

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Molecular diagnosis of bacterial vaginosis – prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in pregnant women

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SUMMARY

Introduction/Objective Bacterial vaginosis (BV) is defined as disequilibrium of vaginal microbiota due to proliferation of Gram-negative/variable anaerobes and reduction/depletion of vaginal lactobacilli. Difficulties in interpreting microscopically categorized findings in diagnosis of BV need a molecular analysis of bacteria present in vaginal discharge of patients. In this regard, we performed real-time qPCR analysis of vaginal discharge samples with the goal to explore in which extent prevalence and amount of anaerobes, *Gardnerella vaginalis* and *Atopobium vaginae*, are related to findings obtained by microscopy.

Methods This study enrolled 111 asymptomatic pregnant women between 24 and 28 weeks of pregnancy. Gram-stained vaginal smears were evaluated microscopically. Afterwards, DNA of bacteria was extracted from Gram slides and real-time qPCR was performed with the aim to detect and quantify *G. vaginalis* and *A. vaginae*.

Results The data of our study showed that 53.2% of patients had normal results, while 20.7% and 26.1% of patients had intermediary (IMD) and BV results, respectively. *G. vaginalis* and *A. vaginae* were more frequently found in IMD and BV than in healthy patients; also, the average bacterial number of *G. vaginalis* and *A. vaginae* were significantly higher in BV and IMD than in the group with normal findings ($p = 0.000$). Comparing mutual relation of *G. vaginalis* and *A. vaginae*, the prevalence and number of *G. vaginalis* were in all groups significantly higher than *A. vaginae*.

Conclusion The data of our study have shown that in distinguishing normal from BV findings, quantification of bacteria may be more important than just molecular detection of bacteria.

Keywords: bacterial vaginosis; real-time qPCR; *Gardnerella*; *Atopobium*

INTRODUCTION

Among disorders affecting female reproductive tract, bacterial vaginosis (BV) is one of the most common causes of vaginal flora disturbance. Bacterial vaginosis is a condition related to the disordered vaginal microbiota of polybacterial origin, characterized with proliferation of Gram-negative/variable anaerobes associated with reduction or almost complete depletion of “protective” vaginal lactobacilli [1].

Bacterial vaginosis prevalence is different between various ethnic groups in North America, Europe, the Middle East, or Asia. The global epidemiology study on this subject has shown that BV prevalence was the highest in some parts of Africa and lowest in most of Asia and Europe [2].

Proper diagnosis of BV is demanding in terms of sensitivity and specificity for precise outlining of the group of patients in need of treatment. The majority of studies have agreed on the fact that is not possible to cultivate microaerophilic or anaerobic residents of the vagina with complete efficiency [3–6]. With the introduction of molecular detection – polymerase chain reaction (PCR) – of the aforementioned bacteria, this problem has been surpassed. Furthermore, molecular analysis has shown that qualitative and quantitative architecture of BV is inconstant, composite, and not completely understood. It may comprise more than 80 various genera and thousands of species such as *Gardnerella vaginalis*, *Prevotella* spp., *Atopobium* spp., *Mobiluncus* spp., etc. [7].

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Microorganisms mostly detected in BV were *Gardnerella vaginalis* and *Atopobium vaginae*, with prevalence in BV ranging between 47.8–99% (*Gardnerella vaginalis*) and 75–95% (*Atopobium vaginae*) without significant difference in prevalence between pregnant and non-pregnant women [4, 8, 9]. In addition, the coexistence of these two microbes was documented in 78–96% of samples with BV [10]. Possible explanation for this was given by Hardy et al. [11]. By analyzing vaginal polymicrobial biofilm, they found that this biofilm is mostly formed by microaerophilic *Gardnerella vaginalis*, which further allows colonization by anaerobic *Atopobium vaginae*.

The importance of BV among pregnant women has been studied recently and it was shown that the rate of preterm delivery in patients with BV reached 30% [12]. Many diagnostic methods have been compared: cultivation of microorganisms mostly connected to BV, various microscopy criteria analyzing Gram-stained slides of vaginal swabs, molecular analysis, and molecular detection and quantification of microbes within the vaginal “ecosystem” [13, 14]. Moreover, it has been shown that microscopy classification of Gram-stained vaginal smears coincided with PCR in great extent, dividing all patients into three groups: normal, intermediary, and patients with BV (4). Nevertheless, although helpful in differing normal and BV findings, microscopy and simple molecular detection of microbes could not give answers on the significance of the intermediary group of patients, apart from its risk for preterm delivery [15, 16]. Due to this issue, Menard et al. [17] quantified by qPCR *Gardnerella vaginalis* and *Atopobium vaginae* in vaginal samples of pregnant women. They found that preterm delivery was not linked to the presence of *G. vaginalis* and *A. vaginae*, but to high concentrations (> 106 copies/ml) of these bacteria, with four times higher prevalence of *Gardnerella* and *Atopobium* in women with preterm delivery than in women with term delivery.

Because of great importance of BV among pregnant women, we performed molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae*, the most common bacteria connected to BV, with the aim of exploring the relation of these microbes to the groups of patients divided by Nugent’s criteria.

METHODS

Study population and design

This retrospective study comprised 111 pregnant and asymptomatic women between 24 and 28 weeks of pregnancy, seen during regularly planned appointments at the Military Medical Academy hospital from 2012 to 2014. Women younger than 18 and older than 40 years, with multiple pregnancies, anomalies of the uterus, cervical colonization, or with previous preterm delivery were excluded from this study. Women who were under any kind of therapy within two weeks before examination, as well as women who had sexual intercourse within a week before appointment, were also not enrolled in the study. The in-

stitutional Ethics Board approved the study protocol and all study subjects agreed to participate through a written informed consent.

Sampling and data collection

The specimens were prepared under standard ethical and laboratory protocols. After clinical examination, vaginal samples were collected by inserting sterile polyethylene terephthalate-tipped swab into the vagina. The swab was rotated 360° against the vaginal wall at the mid portion of the vault and carefully withdrawn to prevent contamination. The swabs were then smeared on a plain-glass slide, air-dried at room temperature, and Gram stained. Using conventional light microscopy (DM 2000 LED microscope, Leica Microsystems, Wetzlar, Germany), the slides were categorized at 1,000 × magnification according to Nugent. DNA extraction was performed from Gram-stained preparations following protocol established by Srinivasan et al. [14] and procedures contained within commercially available kit (QIAamp DNA mini kit, Qiagen, Germantown, MD, USA). Detection and quantification of *Gardnerella vaginalis* and *Atopobium vaginae* was determined using SaCycler-96 by commercially available Bacterial Vaginosis Real-TM Quant test (Sacace Biotechnologies, Como, Italy), according to the instructions of the manufacturer.

Statistical analysis

Complete statistical analysis was conducted using SPSS Statistics, Version 17.0 (SPSS Inc., Chicago, IL, USA). Variables were presented as frequencies of individual parameters (categories), and statistical significance of differences was evaluated using the χ^2 test. Differences among groups of nonparametric data were analyzed by Mann–Whitney and Kolmogorov–Smirnov tests. Receiver operating characteristic (ROC) curve was constructed and used to evaluate whether the number of bacterial DNA copies/ml could be a marker of diagnostic accuracy. Statistical difference of $p < 0.05$ was considered statistically significant.

RESULTS

Using Nugent’s criteria, we found that 26.1% (29/111) of the patients were diagnosed with BV. Of the tested patients, 20.7% (23/111) were classified into the intermediary group, while 53.2% (59/111) were healthy. Prevalence and quantity of *Gardnerella vaginalis* and *Atopobium vaginae* in the vaginal samples of the pregnant women are presented in Table 1.

In addition to the cases with BV (93.1%), *Gardnerella vaginalis* was detected in 95.6% of intermediary patients, as well as in 55.9% of normal specimens. Although *Gardnerella vaginalis* is present in a higher percentage in intermediary and BV patients, the presence of this bacteria is not associated with the diagnosis of bacterial vaginosis (Pearson’s $\chi^2 = 0.668$; $p = 0.716$). *Atopobium vaginae* was also detected in patients with normal findings (16.9%), but

Table 1. Prevalence and quantity of *Gardnerella vaginalis* and *Atopobium vaginae* in the diagnosed groups of patients

Group	Prevalence (total n = 111)			Quantity (DNA copies/ml*)		
	<i>G. vaginalis</i>	<i>A. vaginae</i>	χ^2	<i>G. vaginalis</i>	<i>A. vaginae</i>	KS
N	33/59	10/59	$\chi^2 = 19.4$; $p = 0.000$	1,796	432	$p = 0.000$
IMD	22/23	11/23	$\chi^2 = 13$; $p = 0.000$	27,217	1,413	$p = 0.000$
BV	27/29	14/29	$\chi^2 = 14.1$; $p = 0.000$	35,258,502	5,456,101	$p = 0.004$

N – normal; IMD – intermediary; BV – bacterial vaginosis;

KS – Kolmogorov–Smirnov test;

*mean number

percentage of this bacteria was higher in the intermediary and the BV group, with 47.8% and 48.3%, respectively. However, regarding *Gardnerella vaginalis*, the presence of *Atopobium vaginae* is not associated with the diagnosis of bacterial vaginosis (Pearson's $\chi^2 = 3.480$; $p = 0.175$). Finally, in our samples we showed the coexistence of *Gardnerella vaginalis* and *Atopobium vaginae* (Pearson's $\chi^2 = 14.199$; $p = 0.0005$). In the intermediary and the BV group, this coexistence was seen in 47.8% (11/23) and 48.3% (14/28), respectively, which was almost three times higher than in the normal group (16.9%; 10/59). More importantly, *Atopobium vaginae*, except in one case, were present only in the cases when *Gardnerella vaginalis* was also present.

Using real-time qPCR we found that the number of *Gardnerella vaginalis* and diagnosis are in weak positive correlation ($r = 0.272$; $p = 0.004$). The highest quantity of this bacterium was detected in samples with BV, while the lowest (20,000 times lower than in BV) has been calculated in patients with normal findings. The number of this bacterium in intermediary cases was 15 times higher than in samples with normal findings. Statistical analysis confirmed a significant difference in *Gardnerella vaginalis* quantity among all diagnosed groups of patients ($p = 0.001$) except for intermediary and BV ($p = 0.380$). In addition, as previously shown for *Gardnerella vaginalis*, we found that the number of *Atopobium vaginae* and diagnosis are in weak positive correlation ($r = 0.214$; $p = 0.023$). The largest amount of *Atopobium vaginae* was detected in BV, gradually decreasing in intermediary and normal groups with lesser difference between the normal and the intermediary group (three times only). However, in this case, the differences in the number of *Atopobium vaginae* between BV, intermediary, and normal findings were not statistically significant ($p = 0.072$).

As we found that *Gardnerella vaginalis* was detected in all the groups, that it was at least two times more frequent than *Atopobium vaginae*, and the the average number of *Gardnerella vaginalis* was significantly higher than *Atopobium vaginae* (Table 1), ROC curve was used to evaluate whether the number of DNA copies/ml of *Gardnerella vaginalis* could be a marker for diagnostic accuracy. We found that the number of DNA copies/ml of *Gardnerella vaginalis* is a very good marker for vaginal flora disturbance (AUC = 0.761; $p = 0.0005$). Moreover, using the ROC analysis, we showed that the number of DNA copies/ml of *Gardnerella vaginalis* has the ability to discriminate patients with normal findings from intermediary and BV patients. The defined cut-off value was 2,980 copies/ml, with a sensitivity and specificity of 78.6% and 72%, respectively (Figure 1).

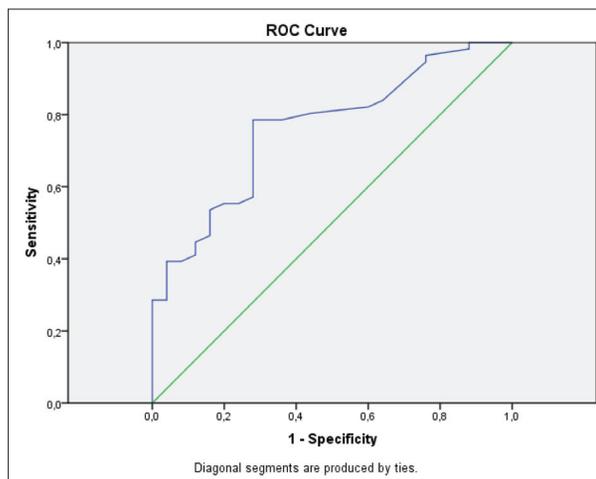


Figure 1. The number of DNA copies/ml was determined by the real-time qPCR method on the SaCycler-96 (Sacace Biotechnologies, Como, Italy); cut-off, sensitivity, and specificity were determined by ROC analysis and shown in the form of ROC curve

DISCUSSION

Bacterial vaginosis does not evolve from a commonly defined bacterial infection caused by one agent, but is a disorder of the vaginal microbiome. Therefore, the appropriate diagnosis of BV is demanding and the decision about the method of choice for its diagnosis requires a review of complexity, cost, and the constancy of samples difficult to interpret [18].

Nugent's criteria are the most widely used diagnostic tool for diagnosing BV, and are considered the gold standard, although their inter- and intraobserver accuracy have been questioned [19]. To avoid demanding and imprecise counting of bacterial morphotypes, qualitative microscopic examination was introduced by Ison and Hay [20] and Verhelst [21].

In daily practice, despite the numerous methods available, clinicians still have difficulties to decide which patient should be treated. This issue becomes further complicated with discrepancies in categorizing intermediate findings. Intermediate flora has been shown to consist of bacteria associated with BV, such as *Gardnerella vaginalis* and anaerobes, as well as lactobacilli, usually associated with normal flora, which is the main reason why this is considered a transitory condition between normal and BV, not yielding all clinical criteria of bacterial vaginosis [20, 22].

In this regard, over the last few years, several studies have been performed aiming to analyze microbial composition of vaginal discharge and quantity of bacteria associated with BV in microscopically categorized samples

using PCR and real-time qPCR [23, 24, 25]. It has been found that the most common bacteria detected in BV was *Gardnerella vaginalis*, but as being insufficiently specific, additional studies suggested *Atopobium vaginae* as the BV marker and an even greater risk factor for preterm delivery than *Gardnerella vaginalis* [26].

For this reason, we performed molecular analysis of vaginal discharge samples of pregnant women targeting these two bacteria. According to the results of our study, *Gardnerella vaginalis* was detected in intermediary patients as well as in patients with normal microscopy findings, which was in accordance with study conducted by Cox et al. [27]. The results of our study have also shown that quantity of *Gardnerella vaginalis* significantly differed between all the groups, representing that *Gardnerella* may be a better marker for BV than *Atopobium*, as well as a better marker in differentiating the intermediary from the normal group of patients. This was not in accordance with the study performed by Bradshaw et al. [28], where *A. vaginae* was found to be more specific for BV. This discrepancy may be explained by the differences between epidemiological characteristics, geographical origin, ethnic affiliation, or PCR assay.

Similarly, *Atopobium vaginae* was also present in healthy patients, but in intermediary and BV patients it was found with frequency almost three times higher. In addition, the quantity of both bacteria was the highest in BV samples. A similar study found that prevalence of *Atopobium vaginae* differed between the normal and the BV group, but not between the normal and the intermediary group [10]. The same group of investigators in additional research performed both molecular detection and quantification of *Gardnerella* and *Atopobium*. They suggested that in addition to the detection of these microbes, quantification is very important in determining patients for treatment since the highest quantities of both bacteria were present in recurrent BV [17]. These data propose that BV is rather related to the disturbance of bacteria ratios as well as to a rise in quantity of the aforementioned anaerobic bacteria. Bretelle et al. [26] suggested that *Atopobium vaginae* is highly important in the reclassification of intermediary

patients. Namely, in their study, analysis of *Atopobium vaginae* helped them to reclassify 57% of intermediary cases as BV [23]. Furthermore, they proved that high concentrations of both *Gardnerella vaginalis* and *Atopobium vaginae* were associated with preterm labor, and they documented the coexistence of both bacteria (78–96% of BV samples), while this association was detected in 5–10% of normal findings [10, 17]. We also observed that coexistence of *Gardnerella* and *Atopobium* was more prevalent in intermediary and BV patients than in patients with normal results. The prevalence of coexistence was almost equal in both the intermediary group and the BV group, which coincides with the observations of Menard et al. [10], who even proposed that the intermediary patients should be considered closer to BV patients than to normal results.

CONCLUSION

In our investigation, we found that the prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* was the highest in patients with BV. In addition, we also observed that quantification of these bacteria may be more important than their detection only, especially in the interpretation of intermediary results.

Our study has certain limitations. Higher number of patients could provide more relevant results and stronger statistics in support of observed phenomena. Additionally, we did not analyze other microorganisms, inhabitants of vaginal microbiome, and their potential link to BV, which future investigations should include.

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Молекуларна дијагноза бактеријске вагинозе – заступљеност *Gardnerella vaginalis* и *Atopobium vaginae* код трудница

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САЖЕТАК

Увод/Циљ Бактеријска вагиноза (БВ) стање је удружено са поремећајем односа лактобацила и анаеробних бактерија у вагини у корист анаероба. Тешкоће у тумачењу микроскопски класификованих налаза у дијагностици БВ захтевају молекуларну анализу бактерија присутних у вагиналном секрету. Циљ овог рада је био да *real-time qPCR* анализом узорака вагиналног секрета испитамо у ком обиму су заступљеност и количина анаероба (*Gardnerella vaginalis* и *Atopobium vaginae*) у вези са микроскопским налазима.

Метод У студију је укључено 111 асимптоматских трудница старости трудноће 24–28 недеља. Грам-препарати вагиналних размаза су категорисани микроскопски, а после тога је са грам-препарата изолована ДНК и изведена реакција идентификације и квантификације (*real-time qPCR*) *G. vaginalis* и *A. vaginae*.

Резултати Резултати наше студије су показали да је 53,2% трудница имало нормалан резултат, док је 20,7% и 26,1% имало интермедијеран (ИМ) и БВ резултат. *G. vaginalis* и *A. vaginae* су били чешће присутни у ИМ и БВ групи него код здравих пацијенткиња, а и просечан број *G. vaginalis* и *A. vaginae* је био значајно виши у БВ и ИМ групама него у групи са нормалним налазом ($p = 0,000$). Поредећи међусобан однос *G. vaginalis* и *A. vaginae*, заступљеност и број *G. vaginalis* је у свим групама био значајно виши од заступљености и броја *A. vaginae*.

Закључак Резултати наше студије су показали да би за разликовање нормалних од БВ налаза квантификација бактерија могла бити значајнија од саме молекуларне детекције.

Кључне речи: бактеријска вагиноза; *real-time qPCR*; *Gardnerella*; *Atopobium*