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Original Article

Quantification of *Gardnerella vaginalis, Atopobium vaginae* and *Lactobacillus* spp. in bacterial vaginosis

Nedzib Numanovic[1], Snezana Ribis[2], Jelena Cukic[3], Dane Nenadic[4], Aleksandar Zivanovic[5,6], Predrag Sazdanovic[5,6], Violeta Ninkovic[3], Dejan Baskic[3,7]

[1] *Department of Gynecology, General Hospital Novi Pazar, Novi Pazar, Serbia*
[2] *Department of Microbiology, University Clinical Center Kragujevac, Kragujevac, Serbia*
[3] *Department of Microbiology, Public Health Institute, Kragujevac, Serbia*
[4] *Department of Gynecology, Military Medical Academy, Belgrade, Serbia*
[5] *Department of Gynecology, Faculty of Medical Sciences, Kragujevac, Serbia*
[6] *Deprtment of Gynecology, University Clinical Center Kragujevac, Krgujevac, Serbia*
[7] *Department of Microbiology and Immunology, Faculty of Medical Sciences, Kragujevac, Serbia*

Abstract

Introduction: The aim of the study was to investigate prevalence of bacteria most frequently associated with bacterial vaginosis using Amsel's criteria as well as to quantify these bacteria by real-time PCR and to explore the difference in their quantity between healthy and bacterial vaginosis samples.

Methodology: For classification of vaginal discharge samples Amsel's criteria have been used. To detect and quantify *Gardnerella vaginalis Atopobium vaginae*, *Lactobacillus* spp. and total vaginal microbiome, real-time PCR has been applied.

Results: According to results of our study Amsel's criteria matched well with real-time PCR diversification of healthy women and women with BV. Nevertheless, real-time PCR has been more sensitive in diagnosis of bacterial vaginosis. DNA quantification of bacteria demonstrated that mutual abundance of *G.vaginalis* and *A. vaginae* was good bacterial vaginosis marker . On the contrary, *Lactobacillus* spp. was present in high amount in both healthy and bacterial vaginosis samples, but ratio of investigated bacteria was different between them. In fact, *G. vaginalis* and *A. vaginae* comprised only 0.1% of total microbiome in healthy, whereas *Lactobacillus* spp. took 99.3% of it. Nonetheless, in bacterial vaginosis, *G. vaginalis* and *A. vaginae* made up 34.4% of total microbiome, while *Lactobacillus* spp. was 21.6%.

Conclusions: According to the results of our study real-time PCR analysis was more sensitive in diagnosis of bacterial vaginosis than Amsel's method, as well as it represented fine tool in making a difference between microbial entities in healthy and bacterial vaginosis samples.

Key words: Bacterial vaginosis; Amsel; real-time PCR; *G. vaginalis*; *A. vaginae*.

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Introduction

Bacterial vaginosis (BV) is the most common form of vaginal discomfort in women of reproductive age. Usually, manifestation of BV is vaginal discharge, but it may be present even without any of symptoms [1]. Substantially, BV is a consequence of vaginal microbiome alteration, when predominant bacterial species of healthy vaginal flora, *Lactobacillus* spp, is replaced by various anaerobic bacteria [2]. Nevertheless, many of these anaerobes are normally present in vaginal flora, but in small amount [3]. Circumstances which can lead to development of anaerobic microbiota and subsequent bacterial vaginosis are multiple. It can be initiated by hormonal changes after puberty, in pregnancy and menopause, but also it can have ethnical background dictated by genetical factors, hygiene and social habits [4]. BV by itself does not represent a disease, but it can mediate spread of sexually transmissible infections as well as it can cause preterm birth [5].

Diagnosis of BV can be achieved by clinical and microscopy criteria, along with using molecular biology techniques. In clinical practice, widely used is Amsel's criterion. According to Amsel, diagnosis of BV is established on the presence of three out of four clinical criteria: vaginal pH > 4.5, homogenous white/grey vaginal discharge, the presence of clue cells (vaginal epithelial cells covered by bacteria) and positive whiff test (fishy odour after addition of potassium hydroxide). Amsel's criterion is

dichotomous, which means that it can distinguish healthy from BV samples [6]. Although easy to perform in clinical practice, it was considered subjective and irreproducible as well as without possibility to give any precise direction in terms of real microbiological composition of vaginal discharge examined.

According to several molecular biology studies, among anaerobes the most frequently associated with BV were *Gardnerella vaginalis* and *Atopobium vaginae* [7-9]. The aim of our study was to investigate presence of healthy and anaerobic flora (since they can be present both in healthy and BV samples), as well as their quantity and relation in healthy and disturbed vaginal microbiome.

Methodology

Population and study design

The case-control study consisted of two groups: (i) healthy women and (ii) women with BV. Criteria for enrollment into study were: women within age range from 20-40 years with confirmed BV by Amsel's criteria [6] and approved by subsequent real-time PCR (RT-PCR) analysis. From the study were excluded patients with other diseases or treatment as well as three cases of intermediary results achieved by RT-PCR assessment. Investigation was conducted in General hospital Novi Pazar, Serbia, at Gynecology and Obstetrics Department during regular visits. All patients provided written informed consent prior to recruitment. This study was approved by Medical Ethics Committee of General hospital Novi Pazar (Nr 3072/19.08.2015) and was conducted according to the principles of the Helsinki Declaration.

Sampling and evaluation by Amsel's criteria

A non-lubricated speculum was placed into the vagina and consistency and color of vaginal discharge was noted (white/gray color considered suspect on BV). Then, four swabs of vaginal discharge were collected. The first cotton swab was taken for making the smear against glass slide which was covered by cover slip after adding two drops of normal saline for microscopy evaluation for the presence of clue cells. Secretions from second cotton swab were put onto pH indicator strips with a pH range from 3.5-6 to determine pH value. On the third cotton swab with collected secretions were added two drops of 10% KOH solution. Appearance of fishy amine odour after adding of 10% KOH solution was considered positive whiff test. Diagnosis of BV was established in patients positive for three out of four criteria. The fourth swab was taken by dacron swab for RT-PCR analysis.

DNA extraction, detection and quantification

Genomic DNA from vaginal samples was extracted following procedures contained within commercially available kit (QIAamp DNA mini kit, Qiagen, Germantown, MD, USA). Detection and quantification of *G. vaginalis*, *A. vaginae*, *Lactobacillus* spp. as well as quantification of total vaginal bacteria was performed by RT-PCR (SaCycler-96, Sacace Biotechnolgies, Como, Italy), by commercially available Bacterial Vaginosis Real-TM Quant test (Sacace Biotechnolgies, Como, Italy) according to the instructions of the manufacturer. Molecular differentiation of normal and BV samples was performed by Microsoft Excel Software algorithm provided by manufacturer.

Genomic DNA extraction, detection and quantification of *G. vaginalis*, *A. vaginae*, *Lactobacillus* spp. as well as quantification of total vaginal bacteria was performed by commercially available Bacterial Vaginosis Real-TM Quant test (Complete Real Time PCR test with DNA purification kit, Sacace Biotechnolgies, Como, Italy) using RT-PCR (SaCycler-96, Sacace Biotechnolgies, Como, Italy) according to the instructions of the manufacturer. Molecular differentiation of healthy and BV samples was performed by Microsoft Excel Software algorithm provided by manufacturer.

Data analysis

Statistical analysis was conducted using SPSS Statistics, version 19.0 (IBM, USA). Variables were presented as frequencies of individual parameters (categories), and statistical significance of differences was evaluated using χ^2 test. Differences among groups of nonparametric data were analyzed by Kolmogorov-Smirnov test. Variation among and between groups was calculated by ANOVA. Statistical difference of $p < 0.05$ was considered statistically significant.

Results

BV diagnosis has been performed by two methods: (i) clinical method by Amsel and (ii) RT-PCR. Since Amsel's clinical categorization considers only two entities, healthy and BV, intermediary results achieved by RT-PCR were excluded from further evaluation. For this reason, 67 women in total have been outlined by investigation (17 healthy and 50 with BV). Using Amsel's method, 67,7% (46/67) of patients were diagnosed with BV, while 31.3% (21/67) were healthy. Real-time PCR analysis has shown that 74,6% (50/67) of patients had BV whereas healthy finding had 25,4%

(17/67) (Figure 1). Although this was not the primary objective of our study, we found high agreement between Amsel's method and RT-PCR with high diagnostic accuracy and balanced sensitivity-specificity rate of Amsel's method (Supplementary Table 1).

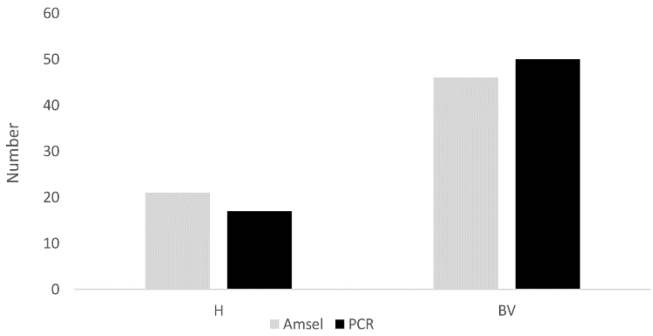
Prevalence of G. vaginalis, A. vaginae and Lactobacillus spp. in BV and healthy findings

G. vaginalis and *A. vaginae* were more frequently present in BV samples (92% and 98% respectively) than in healthy samples (29% and 18% respectively) ($p < 0.01$). *G. vaginalis* or *A. vaginae* were present in all BV samples (100%). Both bacteria were present in 92% of BV cases, while in healthy cases they were exclusively present separately ($p < 0.001$). Compared to this, *Lactobacillus* spp. was present in almost all BV samples (98%) and in 100% of healthy samples ($p > 0.05$). Moreover, while the mutual ratio of bacterial prevalence in BV was almost equal (*Lactobacillus* spp. / *G. vaginalis* = 1.1; *Lactobacillus* spp. / *A. vaginae* = 1), the ratio of BV associated bacteria prevalence and *Lactobacillus* spp. prevalence in healthy samples was several times lower (*Lactobacillus* spp. / *G. vaginalis* = 3.4; *Lactobacillus* spp. / *A. vaginae* =5.7) ($p < 0.001$) (Table 1).

Number of DNA copies of G. vaginalis, A. vaginae, Lactobacillus spp. and total vaginal microbiome in BV and healthy samples

In BV samples number of DNA copies of *G. vaginalis* and *A. vaginae* was considerably higher than in healthy samples ($p < 0.0005$). In contrast to this, in healthy samples, number of DNA copies of *Lactobacillus* spp. was significantly higher ($p < 0.001$). Even though the number of *A. vaginae* DNA copies were higher than *G. vaginalis* DNA copies in healthy samples ($p > 0.05$), in BV samples the number of *G. vaginalis* DNA copies were significantly higher than *A. vaginae* DNA copies ($p < 0.001$). Interestingly, the total number of BV associated bacteria from BV samples were not significantly higher than the number of *Lactobacillus* spp. ($p > 0.05$). Yet, the number of *Lactobacillus* spp. DNA copies in healthy samples was significantly higher compared to total number of both *G. vaginalis* and *A. vaginae* ($p < 0.001$). Although total vaginal mikrobiome (number of DNA copies of all bacteria present in our samples) from BV samples was higher than in healthy samples, observed differences remained below the threshold of statistically significant difference ($p > 0.05$) (Table 2).

Figure 1. Vaginal swabs samples classified by Amsel's criteria and RT-PCR as healthy (H) and bacterial vaginosis (BV).



Relative ratio of number of DNA copies of Lactobacillus spp., G. vaginalis and A. vaginae between BV and healthy samples

In healthy samples relative number of *G. vaginalis* and *A. vaginae* DNA copies, compared to *Lactobacillus* spp., was less than 0.1%. In BV samples, on the contrary, relative ratio of *A. vaginae* and *G. vaginalis* DNA copies was higher than number of *Lactobacillus* spp. DNA copies (159.0%).

Table 1. Prevalence of *Gardnerella vaginalis*, *Atopobium vaginae* and *Lactobacillus* spp. in healthy samples (H) and bacterial vaginosis (BV).

	G. vaginalis	***A. vaginae***	***G. vaginalis* or *A. vaginae***	***G. vaginalis* + *A. vaginae***	***Lactobacilus* spp.**	***Lactobacilus* spp. / *G. vaginalis***	***Lactobacilus* spp. / *A. vaginae***
H	5 (29%)	3 (18%)	8 (47%)	0 (0%)	17 (100%)	3.4	5.7
BV	46 (92%)	49 (98%)	50 (100%)	46 (92%)	49 (98%)	1.1	1.0

Table 2. Average number of DNA copies of *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp. and total bacterial microbiome in healthy samples (H) and bacterial vaginosis (BV).

	G. vaginalis	***A. vaginae***	***G. vaginalis* + *A. vaginae***	***Lactobacilus* spp**	**TVMB**
H	22,061	985,333	1,007,394	778,250,376	783,854,827
BV	224,382,930	119,796,234	344,179,164	216,466,333	1,001,580,079

TVBM: Total vaginal microbiome.

Analyzing the relative ratio of DNA copies between *Lactobacillus* spp., *G. vaginalis* and *A. vaginae* and total vaginal microbiome, statistically significant difference between BV and healthy samples has been observed ($p < 0.001$). In healthy samples *Lactobacillus* spp. made 99.3% of total vaginal microbiome, while contribution of *A. vaginae*, *G. vaginalis* and other microorganisms was minor (around 0.7%). On the other hand, in BV samples *Lactobacillus* spp. made only 21.6% of total vaginal microbiome, while *G. vaginalis* and *A. vaginae* made 12.0% and 22,4% respectively (in sum 34.4% of total vaginal microbiome). According to these results we could assume that in the samples of BV were 44% of DNA copies of bacteria other than those who were under the scope of our research (Table 3).

Discussion

BV represents shift in vaginal homeostasis when "protective" lactobacilli were replaced by more diversified population of bacteria composed from dozens of different anaerobic bacteria [10-12]. Around 50% of women with BV remain asymptomatic, without any need for therapy. Nevertheless, in pregnant women it can lead to preterm delivery [13]. Many methods in BV diagnosis have been used so far. Among them within microscopy methods Nugent, Ison/Hay and Claeys methods were widely applied. In addition to these, Amsel's method has been used in clinical setting, combining microscopy findings and clinical signs [6]. Despite its simplicity and wide utilization, precision and objectiveness of these methods have been questioned, especially after development of molecular assays for BV testing [14]. In this regard, in our study, RT-PCR analysis of vaginal swabs of pregnant women has been performed, along with classification of samples by Amsel's criteria. Subsequently, obtained data have been compared and analyzed.

According to data of our study, higher percentage of BV has been detected by RT-PCR analysis than using Amsel's criteria. Similar results have been obtained in study performed by Menard et al. [15]. The only difference was that in our study, among pregnant women, was higher percentage of patients diagnosed with BV, which was in line with the results of study conducted by Bhavana et al. [16]. Explanation for this discrepancy can be that differences can be influenced by ethnical background and they can vary from 7-70% as it was described before [17].

Furthermore, the data of our study have shown that *G. vaginalis* and *A. vaginae* had higher prevalence in BV than in healthy samples, where both have been present in almost all BV samples. Similar results have been achieved also by other studies [18-22]. Nevertheless, this phenomenon can be explained by ability of *G. vaginalis* to form biofilm which represents protective environment for other vaginal anaerobs such as *A. vaginae* [23]. Like in other studies, the results of our investigation demonstrated that both of bacteria can be present also in healthy samples with low prevalence [18,19]. Nevertheless, according to our findings, dominant species in healthy samples was *Lactobacillus* spp. with prevalence of 100%.

Another finding of our study was that total number of DNA copies of *G. vaginalis* and *A. vaginae* was slightly higher than number of *Lactobacillus* spp. DNA copies in BV, while in healthy samples number of *Lactobacilus* spp. copies was more than 700 times higher than total amount of copies of both *G. vaginalis* and *A. vaginae* (Table 2). Taking into consideration only number of *Lactobacillus* spp. DNA copies in both healthy and BV samples, it has been observed that, although number of DNA copies between BV associated bacteria and *Lactobacillus* spp. in BV samples was not significantly different, number of *Lactobacillus* spp. DNA copies in BV samples was more than three times lover compared to its number in healthy samples. In addition, inverse relationship in numbers of DNA copies of *G. vaginalis* and *A. vaginae* in healthy samples and BV has been noted. However, number of *A. vaginae* DNA copies in healthy samples were around 45 times higher compared to number of *G. vaginalis* DNA copies, while in BV samples number of *G. vaginalis* DNA copies were almost 2 times higher than number of *A. vaginae* DNA copies. Furthermore, the data have shown increase in around 10.000 times in *G. vaginalis* DNA copies in BV, compared with healthy samples. To sum up, according to results of our study, between healthy and BV samples inverse relationship

Table 3. Relative number of DNA copies of *Gardnerella vaginalis*, *Atopobium vaginae and Lactobacillus* spp. in relation with total bacterial microbiome in healthy samples (H) and bacterial vaginosis (BV).

	A. vaginae* / *Lactobacilus	***G. vaginalis* / *Lactobacilus***	***G. vaginalis* + *A. vaginae* / *Lactobacilus***	***Lactobacilus* / TVMB**	***A. vaginae* / TVMB**	***G. vaginalis* / TVMB**	***G. vaginalis* + *A. vaginae* / TVMB**	**Other**
H	0.1%	0.0%	0.1%	99.3%	0.1%	0.0%	0.1%	0.6%
BV	55.3%	103.7%	159.0%	21.6%	12.0%	22.4%	34.4%	44.0%

TVBM: Total vaginal microbiome

of BV associated bacteria and *Lactobacillus* spp. has been observed, similarly to study done by Menard at al. [24]. Additionally, according to our findings, transition to BV was marked by the change in *G.vaginalis* DNA copies than other two bacteria.

Finally, since it has been understood that not only detection and quantity but also ratio of evaluated bacteria was important, it has been found that in healthy samples very high domination of *Lactobacillus* spp. was present compared to BV associated bacteria (Table 3). On the other hand, in BV samples this ratio was different, with higher relative ratio of BV associated bacteria. Moreover, when compared with total vaginal microbiome, in healthy samples *Lactobacillus* spp. had relative ratio 93%, while in BV samples *Lactobacillus* spp. comprised only the fifth part of total vaginal microbiome (21,6%). However, *G. vaginalis* and *A.vaginae* in BV, compared to healthy samples, increased in relative ratio for more than 300 times, making the third of total vaginal microbiome (34,4%). Surprisingly, according to data of our study, it appeared that 44% of total vaginal microbiome from BV samples was made from bacteria not analyzed in this research. As it has been known, vaginal microbiome may be comprised from many different bacterial species [25,26]. It is probable that some of these bacteria, along with bacteria studied in our investigation, could be candidates responsible for microbial transition from healthy vaginal microbiome towards BV. Whether this percentage, of to us unknown bacteria, is mainly modified by single candidate, as it was suggested by some studies [27-29], or it is of multi-bacterial origin, could be an interesting question to answer for some future studies.

Conclusions

The results of present study confirmed that molecular analysis was more sensitive in diagnosis of bacterial vaginosis than Amsel's criteria. Further, in healthy and BV samples inverse ratio of *Lactobacillus* spp. and BV associated bacteria prevalence has been observed. Moreover, it has been found that healthy vaginal microbiome was marked by domination of *Lactobacillus* spp., while transition towards BV has been determined by increase in number of *G. vaginalis* DNA copies. Finally, relative ratio of *G. vaginalis* and *A.vaginae* in BV samples made the third of total vaginal microbiome.

Author's contributions

Authors of the manuscript outline all of the following conditions: 1. Contributing the idea, plan of study, gathering of data, analysis and evaluation of data; 2. Writing the manuscript, critically correcting its intellectual content; 3. Correcting version for publishing.

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Corresponding author

Snezana Ribis, MD, PhD
Department of Microbiology, University Clinical Center Kragujevac, Zmaj Jovina 30, 34000 Kragujevac, Serbia
Phone: +381 64 00 75 471
Email: snezana.ribis@gmail.com

Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Diagnostic accuracy of Amsel's criteria vs RT-PCR.

		AMSEL	
PCR	BV +	BV -	Total
BV +	44	6	50
BV -	2	15	17
Total	46	21	67
	BV +	BV -	
BV +	68.7%	31.3%	
BV -	74.6%	25.4%	
χ^2		$\chi^2 = 0.630$; df = 1; $p = 0.427$	
ORA		0.871	
Kappa		0.699	
Statistic	Value	95% CI	
Sensitivity	88.00%	75.69% to 95.47%	
Specificity	88.24%	63.56% to 98.54%	
Positive Likelihood Ratio	7.48	2.03 to 27.61	
Negative Likelihood Ratio	0.14	0.06 to 0.29	
Positive Predictive Value	76.22%	46.48% to 92.21%	
Negative Predictive Value	94.49%	88.81% to 97.37%	
Youden's index	76.24%		
Youden's index (exp.)	146.95%		
Accuracy	88.16%	77.95% to 94.77%	