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Original article

MULTIPLEX REAL-TIME PCR METHOD AS A RELIABLE TEST IN THE ROUTINE MICROBIOLOGY STUDY OF VAGINAL MICROBIOME IN WOMEN WITH GENITAL TRACT DISCHARGE

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ABSTRACT

Untreated vaginal discharge is a risk factor for complications. Correct diagnosis is crucial. The purpose of this study was to apply Femoflor-16 to study the vaginal microbiome in women with genital tract discharge.

Material/Methods: A total of 45 women were included in the study. Vaginal/cervical swab - one for routine tests and Gram staining and another for PCR Femoflor-16, were tested. Clinical diagnosis of bacterial vaginosis (BV) was done by using Amsel's criteria. Microbiologically, BV was assessed by applying a Nugent score system and Femoflor-16.

Results: A total of 45 women with genital tract discharge were included in the study. Based on Amsel's clinical criteria, 9 (20%) of them were diagnosed with BV. Based on Nugent's score system, 11(24,4%) were categorized as having BV. Only 40 samples were compared, and the results were tested using Femoflor-16 and Nugent score system. Of them, 33 (83%) were in agreement as a result of using both methods. Femoflor-16 detected species such as aerobic bacteria (*Enterobacteriàñeae, Streptococcus spp., Staphylococcus spp.*), *Mycoplasma spp.*, *Ureaplasma spp.* and yeast-like microorganisms (*Candida spp.*).

Conclusions: A reliable, comprehensive, and on-time diagnosis of BV is needed. Nucleic acid amplification tests nowadays complement the "classic" laboratory methods. The real time multiplex PCR test Femoflor-16 can be effectively used in vaginal microbiota evaluation in women with discharge. It can identify a wide range of microorganisms, including bacteria that are difficult to culture, normal *Lactobacilli* microflora and complex of aerobic and anaerobic microorganisms, mycoplasmas and *Candida spp.* In addition, it is able to determine the number and ratio of microorganisms in the total bacterial mass, which further orients to the disease state and the role of the microorganism as an opportunistic or obligate pathogen.

Keywords: Femoflor-16, multiplex PCR, vaginal discharge, bacterial vaginosis,

INTRODUCTION

The healthy vaginal tract in reproductive-aged women is colonized by commensal microflora, which maintains a balanced microbial ecosystem. Women of different races have a unique vaginal microflora with regional variations. This microbiota plays a fundamental role in human physiological mechanisms, such as immunity and nutrition. It lives in a mutualistic relationship with the host vagina, protecting it from potentially pathogenic microorganisms like those causing bacterial vaginosis, urinary tract infections, candida infections, and sexually transmitted diseases (STDs). A shift in the vagina microbiome structure, either through the incorporation of an organism/s or a change in its concentration, alters the homeostasis, resulting in pathology, impaired health status, increased risk of infections and affected quality of women's life. Something else, the impaired vaginal biocenosis promotes co-infections with STI pathogens such as *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Human Immunodeficiency Virus* (HIV) and *Human Papillomaviruses* (HPV) [1] and vaginal complaints such as itching, burning, pain, irritation, dyspareunia, vaginal odor, discharge appear. They are one of the most common reasons for consultation and decreased quality of life.

The genital tract discharge could be physiological or due to vaginitis, cervicitis or pelvic inflammatory disease (PID). Infectious vaginitis refers to the inflammation and infection of the vagina caused by bacterial vaginosis (BV), trichomoniasis, and vulvo-vaginal candidiasis (VVC). They account for 90% of all etiologies among women of reproductive age [2]. So, precise diagnosis is crucial. The gold standard involves the application of Amsel's criteria and Nugent's scoring. Using them is not always possible to detect the pathogen/s, assess the role of opportunistic microbiota, settle the etiological diagnosis, and start adequate treatment. During the past decade, molecular diagnostic methods have been tested and introduced in the practice [3]. The aim of this study was to apply the real-time PCR method (Femoflor-16, DNA-Technology, Russia) to study the vaginal microbiome composition in women with genital tract discharge and to compare these results with the golden standard methods.

MATERIALS AND METHODS

A total of 45 women were enrolled in the study. The study protocol was approved by the Ethical Committee of the Medical University of Sofia, Bulgaria. The inclusion criteria for participation were: (1) female with a regular menstrual cycle (the menstrual cycle that is 21 to 35 days, lasts from 2 to 8 days); (2) aged 20–50 years; (3) no actual pregnancy; (4) without vaginal contraceptives being applied for at least 1 month; (5) without local or oral antibiotics/antiseptics/antimycotics in the previous 3 months; (6) without sexual intercourse within the last 24 hours.

Sample collection

After written informed consent, two sterile vaginal swabs were obtained from each participant and placed in Amies transport medium. The samples were transported and analyzed according to standard procedures.

Clinical diagnosis of bacterial vaginosis (BV) was made by using Amsel's criteria when at least three of the following symptoms or signs were present - thin homogenous vaginal discharge (milklike consistency) that smoothly coats the vaginal walls, fishy odor before or after the addition of 10% KOH in vaginal fluid (i.e., the wiff test), vaginal fluid pH > 4.5, and clue cells (e.g., vaginal epithelial cells studded with adherent bacteria) on wet mount microscopy.

The first swab was used for a Gram stain that was examined microscopically.

Standard microscopy

BV (bacterial vaginosis) was assessed by applaying the Nugent score system. Nugent scoring criteria are based on microscopic visualization of three bacterial morphotypes - large Gram-positive rods (*Lactobacillus* morphotypes; decrease in *Lactobacillus* scored as 0 to 4), small Gram-variable rods *(Gardnerella/Bacteroides* morphotypes; scored as 0 to 4), and curved Gram-variable rods (*Mobiluncus spp.* morphotypes; scored as 0 to 2). Nugent score of 0–3 is characterized as normal; 4–6 as intermediate microbiota, and 7–10 is consistent with bacterial vaginosis.

The microscopic examination of a smear made from the vaginal secretion and stained by Gram gives information about:

• the vaginal epithelium: the presence and predominance of different types of epithelial cells - superficial, intermediate, parabasal or basal; "clue cells"; "pseudo clue cells";

• leukocyte reaction: presence and quantity;

• the ratio between leukocytes and epithelial cells;

• microflora composition: amount and type of bacteria;

• presence/lack of trophozoites;

• the presence of yeast and/or pseudohyphae/hyphae characteristic of a mycotic infection;

Trichomoniasis and candidiasis were diagnosed using the standard microscopy as described.

The second swab was used for DNA extraction and subsequent real time PCR testing with a Femoflor-16 kit.

DNA extraction

Genomic DNA was extracted by using a DNA extraction kit according to the manufacturer's requirements (Sacace Biotechnologies, Como, Italy).

Multiplex Real-Time PCR (Femoflor-16)

Quantitative analysis and detection of the total bacterial mass, normal lactobacilli microflora and complex of aerobic and anaerobic microorganisms was performed. The tested parameters are presented in Table 1.

Table 1. Composition of microorganisms in Femoflor-16 test.

Name of research	Parameter
Controls	Control of sample intake
	Positive control
Diagnosis of Normocenosis	Total bacterial mass
	Lactobacillus spp.
Aerobe microorganisms	*Enterobacteriàñeae*
	Streptococcus spp.
	Staphylococcus spp.
Anaerobe microorganisms	*Gardnerella vaginalis/ Prevotella bivia/ Porphyromonas spp.*
	Eubacterium spp.
	Sneathia spp./ Leptotrihia spp./ Fusobacterium spp.
	Megasphaera spp./ Veillonella spp./ Dialister spp.
	Lachnobacterium spp./ Clostridium spp.
	Mobiluncus spp./Corynebacterium spp.
	Peptostreptococcus spp.
	Atopobium vaginae
Mycoplasmas	*Mycoplasma spp.*
	Ureaplasma spp.
Yeast-like fungi	*Candida spp.*

RESULTS

A total of 45 women aged between 20-50 years were included in the study.

Based on Amsel's clinical criteria, 9 (20%) of them were diagnosed with BV. Based on Nugent's score system and the microscopic visualization of the bacterial morphotypes 11 (24,4%) cases were categorized with BV.

When these 45 samples from women with genital tract discharge were tested with Femoflor-16, 5 (11,1%) of them were excluded from interpretation due to low values of one of the two controls included in the test. Based on this, only 40 samples were compared as results tested with the Femoflor-16 and Nugent score system.

The vaginal state of health or disease is defined as

normocenosis (absolute and relative) or dysbiosis (moderate, severe and mixed aerobic/anaerobic).

Table 2 shows the results obtained by Femoflor-16.

Table 2. The results of the 45 tested samples from women with genital tract discharge were obtained using the Femoflor-16 test.

Femoflor-16	Sample number (%)
Normocenosis	23(51,1%)
absolute	11(24,4%)
relative	12(26,7%)
Dysbiosis	17(37,8%)
severe	8(47,1%)
moderate	5(29,4%)
mixed	4(23,5%)
Total number of tested samples	45

In those11 patients with absolute normocenosis (24.4%), the amount of lactobacilli as an absolute value was 105.8- 107.3, while their relative value as a % of all microorganisms found in the vagina was from 80-85% to 100%.

The other proven bacteria in women with absolute normocenosis are shown in Table 3. Their total amount as an absolute value was from 101 to 104, and the relative value was < 0.1% of the total amount of bacteria found in the vagina.

Table 3. Prevalence of isolates in women with absolute normocenosis.

Isolates in women with absolute normocenosis	Sample number	%
Gardnerella vaginalis / Prevotella bivia / Porphyromonas	8	72,7%
Peptostreptococcus spp.	8	72,7%
Eubacterium spp.	6	54,5%
Atopobium vaginae	5	45,5%
Ureaplasma spp	3	27,3%
Staphilococcus spp.	3	27,3%
Megasphera spp/Veilonella spp/ Dialister spp.	2	18,2%
Streptococcus spp.	1	9,1%
Total sample number	36	

In 12 (26,7%) patients with relative normocenosis, the bacterial isolates that were detected are described in Table 4. In this group of patients, the number of lactobacilli as an absolute value was 106 – 107.8, while their relative value as % of all microorganisms found in the vagina was from 73-80% to 91-100%. The number of proven microorganisms as an absolute value was from 104 to 106.5, and the relative value was 0.1%-0.9%.

Table 4. Prevalence of isolates in women with relative normocenosis.

Isolates in women with relative normocenosis	Sample number	%
Gardnerella vaginalis / Prevotella bivia / Porphyromonas	6	50,0%
Eubacterium spp.	6	50,0%
Candida spp.	6	50,0%
Ureaplasma spp	5	42,0%
Atopobium vaginae	3	25,0%
Streptococcus spp.	2	16,7%
Lachnobacterium spp. / Clostridium spp.	2	16,7%
Mobiluncus spp/ Corynebacterium spp.	2	16,7%
Enterobacteriales spp.	1	8,3%
Staphilococcus spp.	1	8,3%
Total sample number	34	

Mixed infections were found in patients with relative normocenosis: a) Anaerobes; *Candida spp.*; *Ureaplasma spp*. in 7 samples (58.3%); b) Anaerobes; *Candida spp*. in 4 samples (33.3%); c) Anaerobes; Aerobes; *Candida spp*. in 1 sample (8.3%).

Dysbiosis was found in 17 (37.8%) patients out of 40 tested. In 8 (47.1%), it is severe, in 5 (29.4%) moderate, and in 4 (23.5%) – mixed, see Tabl 2.

In the patients with severe dysbiosis, the detected microorganisms were as follow, see Table 5:

Table 5. Prevalence of isolates in women with severe dysbiosis.

Isolates in women with severe dysbiosis	Sample number	%
Gardnerella vaginalis/Prevotella bivia/Porphyromonas	8	87,5%
Atopobium vaginae	6	75,0%
Eubacterium spp.	6	75,0%
Megasphera spp/Veilonella spp/ Dialister spp.	5	62,5%
Sneathia spp /Leptotrihia spp/ Fusobacterium spp.	4	50,0%
Peptostreptococcus spp.	4	50,0%
Ureaplasma spp.	4	50,0%
Lachnobacterium spp./ Clostridium spp.	3	37,5%
Mobiluncus spp/ Corynebacterium spp.	2	25,0%

Candida spp.	2	25,0%
Enterobacteriales spp.	1	12,5%
M. hominis	1	12,5%
Total sample number	46	

The lactobacilli as an absolute value were from 103.6 to 106.1, while their relative value was from 3-9% to 0%. The number of detected microorganisms as an absolute value ranged from >104 to 106.8, and the relative value was >40%-80%.

In the patients with moderate dysbiosis, the detected microorganisms were as follow, see Table 6:

Table 6. Prevalence of isolates in women with moderate dysbiosis.

Isolates in women with moderate dysbiosis	Sample number	%
Gardnerella vaginalis / Prevotella bivia / Porphyromonas	4	80,0%
Atopobium vaginae	3	60,0%
Eubacterium spp.	3	60,0%
Megasphera spp / Veilonella spp / Dialister spp.	2	40,0%
Ureaplasma spp.	2	40,0%
Lachnobacterium spp./ Clostridium spp.	1	20,0%
Mobiluncus spp/ Corynebacterium spp.	1	20,0%
Peptostreptococcus spp.	1	20,0%
Candida spp.	1	20,0%
Total sample number	18	

The lactobacilli as an absolute value were from 106 to 106.7, while their relative value was from 15-20% to 50-70%. The number of detected microorganisms as an absolute value ranged from 104 to 106.3, and the relative value was >10%-40%.

In the patients with mixed dysbiosis, the detected microorganisms were as follow, see Table 7:

Table 7. Prevalence of isolates in women with mixed dysbiosis.

Isolates in women with mixed dysbiosis	Sample number	%
Gardnerella vaginalis / Prevotella bivia / Porphyromonas	3	75,0%
Streptococcus spp.	3	75,0%
Eubacterium spp.	2	50,0%
Atopobium vaginae.	2	50,0%
Enterobacteriales spp.	1	25,0%
Megasphera spp / Veilonella spp / Dialister spp.	1	25,0%
Lachnobacterium spp / Clostridium spp.	1	25,0%
Ureaplasma spp.	1	25,0%
Candida spp.	1	25,0%
Total sample number	16	

In this group of samples, *Streptococcus spp*. and *Mobiluncus spp/Corynebacterium spp*. were also detected, but their quantities were normal.

The lactobacilli's absolute value ranged from 103 to 105.9, while their relative value ranged from 18% to 70%. The number of detected microorganisms as an absolute value ranged from 104 to 106.3, and the relative value was 4%-80%.

Of the 40 materials examined by both methods (Gram staining with subsequent application of Nusgent's criteria and multiplex PCR), 33 (83%) were in agreement. At 7, there was a discrepancy in the interpretation of the result. In 2 of the materials, a disturbed normocenosis was found by microscopy meanwhile, the PCR gave a normal result. In the other 5 materials examined by microscopy, a diagnosis of normocenosis is made, while PCR gives dysbiosis.

DISCUSSION

The vaginal microbiome is a complex dynamic microenvironment which, at any given time, could be influenced by many factors - age, sexual activity, obstetric history, hormonal status, menstrual cycle, antibiotic therapy, gestational status, contraceptive use, accompanying diseases.

The healthy vaginal tract contains more than 50 non-pathogenic microbial species [4]. A big part of these is Lactobacillus species. Nowadays, about 120 species of Lactobacillus have been detected. Twenty of them are known to inhabit the vagina. Lactobacillus iners, Lactobacillus crispatus, Lactobacillus jensenii and Lactobacillus gasseri are the most common and are famous as "the Key4". Generally, the vaginal microbiota of healthy women comprises one or two lactobacilli species [5]. Lactobacillus species produce various antimicrobial compounds - bacteriocins, hydrogen peroxide, lactic acid. They help in the defense against possible invading pathogens in the vagina.

Other representatives of the vagina microbiota are

bacterial genera *Gardnerella*, *Prevotella*, *Fannyhessea*, *Megasphaera*, and *Mobiluncus*. Commonly they are associated with abnormal vaginal constitution [6, 7]. Commonly this abnormal vaginal constitution is called dysbiosis. Bacterial vaginosis (BV) is traditionally defined as dysbiosis - loss of the balance of the vaginal microbiota. Comparing healthy females who have a dominant number of *Lactobacillus* species and low other bacterial diversity with BV-positive females, those last show a 1000-fold higher number of bacteria, greater diversity of anaerobic (facultative and obligate) bacteria, as well as decreased prevalence of *Lactobacillus spp*. [8].

For almost three decades, the gold standard for diagnosing BV has relied on two methods –Amsel's clinical criteria and the standardized system of the Nugent scoring method based on Gram stain detectable morphotypes from the vaginal smear [9]. Both have been evaluated in this study. They showed some discrepancies – with Amsel's clinical criteria, BV was settled in 20% of the tested women, whereas BV was diagnosed in 24,4% with Nugent's score system. Amsel's clinical criteria are rapid and easily performed at the primary examination. The sensitivity and specificity of the Amsel criteria are 37%–70% and 94%–99%, respectively, compared with the Nugent score [10]. The Nugent score system is more time-consuming and depends on the skills and expertise of the microscopist. Maybe that is why, regardless of the more precise results, Amsel's criteria are the more commonly applied technique in practice.

With the development of the knowledge, it became obvious that many different groups of bacteria are involved in the pathogenicity of BV. The vagina is a very heterogeneous ecosystem, colonized by various microorganisms. Both methods can not provide precise information on detectable pathogens. It is not possible reliably to differentiate biofilm-coated epithelial cells (clue cells) and other types of dysbiotic changes in the vaginal microbiota (pseudo clue cells) [11], nor to asses their virulence factors. Something else, when discussing BV that is considered a syndrome, not only vaginal epithelium-adherent biofilm formation but also other non-cell-adherent dysbiotic changes in the vaginal microbiota [12] should be taken into mind. So, previously established diagnostic approaches for the diagnosis of bacterial vaginosis (BV), such as the Amsel criteria or the Nugent scoring system, do not correspond to the modern trends in understanding the etiology and pathogenesis of the polymicrobial condition BV.

Nowadays, more and more frequently, molecular genetics methods are involved in the scientific field of evaluation of BV syndrome and other conditions related to the vagina microbiota. Different polymerase chain reaction (PCR) tests and sequencing techniques, alongside FISH, have been evaluated and try to find a position in the routine diagnostic field. The reason for this is their possibility to detect small changes in the vagina microflora composition both quantitatively and on the taxonomic level.

Molecular diagnosing techniques are objective. Nucleic acid amplification tests, such as PCR, are theoretically capable of detecting as little as one organism in a sample. They even can detect fastidious or uncultivable bacteria. Something else, real-time PCR permits amplification that can be observed in real-time, which eliminates the need for postamplification analysis, use of ethidium bromide and decreases the chances for contamination. Given the polymicrobial nature of BV, it is desirable when PCR is used to amplify more than one target sequence at a time. This could be done by using multiplex real-time PCR. Additionally, some PCR tests not only detect the pathogen but can quantify it.

The molecular technique used in this study is Femoflor-16. The test is designed to detect the DNA of opportunistic bacteria, lactobacilli, and human DNA (as a sampling control). Based on the controls in the kit, 5 samples from the 45 tested were excluded from interpretation. Femoflor-16 is a real time multiplex PCR that allows performing quantitative analysis of the total bacterial mass, urogenital, normal microflora (*Lactobacillus spp*.) and complex of aerobic and anaerobic microorganisms, as well as mycoplasmas and *Candida spp*. that are involved in the disturbance of the vaginal microbiota composition. And yet it was mentioned that when assessing the vaginal state of health or disease, the type and amount of proven microorganisms in it are of great importance. The obtained result from the test compares the amount of opportunistic microbiota with the amount of *Lactobacillus spp*., and normocenosis or dysbiosis are diagnosed. This could not be done by using Amsel's criteria neither by Nugent's score system.

In cases of normocenosis, *Lactobacilli* have a high absolute and relative value. However, in cases of dysbiosis, the absolute value of *Lactobacilli* could be high, while the relative value of *Lactobacillus* may decrease. This means that the presence of some bacteria, their relationship with the other microorganisms in the biocenosis, and their concentration are also important. This gives us the idea that there is a huge difference between the diagnosis of infections caused by opportunistic pathogens, as is the case with the BV syndrome and the diagnosis of infections caused by obligate pathogens.

Something else that attracts attention in cases with dysbiosis is the increased diversity of microorganisms in the patient's sample. The more severe the dysbiosis, the higher the polymicrobial ethiology of the condition. The advantage of the Femoflor-16 test is its ability to distinguish bacteria with the same morphology, which cannot be done with the microscopic examination. It allows for a quick, objective characterization of the vaginal

microbiome. Establishing the exact pathogen gives reason to apply adequate and individual antibacterial therapy.

It is essential to analize the results obtained by Nugent's score system and those obtained by PCR. Of the 40 materials examined by both methods, 33 (83%) were in agreement. However, 17% of inconformities are observed in interpretation. In 2 samples, a disturbed normocenosis was found by microscopy meanwhile, the PCR gave a normal result. In the other 5 materials examined by microscopy, a diagnosis of normocenosis is made, while PCR gives dysbiosis. This leads to the conclusion that this bacterial diversity is not always well valued by using Nugent's score system but by nucleic acid amplification tests. The same conclusions are made by other authors [13, 14].

Another positive of the test is the option to differentiate BV from other inflammatory and/or noninflammatory vaginal conditions. Femoflor-16 detects species such as aerobic bacteria (*Enterobacteriàñeae*, *Streptococcus spp*., *Staphylococcus spp*.), *Mycoplasma spp*., *Ureaplasma spp*. and yeast-like microorganisms (*Candida spp*.).

Although Femoflor-16 is rapid, sensitive and well informative for the presence and the quantity of a huge number of microorganisms from the vagina, the test has some disadvantages. It cannot assess the immunological power of the opportunistic microflora nor determine their sensitivity to different antibiotics. The test cannot discriminate between *Lactobacillus spp*. and *Gardnerella spp*. strains, and so to determine their individual role in the development of the pathological process. For example, the different types of lactobacilli have different protective potential. Lactic acid exists as two isomers, L(+) and D(") lactic acid, and both play a role in reducing the vaginal pH. L-lactic acid originates from both the vaginal epithelial cells (minor contribution to total lactic acid concentration) and vaginal *Lactobacillus spp*., while D-lactic acid is produced by *L. crispatus*, *L. gasseri* and *L. jensenii*, but not *L. iners* [15]. Maybe this is the reason why, in comparison with *L. crispatus*-dominated vaginal microbiota, *L. iners*-dominated microbiota have been associated with increases in BV incidence, HIV acquisition, *C. trachomatis* and VVC incidence and prevalence [16, 17]. Another disadvantage of the test is the lack of the option to detect the virulence factors involved in the syndrome microorganisms. *Gardnerella* is the most common microorganism identiûed from the vaginal samples of women with BV [18]. Recently, a whole genome sequence analysis detected the existence of 13 disparate species of the genus *Gardnerella* [19]. These bacteria show a variety of factors of pathogenicity - sialidase and vaginolysin as the most commonly studied, but also prolidase and glycosulfatase. All of these contribute to the vaginal mucus barrier degradation and pore-forming lysis of epithelial cells. Of course, not all the species demonstrate equal aggressive phenotype, explaining why not all BV-positive persons have *Gardnerella spp*. in the tested specimen sample.

CONCLUSIONS

The shift of normal vaginal microbiota with a loss of protective *Lactobacillus* species and an increase in the abundance of facultative and anaerobic organisms is the reason for vaginal discharge that can not be precisely detected by routine methods. A reliable, comprehensive, and on-time diagnosis of BV is needed. Nucleic acid amplification tests nowadays complement the "classic" laboratory methods. The real time multiplex PCR test Femoflor-16 can be effectively used in vaginal microbiota evaluation in women with BV discharge. It can identify a wide range of microorganisms, including bacteria that are difficult to culture, normal *Lactobacilli* microflora and complex of aerobic and anaerobic microorganisms, mycoplasmas and *Candida spp*. In addition, it is able to determine the number and ratio of microorganisms in the total bacterial mass, which further orients us to the disease state and the role of the microorganism as an opportunistic or obligate pathogen.

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