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Molecular diagnosis of bacterial vaginosis: Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in pregnant women

Молекуларна дијагноза бактеријске вагинозе: Заступљеност *Gardnerella vaginalis* и *Atopobium vaginae* код трудница

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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 goal to explore in which extent prevalence and amount of anaerobs, *Gardnerella vaginalis* and *Atopobium vaginae*, are related to findings achieved by microscopy.

Methods This study enrolled 111 asymptomatic pregnant women between 24-28 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 aim to detect and quantify *G. vaginalis* and *A. vaginae*.

Results The data of our study have shown that 53,2% of patients had normal result, 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, as well as the average bacterial number of *G. vaginalis* and *A. vaginae* were significantly higher in BV and IMD than in 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 diversifying of 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*.

САЖЕТАК

Увод/Циљ Бактеријска вагиноза (БВ) је стање 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 испитамо у ком обиму су заступљеност и количина анаероба (*Gardnerella vaginalis* и *Atopobium vaginae*) у вези са микроскопским налазима и то *real-time qPCR* анализом узорака вагиналног секрета.

Методе У студију је укључено 111 асимптоматских трудница старости трудноће 24–28 недеља. Грам препарати вагиналних размаза су категорисани микроскопски. Након 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 респективно. *G. vaginalis* и *A. vaginae* су били чешће присутни у ИМ и БВ групи него код здравих пацијенткиња, а такође 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*

INTRODUCTION

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

BV 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 the group of patents in need for treatment. The majority of studies have agreed on the fact that is not possible to cultivate microarephilic or anaerobic residents of vagina with complete efficiency [3-6]. Introducing molecular detection (PCR) of 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].

Microorganisms the mostly detected in BV were *Gardnerella vaginalis* and *Atopobium vagine* with prevalence in BV ranging from 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 to this has been given by Hardy et al [11]. By analysing vaginal polymicrobial biofilm they found that this biofilm is mostly formed by microarephilic *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 was even 30% [12]. Many diagnostic methods have been compared: cultivation microorganisms mostly connected to BV, various microscopy criteria analysing Gram stained slides of vaginal swabs, molecular analysis as well as molecular detection and quantification of microbes within 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 in 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 significance of intermediary group of patients albeit its risk for preterm delivery [15, 16]. Due to this issue, Menard et al. quantified by qPCR *Gardnerella vaginalis* and *Atopobium vaginae* in vaginal samples of pregnant women [17]. 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 aim to explore relation of these microbes to do 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 in 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 not enrolled in the study as well. The institutional Ethical 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 dacron-tipped swab into vagina. The swab was rolled round through 360 degrees against the vaginal wall at the mid portion of the vault and carefully withdrawn to prevent contamination. Swabs were then smeared on a plain glass slide, air-dried at room temperature and Gram stained. Using conventional light microscopy (Leica DM 2000 LED) slides were categorized at 1000x magnification according to Nugent. DNA extraction was preformed from Gram stained preparations following protocol established by Srinivasan et al [16] and procedures contained within commercially available kit (QIAamp DNA mini kit, Qiagen, 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 instructions of manufacturer.

Statistical analysis

Complete statistical analysis was conducted with commercially available statistical software SPSS v17.0. Variables were presented as frequencies of individual parameters (categories), and statistical significance of differences was evaluated using chi-squared test. Differences among groups of nonparametric data were analyzed by Mann–Whitney and Kolmogorov Smirnov test. Receiver operating characteristic (ROC) curve was constructed and used to evaluate whether the number of bacterial DNA copies/ml could be a marker of the diagnostic accuracy. Statistical difference of $p<0.05$ was considered significant.

RESULTS

Using Nugent's criteria we found that 26.1% (29/111) of patients were diagnosed with BV. Into the intermediary group were classified 20.7% (23/111) of tested patients, while 53,2% (59/111) of patients were healthy. Prevalence and quantity of *Gardnerella vaginalis* and *Atopobium vaginae* in vaginal samples of pregnant women are presented in table 1.

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

	Prevalence (n = 111)			*DNA copies/ml		
	G. vaginalis	A. vaginae	χ^2	G. vaginalis	A. vaginae	KS
N	33/59	10/59	x^2=19,4; p=0.000	1.796	432	p=0.000
IMD	22/23	11/23	x^2=13,0; p=0.000	27.217	1.413	p=0.000
BV	27/29	14/29	x^2=14,1; p=0.000	35.258.502	5.456.101	p=0.004

mean number of DNA copies/ml, **N – Normal. **IMD** – intermediary, **BV** – bacterial vaginosis, **KS** – Kolmogorov-Smirnov test*

Besides 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 IMD and BV patients, the presence of this bacteria is not associated with the diagnosis of bacterial vaginosis (Pearson Chi-Square=0,668; *p*=0,716). *Atopobium vaginae* was also detected in patients with normal findings (16,9%), but percentage of this bacteria was higher in intermediary and BV groups, 47,8% and 48,3 %, respectively. However, as for the *Gardnerella vaginalis*, the presence of *Atopobium vaginae* is not associated with the diagnosis of bacterial vaginosis (Pearson Chi-Square=3,480; *p*=0,175). Finally, in our samples we showed coexistence of *Gardnerella vaginalis* and *Atopobium vaginae* (Pearson Chi-Square=14,199; *p*=0.0005). In intermediary and BV groups this coexistence was seen in 47,8% (11/23) and 48,3% (14/28) respectively, which was almost three times higher than in normal group (16,9%; 10/59). More importantly, *Atopobium vaginae*, except in one case, were present only in cases when *Gardnerella vaginalis* was present.

Using real-time qPCR we found that the number of *Gardnerella vaginalis* and diagnosis are in week positive correlation (r=0.272; *p*=0.004). The highest quantity of this bacterium was detected in samples with BV, while the lowest (20 thousand times lower than in BV) has been calculated in patients with normal findings. The numbers of this bacterium in intermediary cases were 15 times higher than in samples with normal findings. Statistical analysis confirmed significant difference in *Gardnerella vaginalis* quantity among all diagnosed groups of patients (*p*=0.001) except for IMD 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 week 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 normal and intermediary groups (3 times only). However, in this case, differences in the number of *Atopobium vaginae* between BV, IMD and normal findings were not statistically significant (*p*=0.072).

As we found that *Gardnerella vaginalis* was detected in all groups, at least two times more frequent than *Atopobium vaginae,* as well as 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 of the 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

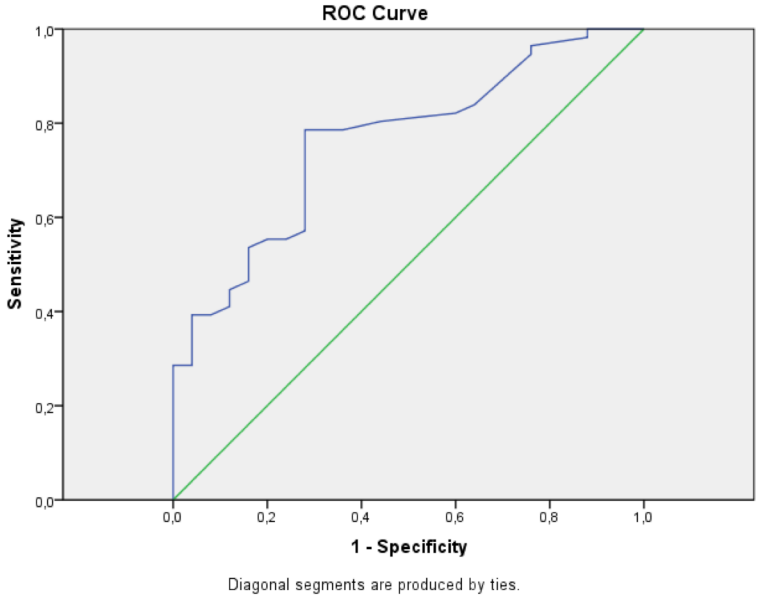

Figure 1. The number of DNA copies/ml was determined by real-time qPCR method on the SaCycler-96 (Sacace Biotechnologies, Como, Italy). Cut off, sensitivity and specificity were determined by ROC analysis and shown in the form of ROC curve.

number of DNA copies/ml of *Gardnerella vaginalis* has the ability to discriminate patients with normal findings from IMD and BV patients. The defined cut-off value was 2980 copies/ml, with a sensitivity and specificity of 78,6% and 72,0%, respectively (Figure 1).

DISCUSSION

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

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

In daily practice, despite the numerous methods available, clinicians still have difficulties to decide, which one of patients 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 condition is considered to be a transitory condition between normal and BV [22], not yielding all clinical criteria of bacterial vaginosis [20].

In this regard, during 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 [23-25]. 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 BV marker as well as even greater risk factor of 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 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 among all groups representing that *Gardnerella* may be better marker for BV than *Atopobium*, as well as better marker in differing intermediary from normal group of patients. This was not in accordance with study performed by Bradshaw et al. where was found that *A. vaginae* was more specific for BV [28]. This discrepancy may be explained by differences between epidemiological characteristics, geographical origin, ethnic affiliation or PCR assay.

Similarly, *Atopobium vaginae* was also present in healthy patients, but in almost three times higher frequency in intermediary and BV patients. In addition, the quantity of both bacteria was the highest in BV samples. In similar studies has been found that prevalence of *Atopobium vaginae* differed between normal and BV group but not between normal and intermediary group [10]. The same group of investigators in additional research performed both molecular detection and quantification of *Gardnerella* and *Atopobium*. They suggested that besides detection of these microbes, quantification is very important in differing patients for treatment since the highest quantities of both bacteria were present in recurrent BV [17]. This data propose that BV is rather related to disturbance of bacteria ratios as well as a rise in quantity of the aforementioned anaerobic bacteria. Bretelle at al [26] suggested importance of *Atopobium vaginae* as highly sensitive in reclassification of intermediary patients. Namely, in their study, analysis of *Atopobium vaginae* helped them to reclassify 57% of intermediary cases into BV [23]. Furthermore, they have proved that high concentrations of both, *Gardnerella vaginalis* and *Atopobium vaginae* were associated with preterm labor [10, 17] as well as they documented coexistence of both bacteria (78-96% of BV samples), while this association was detected in 5-10% of normal findings. We also observed that coexistence of *Gardnerella* and *Atopobium* was more prevalent in intermediary and BV patients than in patients with normal results. Prevalence of coexistence was almost equal in both intermediary group and BV, which coincides with observation of Menard et al [10] who even proposed intermediary group to be considered more close to BV than normal result.

CONCLUSION

In our investigation we found that prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* was the highest in patients with bacterial vaginosis. In addition, we also observed that quantification of these bacteria may be more important than their detection only, especially in interpretation of intermediary results. Nevertheless, we agreed on some limitations of our study. Higher number of patients could give more relevant results and stronger statistics in support of observed phenomena.

Additionally, we did not analyse other microorganisms, inhabitants of vaginal microbiome and their potential link to BV, which should include future investigations.

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