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Study of Femoflor-16 for Evaluation of Vaginal Microbiocenosis in Women with Inflammatory Diseases of the Genitals

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Abstract The article describes bacterial vaginosis (BV) — a violation of the balance of the vaginal microflora associated with a number of infectious diseases of the urogenital tract. The Femoflor-16 test is widely used to detect dysbiotic conditions of the vagina, but the management of patients with this test does not include the BV category. The study was conducted in the city maternity complex No.3 of the city of Samarkand 2018-2021.

Keywords Bacterial vaginosis, Femoflor-16, Lactobacilli, Anaerobic bacteria

1. Introduction

Bacterial vaginosis (BV) is the main cause of pathological vaginal discharge in women of reproductive age. Recent studies show that BV is associated with a number of inflammatory diseases of the genitourinary organs [1-4]. The prevalence of the disease varies widely (from 7 to 68%) depending on the region, ethnicity/race, as well as the surveyed population [5].

The main method of clinical diagnosis of BV is the Amsel method [6]. For laboratory diagnostics, the Nugent method is mainly used, based on the determination of bacterial morphotypes by microscopy of Gram-stained preparations [7]. In our city I do not use these methods are used very rarely.

Bacterial vaginosis is a violation of the balance between the physiological microflora of the vagina, represented mainly by lactobacilli, and conditionally pathogenic microflora, normally found in the vagina in small quantities. In relation to such conditions, the term "vaginal dysbiosis" is often used in Russian literature. Another well-known vaginal dysbiosis is aerobic vaginitis (AV). The similarity of BV with AB is that in both conditions there is a decrease in the number of lactobacilli, leading to a decrease in the concentration of lactic acid and, accordingly, an increase in the pH of the vaginal environment, and their replacement by conditionally pathogenic microorganisms. The differences between these diseases are significant. BV is characterized by the absence of inflammation and the presence of a large amount of anaerobic microflora (Gardnerella vaginalis, Atopobium vaginae, representatives of the genera Prevotella, Megasphaera, Leptotrichia, Sneatia, Mobiluncus, etc. are typically detected). Aerobic vaginitis in its typical manifestation is characterized by an increased inflammatory response and/or pronounced signs of atrophy of the vaginal epithelium and the presence of a moderate amount of commensal intestinal microflora [8]. Streptococci (up to 59% of cases), Staphylococcus aureus (up to 42%), coagulase-negative staphylococci (up to 37%), Escherichia coli (up to 23%) are most often detected in AV [9]. In recent years, the domestic Femoflor-16 test has been widely used to detect dysbiotic conditions of the vagina. The test is based on real-time multiplex quantitative PCR. With pomothe test is used to determine the total concentration of bacterial DNA — the total bacterial mass (MBM) and the concentration (absolute and relative) of the following types of genera of microorganisms: *Lactobacillus, Enterobacteriaceae, Streptococcus, Staphylococcus, Gardnerella vagina- lis/ Prevotella bivia/ Porphyromonas, Eubacterium, Sneathia/ Leptotrichia/ Fusobacterium, Megasphaera/ Veillonella/ Dialister, Lachnobacterium/ Clostridium, Corynebacterium/ Mobiluncus, Peptostreptococcus, Atopobium vaginae.* The ratio of these bacteria determines the state of vaginal microbiocenosis — normocenosis or dysbiosis. Dysbiosis, in turn, is assessed by the degree of severity (moderate or severe dysbiosis) and the predominance of aerobic or anaerobic conditionally pathogenic microflora (aerobic or anaerobic dysbiosis, respectively).

The results of the Femoflor-16 test do not include the BV category. However, for the management of a patient based on the principles of evidence-based medicine, a laboratory report should provide information that allows to establish or refute a certain generally accepted diagnosis with high accuracy.

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The aim of the study was to study the diagnostic criteria for determining BV during the examination of the vaginal discharge with the Femoflor-16 test.

2. Materials and Methods

The study involved women of different age groups who applied to the maternity complex of Samarkand to the gynecologists department with complaints of discomfort and discharge from the genital tract.

The material for the study was a vaginal discharge, which was obtained using two dacron tampons. The contents of one tampon were applied to a slide for microscopic examination. The contents of the second swab were used for analysis using the Femoflor-16 test.

For the clinical diagnosis of BV, the Amsel criteria were used [6] with a slight modification, which consisted in the fact that a Gram-stained preparation was used to determine the key cells in the vaginal discharge. When at least three of the four criteria were met, namely, the presence of specific vaginal discharge, increased vaginal pH (>4.5), a positive amine test; the presence of "key" cells during microscopic examination of the vaginal discharge, the diagnosis of BV was established.

Laboratory analysis for BV was performed by examining the vaginal discharge using the Nugent method [7]. The following bacterial morphotypes were determined in Gram-stained preparations: large gram-positive rods (lactobacillus morphotype), small gram-negative or gram-variable cocci and coccobacilli (Gardnerella and Bacteroides morphotype) and gram-negative or gram-variable curved rods (Mobiluncus morphotype). Depending on the amount of points, the samples were regarded as normal microflora (number of points from 0 to 3), intermediate microflora (number of points from 4 to 6) and BV (number of points from 7 to 10).

The analysis of samples of the vaginal discharge using the Femoflor-16 test was carried out in accordance with the manufacturer's instructions. When developing diagnostic criteria for BV, the number of all microorganisms, with the exception of M. hominis, Ureaplasma and Candida, was presented as the ratio of their concentration in fractions of one. The amount of M. hominis, Ureaplasma and Candida was represented in absolute concentration values (genome equivalents in the sample). To study the association of microorganisms/groups of microorganisms determined by the Femoflor-16 test with BV, the correlation of their number with clinical and microscopic indicators of BV was analyzed: the number of points on the Nugent scale, the sum of Amsel's positive criteria and with vaginal pH values. To assess the ability of potential bacterial markers determined using the Femoflor-16 test to correctly classify samples with normal microflora and BV, ROC analysis (ROC - receiver operating characteristic) was used. The optimal threshold of bacterial content was determined by the maximum proportion of correctly classified samples. Statistical analysis of the results was carried out using statistical packages Statistica (StatSoft) and SPSS (IBM).

3. Results

The study included 80 women aged 20 to 45 years (29 ± 5.7 years). Using the Amsel criteria, BV was detected in 65 women, 15 women did not have BV.

According to the classification of vaginal samples by the Nugent method Samples from all women were tested using the Femoflor-16 test.

The results of 10 samples were considered invalid due to the low value of the control of the material collection and excluded from the analysis. Thus, 80 samples were included in the analysis. Of these, 15 samples were from the category of normal microflora by Nugent, 25 — intermediate microflora and 40 — BV.

To study the association of microorganisms of groups of microorganisms determined by the Femoflor-16 test with BV, an analysis of the correlation of their number with clinical and microscopic indicators of BV, namely, the number of points on the Nugent scale, the sum of positive Amsel criteria and with vaginal pH values was carried out.

A negative correlation with BV was established for the Lactobacillus/OBM ratio. Anaerobic bacteria of the bacterial group predictably showed a positive correlation with BV, while the highest correlation coefficients were observed for the groups *Gardnerella vaginalis/ Prevotella bivia/ Porphyromonas, Sneathia/ Leptotrichia/ Fusobacterium* и *Megasphaera/ Veillo- nella/ Dialister*. A positive correlation with BV was also observed for M. hominis. Of the bacteria traditionally associated with BV, Mobiluncus did not show a significant correlation with any of the BV indicators, which may be due to the fact that these bacteria in the Femoflor-16 test are detected in conjunction with phylogenetically related, but unrelated to BV bacteria of the genus *Corynebacterium*. The DNA content of aerobic bacteria of the intestinal group (Enterobacteria, streptococci and staphylococci), as well as ureaplasmas and yeast-like fungi of the genus Candida, was not expected to correlate with the indicators of BV. The next stage of our analysis was to assess the ability of bacterial markers determined using the Femoflor-16 test to correctly classify samples with normal microflora and BV. ROC analysis was used for this purpose. Since the determination of diagnostic characteristics requires a binary classification of cases ("there is a disease" or "there is no disease"), cases of intermediate microflora by Nugent were excluded from the analysis. The analysis included MBM and the relative DNA concentration of bacteria that showed a significant correlation with BV, namely lactobacilli and all groups of anaerobic bacteria, with the exception of Corynebacterium / Mobiluncus. The values of the area under the ROC curve were calculated, as well as the values of sensitivity and specificity at the optimal threshold of relative concentration. The highest diagnostic accuracy (the area under the ROC curve exceeds the value of 0.9, which is considered as

excellent diagnostic accuracy) was demonstrated by the following markers: the ratio of lactobacilli and MBM (the area under the ROC curve is 0.996), *Gardnerella vaginalis/ Prevotella bivia/ Porphyro- monas/ОБМ* (0,975), *Eubacterium/* ОБМ (0,942), *Sneathia/ Leptotrichia/ Fusobacterium/ ОБМ* (0,907), *Megasphaera/ Veillonella/ Dialister/ОБМ* (0,934) и *Atopobium vaginae/ОБМ* (0,908). The markers Lachnobacterium/Clostridium/OBM and Pepto-streptococcus/OBM had satisfactory (area under the ROC curve 0.775) and high (0.850) diagnostic accuracy, respectively. The total bacterial mass and concentration of M. hominis showed low diagnostic accuracy (the area under the ROC curve is below 0.7). For markers with an area under the ROC curve above 0.7, the optimal threshold of relative concentration and sensitivity and specificity indicators at this threshold were calculated. The highest indicators of sensitivity and specificity were shown for the ratio of lactobacillus concentration to AML. At a threshold < 0.1 (in other words, if the lactobacillus content in the vaginal discharge is below 10%), this indicator predicts BV with sensitivity and specificity equal to 99%. The marker had the highest indicators of sensitivity and specificity *Gardnerella vaginalis/Prevotella bivia/ Porphyromonas/*— 95 и 94 % accordingly, at a threshold > 0.01 (i.e. at a content above 1%). The sensitivity of the remaining markers varied from 39 to 80%, specificity — from 93 to 99%.

Thus, the low content of lactobacilli is the most sensitive and specific criterion of BV. However, it should be borne in mind that when calculating diagnostic characteristics, we excluded the intermediate category for Nugent, which includes most of the samples from the category "pronounced aerobic and mixed dysbiosis with a high content of aerobic bacteria", also characterized by a low content of lactobacilli. In this regard, the use of the ratio of lactobacilli to MBM as the only criterion can lead to false positive results, although few. Taking into account this fact, it seems appropriate to consider the vaginal microflora as corresponding to BV if two conditions are met: a low content of lactobacilli and an increased content of at least one of the bacterial markers of BV.

In order to assess the sensitivity and specificity of the proposed approach to the diagnosis of BV, we included the intermediate microflora according to Nugent in the analysis, combining it with the normal microflora. In other words, all cases were divided into BV (n = 80) and the absence of BV (n = 190). if only a low lactobacillus content was used as a BV criterion, the sensitivity and specificity of BV detection were 99 and 89%, respectively. When a low content of lactobacilli in combination with an increased content of anaerobic bacteria was used as a criterion for BV, the number of false-positive cases was expected to decrease (from 20 to 13), which led to an increase in specificity to 93%.

The final stage of our work was the comparison of the criteria we developed for the detection of BV with the criteria of the test manufacturer for severe anaerobic dysbiosis, which is informally equated to BV. Of the 80 samples interpreted by us as BV, 40 samples were classified as severe anaerobic dysbiosis, 2 — severe aerobic dysbiosis, 3 — severe mixed dysbiosis. At the same time, 3 out of 80 samples from the category of severe aerobic dysbiosis were interpreted by us as the absence of BV.

Thus, the diagnostic criteria of BV developed in this study and the criteria developed by the manufacturers of the Femoflor-16 test largely describe the same category of vaginal microbiocenosis.

4. Discussion of the Results

In recent years, molecular methods have been actively introduced into the diagnosis of urogenital infections, including those associated with conditionally pathogenic microorganisms. Conditionally pathogenic vaginal microflora is part of the endogenous (normal) microflora, therefore, when analyzing dysbiotic conditions associated with it, it is necessary to take into account its quantitative composition. The Femoflor-16 test, based on quantitative real-time PCR, has been introduced into clinical laboratory diagnostics and has found quite wide application. The test has a number of advantages over traditional methods of detecting violations of the micro—biocenosis of the vagina - bacteriological and microscopic. its advantages over both methods are a high level of standardization of analysis and interpretation of results, exclusion of the subjectivity factor, accurate quantitative assessment. The Femoflor-16 test also differs from the bacteriological method in the speed of analysis, the ability to identify uncultivated and difficult-to-cultivate microorganisms. Further, the test makes it possible to differentiate bacteria having similar morphotypes and therefore indistinguishable during bacterioscopy. Summarizing the above, it can be argued that the test makes it possible to quickly, objectively, standardized characteristics of vaginal microbiocenosis. The laboratory conclusion of the study of the vaginal discharge with the Femoflor-16 test includes the following main categories of vaginal microflora: normocenosis (including conditional normocenosis), moderate dysbiosis (anaerobic and aerobic) and pronounced dysbiosis (anaerobic and aerobic). Initiating this study, we proceeded from the belief that the Femoflor-16 test has the potential to accurately identify two main disorders of the balance of the vaginal microflora — BV and AB. We devoted this work to the development of criteria for the diagnosis of BV. In our opinion, with the help of the test, it is possible, after the development of appropriate criteria, to identify with a high degree of certainty the type of vaginal microflora corresponding to aerobic vaginitis (for an accurate diagnosis of aerobic vaginitis, an assessment of the inflammatory reaction is also necessary).

The first stage of our analysis was to assess the association of detected microorganisms/groups of microorganisms with BV. All groups of anaerobic bacteria, with the exception of

the Corynebacterium/Mobiluncus group, correlated to one degree or another with BV. Both bacterial genera belong to the order Actinomycetales of the class Actinobacteria, however Mobiluncus spp. it is associated with BV, and Corynebacterium spp. - no. The results of metagenomic studies of the vaginal microflora indicate that the content of bacteria of the genus Corynebacterium in some cases, although few, may be significant, higher than 10%. In this regard, the combined detection of Mobiluncus spp. and Corynebacterium spp. it may lead to an unreasonable conclusion about the dysbiotic state of the vagina. Further, we have developed criteria that allow with 99% sensitivity and 93% specificity to establish a diagnosis of BV or exclude it. It should be noted that when evaluating the developed criteria, we combined the categories of normal and intermediate microflora according to Nugent, defining this combined category as the absence of BV. It is known that the category of intermediate microflora by Nugent is heterogeneous in its composition, and to date there is no consensus on the management of patients with this category of vaginal microflora. It is believed that this category contains mostly microflora corresponding to aerobic vaginitis, as well as really intermediate microflora, i.e. transitional from normal to BV. Our results serve as an indirect confirmation of this: 5 out of 8 cases (63%) of pronounced aerobic dysbiosis, determined by the Femoflor-16 test, entered the category of intermediate microflora according to Nugent. It is important to note that when comparing the results of testing for BV using the criteria developed by us and testing for pronounced anaerobic dysbiosis (informally equated to BV) using the criteria of the test manufacturer, the number of discordant results was relatively small. One motivation for this study was the difficulties experienced by clinicians in interpreting the category of moderate dysbiosis, which implies an intermediate state between normocenosis and severe dysbiosis. The category of moderate anaerobic dysbiosis is particularly problematic, since it is detected quite often. So, in our study (16%) were assigned to this category. Comparison of the results of the Femoflor-16 test and the Nugent method showed that the vast majority of samples from the category of moderate anaerobic dysbiosis 84% were classified as normal microflora on the Nugent scale and only 14% of cases were classified as intermediate microflora, and one case 2% was classified as BV. In our opinion, the category of intermediate state, defined by the Femoflor-16 test as moderate dysbiosis, should be narrowed and include only borderline, difficult-to-interpret cases. By way of discussion, I would like to note the significant potential of the Femoflor-16 test for the analysis of vaginal microflora for scientific purposes, which, in our opinion, is somewhat limited by the fact that Gardnerella vaginalis — a facultative anaerobe, a representative of the order Bifidobacteriales of the Actinobacteria class — is detected in conjunction with Prevotella bivia and Porphyromonas spp. — obligate anaerobic bacteria of the order Bacteroidales of the class Bacteroidetes. Limitations of this kind can also include the already mentioned joint detection of bacteria of the genus Corynebacterium with bacteria of the genus Mobiluncus.

Thus, we have developed criteria for the diagnosis of BV using the Femoflor-16 test, designed to characterize the microbiocenosis of the vagina. Low content of lactobacilli (< 10% of the total bacterial mass) in combination with an increased content *Gardnerella vaginalis/Prevotella bivia/ Porphyromonas* (>1%), and *Eubacterium* (> 2%), and *Sneathia / Leptotrichia / Fusobacterium* (> 0,1%), and *Megasphaera / Veillonella / Dialister* (> 0,1%), and *Lachnobacterium / Clostridium* (> 0,1%), and *Peptostreptococcus* (> 0,1%), and *Atopobium vaginae* (> 0,2%) BV is determined with a sensitivity of 99% and a specificity of 93%. These BV criteria and the criteria developed by the manufacturers of the Femoflor-16 test for the category of severe anaerobic dysbiosis largely describe the same category of vaginal microbiocenosis.

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