ORIGINAL ARTICLE

Multiplex PCR, Clinical Amsel's Criteria and Microbiological Nugent Score for Diagnosis of Bacterial Vaginosis and Detection of its Virulence

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ABSTRACT

Background: *bacterial vaginosis is universally the commonest vaginal infection in reproductively active females. It causes major consequences as preterm labor, predisposing to sexually-transmitted infections and HIV infections. Although it is a public health concern, no one knows exactly its pathogenesis when some say that it is just disturbance in vaginal floral balance predisposing to clinical symptoms and signs, where the predominant Lactobacilli in vagina become replaced by other facultative and anaerobic bacteria.* ***Objective****: To evaluate different diagnostic tests, Amsel's criteria and PCR and their ability to diagnose bacterial vaginosis in comparison to the gold standard test, Nugent score in terms of sensitivity, specificity, NPV and PPV. Also test for prevalence of BV among included females.* ***Methodology****: screening was done for all females in presence or absence of Amsel's criteria. Wet mount along with Gram stained films were examined in Microbiology lab and Nugent score was calculated for every patient. Cervico-vaginal aspirate samples were collected for detection of G. vaginalis, Lactobacilli and Sialidase enzyme by multiplex PCR.* ***Results****: Using Nugent score patients were categorized into bacterial vaginosis (BV) group (32%), non-BV group (51%) and 17 % were in intermediate group. Sensitivity, specificity, PPV and NPV of whole Amsel's criteria (100, 80.4, 76.2 and 100 % respectively) were better than using any criterion alone. Using multiplex PCR, detection of G. vaginalis (100%) and sialidase gene (93.7%) were higher in BV group and Lactobacilli gene (100%) higher in non-BV group with statistically high significant difference. Multiplex PCR detection of G. vaginalis has sensitivity (100%), specificity (92.2%), PPV (88.9%) and NPV (100%) for diagnosis of BV in relation to the gold standard test, Nugent score.* ***Conclusion****: using Amsel's criteria as a whole is better than using individual criteria for diagnosing BV with highest sensitivity, specificity, NPV and PPV. Combined PCR detection of G. vaginalis and sialidase gene can predict occurrence of virulent BV infection. BV is associated with significant loss of protective Lactobacilli.*

INTRODUCTION

Bacterial vaginosis (BV) is considered the commonest reason for women's vaginal discomfort and it is claimed that bacterial vaginosis is the commonest cause of vaginal infections both in pregnant and non-pregnant females. The published prevalence in European and American societies is from 4.9 to 36%. The presenting symptoms for BV typically include abnormal vaginal discharge and unpleasant fishy odor[1].

Although BV is the commonest cause of changed pattern of vaginal discharge in females during reproductive age as shown in most of the published studies, about 50 % of cases are non-symptomatizing and accidentally discovered during routine clinical assessment[2]. BV is associated with many complications especially in pregnant females resulting in poor reproductive outcome e.g. chorioamnionitis, preterm delivery, low birth weight, pelvic inflammatory disease and endometritis (post-delivery or post-surgical)[3], also females having BV are at higher risk to be infected with human papilloma virus, Herpes simplex virus type 2, *Trichomonas vaginalis* infection, *Neisseria gonorrhoeae* infection and HIV[4]. So, early prompt treatment is crucial to relieve symptoms for symptomatic patients and to prevent serious complications that may happen[2].

Vagina is normally inhabited by large non-motile Gram positive, hydrogen peroxide- producing Lactobacilli. Bacterial vaginosis is characterized by decreased concentration of lactobacilli and predominance of other facultative and strict anaerobic bacilli e.g. *G. vaginalis*, *Mycoplasma hominis*, Mobiluncus, Prevotella, Porphyromonas and

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Bacteroides and even anaerobic Gram positive cocci; *Peptostreptococcus* species[5].

The presence of anaerobic bacteria which heavily infest the vagina leads to production of carboxylase enzymes which in turn cleave peptides present in vagina into different amines. These amines, primarily trimethylamine are responsible for fishy odor associated with BV as they become volatile in presence of high PH; which is the first character to diagnose BV. They also increase transudate production along with exfoliation of vaginal squamous epithelia cells that results in production of characterized vaginal discharge associated with BV; this is the second character to diagnose BV. A third character to diagnose BV is clue cell detection in vaginal discharge which is caused by enhanced adherence of *G. vaginalis* to exfoliated squamous epithelia cells caused by elevated vaginal PH[6].

Of the well-defined risk factors predisposing to development of BV were: using the intrauterine devices, douching, nonwhite ethnicity, prior pregnancy[6]. Also epidemiological studies correlated BV to previous STDs, more than one sexual partner in a short period of time, or a new sexual partner one month prior to complaining from BV[7].

Laboratory and Microbiological diagnosis of the etiological agent responsible for BV is not clearly recognized as there is no single organism was solely linked to the development of BV. *G. vaginalis* has been isolated from approximately all females presenting with BV and also from non-symptomatizing females who didn't even meet clinical diagnostic criteria[7].

There are 4 common diagnostic methods for BV, Amsel's criteria (clinical diagnosis), Nugent Score (Gram staining score), culture-based techniques for isolation of bacteria causing BV and molecular diagnosis of BV. Amsel's criteria, are clinical criteria where BV is clinically diagnosed if 3 of 4 criteria are present: elevated vaginal PH more than 4.5, fishy odor of vaginal discharge after adding KOH to it, presence of characteristic vaginal discharge and presence of clue cells upon wet mount examination of vaginal discharge[8]. Nugent score is calculated after scoring of the present lactobacilli morphotypes (large Gram-positive bacilli) scored from 0 to 4, *G. vaginalis* morphotypes (small gram-variable bacilli) score from 0 to 4 and Mobiluncus morphotypes (curved gram-variable bacilli) scored from 0 to 2. Then Nugent score is ranging from 0 to 10, where patients scored from 7 to 10 are diagnosed positive BV, patients scored 0 to 3 are negative for BV, patients scored 4-6 are intermediate group[9]. Culture-based techniques are found to be less helpful, as 50-60 % of females are not meeting other diagnostic criteria for BV are positive for *G. vaginalis* culture, so specificity is poor. Isolation of Bacteroides species, *Peptostreptococcus* species and *M. hominis* by culture-based techniques was found to be specific but not sensitive and isolation of *Mobiluncus* species from cultures is difficult. So, it was concluded that culture-based techniques for diagnosis of BV is of less value. Now there are multiple nucleic acid-based techniques for diagnosis of BV quantifying the concentration of *M. hominis*, *G. vaginalis*, and lactobacilli in Cervico-vaginal lavage (CVL) samples and correlating the concentrations with the development of BV[10].

Part of BV pathogenesis is virulence factors of *G. vaginalis* including in *vivo* and in *vitro* biofilm formation, sialidase enzyme production, epithelial cells exfoliation, cytolysin and vaginolysin production [5].

Bacterial vaginosis is basically treated with Metronidazole orally; it is the standard treatment option as it is broadly affecting all anaerobic bacteria with limited effect on Lactobacilli and other facultative anaerobic bacteria. Also, bacteria rarely develop Metronidazole resistance. But it is observed that treatment with Metronidazole is associated with high recurrence rate within 12 months whether it is a relapse or reinfection[11]. Recurrence is identified if the patient got 3 or more infection periods per year and it should be treated with long term treatment course. Failing to respond to treatment may be due to bacterial biofilms associated with BV[1]. New treatment approaches are dependent on using probiotics using vaginal microbiome transplantation (VMT) from healthy volunteers for treating patients with recurrent intractable BV[12].

The aim of our study is to screen for bacterial vaginosis among symptomatic and asymptomatic females visiting a health care facility for gynecological consultations and to test for sensitivity and specificity of different clinical, laboratory and PCR methods for diagnosis of BV.

METHODOLOGY

The study is a cross-sectional study, conducted in the period from February 2020 to January 2021 on females in reproductive age group visiting clinic of the Obstetrics and Gynecology of Menoufia University Hospitals, Menoufia, Egypt. The study protocol was approved by ethics committee of the Faculty of Medicine, Menoufia University, Egypt.

Study Population:

Samples were collected from 100 females in reproductive age period (18-48 years) attending the Clinic of Obstetrics and Gynecology complaining or not complaining of symptoms of BV, with primarily abnormal vaginal discharge with amine fishy odor.

Exclusion Criteria: previous history of genital tract related cancer, use of antibiotics or medication with vaginal route 3 weeks before the visit or had sexual intercourse 2 days prior to the visit or postmenopausal women[4].

Specimen Collection:

Pelvic examination was done by water only lubricated vaginal speculum where samples were collected from posterior fornix or lateral vaginal wall[2] i. vaginal secretions for vaginal pH; ii. three microscopy slides (for detection of clue cells, whiff test and Gram stain) prepared by using sterile cotton tipped swabs and assessment of vaginal discharge character during examination.

Cervico-vaginal lavage (CVL) samples: were collected by injecting 10 ml of sterile saline into cervix doing irrigation of both cervix and vagina then do aspiration from poster vaginal fornix. The aspirate was kept on ice till transported to laboratory to be processed within 6 hours[13]. All samples were immediately transported to Microbiology laboratory.

Diagnostic Methods for BV:

Amsel's Criteria: (clinical assessment criteria) were used for patient's evaluation who were evaluated for presence of 3 out of 4 criteria to be diagnosed as positive for BV. These criteria are i. presence of thin homogenous discharge adhering to vaginal wall and this was evaluated by the gynecologist. ii. Whiff test: adding few drops of 10 % KOH to one of the slides streaked with vaginal discharge that leads to production of fishy amine odor with BV. iii. Elevated vaginal PH: detected by PH indicator strip. Elevated vaginal PH above 4.5 is a character of BV[4]. iv. Presence of Clue cells in vaginal wet mount: the second slide was used for wet mount examination of vaginal discharge. Clue cells are large squamous epithelial cells seen under high-power microscopy with lost cell boundaries due to adherent bacteria[14].

Nugent Score: Gram-stained film of vaginal discharge (third slide) and counting the numbers of three main morphotypes: Lactobacillus morphotype (large Gram-positive bacilli), Gardnerella morphotype (small Gram positive or Gram variable bacilli), and Mobiluncus morphotype (curved Gram-negative or Gram variable bacilli) was done. Scoring was done according to table 1.

Table 1: Scoring different morphotypes in Gram stain

Score	Lactobacillus morphotype (a)	Gardnerella morphotype (b)	Mobiluncus morphotype (c)
0	+4	0	0
1	+3	+1	+1 or +2
2	+2	+2	+3 or +4
3	+1	+3	
4	0	+4	

Where +1 = less than 1 morphotype / 1000 x, +2 = 1-5 morphotypes / 1000x
+3 = 6-30 morphotype / 1000x, +4 = >30 morphotype / 1000x

Nugent scoring system for diagnosis of bacterial vaginosis was done according to morphotypes scoring in table 2.

Table 2: Nugent score for diagnosis of bacterial vaginosis

Score	Group
0-3	Normal flora (negative BV)
4-6	Intermediate
7-10	Bacterial vaginosis

One of the prepared slides was fixed and stained using Gram stain and then examined microscopically using oil emersion lens (1000x) where number of morphotypes mentioned in table 1 is counted per high power field to give points for every morphotype. If less than one morphotype was detected per field this means +1 score, detection of 1-5 morphotypes/ field means +2 score, detection of 6-30 morphotype/ field means +3 score, detection of >30 morphotype / field means +4 score. A score was given to the 3 morphotypes according to table 1 and final score was calculated by sum of score of all morphotypes (total score = a+b+c) and it is ranging from 0 to 10. Then according to table 2 the patients were categorized into 3 groups: patients with score from 0-3: are normal flora group, patients with score 4-6: intermediate group, patients with score 7-10: are BV group[15, 16].

Cervico-vaginal aspirate samples were mixed using a gentle vortexing then stored at -70 0C till PCR time.

Multiplex PCR:

- Genomic DNA extraction: from thawed Cervico-vaginal aspirate samples was done using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to manufacturer instructions.
- DNA amplification for detection of *G. vaginalis* and lactobacilli bacteria and sialidase enzyme (virulence factor) of *G. vaginalis* was done by multiplex PCR study where the sequences of used forward and reverse primers and their sizes in Pb were listed in table 3.

Table 3: Primers used for detection and amplification of *G. vaginalis*, lactobacilli and sialidase enzyme genes:

Primer	Primer Sequence (5´→3´)	Size, bp	Reference
G. vaginalis	F: CTCTTGGAAACGGGTGGTAA	301	18
	R: TTGCTCCCAATCAAAAGCGGT		
Lactobacillus	F: TGGAAACAGGTGCTAATACCG	233	18
	R: GTCCATTGTGGAAGATTCCC		
Sialidase (sld)	F: AGCCCGCATATCCCGTATCG	454	17
	R: GGACCTGGCCAACATGGAGT		

Reaction content: a PCR reaction volume 20 µl was used consisting of 1U Taq polymerase, 1 × PCR buffer which contained 1.5 µM MgCl2 (25 mM) (QIAGEN, USA) and 0.7 µM of every dNTPs. 0.5 µM of each primer and target template DNA concentration of ~4 ng/µL, the remaining volume is DNAase, RNAse free H2O. Multiplex-PCR amplification was done on thermal cycler (ThermoFisher, USA). First denaturation step temperature was 94°C for 2 minutes, followed by 29 amplification cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds followed by extension step at 72°C for 1 minute. The final extension step was done at 72°C for 5 minutes [18]. 4 µL of Amplified PCR product was separated by 1.5% (w/w) agarose gel electrophoresis using 0.1% ethidium bromide staining at 80V for 2h.

RESULTS

For 100 women came to obstetrics and gynecology clinics during the period from February 2020 to January 2021, 51(51%) had normal vaginal microbiota, 17 (17%) had intermediate vaginal microbiota and 32 (32%) had bacterial vaginosis according to Nugent score. 58 (58%) showed no BV and 42 (42%) had BV using Amsel's criteria as shown in table 4. Sialidase enzyme gene was detected in 41 patients; two were in Non-BV group, 9 in intermediate group and 30 were in BV group as shown in table (4) and figure (1). Sensitivity, specificity, PPV and NPV of Amsel's criteria as a whole and of every component; vaginal PH, whiff test, Clue cells and characteristic vaginal discharge were done in comparison to Nugent score as the gold standard test. Clue cell detection has higher sensitivity and specificity for diagnosing BV compared to the other three components of the Amsel's criteria, while using Amsel's criteria as a whole showed the highest sensitivity, specificity, PPV and NPV rather than using separate parameters as shown in table 5. *G. vaginalis* and sialidase gene were detected with statistically significant difference in women with BV than without, Lactobacilli was detected more in women without BV than with BV with statistically significant difference as shown in table 6. Multiplex PCR showed 100% sensitivity & NPV and 92.2 % specificity in comparison to Nugent score in detection of *G. vaginalis* among BV and Non-BV patients as shown in table 7.

Table 4: No and % of Positive and Negative Cases of Different Tests Used for Diagnosis of Bacterial Vaginosis

Nugent Score Groups	Amsel's Crireia No (%)	*G. vaginalis* Gene by PCR	Lactobacilli Gene by PCR	Both *G. vaginalis* and lactobacilli Genes by PCR	Salidase gene by PCR
Non BV n=51 (51%)	58 (58%)	4 (8.9%)	51 (85%)	4 (28.6%)	2 (4.9%)
Intermediate N=17 (17%)	-----	9 (20%)	7 (11.7%)	7 (50%)	9 (21.9%)
BV N=32 (32%)	42 (42%)	32 (71.1%)	2 (3.3%)	3 (21.4%)	30 (73.2)
Total 100 (100%)	100 (100%)	45 (100%)	60 (100%)	14 (100%)	41 (100%)

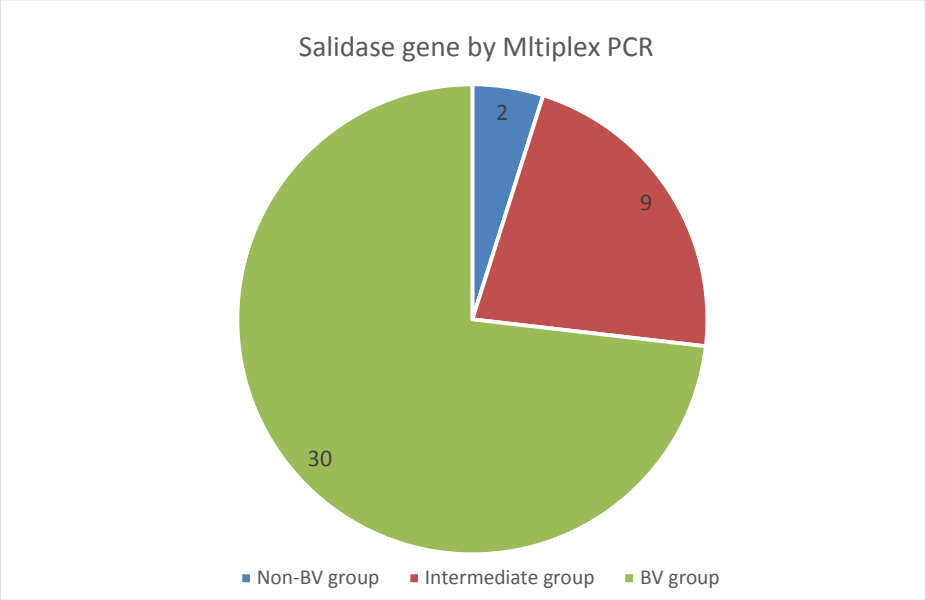

Fig. 1: Sialidase gene among different Nugent score groups

Table 5: Sensitivities, specificities, NPV and PPV of Amsel's Criteria as a Whole and of Every Component in Comparison to Nugent Score for the Diagnosis of Bacterial Vaginosis

Amsel's criteria	Nugent Score		Sensitivity (%)	Specificity (%)	PPV	NPV
	Positive (n= 32)	Negative (n= 51)				
Vaginal pH More than 4.5(n = 46) Less than 4.5(n= 54)	 29 3	 17 34	90.6	66.7	63	91.9
Clue Cells Present (n= 42) Absent (n= 58)	 31 1	 11 40	96.8	78.4	73.8	97.6
Whiff Test Positive (n= 42) Negative (n= 58)	 25 7	 17 34	78.1	66.7	59.5	82.9
Vaginal Discharge Present (n= 39) Absent (n= 61)	 27 5	 12 39	84.3	76.5	69.2	88.6
Total Amsel's criteria Positive (n= 42) Negative (n=58)	 32 0	 10 41	100	80.4	76.2	100

Table 6: Multiplex PCR Detection of Bacterial Vaginosis and Virulence genes in comparison to Nugent Score

PCR genes	Nugent Score			Chi-square	P value
	Non-BV (n=51)	Intermediate (n=17)	BV (n=32)		
***G. vaginalis* positive**	4 (7.8 %)	9 (52.9 %)	32 (100 %)	95.15	< 0.001
Lactobacilli positive	51(100%)	7 (4.1%)	2 (6.3%)		
Sialidase positive	2 (3.9 %)	9 (52.9 %)	30 (93.75 %)		

Table 7: Sensitivity, specificity, NPV and PPV of Multiplex PCR for Detection of *G. vaginalis* in Comparison to Nugent Score for Diagnosis of Bacterial Vaginosis

PCR detection of *G. vaginalis*	Nugent Score		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	BV Group (n=32)	Non-BV group (n=51)				
Positive for *G. vaginalis*	32	4	100	92.2	88.9	100
Negative for *G. vaginalis*	0	47				

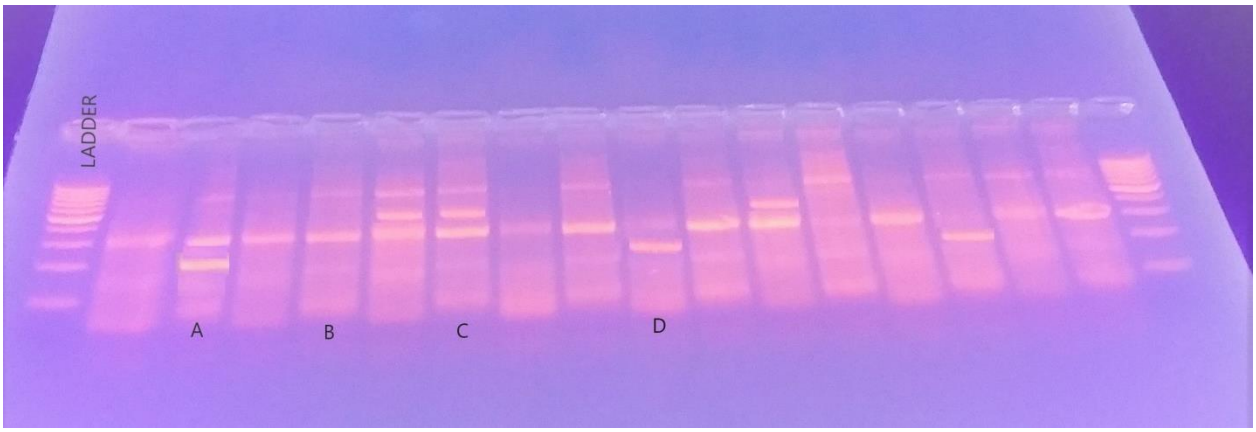

Fig. 2: Multiplex conventional PCR showing: A both lactobacilli and *G. vaginalis* genes. B *G. vaginalis* gene only. C both *G. vaginalis* and Sialidase genes. D lactobacilli gene only

DISCUSSION

BV is the commonest leading factor of vaginitis. In the present study Nugent scoring, Amsel's criteria and multiplex PCR were evaluated for diagnosing BV. Because the vaginal microbial environment consists of diverse bacterial species present in equilibrium but this microbiota are not static, there is continuous transition alternating between healthy and BV environment. So, we selected two BV-marker organisms to include in our multiplex PCR; *G. vaginalis* and *Lactobacillus* spp., the commonest morphotypes detected in Gram stained film used for diagnosing BV.

In the present study 51% of women had normal vaginal microbiota, 17% had intermediate phase vaginal microbiota and 32% had BV according to the Nugent score system. This agreed with Kusters et al.[19] who found 55 % had normal vaginal microbiota, 9 % intermediate and 36 % had bacterial vaginosis using Nugent score. While higher percent (69.2 %) had normal vaginal microbiota, 11.5 % had intermediate phase vaginal microbiota and slightly lower percent (19.2 %) had bacterial vaginosis using Nugent score by Cox et al.[9]

Multiple studies stated that intermediate group can be categorized under BV group as most of patients develop BV later on. So, this scoring system must correlates well with clinical disease. The clinical evaluation is clinically helpful as it provides timely decision, however Nugent scoring is more accurate [20]. In study conducted by Anderson et al.[21] 30% of symptomatic patients categorized in intermediate group were underscored after full evaluation. Subsequently 53% of patients developed BV after sometime and 47% revert back to normal flora. For that reason we reported all patients with intermediate score to treating physician who should consider clinical evaluation for patient management. Also, we used Amsel's criteria and multiplex PCR to complete the diagnosis of this category.

The normal vaginal microbiota consists mainly of lactobacilli throughout the reproductive period. BV represents a complex change in the flora, characterized by a marked reduction in lactobacilli and an increase in other microorganisms including; *G. vaginalis*, Peptostreptococcus, *Mycoplasma hominis* and anaerobic gram-negative bacilli including Prevotella, Porphyromonas, Bacteroides and Mobilincus. This alteration causes changes in consistency, color, volume, pH, odor and metabolic components of vaginal secretions[19].

In the present study; 58% showed no BV and 42% had BV using Amsel's criteria. By using Nugent score as the gold standard for diagnosing BV, we assessed Sensitivity, specificity, PPV and NPV of vaginal PH, Whiff test, Clue cells and Characteristic vaginal discharge and whole Amsel's criteria in comparison to Nugent score. Vaginal PH had 90.6%, 66.7%, 63% &

91.9% respectively. Clue cells had 96.8%, 78.4%, 73.8% &97.6 respectively. Whiff test had 78.1%, 66.7%, 59.5% & 82.9% respectively. Characteristic vaginal discharge had 84.3%, 76.5%, 69.2% &88.6% respectively. Results showed clue cells were the best criterion of Amsel's criteria. Comparable results were shown by Sha et al.[8] who reported sensitivity and specificity of vaginal PH 83% and 69%, for clue cells 32% and 98%, for whiff test 44% and 96% and for the characteristic vaginal discharge 33% and 85% respectively, on the other hand they found that vaginal pH higher than 4.5 improved the sensitivity but with poor specificity for BV diagnosis. Schwebke et al.[22] reported poor sensitivity and specificity of Amsel's criteria 70% and 94% respectively. We can consider subjective clinical evaluation by different physicians may play a role in that variability.

Multiplex PCR used for detection of *G. vaginalis* and lactobacilli significantly correlated with the Nugent score in our study where *G. vagimalis* was detected more among BV group of Nugent score, and lactobacilli was detected more among non BV group, with high statistically significant difference. Multiplex PCR showed 100% sensitivity & NPV, 92.2% specificity and 88.9% PPV in diagnosis of BV compared to Nugent score (gold standard test). This agreed with Kusters et al.[19] who reported PCR sensitivity 92 % and specificity 96 % for diagnosis of BV, confirming the potential diagnostic value of molecular diagnosis of BV. Our results agreed with findings of Hillier et al.[23] and sha et al.[8] findings where there was significant loss of lactobacilli in patients with Nugent scoring 7 to 10 compared to patients with Nugent scoring 4 to 6 and 0 to 3 groups.

While PCR is labor-intensive and costly than the Nugent Gram stain scoring method for clinical diagnosis, it is less cumbersome than older quantitative culture techniques utilized in research settings where assessing for changes in specific bacteria could aid in understanding the pathogenesis and/or consequences of BV [24].

Many virulence factors, such as the ability to adhere and degrade mucin, biofilm formation and sialidase activity, contribute to the pathogenic potential of *G. vaginalis* [25]. Sialidase is a significant virulence factor of G. vaginalis as in addition to lysis of mucin, it interfers with IgA action[26].

In our study we correlated the detection of *G. vaginalis* with the expression of sialidase gene where presence of both of them can be a positive index of BV caused by virulent *G. vaginalis*. Salidase gene was detected in 30 out of 32 *G. vaginalis* positive patients. In Pleckaityte et al.[27] study sialidase gene was detected in all *G. vaginalis* positive patients. Moncla and Pryke [28] found that sialidase-positive strains varied from 20% to 40% of *G. vaginalis* isolates.

CONCLUSION

Using Amsel's criteria as a whole is better than using individual criteria for diagnosing BV with highest sensitivity, specificity, NPV and PPV. Combined PCR detection of *G. vaginalis* and sialidase gene can predict occurrence of virulent BV infection. BV is associated with significant loss of protective Lactobacilli.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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