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Diagnosis of bacterial vaginosis: comparison of Nugent's and novel microscopic method

Dijagnoza bakterijske vaginoze: poređenje Nugent-ove i nove mikroskopske metode

Dane Nenadić*†, Snežana Ribis‡, Miloš Pavlović§, Dejan Baskić‖

***Military Medical Academy, Department of Gynecology, Belgrade, Serbia; †University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia; ‡University Clinical Center of Kragujevac, Department of Microbiology, Kragujevac, Serbia; §Infinity Family Medicine Clinic, Dubai, UAE; ‖University of Kragujevac, Faculty of Medical Sciences, Center for Molecular Medicine and Stem Cell Research, Kragujevac, Serbia**

Abstract

Background/Aim. Bacterial vaginosis (BV) is a common cause of vaginal discomfort in women. The aim of this study was to compare Nugent's scoring system and novel microscopy method, introduced in our laboratory and used in BV diagnosis. **Methods.** This study included 705 pregnant and asymptomatic women between 24 and 28 weeks of pregnancy. The degree of agreement between methods was determined by the *kappa* (κ) index. The sensitivity, specificity, positive and negative predictive value of the novel microscopy method was compared to Nugent's score as standard. **Results.** Based on the scoring system of both methods, Nugent's and novel microscopy method, BV was diagnosed in 21% and 25% of women, respectively. Despite the disparities among diagnostic criteria, which mainly concerned classification of intermediary samples, the degree of agreement between categories, determined by κ index, was satisfactory: Nugent's vs. novel microscopy method (κ = 0.68; good agreement), and Nugent's vs. novel microscopy method without intermediary results (κ = 0.83; very good agreement). We also demonstrated that compared to Nugent's method, as the golden standard, the novel microscopy method had high sensitivity and specificity (ranging from 75%–99.3%) and positive and negative predictive values (ranging from 88.8%–99.5%). Our method is based on a relative number of bacterial morphotypes, either rod forms (> 1.5 μm, *lactobacilli*) or non-rod forms (< 1.5 μm, bacterial vaginosis associated bacteria) under 200× magnification, which extends the surface of examined preparation, but without prolongation of observer's working time. **Conclusion.** The novel microscopy method in diagnosing BV corresponded well with Nugent's scoring system which allows it to be an alternative method in diagnosing BV. Furthermore, the novel microscopy method is based on a relative number of bacterial morphotypes that appeared to be flexible and can be reorganized in the way to categorize findings into only two groups: normal and BV, which makes it comparable to dichotomous Amsel's clinical criterion.

Key words:
diagnosis; microscopy; vaginal smears; vaginosis, bacterial.

Apstrakt

Uvod/Cilj. Bakterijska vaginoza (BV) je čest uzrok vaginalne nelagodnosti kod žena. Cilj ovog istraživanja bio je poređenje Nugent-ovog sistema vrednovanja mikroskopskih preparata i nove mikroskopske metode koju smo uveli u našoj laboratoriji radi dijagnoze BV. **Metode.** Istraživanjem je obuhvaćeno 705 asimptomatskih trudnica između 24. i 28. nedelje trudnoće. Stepen slaganja između metoda je određivan *kappa* (κ) indeksom. Senzitivnost, specifičnost, pozitivna i negativna prediktivna vrednost nove mikroskopske metode su poređene sa Nugent-ovom metodom kao standardom. **Rezultati.** Na osnovu sistema vrednovanja obe metode, po Nugent-u i nove mikroskopske metode, BV je dijagnostikovana kod 21%, i 25% žena, redom. Bez obzira na razlike između dijagnostičkih kriterijuma, koje su se uglavnom odnosile na klasifikaciju intermedijarnih rezultata, stepen slaganja između kategorija, određen *kappa* indeksom, bio je zadovoljavajući: Nugent-ov i novi mikroskopski metod su pokazali dobro slaganje (κ = 0,68), dok su Nugent-ov i novi mikroskopski metod bez intermedijarnih rezultata, pokazali veoma dobro slaganje (κ = 0,83). Takođe, pokazali smo da je u poređenju sa Nugent-ovom metodom, kao zlatnim standardom, nova mikroskopska metoda imala

Correspondence to: Snežana Ribis, University Clinical Center of Kragujevac, Department of Microbiology, Miloja Pavlovića 2/35, 34 000 Kragujevac, Serbia. E-mail: enci2001@gmail.com

visoku senzitivnost i specifičnost (od 75% do 99,3%), kao i dobru pozitivnu i negativnu prediktivnu vrednost (od 88,8% do 99,5%). Naša metoda je bazirana na relativnom broju bakterijskih morfotipova, bilo štapićastih formi (> 1,5 μm, *lactobacilli*), ili neštapićastih formi (< 1,5 μm, bakterije udružene sa BV) pod 200× uvećanjem, što povećava površinu preparata koji se pregleda, ali bez produžavanja vremena za koje posmatrač pregleda preparat. **Zaključak.** Nova mikroskopska metoda se dobro podudarila sa Nugent-ovim sistemom skorovanja ukazujući na to da se može koristiti kao alternativna mikroskopska metoda u dijagnostici BV. Novi mikroskopski metod je baziran na relativnom broju bakterijskih morfotipova i pokazao se fleksibilnim u smislu reorganizovanja tako da se sve kategorije uzoraka klasifikuju u samo dve grupe: normalan nalaz i BV, što ga čini komparabilnim dihotomnom kliničkom kriterijumu po Amsel-u.

Ključne reči:
dijagnoza; mikroskopija; vaginalni brisevi; vaginoza, bakterijska.

Introduction

The main constituents of a healthy vaginal microbiome are *lactobacilli*. The protective role of lactobacilli is reflected in their ability to antagonize with other bacteria for adherence to the vaginal epithelium as well as to synthesize antimicrobials (hydrogen peroxide, lactic acid, bacteriocins) which suppress the growth of pathogenic microbes [1–3]. Any decrease in the number of *lactobacilli* can result in disturbance of vaginal microflora and subsequent development of bacterial vaginosis (BV). The composition of BV is complex. Molecular analysis has shown that BV is not a monobacterial disorder but can be caused by many microbes such as *Gardnerella vaginalis, Prevotella spp, Atopobium spp, Mobiluncus spp, Sneathia sanguinegens* [4]. Quite often, BV can be asymptomatic, which can make this disorder insidious in regard that it can cause obstetric and gynaecological complications without warning. Some of the consequences of BV can be premature birth, or increased risk to encounter additional infection (*Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, HSV2, and HIV) [5–7].

This disorder can be diagnosed under various criteria (clinical or microscopic) introduced by Amsel et al. [8], Nugent et al. [9], Ison and Hay [10], and Verhelst [11] (the first two of four are widely accepted as "golden" standards in BV diagnosing, clinical and microscopic, respectively). The method of Amsel et al. [8] was mainly based on clinical findings and properties of vaginal discharge. According to the Amsel's criterion, a patient is positive for BV when 3 of 4 criteria are present (vaginal pH above 4.5, "milk-like" white-grayish vaginal discharge, positive whiff test, and clue cells on microscopic examination). Nugent et al. [9] categorize the microscopic findings of Gram-stained vaginal smears by quantification of some of the present morphotypes, *Lactobacillus*, *Gardnerella-Bacteroides*, and *Mobiluncus* into: normal, intermediary, and BV. The Ison/Hay system is based on the observation of Gram stains to estimate the qualitative ratios of the observed morphotypes rather than the exact number of bacteria. In order to obtain a more precise classification, two additional categories have been introduced to Ison/Hay criteria, group 0 – without bacteria, and group IV – with a large amount of Gram-positive cocci [10]. Further modification by Verhelst et al. [11], using culture and molecular identification of vaginal microbiota, introduced even more categories, subdividing grade I samples in several additional categories: Ia, Ib, Iab, I-like, I-PMN, regarding the relative concentration of Gram-positive rods (*lactobacilli*) and BV-associated bacterial morphotypes [11, 12].

Although widely used, all of these methods mentioned above had certain insufficiencies. The method of Nugent et al. [9] categorizes the smears by quantification of bacterial morphotypes, which demands noticeable time and skill of an observer (experienced microbiologist). Additionally, the Nugent-scoring system includes only three bacterial morphotypes and, therefore, it may not match the heterogeneity and complexity of the vaginal microflora. Albeit that Ison and Hay [10] and Verchelt et al. [11] had overcome some deficiencies of the method of Nugent et al. [9] by introducing qualitative assessment of vaginal smears, their method is still based on observation of small slide area (under 1,000× magnification). Observing 5–20 fields of view under the 1,000× magnification the actual scanned surface makes only a tiny fraction of the slide surface, thus being a source of sampling error [13, 14].

In regard to overcoming some insufficiencies of previously mentioned criteria: time-consuming, a complicated numerical summing with narrow intervals, a need for experienced personnel, a demand for standardizing surface of the microscopic field of view, and evaluation of only three bacterial morphotypes, we established a novel method of microscopic examination of Gram-stained vaginal smears based on qualitative examination of preparations under 200x magnification [15]. The categorization system of our method refers to six groups: three normal and three BV, which can make an easier comparison of microscopic method and dichotomous clinical assessment of samples such as the method of Amsel et al. [8]. To test its value, we compared our method to the already established Nugent's method.

Methods

Study population and design

This prospective study comprised of 705 pregnant and asymptomatic women between 24 and 28 weeks of pregnancy, seen during regularly planned appointments at the Military Medical Academy, Belgrade, from 2012 to 2014. Patients younger than 18 and older than 40 years, patients with multiple pregnancies, anomalies of the uterus,

cervical conization, or patients with previous preterm delivery were excluded from this study. Patients who were under any kind of therapy two weeks before the examination, as well as patients who had sexual intercourse a week before the appointment, were also excluded from the study. The institutional Ethics Committee approved the study protocol, and all study subjects agreed to participate through 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 a sterile dacron-tipped swab into the 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 and air-dried at room temperature. The slides were Gram-stained and categorized according to Nugent's criteria (viewed under immersion, 1,000× magnification) and novel method of microscopic examination (viewed under immersion, 200× magnification), which will be further denoted here as the criterion of Nenadić et al. [15].

Analysis of data

Nugent scoring system implies categorization of Gram-stained smears into three groups regarding morphotypes of bacteria under microscope 1,000× magnification. Morphotypes are scored by their presence/absence as the average number seen per oil immersion field (5–20 fields) [9]. For example, if more than 30 *lactobacilli* are recognized in the visual field, the score is 0; if no lactobacilli are detected, the score will be 4 points. If *Gardnerella*-like bacteria are absent, the score is 0; if more than 30 are observed, the score will be 4. The presence of other microorganisms, such as *Mobiluncus*, can add additional 2 points. According to the final score, all findings are designated as follows: I-normal (0-3), II-intermediate (4-6), and III-bacterial vaginosis (7-10). The scoring system of Nenadić et al. [15] is based on the examination of Gram-stained vaginal smears under 200× magnification and their categorization depending on the presence of either rod forms (RFs) or non-rod forms (NRFs). The shortest length still observable as a rod at the 200× magnification is 1.5 μm. Based on this fact, under 200× magnification, there are no obstacles to recognizing the predominance of either RFs (> 1.5 μm, *lactobacilli*) or NRFs (< 1.5 μm, bacterial vaginosis associated bacteria). The number of RFs and NRFs was estimated semi-quantitatively in the following way: numerous bacteria, covering the most of slide surface between, around, and over epithelial cells, were labelled as "full"; bacterial forms rare or absent between, but found mostly around and on epithelial cells were designated as "mid"; the absence of bacterial forms with only rare elements seen around and on epithelial cells were termed as "empty". According to the predominance of either RFs or NRFs, each of these three categories was additionally subdivided into a normal (N) and bacterial vaginosis (BV) subgroup, respectively. In this way, all slides were categorized into 6 groups. Three out of those six were normal: normal full – NF, normal mid – NM, and normal null – NN. The other three were bacterial vaginosis varieties: BV full – BVF, BV mid – BVM, and BV null – BVN. For the purpose of the study, Nugent's score was taken to be the gold standard.

With the aim to compare our results with Nugent's as "golden" standard, we grouped our findings into the following groups: six groups by novel microscopy method (NMM): NF, NM, NN, BVF, BVM, BVN, three groups by NMM (NF and NM were considered as normal, NN and BVN as intermediate, and BVM and BVF as BV), and two groups by NMM (N-normal: NF+NM+NN, and BV-bacterial vaginosis: BVF+ BVM+BVN).

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 the statistical significance of differences was evaluated using the χ^2 test. The degree of agreement between categories (scale of measurement) was determined by the *kappa* index. Sensitivity and specificity were calculated in an ordinary manner. Statistically, a significant difference was evaluated on a minimal level $p < 0.05$.

Results

Based on NMM and Nugent's scoring, bacterial vaginosis was diagnosed in 25% and 21% of women, respectively. The normal finding was observed in 75% of women by NMM, and 63% by Nugent, while 16% of patients were classified as intermediate under Nugent.

According to the χ^2 test association has been found between NMM and Nugent et al. [9] categorization ($\chi^2 = 669.800$; df = 10; $p < 0.001$). When we observed the group with normal findings, the best association has been found between groups with the intermediary result by Nugent et al. [9] and NF (normal full by NMM, 96.0%), and between intermediary group by Nugent et al. [9] and NM (normal mid by NMM, 80%) (Figure 1). The group with the intermediary result by Nugent et al. [9] was in a significant association with BVN (bacterial vaginosis normal, 57%) and NN (normal null, 44%). BV group has shown the best association with BVF (bacterial vaginosis full, 99%) and BVM (bacterial vaginosis mid, 78%). It can be observed that around half of patients from Nugent's intermediary group were grouped as NMM's NULL groups (hypocellular: NN+BVN).

In Figure 2, it can be seen that the intermediary group under NMM was formed by adding NN (normal null) to BVN (bacterial vaginosis null), considering that the majority of intermediary patients by Nugent et al. [9] were contained within these groups (Figure 1). It was shown that the best association

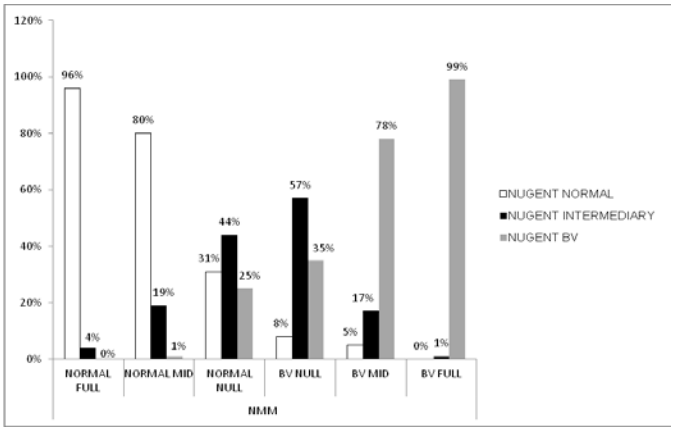


Fig. 1 – Comparison of six groups of results classified by the novel microscopy method (NMM) and Nugent's criteria. BV – bacterial vaginosis.

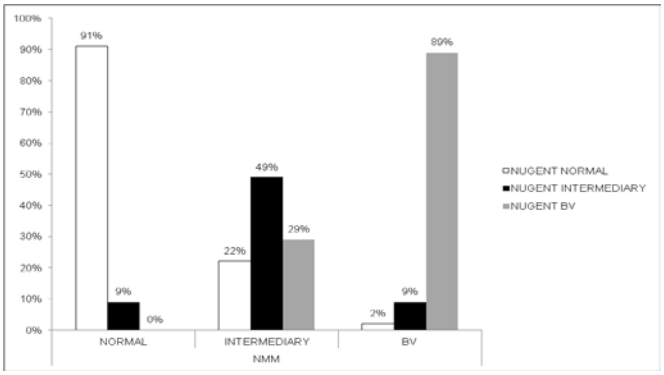


Fig. 2 – Comparison of results classified by the novel microscopy method (NMM) into 3 groups [normal, intermediary, and bacterial vaginosis (BV)] and Nugent's criteria. The intermediary group of NMM was formed by adding normal null (NN) to the BV null group of NMM.

($\chi^2 = 634.442$; df = 4; $p < 0.001$) was found between patients with normal finding (91%) and those with bacterial vaginosis (89%) (Figure 2). On the other hand, the weakest association was observed in patients with the intermediary result (49%).

When we observe results presented in Figure 3, the best association was found in groups with normal finding (82%) and groups categorized as BV (74%) ($\chi^2 = 437.40$; df = 2; $p < 0.001$). Twenty-two percent of the intermediary group determined by the method of Nugent et al. [9] was categorized as BV according to NMM, while 14% of intermediary findings determined by the method of Nugent et al. [9] was classified as NMM's normal group.

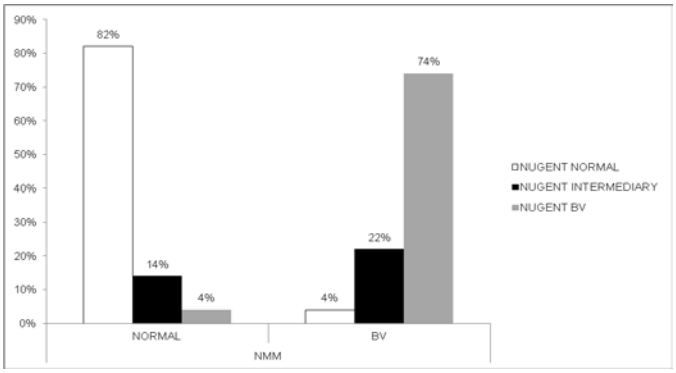


Fig. 3 – Comparative results of categorization by the novel microscopy method (NMM) and the Nugent's method. NMM reorganized results into two groups: patients with normal findings and bacterial vaginosis (BV).
The group with normal findings, under NMM, was formed by summarizing all groups with normal findings: NN (normal null) +NM (normal mid) +NF (normal full).

Table 1 shows mutual agreement of overall results from our study under Nugent's criteria and NMM presented through the kappa index ($\kappa = 0.68$; $p < 0.001$, good agreement) (Table 1) and mutual agreement of results classified under NMM and Nugent's criteria, when IMD (intermediary) group was excluded (Table 1). The greatest discrepancy was observed among IMD results. Almost half of IMD cases, according to Nugent et al. [9], 41/113 (36.3%) and 11/113 (9.7%), were placed into the normal and BV group by NMM, respectively. Furthermore, 35/148 (23.6%) of BV findings determined by the method of Nenadić et al. [15] were designated as IMD, while 26/444 (5.9%) of normal cases determined by the method of Nugent et al. [9] were grouped as IMD, according to NMM. Moreover, three normal cases determined by the method of Nugent et al. [9] were classified as BV by NMM, and two BV according to the method of Nugent et al. [9] were categorized as normal according to the method of Nenadić et al. [15]. On the whole, Nugent's and NMM criteria diverged in 118/705 (16.7%) of cases. Finally, when the results were analyzed after removal of the IMD group (Table 1), an increase in agreement among two different criteria was observed (the kappa index rose from 0.68 to 0.83).

Sensitivity, specificity, positive and negative predictive value of NMM, compared to the Nugent's score as standard, is given in Table 2. The intermediary score, grade II, was considered either positive, negative, or excluded. In the case when the intermediary score was considered negative/normal, the sensitivity and specificity of the Nenadić et al. [15] criterion was lower, but still high, with high positive and negative predictive values. When IMD and BV samples were analyzed as one group, the sensitivity of NMM increased from 75.0% to 94.6% without significant loss of specificity. The positive and negative predictive values remained high. Finally, when we excluded IMD findings from our analysis, we found that 111 of 113 BV samples according to the method of Nugent et al. [9] and three of 419 N samples according to this method met positive NMM criteria for BV. Thus the sensitivity and specificity of NMM were very high, (98.2% and 99.3%, respectively), as well as positive and negative predictive values (97.4% and 99.5%, respectively).

Discussion

The human vaginal microbiome is very important for the health of women. It can be changed by hormonal status (it is not the same before puberty, during the reproductive period, or among menopausal women), certain sexual behavior, and it varies according to ethnic affiliation. Nevertheless, although BV may appear at any age, it is the most frequent in the reproductive period. The

Table 1

Mutual agreement of overall results classified by the Nugent's method and novel microscopy method (NMM) presented through the Kappa index (κ), and mutual agreement of the Nugent's method and NMM, when intermediary (IMD) findings were excluded from the Nugent's categorization presented through the Kappa index (κ) as well

Nugent's method	NMM			NMM without IMD	
	N	IMD	BV	N	BV
N	415	26	3	416	3
IMD	41	61	11		
BV	2	35	111	2	111
	$\kappa^7 = 0.68$, $p < 0.001$			$\kappa^7 = 0.83$, $p < 0.001$	

N – normal finding; BV – bacterial vaginosis.

Table 2

Sensitivity (SN), specificity (SP), positive and negative predictive values (PPV and NPV, respectively) of the novel microscopy method (NMM), compared to the Nugent's method as standard, in the next order: when intermediary (IMD) findings were added to normal (N) Nugent's findings, when IMD findings were added to the bacterial vaginosis (BV) findings determined by the Nugent's method, and when Nugent's IMD findings were excluded from data analysis

Nugent's method	NNM						
	N	BV	Total	SN (%)	SP (%)	PPV (%)	NPV (%)
IMD considered N							
IMD/N	543	14	557				
BV	37	111	148	75.0	97.5	88.8	93.6
Total	580	125	705				
IMD considered BV							
N	416	29	445				
BV/IMD	43	217	260	94.6	93.5	89.5	96.7
Total	459	246	705				
IMD excluded							
N	416	3	419				
BV	2	111	113	98.2	99.3	97.4	99.5
Total	418	114	532				

most striking event in shifting of healthy vaginal environment towards BV is the substitution of dominant *lactobacilli* by a mixture of mainly anaerobic bacteria such as *Gardnerella vaginalis*, *Atopobium vaginae*, and *Prevotella spp* [16–18]. Adequate diagnosis of BV is demanding, and choosing the right method for its diagnosis requires a review of hardly explicable results such as intermediary results [19]. Although there are many criteria and mutually comparable scoring systems, it is not convincing that they will always classify the same category of patients. As is well known, demonstration of infectious agent existence is often a basic criterion in diagnosing the infective disease. This is not the case with BV since the real cause of the disorder is not yet defined. Thus, the patient must meet clinical or laboratory criteria which do not consider the presence or quantity of a specific bacterium. It is important to keep this in mind when comparing different diagnostic methods. BV does not evolve from a commonly defined bacterial infection caused by one agent but can rather be compared to consequences caused by anaerobic mixed flora in other parts of the organism. Diagnosis based on diagnostic criteria is actually the weighting of criteria to provide the best possible agreement between the criteria and the presence of BV. It is important that the examiner, whether a clinician or laboratory technician, is well trained and able to evaluate the clinical adequacy of the diverse methods available for BV diagnosis.

Clinically, BV is usually diagnosed by physical examination, pH of vaginal discharge, whiff test, and presence of clue cells which represents the diagnostic system proposed by Amsel et al. [8] in the early 1980s, the Nugent's criterion is the method mostly used for diagnosing BV, and it is considered to be the golden standard among microscopic methods [13]. However, its score intervals are very narrow, differing in only a few bacteria, and the observed number of bacterial morphotypes may vary depending on the examiner. The homogeneity and thickness of the specimen may be influenced by the way of spreading the sample on the glass slide [14]. To avoid demanding counting of bacterial morphotypes, a qualitative microscopic examination was introduced by Ison and Hay [10] and Verhels et al. [11]. These methods give an advantage in saving the observer's time and more precise differentiation of *lactobacillus* morphotypes, but on the other hand, they examine small microscopy fields, which can influence the results (because of unequally scattered smears over the slides).

In our institutions, clinical examinations, as well as microscopy, are in routine use in the diagnosis of BV, but often there is neither sufficient time nor expertise available to practice the quantitative scoring systems. Therefore, the main goal of our study was to validate simpler grading schemes for microscopic diagnosis of BV, previously described by Nenadić et al. [15] (novel microscopy method), against the established reference method introduced by Nugent et al. [9].

Comparing the novel microscopy method with the reference method by Nugent et al. [9], we also demonstrated high sensitivity, specificity, positive and negative predictive values, and kappa indexes for the novel microscopy method. Generally, results of the present study indicated that both methods, that of Nugent et al. [9] and novel microscopy method are factually similar and nominate our qualitative assessment of the vaginal microbial flora as an alternative method in diagnosing BV.

What is more, the novel microscopy method has several advantages compared to other methods of microscopic diagnosis of BV. The principal difference between previous methods and the novel microscopy method is that previously established criteria use microscopic observation under 1,000× magnification, while the novel method is based on slide examination under 200x magnification. It is obvious that examining Gram-stained samples under 200× magnification comprises a much greater area than under 1,000× magnification. Actually, according to Nugent et al. [9] (1,000× magnification), we observe 5–20 fields from a total of 17,143 fields, while when viewed under 200×, according to novel microscopy method, we monitor 100–150 fields from a total 686 fields [15]. Besides observing a bigger surface, analysis can be done in 5–10 min, and we do not need to include burdensome counting of individual bacteria like under Nugent's criteria.

However, regardless of the microscopic method used for diagnosing BV, for an accurate diagnosis of the disease, it is necessary to evaluate the clinical aspects and clinical adequacy of diverse methods available. Besides various methods currently used, clinicians still have difficulties deciding for patients about patients with BV that should be treated. What makes this decision even more difficult are discrepancies in the classification of intermediary findings. It has been shown that the composition of intermediary flora is divided among *lactobacilli* and bacteria associated with BV, which is the main reason why the intermediary "phase" is considered the "transient phase" between the healthy vaginal microbiome and BV [20, 21]. From our study, we could indirectly assume that most of the patients with intermediary findings, according to Nugent et al. [9], actually belonged to the group with a low number of bacterial forms. A possible explanation for these "illogical" results lies in the narrow intervals in Nugent's categorization criterion. For example, in the original Nugent's criterion, counting is performed on 5–10 visual fields under the magnification 1,000x, notified as an interval on an ordinary scale (in the range from 0–1,000,000 bacteria *per* visual field). Evaluation of bacterial numbers in intervals is carried out assuming that the number of bacteria from 1–30, counted on part of the visual field, can be used for approximate bacterial number estimation on the entire visual field. What was illogical in Nugent's categorization is that patients with 4 or fewer bacterial forms were assigned as 0, 1, or 2 points, while patients with the bacterial number above 4 and above 30 were assigned with 3 and 4 points, respectively. Therefore, 0 points will be given only to those patients with the finding of 0 bacterial numbers on 5 observed visual fields. Accordingly, if we imagine a finding that is "clean" under the method by Ison and Hay [10] and if we did not find any *lactobacillus* on 5 visual fields, the

patient would receive 4 points. If we also observed *G. vaginalis* in the latter patient case, with an average number of > 4 *per* field, this finding would be assigned with 3 additional points (total of 7 points) and categorized as BV. There is a possibility in reobservation of aforementioned Gram-stained smear to count bacteria slightly different with the average number of exactly 4 bacterial forms *per* visual field. In this case, a patient will be assigned 2 points instead of 3 points and categorized as an intermediary group by Nugent's criteria. Taking all previously mentioned into consideration, we can conclude that cases with small bacterial numbers could be tricky for observers, which is furthermore complicated by narrow diagnostic criteria. Given that a low number of bacteria does not mean the absence of disorder (inflammation and potential risk of miscarriage), we should pay more attention to these groups: "clean" under methods by Ison and Hay [10], intermediary according to Nugent et al. [9], and NN and BVN under the novel microscopy method. The reasons could be various, but we will try, on practical examples, to discuss some of them.

In the repeated observation of preparations, probability of analyzing the same 10 visual fields is almost nonexistent. During the first examination, we can see small bacterial numbers and categorize patients as intermediary results. While we observe the same sample again, there is a possibility to assign preparation with 7 points (BV), which means – the lesser the cellularity, the greater the chance to misinterpret the finding through repeated observation. If we involve additional observers, the likelihood of different preparation "reading" can become even higher. It is important to stress another yet observed rule from our study: homogeneity of preparation is proportional to cellularity. For example, according to the novel microscopy method, the highest homogeneity is noted in patients with BVF and NF. According to our investigation, the probability of finding BVN areas in BVF preparations was very low (under 5%), while the possibility of finding BVN areas in BVM preparations was slightly higher (10%–15%). However, in our opinion, the Nugent's criterion has two crucial advantages concerning other diagnostic criteria: first, as we said before, it is well established and widely used criterion because of its simplicity (golden standard); and second, Nugent et al. [9] have an intermediary group (compared to Amsel et al. [8]). We cannot diminish the significance of the intermediary group without an explanation.

In accordance with presented findings from our study, we have clearly shown that tested methods are reliable in diagnosing extreme categories, either BV negative or positive, but the problem arises in the classification of the intermediary group, the most difficult to interpret. As we have shown, the difference in the percentage of IMD patients is significant (16% according to Nugent et al. [9]). Besides the fact that the largest number of disagreements was observed in IMD samples, the kappa value was not low because differences occurred between successive categories but not between extreme categories. Therefore, excluding IMD patients from our analysis, we found nearly perfect agreement between tested criteria, with very high sensitivity, specificity, positive and negative predictive values. By shifting criteria, in order to conjoin IMD findings to normal or BV, both sensitivity and specificity have been decreased, confirming that IMD samples truly represent an intermediary status between normal and BV.

These findings suggest that the Nugent's method seems to recognize a higher number of positive cases compared to other methods for scoring Gram-stained samples. Moreover, other studies indicated that, compared to the method by Amsel et al. [8], the Nugent's criterion can overestimate the real prevalence of BV and may even interpret healthy individuals to be diseased [22–24]. Nevertheless, measuring agreement between two sets of criteria usually take one set as a working definition of the disease, but it is unable to determine superiority between them because of the basic differences between these two methods. What further complicates the problem of BV diagnosis is that the Amsel's method, mainly based on clinical findings, is dichotomous, having only two categories, whereas microscopic methods allow assessment of variation in vaginal microflora as a continuum and have three or more categories. Therefore, it is of great importance to provide the best possible agreement between clinical and/or laboratory criteria and the presence of BV. In line with that, to improve clinical adequacy of BV diagnosis revised set of criteria that combines clinical and microbiological parameters is needed.

Conclusion

The novel microscopy method scoring system seems to constitute a good classification method, as it allows the microscopist to formulate an impression based on the relative numbers of RFs and NRFs morphotypes while the influence of the surface area and bacterial density are lessened. Furthermore, the novel microscopy method is flexible and can be reorganized in the way to categorize findings into only two groups: normal and BV, the fact that may have important clinical implications.

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