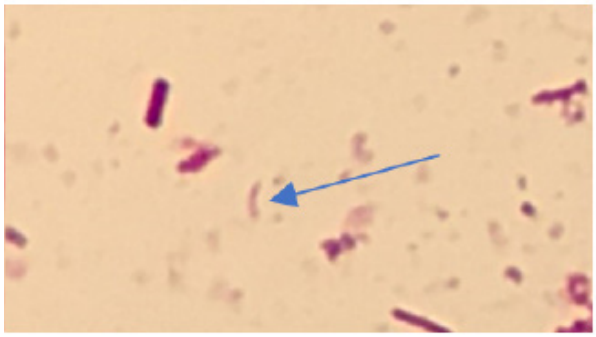

Figure 3. *Mobiluncus* spp

According to the Nugent scoring criteria and Alberta guideline, the patients examined in this study were classified into three groups shown in Table 5. As shown in this table, bacterial vaginosis was positive in three (4.29%) cases, while it was negative in five (7.14%) cases. In addition, 62 (88.57%) cases were identified with intermediate BV. In this study, the prevalence of *G. vaginalis*, *A. vaginae*, and *M. curtisii* in vaginal discharge samples was investigated by PCR method. According to the PCR test results, *G. vaginalis* was detected in 50 (71.43%) samples, *A. vaginae* in 21 (30%) samples, and *M. curtisii* in 45 (64.29%) samples.

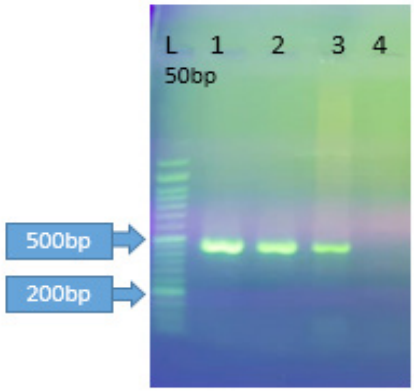

Figure 4) 1% agarose gel electrophoresis for PCR results of *G. vaginalis*. Well L: ladder with a size of 50 bp, Wells 1, 2, and 3: *G. vaginalis* (465bp) in a vaginal discharge sample of pregnant women, Well 4: negative control

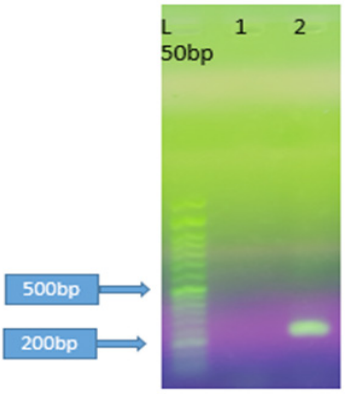

Figure 5) 1% agarose gel electrophoresis for PCR results of *A. vaginae.* Well L: ladder with a size of 50 bp, Well 1: negative control, Well 2: *A. vaginae* (248bp) in a vaginal discharge sample of pregnant women

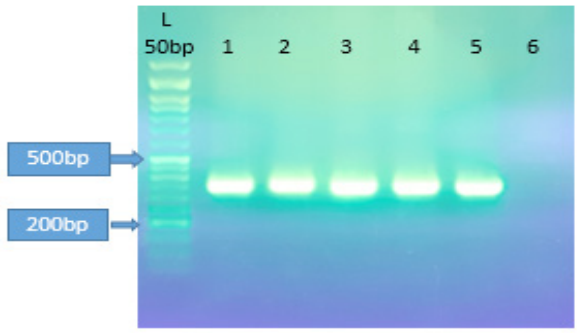

Figure 6) 1% agarose gel electrophoresis for PCR results of *M. curtisii.* Well L: a 50 bp ladder, Wells 1, 2 , 3, 4, and 5: *M. curtisii* (361bp) in a vaginal discharge sample of pregnant women, Well 6: negative control

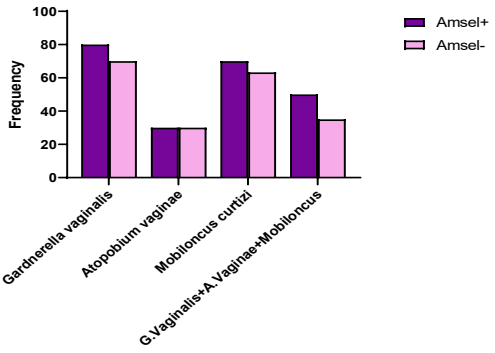

Chart 1) Frequency of bacteria detected by PCR based on Amsel criteria. In the group diagnosed as positive based on Amsel criteria, the highest frequency of bacteria was related to *G. vaginalis* (80%), and the probability of simultaneous infection with three bacteria was higher (50%).

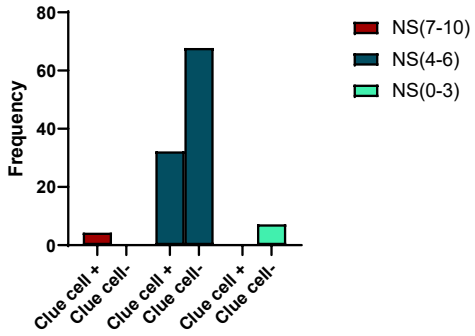

Chart 2) Frequency of clue cells in the Nugent method. According to the Nugent criteria, all subjects with positive vaginosis had clue cells, but most of the individuals with intermediate vaginosis were negative for the presence of clue cells, and there were no clue cells in healthy individuals.

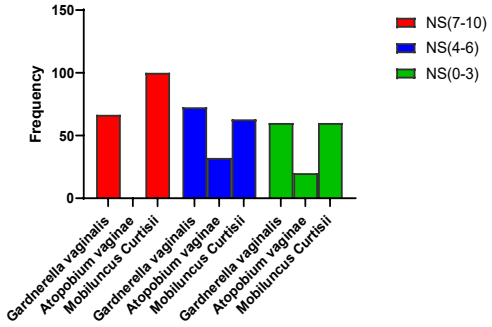

Chart 3) Frequency of bacteria detected by PCR based on the Nugent method. According to the Nugent criteria, the highest frequency of bacteria in patients with positive bacterial vaginosis (7-10) was related to *M. curtisii* (100%). In patients with intermediate bacterial vaginosis (4-6), the highest frequency was related to *G. vaginalis* (72.58%), and the highest frequency in people with negative bacterial vaginosis (0-3) was related to *G. vaginalis* (60%) and *M. curtisii* (60%).

Table 5) Frequency of bacteria detected by PCR in the groups based on Nugent scoring and Alberta guideline

Nugent Scoring	Frequency (%)	Clue Cell (%)	Polymorphonuclear Cells (PMNs)[a](%)	Bacteria (PCR)		
				Gardnerella vaginalis (%)	*Atopobium vaginae* (%)	*Mobiluncus curtisii* (%)
Negative bacterial vaginosis NS (0-3)	5 (7.14)	0	3 (60)	3(60)	1 (20)	3 (60%)
Intermediate bacterial vaginosis NS (4-6)	62 (88.57)	Clue cell - : 42 (67.74)	19 (45.2)	34 (80.9)	13 (30.9)	27 (64.2)
		Clue cell + : 20 (32.26)	8 (40)	11 (55)	7 (35)	12 (60)
Positive bacterial vaginosis NS (7-10)	3 (4.29)	3 (4.29)	3 (100)	2 (66.6)	0	3 (100)

Discussion

Abnormal vaginal discharge is a common complication during pregnancy, which causes 5 to 10 million pregnant women to consult a doctor every year. This problem in pregnant mothers could lead to complications such as premature birth, abortion, etc [28-30].

In the present study, all subjects were pregnant women referring to a gynecology and obstetrics clinic with complaints of vaginal discharge and clinical symptoms such as burning, itching, etc. Most patients were in the age range of 25-29 years with a gestational age of 28-36 weeks. Also, most women used natural methods to control pregnancy, and most of whom were experiencing their first pregnancy. Among the patients, 7.14% had recurrent miscarriages, and 18.5% had a history of infertility.

These demographic characteristics are consistent with those reported in other studies conducted by Rahimi and colleagues (2011) in Ardabil province [31] and Golmohammadlou et al. (2013) in Urmia [32]. In the present study, most of the pregnant women belonged to the age group of 20-25 years, this finding is inconsistent with the finding of another study by Roohbakhsh et al. (2019) in Rasht [12].

In this study, most of the pregnant women who referred to the physician were at 28-36 weeks of gestation, which is consistent with the finding of another study by Redelinghuys et al. (2017), reporting that most of the pregnant women referring to the physician were at the gestational age of 26-32 weeks [33].

In a study by Rahimi and colleagues (2011), among 507 pregnant women, most of the patients used the contraceptive tablet method (61%), while in the present study, most of the patients used the natural contraceptive method [31].

The subjects included in this study were examined in terms of delivery frequency, the results showed that there was no significant difference in the delivery frequency between patients with and without bacterial vaginosis, which is consistent with the result of Rahimi's study [31]. However, some studies, such as the study by Sherrard et al. (2018), have shown an association between a higher number of deliveries and a higher likelihood of bacterial vaginosis [34].

Amsel method is one of the most important tests used to diagnose bacterial vaginosis. In this study, epithelial cell shedding was observed as a common factor in most of the healthy pregnant women studied. Therefore, it is difficult to diagnose this disease, especially clue cells, in pregnant women only through the Amsel test. Of the

four factors included in the Amsel criteria, the most errors are related to the clue cell factor. The probability of error in the other factors is very low or zero because among the other factors, the type of discharge is approved by the physician, the whiff test is confirmed by the specific smell of the amine, and the pH level is controlled by the buffer. Considering all these issues, these tests were used as the primary screening method in this study. Based on the Amsel test results, out of 70 vaginal discharge samples, bacterial vaginosis was positive in ten (14.29%) cases. This result is consistent with the result of another study by Menard et al. (2010), showing that out of 163 pregnant women, bacterial vaginosis was positive in 21 samples (12%) [35]. In a study by Srinivasan et al. (2012) in America, out of 220 vaginal discharge samples examined, 98 (43%) cases were diagnosed with BV by Amsel criteria, which is not consistent with the finding of this research [36].

In this study, Nugent scoring assay according to Alberta guideline was used to confirm the Amsel test results. The morphologies of *Lactobacillus* species, which are long and sometimes filamentous Gram-positive bacilli, and *Mobiluncus* species, which are curved Gram-variable or negative bacilli, are almost clear and easily distinguishable from other bacteria, but bacteria such as *G. vaginalis*, which are in the form of Gram-negative coccobacilli, are very similar to other bacteria, including *Bacteroides*, *Prevotella*, and *Peptostreptococcus*; thus, experience is required to observe and distinguish Gram slides of these specimens. In this study, three (4.29%) patients were positive for bacterial vaginosis based on Nugent diagnostic criteria; this result is not consistent with the findings of other studies by Srinivasan et al. (2012) and Malaguti et al. (2015), showing that out of 223 and 220 samples, 45 and 117(53%) samples were positive for bacterial vaginosis based on Nugent criteria, respectively [36, 37].

According to the Nugent criteria and Alberta guideline, out of 70 patients, three (4.29%) cases were positive for BV (with smear samples consistent with bacterial vaginosis), 20 (32.26%) cases were identified with intermediate BV with clue cells (suggesting the transition of vaginal flora towards bacterial vaginosis), 42 (67.74%) cases were identified with intermediate BV without clue cells (with smear samples showing altered vaginal flora), and five (4.29%) cases were negative for BV (with smear samples negative for bacterial vaginosis). The frequency of the three studied bacteria in vaginal discharge samples in different groups of patients based on Nugent criteria is shown in Table 5.

According to Table 2, the presence of +3 to +4 PMNs in the microscopic slide is considered as inflammation by other infectious agents, which requires additional testing to detect *Neisseria gonorrhoeae*, *Chlamydia trachomatis,* and *Trichomonas vaginalis* [27]. In the present study, according to the PCR test results, *G. vaginalis* was detected in 50 (71.43%) vaginal samples, *A. vaginae* in 21 (30%) samples, and *M. curtisii* in 45 (64.29%) samples. In a study by Dirani et al. (2017), among 36 vaginal discharge samples examined in the laboratory by PCR, *G. vaginalis* was detected in 29 (80.5%) samples, and *A. vaginae* was detected in 20 (55.5%) samples [38]. In another study by Raykova and colleagues (2017), among vaginal discharge samples of 98 women (74 symptomatic and 24 control patients) evaluated, *A. vaginae* was detected in 21 samples (28.4%), which is not consistent with the present study result [39]. In another study by Menard et al. (2010), 34 patients were identified with bacterial vaginosis using PCR. According to the PCR test results, the presence of two bacteria *G. vaginalis*

and *A. vaginae* was detected simultaneously in 21% of cases, and the presence of three bacteria *G. vaginalis*, *A. vaginae*, and *Mobiluncus* was simultaneously detected in 37.14% of cases [35].

In the group that was positive for BV in the Amsel test, the highest frequency of bacteria was related to *G. vaginalis* (80%) and *M. curtisii* (70%). In this study, only two of the ten cases that were positive in the Amsel test were also confirmed by the Nugent method, while eight cases were not confirmed by this method. The frequency of *G. vaginalis* and *M. curtisii* bacteria increased from BV negative to BV positive individuals, respectively, but *A. vaginae* was not observed in BV positive individuals.

In a study by Bradshaw et al. (2006), among 358 participants examined using PCR, 103 cases (60%) were assigned to the healthy group, 33 cases (92%) to the intermediate group, and 138 cases (99%) to the bacterial vaginosis group. *A. vaginae* was detected in 20 (12%) healthy individuals with negative BV, 28 (78%) patients with intermediate BV, and 133 (96%) patients with positive BV. The frequency of *G. vaginalis* is consistent with the present study results, but the frequency of *A. vaginae* is not consistent with the present study results [40] Discrepancies between the results of other studies and the present study may be related to the type of samples, sampling time, sampling method, sample transfer method to the laboratory, or even history of antibiotic use.

Conclusion

Vaginal discharge could be caused by three very similar diseases that are difficult to distinguish from each other, including bacterial vaginosis, desquamative inflammatory vaginitis [41] and cytolytic vaginosis[42-44].

Bacteria involved in bacterial vaginosis are present in the normal flora of some women, and since more than 40-50% of BV cases are asymptomatic [46,45,24], they could not be diagnosed by the Amsel test alone. This test does not have sufficient diagnostic features and identifies only a small number of patients. Therefore, it is better to use clinical and microscopic criteria simultaneously to confirm the Amsel test results and make a more accurate diagnosis. On the other hand, Gram staining is a quantitative method for measuring white blood cells, clue cells, lactobacilli, *Gardnerella,* and *Mobiluncus*, which is very useful in assessing infection. Studies have shown that Nugent diagnostic method could not confirm all cases diagnosed with Amsel test because there are cases where clue cells and decreased pH are not due to bacterial vaginosis[48,47,1]. This result is consistent with the present study result considering that the Nugent criteria failed to confirm all ten cases diagnosed by the Amsel criteria. Therefore, to confirm intermediate bacterial vaginosis according to the Nugent criteria, the type and load of microorganisms involved in the disease should be considered. For this purpose, the PCR method is more accurate and could easily detect bacteria that could not be detected by Gram staining; thus, this test is recommended to be used [37, 49]. But PCR also has drawbacks because it only shows the presence or absence of bacteria. Since these bacteria are present in both healthy and sick women, bacterial load should be considered as a diagnostic criterion. According to experimental findings, if the bacterial load exceeds the normal flora, it could cause the disease. Quantitative methods such as real time PCR could be used for better diagnosis. However, common causative agents of bacterial vaginosis, such as *A. vaginae*, were not observed in most of the subjects in this and other studies; therefore, given the more logical classification of Alberta, both quantitatively and qualitatively, it is

recommended to use the Alberta guideline [27] to diagnose bacterial vaginosis.

Due to the conditions and limitations in clinical laboratories, access to molecular methods such as real time PCR may be difficult. On the other hand, PCR method alone could not be used for interpretation and could not meet our needs; thus, it is recommended to use a standard method such as the Alberta guideline.

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