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PCR in the Bacterial Vaginosis Diagnostic Algorithm

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Abstract

Bacterial vaginosis (BV) is the most common vaginal infection worldwide. The objective of this study was to apply a PCR-based method for detection of BV-associated organisms in symptomatic and asymptomatic women and to assess the need of PCR in the diagnostic algorithm of BV. Two vaginal samples were taken from 98 women (74 symptomatic, 24 controls). Amsel criteria and Nugent scoring were used together with tests such as: vaginal pH, Gram staining, routine culture, culture on dextrose agar with Gentamycin and Chromagar candida, and PCR for identification of *Gardnerella vaginalis*, *Atopobium vaginae*, *Eggerthella-like bacterium*, *Leptotrichia*, BVAB1, BVAB2, and *Megasphera* type1. In the symptomatic group 17 swabs were with vulvovaginal candidiasis (VVC); 24 with BV, 8 with intermediate BV, 13 with co-infections, and 12 with other infections. In the control group 2 swabs were with VVC, 1 with BV and 1 with other infection. Using PCR in the group with complaints *G. vaginalis* was found in 59 samples (79.7%), *A. vaginae* in 21 (28.4%), *Eggerthella-like bacterium* in 15 (20.3%), *Leptotrichia* in 21 (28.4%), BVAB1 in 3 (4.1%), BVAB2 in 16 (21.6%), *Megasphera* type1 in 25 (33.8%). In the control group *G. vaginalis* was identified in 4 samples, *A. vaginae* in 2, PCR for other bacteria remained negative. *Eggerthella*-like bacterium, *Leptotrichia*, BVAB2, and *Megasphera* type1 could be used as strong markers of BV meanwhile *G. vaginalis* could not. PCR-based technique is sensitive and specific, but a combined approach is needed in the diagnosis of vaginal discharge conditions.

Key words: bacterial vaginosis, PCR, BV-associated bacteria

Резюме

Бактериалната вагиноза (BV) е най-често развиващата се вагинална инфекция. Целта на настоящето изследване е приложението на PCR методиката за откриване на BV-свързани организми в симптоматични и безсимптомни жени и оценка на необходимостта от използването ѝ в диагностичния алгоритъм на BV. Изследвани са по две вагинални проби, взети от 98 жени (74 симптоматични, 24 контроли). За оценка на секретите бяха използвани критериите на Amsel и скалата на Nugent, определяне на вагалищното pH, оцветяване на натривки по Грам, рутинно култивиране, както и култивиране върху декстрозен агар с Gentamycin и Chromagar candida. Бе проведена и PCR идентификация на *Gardnerella vaginalis*, *Atopobium vaginae*, *Eggerthella*-подобни бактерии, *Leptotrichia*, BVAB1, BVAB2 и *Megasphera* тип1. В симптоматичната група 17 материала бяха с вулвовагинална кандидоза (VVC); 24 с BV, 8 с интермедиерна форма на BV, 13 с ко-инфекции и 12 с други инфекции. В контролната група 2 секрета бяха са с VVC, 1 с BV и 1 с друга инфекция. Използвайки PCR в групата с оплаквания *G. vaginalis* се открива в 59 проби (79.7%), *A. vaginae* в 21 (28.4%), *Eggerthella*-подобни бактерии в 15 (20.3%), *Leptotrichia* в 21 (28.4%), BVAB1 В 3 (4.1%), BVAB2 в 16 (21.6%), *Megasphera* тип 1 в 25 (33.8%). В контролната група *G. vaginalis* бе идентифицирана в 4 проби, *A. vaginae* в 2, PCR за други бактерии остана отрицателна. За разлика от *G. vaginalis Eggerthella* подобните бактерии, *Leptotrichia*, BVAB2 и *Megasphera* тип1могат да се използват като сигурни маркери за наличието на BV. Технологията, базирана на PCR, е чувствителна и специфична, въпреки това е необходим комбиниран подход при диагностиката на всички случаи.

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Introduction

Bacterial vaginosis (BV) is an aberrant state of the vaginal microbiota which is characterized by loss of the normal protective lactobacilli and overgrowth of diverse anaerobe bacteria (Dols *et al*., 2016). BV is the most prevalent lower genital tract infection in women of reproductive age worldwide (Schwebke, 2009). Nevertheless, decades of microbiological analyses have not established the exact etiology of the condition (Bohbot and Lepargneur, 2012). BV is often diagnosed clinically based on the criteria described by Amsel *et al.* (1983). Another method that is widely used for BV diagnosis is based on grading or scoring the microbiota in Gram-stained smears of vaginal fluid - Nugent scoring (Nugent *et al.*, 1991). Conventional microbiological approaches such as culture-based analyses of the vaginal microbiota could not serve as a reliable assay in evaluating patients for BV. Many of the key organisms that are associated with the condition are obligate anaerobes that are either difficult to recover or unrecoverable. Several fastidious bacteria have recently been associated with BV using PCR methods (Malaguti *et al*., 2015) and appear to be highly specific markers of BV (Fredricks *et al*., 2007).

We report a PCR diagnostic assay for detection of BV-associated bacteria (*Gardnerella vaginalis*, *Atopobium vaginae*, *Eggerthella-like bacterium*, *Leptotrichia*, BVAB1, BVAB2, and *Megasphera* type1) performed together with the most commonly used laboratory tests for BV in order to assess the need of PCR in the diagnostic algorithm of BV.

Materials and Methods

Between May 2016 and February 2017 a total of 98 women in reproductive age were included in the study after completion of a written informed consent form. Twenty four of them were controls (all had undergone their regular gynecological examination) and the remaining 74 persons were with complaints of pruritus, burning, dyspareunia or signs of inflammation of the genitals and with symptoms of abnormal vaginal discharge. Exclusion criteria for participation in the study were ingestion of hormonal contraceptives, cervical cancer and antibiotic therapy in the previous four weeks. Two vaginal samples from the vaginal side wall and/or the pool were obtained by using sterile cotton swabs after the insertion of a speculum. The first sample was used for routine microbiological study, the second for molecular diagnostic procedures. The samples were collected in Amies media and examined afterwards.

Culture

The samples were cultivated in aerobic conditions on nonselective sheep blood agar and MacConkey agar (for resident microflora) and Sabouraud's agar (SDA) with Gentamycin and Chromagar candida medium (Becton Dickenson) for 72 hours at 35°C. For detection of *G. vaginalis* we used Columbia blood agar base with Selective Supplement SR0119E, Oxoid (with gentamicin and nallidixic acid) in a microaerophilic atmosphere (5–10% CO_2) at 36°C for 48–72 h. The Gram-negative or Gram-variable short rods, transparent colonies, β-hemolytic on human blood agar, catalase-negative, Glucose, Prolin, ONPG positive, were presumptively identified as *G. vaginalis* using Remel RapID NH.

Diagnosis of BV and vulvovaginal candidiasis

To determine if women met the criteria for BV or had vulvovaginal candidiasis (VVC), microscopic scoring was performed (potassium hydroxide (KOH) preparation and Gram-stain). The smears were assessed for the quantity of lactobacilli, epithelial cell morphology, absence/presence of clue sells and *Candida spp.* A vaginal smear was examined using the Nugent scale, which includes the scores 0–3 as Normal; 4–6, Intermediate; and 7–10 for BV. The diagnosis of BV was based on the Nugent Gram stain and the presence of three Amsel criteria, characteristic vaginal discharge, clue cells, and positive amine test. Measurement of the pH was performed using pH indicator paper (Merck, Germany).

DNA isolation

Total DNA from vaginal samples was isolated using the DNAsorb-AM nucleic acid extraction kit (AmpliSens) according to the manufacturer's guidelines.

Polymerase Chain Reaction (PCR)

Purified DNA in an amount of 2.5µl was used for PCR reactions. The total reaction volume was 25µl. In the reaction specific primer pairs were used, providing amplification with respective product sizes: 207bp for *G. vaginalis*, 597bp for *A. vaginae*, 238bp for *Eggerthella*-like bacterium, 251bp for *Leptotrichia*, 261bp for BVAB1, 406bp for BVAB2 and 211bp for *Megasphera* type1.

The amplification protocol methodology for proving *A. vaginae*, *Eggerthella*-like bacterium, *Leptotrichia*, BVAB1, BVAB2, and *Megasphera* type1, was identical and was as follows: initial denaturation (95ºC, 10min), followed by 40 cycles of denaturation (95ºC, 30sec), annealing (55ºC,

30 sec) and extension (72ºC , 30 sec), with a final elongation of the chain at 72ºC for 7 min. The protocol for *G. vaginalis* was: initial denaturation (95ºC, 10min), followed by 40 cycles of denaturation (95ºC, 30 sec), annealing (62ºC, 30 sec) and extension (72ºC, 30 sec), with a final extension of the chain at 72ºC for 7 min.

Results

Out of the 98 women tested 74 were with genital complaints and vaginal discharge, and 24 were controls. Based on the results from the routine cultivation, the Amsel criteria and Nugent scoring (the both showing very similar results) VVC was detected in 19 samples - 17 from the group with complaints and 2 from the control. BV was found in 25 swabs - 24 in the group with complaints and 1 in the control. Eight samples were with an intermediate form of bacterial vaginosis (IBV) all in the group with complaints. Co-infections were diagnosed in 13 swabs - VVC plus anaerobes; VVC plus Streptococci; other combinations. Samples with plenty of leucocytes and without the possibility of identifying other microorganisms with routine laboratory tests were 13 - 12 in the group with complaint and 1 in the control. In these last 13 samples we suspected the presence of *Mycoplasma*, *Ureaplasma* or *Chlamydia* infection and extra tests were needed for their identification.

The distribution of *G. vaginalis*, *A. vaginae*, *Eggerthella-like bacterium*, *Leptotrichia*, BVAB1, BVAB2, and *Megasphera* type1 detected by PCR in the samples is shown in Table1.

PCR performed in the group of women with complaints found *G. vaginalis* in 59 samples (79.7%), *A. vaginae* in 21 (28.4%), *Eggerthella-like bacterium* in 15 (20.3%), *Leptotrichia* in 21 (28.4%), BVAB1 in 3 (4.1%), BVAB 2 in 16 (21.6%), *Megasphera* type1 in 25 (33.8%). In the control group *G. vaginalis* was identified in 4 samples, *A. vaginae* in 2, the rest of the tested bacteria were not detected. Using a selective agar medium the isolation rate of *G. vaginalis* was significantly lower in comparison with the PCR technique – only in half of the cases.

Discussion

BV is the most prevalent lower genital tract infection in women of reproductive age worldwide (Schwebke, 2009). Nevertheless, its etiology is not completely understood. No single etiological agent is the known cause of BV, and the syndrome is considered an ecological disorder of the vaginal microbiota. These difficulties result in part from the approximately 40% asymptomatic cases (Karla *et al*., 2007), and in part from the complex microbiota of the vagina, composed of hundreds of bacterial species, with titers ranging from billions to fewer than 100 cells, many of which are fastidious, unculturable, or difficult to identify (Hillier, 2005; Srinivasan and Fredricks, 2008).

Typically, BV is characterized by a reduction of lactic acid-producing bacteria (mainly *Lactobacillus* spp.) and an increase in the number and diversity of facultative and strictly anaerobic bacteria (Malaguti *et al*., 2015) with acquisition of diverse communities of anaerobic and facultative bacteria and depletion of the usually dominant

Table 1. The distribution of *G. vaginalis*, *A. vaginae*, *Eggerthella-like bacterium*, *Leptotrichia*, BVAB1, BVAB2, and *Megasphera* type1 detected by PCR in the group of patients with complaints and in the control group.

№ of persons	With complaints						Controls					
	74						24					
Vaginal condition	VVC	BV	IBV	Coinfect.	Other	Total	VVC	BV	IBV	Coinfect.	Other	Total
№ of + patients	17	24	8	13	12	74	2	1	0	0	1	4
G. vaginalis	15	16	8	12	8	59	2	1	0	0	1	4
A. vaginae	0	12	4	4	1	21	0	1	0	0	1	2
Eggerthella-like	0	11	0	4	0	15	0	0	0	0	0	0
Leptotrichia	0	15	1	5	0	21	0	0	0	0	0	0
BVAB1	1	1	0	1	0	3	0	0	0	0	0	0
BVAB2	0	10	2	4	0	16	0	0	0	0	0	0
Megasphera	0	19	2	4	0	25	0	0	0	0	0	0

lactobacillus flora. This leads to the appearance of malodorous vaginal discharge. The discharge is not the main consequence. BV is a risk factor for pelvic inflammatory disease (PID) (Peipert *et al*., 2001; Haggerty *et al*., 2004) and subsequent infertility (Klebanoff *et al*., 2005), increased risk of preterm labor and delivery (Klebanoff *et al*., 2005; Ling *et al*., 2010), amniotic fluid infections (Silver *et al*., 1989), chorioamnionitis (Hillier *et al*., 1988), low birth weight (Leitich *et al*., 2003), endometritis (Sweet, 2000), cervicitis (Ugwumadu, 2002), and an increased risk of acquiring sexually transmitted infections (STIs) and HIV (Cohen *et al*., 2012; Hay, 2014). Therefore, the diagnosis of BV is essential.

The diagnosis of BV is usually made clinically based on the criteria described by Amsel *et al.* Three of the following four signs must be evident: vaginal fluid pH greater than 4.5; presence of clue cells (>20%); homogeneous vaginal discharge on examination; and detection of a fishy odour upon addition of 10% potassium hydroxide to the vaginal fluid. The use of clinical criteria to diagnose BV has the advantage of rapid diagnosis at the point of care but requires the performance of vaginal pH and microscopy of vaginal fluids by a skilled person. Moreover, some patients without BV may manifest clinical findings similar to those of BV, such as those with *Trichomonas vaginalis* vaginitis or those with cytolytic vaginosis.

Another method that is widely used for BV diagnosis is based on grading or scoring the microbiota in Gram-stained smears of vaginal fluid (Nugent scoring). BV diagnosis in research and laboratory settings depends on traditional methods, such as culture and Gram-staining vaginal smears. Conventional microbiological approaches have only limited utility in the clinical evaluation of patients suspicious for BV. First, Gram staining provides very limited information regarding the identities and the relative variety of microorganisms found in the sample. Second, culture-based identification of single "marker" organisms lacks sensitivity and specificity (Cartwright *et al*., 2012). Additionally, many of the key organisms that are associated with BV are obligate anaerobes that are either difficult to recover or unrecoverable using conventional culture methods, which makes a true evaluation of vaginal microbiota using culture impossible (Ravel *et al*., 2011).

In our study for the evaluation of BV together with Amsel criteria and Nugent score we used a broad-range 16S rRNA gene PCR - a cultivation-independent method. The reason for performing PCR was that patients with BV have complex communities of vaginal bacteria and because culture-based analyses of the vaginal microbiota identify far fewer organisms than broad range molecular methods (Hill, 1993; Oakley *et al*., 2008; Ravel *et al*., 2011).

Our assay was applied to vaginal samples obtained from women with and without BV in order to assess the prevalence of each bacterial species and to evaluate the potential of PCR for the microbiological diagnosis of BV. To our knowledge, this is the first study to simultaneously screen for BV-associated microorganisms as *G. vaginalis*, *A. vaginae*, *Eggerthella-like bacterium*, *Leptotrichia*, BVAB1, BVAB2, and *Megasphera* type1 in symptomatic and asymptomatic Bulgarian women using a PCR-based technique. Several studies have used broad-range 16S rRNA gene to characterize the community of vaginal bacteria (Verhelst *et al*., 2004; Fredricks *et al*., 2005; Malaguti *et al*., 2015). These molecular assays have detected a large number of novel fastidious bacterial species. In the present study we have performed PCR only to some of the BV-associated bacteria that were previously shown to be good indicators of BV (Fredricks *et al*., 2007; Kalra *et al*., 2007).

The analysis of the results from all the samples gave us the information that the most commonly detected bacteria as a single or simultaneous agent was *G. vaginalis*. Our study showed that *G. vaginalis* is a very common microorganism in samples from patients with complaints. Historically, *G. vaginalis* was thought to play the leading role in BV. Recent published findings have suggested that *G. vaginalis* biofilms may be critical in BV pathogenesis and symptomatology (Menard *et al*., 2008). It was interesting to find out that almost all cases with VVC were also positive for the presence of *G. vaginalis.* The microorganism could not be used as a reliable indicator of BV, as demonstrated by the high rate of detection in subjects without BV in this study. However, *G. vaginalis* surely is closely related with the condition and with others in which disturbances in the vagina microbiota appear.

Our PCR was helpful in determining the frequencies of novel, fastidious, or uncultivated bacterial species. Very common identified microorganisms in patients with symptoms were *A. vaginae*, *Eggerthella-like bacterium*, *Leptotrichia*, BVAB 2, and *Megasphera* type1. It is worth noting that these bacteria were detected only in the groups of samples from patients with BV, IBV and co-infections. BV-associated bacteria were indeed

uncommon in subjects without BV. The prevalence of these bacteria in BV patients was as follows: *A. vaginae* 50%, *Eggerthella-like bacterium* 45.8%, *Leptotrichia* 62.5%, BVAB 2 41.7%, and *Megasphera* type1 79.2%. The prevalence of the same bacteria in patients with IBV was as follows: *A. vaginae* 50%%, *Eggerthella-like bacterium* 0%, *Leptotrichia* 12.5%, BVAB2 25%, and *Megasphera* type1 25%; meanwhile in patients with co-infections it was: *A. vaginae* 30.8%%, *Eggerthella-like bacterium* 30.8%, *Leptotrichia* 38.5.5%, BVAB2 30.8%, and *Megasphera* type1 30.8%.

The PCR test data received helped us to establish more precisely the composition of the vaginal microbiota in different subject groups. Furthermore, different bacterial agents could be detected independently of the clinical status of the patient – symptomatic or asymptomatic. And by detecting these new BV-associated bacteria we could apply a more convenient treatment for our patients. The reason for this is the fact that some of the BV-associated bacteria are resistant to the most commonly used medication – metronidazole, and this resistance could not be detected either with the conventional methods of cultivation, or with the Gram staining procedure.

Our PCR study has some limitations. Patients with BV have complex communities of vaginal bacteria and in order to identify all of them a lot of reactions have to be performed and we have used different assays for each microorganism. This disadvantage could be avoided with the application of multiplex PCR. Another limitation of the study was that the used PCR was qualitative and did not provide information about the quantities of the bacteria involved in the BV process. Surely, in some cases the quantity may be the more important marker of disease than the qualitative presence or absence of bacteria. As it is very well known, the infectious process is the interaction of the pathogenic microorganism with the macroorganism under certain environmental conditions. Further investigations are needed in order to determine precisely the real role of the bacteria called BV-associated, their combinations that could be more or less aggressive and thus use this information for choosing the best treatment that will diminish the complications of BV and of other vaginal diseases.

Conclusions

This is the first Bulgarian study for detection of six different BV-associated bacteria using PCR technique. Our results show that *G. vaginalis*, *A. vaginae*, *Eggerthella*-like bacterium, *Leptotrichia*, BVAB 2, and *Megasphera* type1 are frequent in Bulgarian symptomatic women. *G. vaginalis* could not be used as marker of BV but surely is closely related with the condition and with others when disturbances in the vagina microbiota appear. *Eggerthella*-like bacterium, *Leptotrichia*, BVAB 2, and *Megasphera* type1 are found only in samples from patients with BV or its intermediate form. So, they could be used as strong markers of BV. PCR-based techniques are rapid with high sensitivity and specificity, possibility for identification of fastidious microorganisms. Nevertheless, a combined approach including microscopy, culture and molecular techniques is needed in the diagnosis of the different vaginal discharge conditions.

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