ACTA MICROBIOLOGICA BULGARICA

Microscopic and Molecular Genetic Methods in the Detection of Bacterial Vaginosis

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Abstract

Every obstetrician or microbiologist encounters bacterial vaginosis (BV) in his/her practice. 50% of the cases are asymptomatic. The percentage of affected women varies. The aim of this study is interpretation of microscopic and molecular diagnostic techniques for diagnosing fastidious microorganisms associated with BV. 234 women were tested. Vaginal secretions were collected, after medical history and detailed gynecological status were completed. Smears were prepared and stained by Gram. Identification of fastidious *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* sp. type 1, and BV associated bacterium type 2 (*BVAB2*) was performed by PCR. 112 of the 234 tested women had microscopic evidence of some vaginal problem. 65 of them, had anaerobic infection found on the basis of the microscopic data. Materials from women suspected for anaerobic infection were subjected to PCR for proving of specific anaerobes. In 63 patients *G. vaginalis* was found, in 27- *A. vaginae*, in 18 - *Megasphaera* sp. type 1, and in 15 - *BVAB2*. The detected anaerobe in 25 swabs was single. Combination of several pathogens was demonstrated in 40 women. Gram stain is a fast, easily performed, and specific method for diagnosing BV. Methodology limitation is the inability to distinguish visually the different microorganisms involved in the condition. The PCR method provides possibilities for the sensitive, highly specific, and rapid identification of bacteria-specific markers for BV. Accurate determination of microorganisms involved in this syndrome provides possibilities for an adequate and complete treatment of the women affected.

Key words: Bacterial vaginosis, Gram stain, polymerase chain reaction, *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* sp. type 1 and *BVAB2*;

Резюме

Всеки гинеколог и микробиолог срещат в практиката си бактериална вагиноза. В 50% от случаите състоянието е безсимптомно. Процентът на засегнатите жени варира. Цел на проучването е интерпретация на микроскопските и молекулярно диагностичните методи за доказване на трудно култивируеми причинители на БВ. Изследвани са вагиналните секрети на 234 жени, след снемане на анамнеза и статус. Изготвени бяха натривки, оцветени по Грам. Бе проведена идентификация на *Gardnerella vaginalis, Atopobium vaginae, Megasphaera* type 1 и *BVAB2*чрез PCR. От 234 изследвани жени при 112 се установиха микроскопски данни за вагинален проблем. От тях при 65 въз основа на натривката бяха получени данни за анаеробна инфекция и съответно проведена PCR за доказване на конкретни анаеробни причинители. Получени резултати: при 63 пациентките бе доказано наличието на *G. vaginalis*, при 27 на *Atopobium* spp., при 18 на *Megasphaera* type 1 и при 15 на *BVAB2*. При 25 причинителят бе един. Коинфекция бе доказана при 40 от пациентнките. Оцветяването по Грам е бърз, лесен и специфичен метод за диагностика на БВ. Ограничение е невъзможността за визуално разграничаване на микроорганизмите, въвлечени в етиопатогенезата на състоянието. PCR позволява чувствително, високо специфично и бързо определяне на бактериите-маркери, характерни за БВ. Точното определяне на участващите в БВ микроорганизми дава възможност за адекватна терапия на засегнатите жени.

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Introduction

Bacterial vaginosis (BV) is an infectious, non-inflammatory, polymicrobial syndrome with yet undetermined etiology and pathogenesis that affects the lower portions of the genital tract in women (Sobel, 1997). There is currently no test that is reliable to the extent of providing a correct diagnosis. That is why, a lot of scientific teams continue to search for methods and combinations thereof, which can be relied upon to be specific and sensitive, and to minimize possible false diagnoses, which is also the aim of the present study.

Materials and Methods

A total of 234 vaginal secretions of women were examined in the age range from 18 to 64 years. At first, each woman signed an informed consent. After thorough medical history was taken and detailed gynecological status was completed, most common subjective complaints found were vaginal discharge and discomfort in genital area. Two cotton tips were used for obtaining the smear from the lateral side of the vagina, placing the secretion into Aimes transport medium for culturing.

The first swab was used for preparing Gram stain. The second one was used for DNA extraction and molecular diagnostic examination of *G. vaginalis, A. vaginae, Megasphaera* sp. type 1 and *BVAB2*. The extraction was performed with commercial kit DNA-Sorb-A (Sacace Biotechnologies Srl, Italy) in compliance with the manufacturer's instructions.

Purified DNA of the quantity of 2.5 µl was used for PCR reactions. The total reaction volume was 25 µl. Specific primer pairs providing amplification were used in the reaction with respective product size: 207 base pairs for *G. vaginalis*, 597 base pairs for A. *vaginae*, 211 base pairs for *Megasphaera* sp. type 1, and 406 base pairs for *BVAB2*. The amplification protocol methodology for proving *BVAB2*, *A. vaginae*, and *Megasphaera* sp., was identical; it was as follows: initial denaturation (95ºC, 10 min), followed by 40 cycles of denaturation (95ºC, 30 sec), annealing (55ºC, 30 sec), and extension (72ºC, 30 sec), with a final elongation of the chain under conditions of 72ºC for 7 min. The protocol for *G. vaginalis* was as follows: initial denaturation (95ºC, 10 min), followed by 40 cycles of denaturation (95ºC, 30sec), annealing (62ºC, 30 sec), and extension (72ºC, 30 sec), with a final extension of the chain under conditions of 72ºC for 7 min.

Results

No microscopic evidence of disease was found in 122 (52%) of all 234 women studied. The remaining 112 (48%) women had microscopic evidence of some vaginal problem. 65 (28%) of them were suspect, based on the smear anaerobic infection. These materials were subjected to DNA extraction, and subsequent polymerase chain reaction was performed for detection of any anaerobic bacteria that were possible causative agents of BV. The results obtained were as follows: 63 of the patients demonstrated presence of *G. vaginalis* (96.9%), 27 (41.5%) - of *A. vaginae*, 18 (27.7%) - of *Megasphaera* sp. type 1, and 15 (23.1%) - of *BVAB2*. Only one causative agent was detected in 25 (38%) smears. Coinfections were demonstrated in 40 (62%) tested swabs.

Discussion

BV disease was first described in 1955 by Gardner and Dukes as rarely occurring watery, homogeneous discharge with unpleasant odour (Spiegel, 1991). Westrdum and co-authors introduce the concept BV in 1984. The International Statistical Classification of Diseases and Related Health Problems (10th Revision Version) for 2006 does not emit BV as an independent disease. At this stage, it is statistically identified under the heading of non-inflammatory diseases of the vagina. According to the World Health Organization recommendations from 2005, BV relates to endogenous infections affecting the reproductive tract in humans.

The different reasons that may lead to this condition still remain unexplained. Various studies demonstrated the conditions that could provoke BV: promiscuity or having a new partner; use of intrauterine contraception; frequent vagina washes, etc. It was found that women cannot become infected with BV when using the toilet, swimming pools, or mutual bed linen. Studies have demonstrated that women without active sexual life rarely develop BV.

In healthy women, vaginal ecosystem is balanced due to different bacteria (Zhou *et al.*, 2004; Sobel, 1999): *Staphylococcus, Streptococcus, Enterococcus, Corynebacterium, Escherichia, Ureaplasma, Mycoplasma, Peptostreptococcus, Gardnerella, Bacteroides, Veillonella, Bifidobacterium*, and *Candida* (Marrazzo *et al.*, 2002). The balance is maintained mainly by representatives of genus *Lactobacillus*, which convert glycogen in the epithelial cells of the vagina into lactic acid, through the production of hydrogen peroxide, and

provide acidic pH in the vagina. The acidic environment of the vagina inhibits the development of pathogenic and commensal microflora. In addition, the normal flora of the vagina synthesizes antimicrobial peptides that ensure the balance and provide prevention from invasion of atypical microorganisms (Hiller, 1998). Despite these and other defensive mechanisms, characteristic of the vaginal mucosa, each woman, at least once in her life, obtains such an infection. Even repeated (recurrent) infections could be registered in some 5-10% of the female population.

In more than 50% of the cases of BV no characteristic signs are detected. The condition is determined as asymptomatic bacterial vaginosis and requires the use of microscopic and molecular biological methods for the diagnosis specification. Many countries have developed screening BV programs especially for young women in order to prevent possible health complications such as pelvic inflammatory disease (PID), premature birth, coinfections with other sexually transmitted pathogens, etc.

The existence of imbalance in the vaginal ecosystem is typical for BV. Another feature is the abruptly reduced number of representatives of genus *Lactobacillus* (especially *L. acidophilus*). As a result, change in the pH occurs and proliferation of anaerobic bacteria such as *Ureaplasma, Mycoplasma, Gardnerella, Mobiluncus* spp., *Atopobium* sp., *Megasphaera* sp. type1, *BVAB1-3, Leptotrichia*, etc., is stimulated. The amount of bacteria in volume of 1 ml from 10^6 (in healthy woman) typically reaches amounts of 10^{13}. These organisms produce a number of organic products that lead to the exfoliation to the vaginal mucosa. It is interesting that more than 50% of women with such changes in the vagina have no presence of any clinical manifestation.

The microbiological diagnosis of BV is a challenge. That is due to the following reasons: the large amount and variety of bacterial etiologic agents; the detection of new species recently accepted as involved in the pathogenetic process; the lack of clear bacterial markers for the condition, etc. For example, one of the bacterial pathogens for years associated with the etiology of BV - *G. vaginalis*, was isolated in 83% - 94% of women with clinical symptoms, but is also evidenced in 36% - 55% among the healthy female population or, more precisely, among women without clinical signs (Hiller, 1993). Another example is *A. vaginae*, also isolated from materials of patients with complaints, but also from women with regular gynecological examination without any symptoms.

Therefore, the correct detection of that medical condition requires a complex approach in the diagnostic algorithm of BV. There are two main approaches - clinical criteria and application of laboratory tests.

The clinical approach is based on the so-called Amsel (Pheifer *et al*., 1978, Amsel *et al*.,1983) criteria, that include the following: vaginal pH > 4.5; thin, gray, and homogenous discharge; positive whiff test - in addition to the mucus of 10% KOH, smell of "amines or fish" occurs; demonstration of clue cells on a saline smear. The presence of 3 of the 4 criteria is indicative of BV (Amsel *et al*., 1983).

The laboratory tests include preparation of native smears or coloring with Gram, methylene blue, or PAP staining, plus one of the next methods- culturing with subsequent identification of the species; molecular genetic methods; liquid-gas chromatography.

Methods, that are still in investigation stage and, however, are already embarked on the practice in some countries, are:

OSOM® BVBlue® chromogen system- a diagnostic test, which enables determining in vaginal secretions of the presence and level of the enzyme sialidase. It was found that this enzyme is produced by a number of bacteria such as *Gardnerella*, *Bacteroides*, *Prevotella*, and *Mobiluncus*, all associated in the pathogenesis of BV. The sensitivity of the metnod is 88% - 94%; the specificity amounts to 91% - 98% (Myziuk *et al*., 2003; Sumeksri *et al*., 2005; Bradshaw *et al*., 2005).

FemExam G. vaginalis PIP Activity is another type of test. It detects proline aminopeptidase activity of the anaerobic bacteria, especially in *G. vaginalis*. The sensitivity of the test is 89% - 92%, and the specificity is 94% - 96% (Nelson *et al.*, 1994; Calderón *et al*., 1997).

FemExam pH - allows the determination of pH and the presence of trimethylamine (Gutman *et al*., 2005; West *et al*., 2003).

Different quantitative PCRs for *G. vaginalis*, *A. vaginae*, and other bacterial participants in the pathogenesis of BV are at some stage of development (Menard *et al*., 2008; Cartwright *et al*., 2012). At least 17 species undergo research.

Fluorescence in situ hybridization (FISH) is applied for the analysis of urine, wherein BV is determined as biofilm desquamation of the epithelium of the vaginal mucosa in sedimintation (Swidsinski *et al*., 2010).

In the present study, BV was determined using the combination swab stained by Gram's method, and PCR for detection of *G. vaginalis, A. vaginae, Megasphaera* sp. type 1 and *BVAB2.*

Culture method with subsequent identification was not used due to the fact that the method process is laborious and time-consuming. On the other hand, the presence of fastidious bacteria such as *Atopobium* spp., *Megasphaera* sp. type 1, *BVAB 1-3, Leptotrichia, Ureaplasma* and *Mycoplasma* cannot be subjected to cultivation and, thus, it limits the application of the method. Their detection is crucial for adequate therapy. It is due to the fact that most of the *Atopobium* spp. strains are resistant to treatment with metronidazole, frequently used in the therapy of BV. When resistance is present recurrent forms of BV occur.

In this study the prepared and stained by Gram's method smears were assessed, based on a standard scoring system (Tab. 1). The diagnostic criteria of this scoring system were introduced by Spigel et al and later modified by Nugent *et al.* (Spiegel, 1991). The slides were observed with immersion (1000 × magnification) and were evaluated over 20 visual fields for the following types of bacteria:

- long Gram-positive rods (Lactobacillus spp.)
- small Gram-variable rods (Gardnerella vaginalis)
- small Gram-negative rods (Bacteroides spp.)
- curved Gram-variable rods (Mobiluncus spp.)

Gram-positive cocci were not a part of the scoring system.

When Nugent Score system is applied it is important to pay attention on the following: the number of polymorphonuclear leukocytes in BV has to be low (Holmes *et al*., 1983); and the „clue cells" are very specific BV indicator, if they are more than 20% of the epithelial cells on the smear (Eshenbach *et al*., 1988).

PCR is one of the main methods by which accurate and quick identification of bacterial markers for BV could be performed. It is important to determine correctly and detect all the microorganisms involved in the etiopathogenesis of BV. This will allow appropriate treatment of the condition. The amplification technologies are characterized by high specificity and sensitivity (Ferris *et al*., 2004). The presence of different microorganisms does not suppress the test performance. Even more coinfections could be detected, based on the reagents included in the reaction system. Advantage of the latter method is the possibility of using different types of biological samples comprising the already dead microorganisms in them. This eliminates the problem of the great instability of some of the microorganisms associated with BV. Many PCR techniques have been developed, some of which were used in this study.

No microscopic evidence of the disease was found in 122 (52%) of all of the 234 examined women. Microscopic evidence of vaginal problem was detected in the remaining 112 (48%) women. In 65 (28%) of the latter 112 women, anaerobic infection was suspected, based on Gram stained smear. These materials were subjected to DNA extraction and subsequent polymerase chain reaction for detection of anaerobic bacteria. The obtained results were as follows: *G. vaginalis* was found in 63 smears (96.9%), *A. vaginae* in 27 (41.5%), *Megasphaera* sp. type 1 in 18 (27.7%), and *BVAB2* in 15 (23.1%). 25 (38%) materials had only one causative agent. Coinfections by different pathogens was demonstrated in 40 (62%) vaginal swabs.

Conclusions

Gram staining is a quick, cheap, and sensitive method for the detection of BV. It allows obtaining of information for "clue cells", lactobacilli, and other microflora, presence or absence of polymorphonuclear leukocytes. Disadvantage of the methodology is its inability of visual differentiation between the microorganisms involved in the etiopathogenesis of the condition. The method is subjective, and requires experience and competence of the observer. The PCR provides possibilities for the sensitive, highly specific, and rapid identification of bacteria-markers of BV. Accurate detection of the involved in BV microorganisms provides possibilities for an adequate treatment of the affected women.

Acknowledgements

This work was supported by a Grant entitled "The Use of Molecular Diagnostic Techniques for Determining Anaerobic Pathogens Involved in the Etiopathogenesis of Bacterial Vaginosis" from 2015 at the Medical University of Sofia, Bulgaria.

Table 1. Nugent Score (a Gram stained scoring system) for evaluation of bacterial vaginosis

Score	Morphotype		
	Lactobacilli Gram-positive rods	*Gardnerella* Gram-variable rods	*Mobiluncus* Gram-variable rods
0	> 30	0	0
1	5–30	< 1	1–5
2	1–4	1–4	> 5
3	< 1	5–30	–
4	0	> 30	–

Interpretation of the Nugent Score system: 0 - 3 - normal; 4-6 - intermediate form; 7-10 - bacterial vaginosis;
„clue cells" - with more than 20% of the epithelial cells.

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