ORIGINAL ARTICLE

UDC: 579.61:616-078]:618.15-022.7-076
DOI: 10.2298/VSP140612065N

A novel microscopic method for analyzing Gram-stained vaginal smears in the diagnosis of disorders of vaginal microflora

Nov mikroskopski metod za analizu preparata bojenog po Gramu u dijagnozi poremećaja vaginalne mikroflore

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Abstract

Background/Aim. The Nugent's score is still the gold standard in the great majority of studies dealing with the assessment of vaginal flora and the diagnosis of bacterial vaginosis (BV). The aim of this study was to show that the analysis of Gram-stained vaginal samples under microscope at the magnification of ×200 (a novel microscopic method – NMM), as a fast and simple tool, easily applicable in everyday practice, better reflects complexity of vaginal microflora than the Nugent's methodology (×1000). **Methods.** Gram-stained vaginal smears from 394 asymptomatic pregnant women (24–28 week of pregnancy) were classified according to the Nugent's microscopic criteria (immersion, magnification ×1000). The smears were then reexamined under immersion but at magnification ×200. All samples were classified into 6 groups according to semiquanititative assessment of numbers (cellularity) and the ratio of rod (length < 1.5 μm) and small bacterial (< 1.5 μm) forms: hypercellular (normal full – NF), moderately cellular (normal mid – NM), hypocellular (normal empty – NE), bacterial vaginosis full (BVF), bacterial vaginosis mid (BVM), and bacterial vaginosis empty (BVE). Also yeasts, *coccae*, bifido and lepto bacterial forms as well polymorphonuclear (PMN) leukocytes were identified. **Results.** According to the Nugent's scoring, BV was found in 78, intermediate findings in 63, and yeasts in 48 patients. By our criteria BV was confirmed in 88 patients (37 BVF, 24 BVM, and 27 BVN). Generally, both tools proved to be highly concordant for the diagnosis of BV (Lin's concordance correlation coefficient = 0.9852). In 40% of the women mixed flora was found: yeasts in 126 (32%), *coccae* in 145 (37%), bifido forms in 32 (8%) and lepto forms in 20 (5%). Almost a half of BV patients had also yeasts (39/88). Elevated PMN numbers were found in 102 (33%) patients with normal and in 36 (41%) women with BV. **Conclusion.** The newly described methodology is simpler to apply and much better reflects diversity of vaginal microflora. In this way it may be more valuable to molecular biologists and their attempts based on quantitative polymerase chain reaction (PCR) to define formulas for molecular diagnosis of bacterial vaginosis.

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

Apstrakt

Uvod/Cilj. Nugentov skor još uvek važi za zlatni standard u velikoj većini studija o proceni vaginalne flore i dijagnozi bakterijske vaginoze (BV). Cilj ovog rada bio je da se ustanovi da je mikroskopska analiza vaginalnog brisa preparata bojenog po Gramu na uvećanju ×200, brza i jednostavna tehnika, lako primenljiva u svakodnevnoj praksi i da bolje odražava kompleksnost vaginalne flore od metodologije po Nugentu. **Metode.** Preparati bojeni po Gramu kod 394 asimptomatske trudnice (24–28 nedelja trudnoće) klasifikovani su na osnovu Nugentovih mikroskopskih kriterijuma (imerzija, uvećanje ×1000). Slajdovi su ponovo analizirani pod imerzijom na uvećanju ×200. Na osnovu polukvantitativne procene broja (celularnost) i odnosa štapićastih (dužina > 1,5 μm) i malih bakterijskih formi (< 1.5 μm) sve ispitanice bile su podeljene u 6 grupa: hipercelularni (*normal full* – NF), srednje celularan (*normal mid* – NM), hipocelularan (*normal empty* – NE), „*bacterial vaginosis full*" (BVF), „*bacterial vaginosis mid*" (BVM), i „*bacterial vaginosis empty*" (BVE). Takođe, identifikovane su gljivice, koke, bifido i lepto bakterijske forme kao i polimorfonuklearni leukociti (PMN). **Rezultati.** Na osnovu Nugentovih kriterijuma, BV nađena je kod 78 ispitivanih trudnica, intermedijerni nalaz kod 63, i gljivice kod 48. Na osnovu naših kriterijuma BV je potvrđena kod 88 ispitanica (37 BVF, 24 BVM, i 27 BVN). Generalno, oba pristupa pokazala su visoku podudarnost u dijagnozi BV (Linov koeficijent podudarnosti korelacije =

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0,9852). Kod 40% žena pronađena je mešovita flora: gljivice kod 126 (32%), koke kod 145 (37%), bifido forme kod 32 (8%) i lepto forme kod 20 (5%). Skoro polovina žena sa BV imala je, takođe, gljivice (39/88). Povećan broj PMN je nađen kod 102 (33%) trudnica sa normalnim nalazom i kod 36 (41%) trudnica sa BV. **Zaključak.** Nova metodologija jednostavnija je za primenu i mnogo bolje odražava raznolikost vaginalne mikroflore. Ovakav pristup možda bi bio korisniji molekularnim biolozima i njihovim pokušajima da na osnovu kvantitativne lančane reakcije polimeraze (*polymerase chain reaction – PCR*) dođu do molekularne „formule" za dijagnostiku BV.

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

Introduction

The Nugent's score is still the gold standard in the great majority of studies dealing with the assessment of vaginal flora and the diagnosis of bacterial vaginosis (BV) [1–8]. This being despite many, more or less obvious, shortcomings of the test: time consuming, a complicated numerical summing with narrow intervals, a need for experienced personnel, inutil in everyday practice, a need for standardizing surface of the microscopic field of view, and evaluation of only three bacterial morphotypes. In our opinion, the most important of the shortcomings is the inability to distinguish between *Lactobacillus iners* and *Gardnerella vaginalis* due to their great phenotypic resemblance (length, shape, Gram-staining properties) [9, 10]. Yet, the two bacteria make the mainstay of the Nugent's score. Not of less importance is the fact that observing 5–20 fields of view under the magnification ×1000, the actual scanned surface makes only a tiny fraction of the slide surface thus being a source of sampling error.

Over the past 2 years we have developed a novel method of viewing Gram-stained slides at magnification of ×200 in an attempt to eliminate most of the above cited drawbacks of the Nugent's score. It very simple, learning curve is steep and does not require any special microbiologic or gynecologic knowledge.

Introduction of new identification techniques (in particular, nucleic acid-based tests) independent from culture led to a true revolution in our understanding of the vaginal microflora. These studies discovered new species of bacteria, showed that the vaginal microbiome is more heterogenous and dynamic than thought previously [11–17]. Results of the Ravel's seminal study were surprising showing that lactobacilli are not prevailing bacteria in over 27% asymptomatic patients [18]. Moreover, in their cohort 97 had BV (according to Nugent's criteria) and 48% of examined women had vaginal pH > 4.5. Many questions need to be answered. First, are the women who do not have lactobacilli as a dominant flora, healthy or have asymptomatic BV? If they are healthy, then diagnosis of BV based on Nugent's criteria is frequently false, but if have asymptomatic BV, then the prevalence of this entity is much higher than we think. Second, is the acidic vaginal environment a prerequisite for a healthy vagina: what is then the value of Amsel's criteria in diagnosing BV if almost a half of asymptomatic women have pH > 4.5 [18, 19]? In this work we shall try to answer indirectly to the first question considering the value of Nugent's criteria in diagnosis of BV.

Methods

Gram-stained vaginal smears of 394 asymptomatic pregnant women (24–28 weeks of pregnancy) originally classified according to the Nugent's criteria (viewed under immersion, magnification ×1000) were reviewed and reclassified according to our new protocol (immersion, magnification ×200). The Nugent's scoring system was described previously [1]. The diameter of the image areas on our microscope (Leica DM 2000 LED, Ocular 10 × 22, Lens 100× 1, 25), was measured using a stage micrometer with a 0.01 mm interval scale (D = 0,21 mm) and the area was calculated using the formula $A = r^2 xphi = 0.35\ mm^2$. Calibrations of Nugent scoring system and counting of bacterial morphotypes were done as previously described Larson et al. [20]. In brief, score intervals 0–3 represented normal flora, 4–6 intermediate and 7–10 BV. The scoring system was based on counting three morphotypes: *Lactobacillus* spp., *Garduerella vaginalis* or *Bacteroides* (small Gram-variable rods or Gram-negative rods) and curved Gram-variable rods. As the slides were first viewed under immersion (magnification ×1000), in order to get a clear view at repeated viewing at magnification ×200, we had to put a drop of immersion oil – obviously not necessary if slides are viewed for the first time. The slides were viewed at two ends and in the middle along the shorter axis, e.g. perpendicularly to the direction of smear: 100 to 150 fields of view were viewed and it took at most 5–10 minutes. Apart from epithelial cells and above described bacterial morphotypes, polymorphonuclear leukocytes, yeasts, trichimonas, coccae, lepto forms, the degree of cytolysis, spermatozoids etc. were also identified. The shortest length still recognizable as a rod at the magnification ×200 is 1.5 µm. Based on this fact, under magnification ×200, it is easy to recognize the predominance of either rod forms (RFs, > 1.5 µm, laktobacilli) or non-rod forms (NRFs, < 1.5µm, Bactrerial Vaginosis Associated Bacteria – BVAB). The former is considered a normal finding. Numbers of RFs and NRFs were assessed semiquantitatively in this way: numerous bacteria covering most of the slide surface (in between, around and over epithelial cells – EC) were designated as "full"; bacterial forms rare or absent in between EC but found mostly around and at EC were designated as "mid"; and almost absent bacterial forms with only rare elements seen around and at EC were designated as "empty". Depending upon the ratio of RFs: NRFs, these three categories were further subdivided each into "normal" and "bacterial vaginosis" subgroups: the predominance of either RFs or NRFs, respectively. In this way all slides may be categorized into 6

groups: three normal (normal full – NF; normal mid – NM; and normal empty – NE) (Figure 1a, b, c) and three bacterial vaginosis varieties (BV – full, BVF – BV mid, BVM, and BV – empty – BVE) (Figure 1d, e, f) *Coccae* are generally Gram-positive, round, measuring 0.2 to 2 μm, but may be also Gram-negative, of irregular shape, larger but may always be distinguished from bacterial vaginosis/associated bateria (BVAB). We also had a group designated "*coccae*" into which were classified women with numerous, strongly Gram-positive, round and usually small bacteria, resembling dots, easily distinguished from BVAB (Figure 1g). Bifido forms (as in I-like group by Verhelst et al. [21]) were identified as Gram-positive forms, irregularly stained, shorter or longer, often irregular in shape with a tendency of branching with clubbed or curved endings (Figure 1h). All forms longer than 20 μm irrespective of Gram-staining were classified into the lepto forms (Figure 1i). PMN numbers were determined also semiquantitatively at ×200 during the same slide analysis and the women were divided into 4 categories: group 0 – PMN absent or much less numerous than EC; group 1 – PMN seen on more than 50% of field of view (FV) but their numbers still less than that of EC; group 2 – PMN seen on most FV and their numbers equal or higher than numbers of EC; group 3 – PMN seen on most FV and their numbers much higher than numbers of EC. The groups 0 and 1 were considered normal as to PMN number, and the other two groups were considered pathological.

We believe that our semiquantitative classification into 6 groups avoiding any intermediate group better reflects the complexity of vaginal microflora than the Nugent's criteria (Figure 1a-i).

Differences between the groups were calculated by paired *t*-test. The concordance between Nugent's and our classification systems was determined by the Lin's concordance test [22].

Results

According to the Nugent's criteria, BV was confirmed in 78 patients, 63 women had intermediate scores, and 253 patients had normal findings. At ×1000 magnification we detected yeasts in 48 women: 15 had BV, 7 were in the intermediate group and 26 had normal findings. When we used our own criteria, the diagnosis of BV was made in 88 patients, whereas normal findings were ascribed to 306 patients. Comparative results of microscopic analysis of Gram-stained specimens viewed under immersion at ×200 and ×1000 are given in Figure 2.

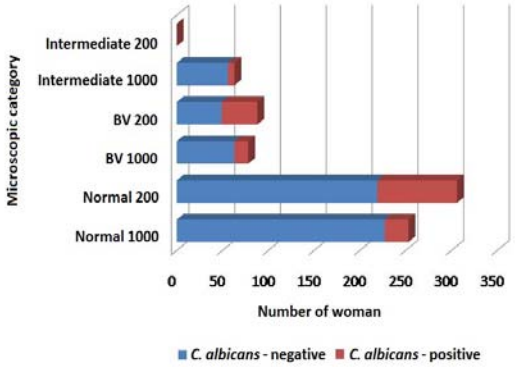

Fig. 2 – Microscopic categories of findings (BV – bacterial vaginosis)

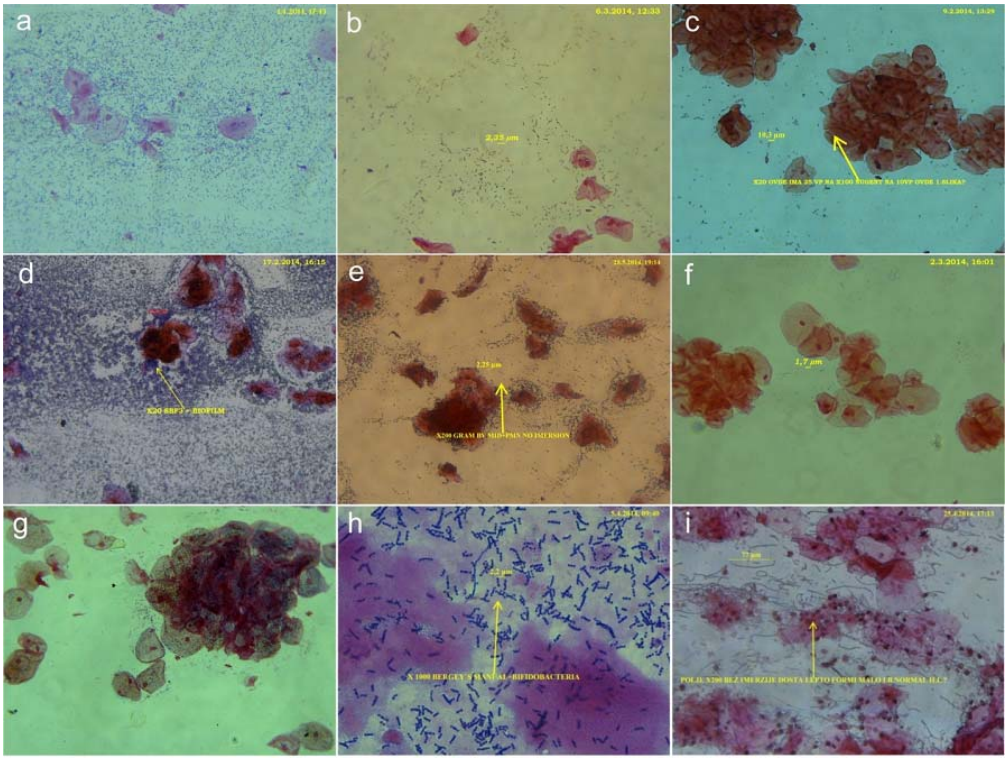

Fig. 1 – Characteristic microscopic classes of Gram-stained vaginal smears viewed under immersion with ×200 magnification:
a) normal full (NF); b) normal mid (NM); c) normal empty (NE); d) bacterial vaginosis full (BVF); e) bacterial vaginosis mid (BVM); f) bacterial vaginosis empty (BVE); g) *Coccae*; h) Bifido forms (×1000 magnification); i) Lepto forms.

When different classes of women, identified with our method of observation at ×200 magnification, were compared to diagnoses made by the Nugent's criteria, a substantial agreement was calculated by the Lin's method [concordance correlation coefficient (ρc) = 0.9852] (Figure 3).

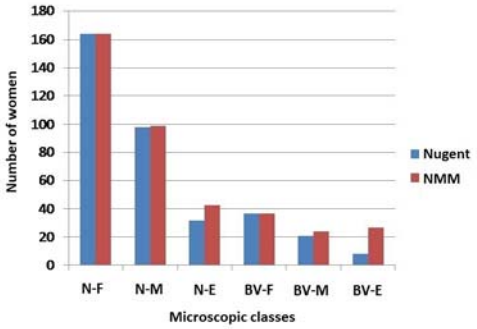

Fig. 3 – Comparison of microscopic classes.
N – normal; F – full; M – mid; E – empty; BV – bacterial vaginosis. NMM – our method.

As seen in Figure 3, the lowest concordance was found for cell-poor samples ("empty"), both normal and BV. Roughly, 40% of women (with both normal and BV findings) had mixed infections: yeasts (32%), *coccae* (37%), bifido (8%) and lepto forms (5%). The highest proportion of mixed infections was detected in cell-poor samples ("empty") (63%), and the lowest proportion in hypercellular ("full") (27%) samples with normal findings (p = 0.032) (Figure 4).

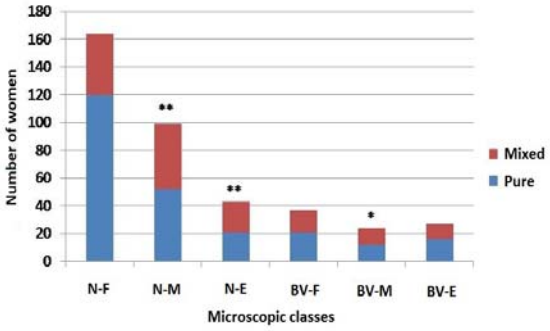

Fig. 4 – Microscopic classes of vaginal smears of women assessed by our method.
Cases of mixed infections or coinfections are presented within each group. With asterisks are denoted classes in which the ratio of mixed infections is significantly higher than in women with normal hypercellular smears (the first bar).
****p < 0.01, *p < 0.05. N – normal; BV – bacterial vaginosis; F – full; M – mid; E – empty.**

When the intermediate group identified by Nugent was analyzed across our criteria (Figure 5), it was clear that the great majority of the women belong to the hypocellular ("empty") samples but not a single to the group with a full-blown BV. As for elevated PMN numbers, there was no significant difference between the women with normal (33%) and BV samples (41%) (p = 0.205). However, significantly more PMN had women with both normal and BV moderately cellular samples ("mid") in comparison to those with normal hypercellular ("full") specimens (Figure 6). More than 50% of patients whose Gram-stained samples contained coccae, bifido and lepto forms had also elevated PMN numbers.

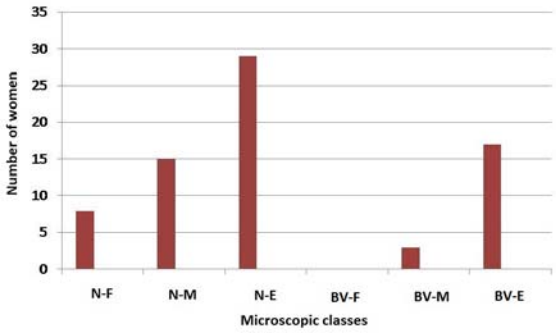

Fig. 5 – Distribution of women of the intermediate group by Nugent across microscopic classes determined by our method.
N – normal; F – full; M – mid; E – empty; BV – bacterial vaginosis.

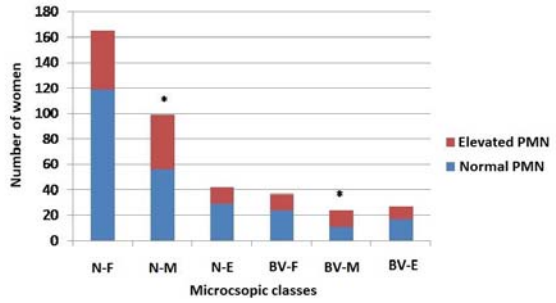

Fig. 6 – Distribution of women with normal and elevated polymorphonuclear leukocyte (PMN) numbers across microscopic classes determined by our method.
With asterisk are denoted microscopic classes with significantly higher proportion of elevated PMN numbers in comparison to normal (N) and bacterial vaginosis (BV) classes with hypercellular full (F) or paucicellular empty (E) samples.

Discussion

Our observations confirm both quantitative and qualitative variety of Gram-stained samples calling for more precise classifications based not solely on three bacterial morphotypes but extended to other important bacteria types seen under a microscope as well as PMN. Among our 88 women with BV, in 39 (44%) coinfection or mixed infection with *Candida albicans* was identified. We endorse the terms coinfection or mixed infection as proposed by Sobel et al. [23] as his definition of mixed vaginitis implies that at least two or more pathogenic processes, rather than two pathogens *per se*, coexist in the vagina, each contributing to symptoms and signs. This is very plausible as a means to distinguish between coinfections and mixed infections. But from a practi-

cal standpoint it is not much helpful as among these women we may encounter all possible combinations of clinical and laboratory signs: homogenous whitish vaginal discharge; granular, cheesy vaginal discharge; completely asymptomatic women; pruritus and burning; either positive or negative test with 10% potassium hydroxide (KOH); vaginal pH either lower or higher than 4.5; microscopy for yeasts negative but culture positive and *vice versa*. There are so many combinations making distinction between coinfection and mixed infection on these grounds a daunting task.

It is obvious that scanning Gram-stained samples at ×200 includes a much larger area than at x1000 so that things are seen differently, both quantitatively but also qualitatively. For example, more yeasts are detected if slides were viewed under ×200 according to our methodology. Further if women without BV, yeasts or *T. vaginalis* are considered "healthy", within this group we identified 66 patients with *coccae*, 30 with bifido forms and 18 with lepto forms. We feel these findings should not be ignored in future analyses. Besides much higher surface scanned, it can be done in 5–10 minutes as we do not need to include cumbersome counting of individual bacteria as in the Nugent's method. Hence, it is pretty much faster.

In hypercellular and moderately cellular samples (F and M groups) there is a rather high concordance in BV diagnosis according to the Nugent's and our criteria, 100% and 88%, respectively (Figure 2). However in cell-poor ("empty") samples for both BV and normal findings, concordance is much lower, 30% and 28%, respectively. A previous interobserver study draw attention to these patients: "however, some issues need to be looked at carefully. First, we found major discrepancies in scoring when lactobacilli morphotypes were few in number. This is of major importance in the scoring system since the score intervals are very narrow with a difference of only a few bacteria" [24]. In general, analyzing Gram-stained samples for BV with ×200 magnification is comparable to the Nugent's method with ×1000 magnification (Lin's concordance correlation coefficient 0.9852).

The most problematic are actually women with cell-poor samples where the concordance is lowest. Avoiding counting of bacterial morphotypes on a tiny fraction of a sample as in the Nugent's method may actually be helpful. It is known that two very important bacteria (*Lactobacillus iners* and *Garduerella vaginalis*) cannot be distinguished microscopically – it may be just one of the reasons to question the value of the Nugent's score as the gold standard [9–10]. Our poor-cell and, often, Nugent's intermediate group samples are characterized by conspicuous inhomogeneity of the smear on the slide. The area of our slide is 25.4 × 76.2 mm = 1935 mm^2, and if we assume that the cell smear takes up one third of the area slide (about 600 mm^2), when these samples are viewed under immersion at ×1000 we scanned 5–20 fields (Nugent) from a total of 17 143 fields (600/0.035). When viewed under ×200 according to our methodology, we scanned 100-150 fields from a total 686 fields (600/0.875), or ¼ of all stained slide surface [20]. When coupled with the smear inhomogeneity may explain a vast source of error [20]. Moreover, recent PCR assays report the heterogeneous character of intermediate flora, with some of them suggesting a molecular profile more similar to that of BV than to normal samples [25, 26]. That's why we should investigate other, more appropriate reference (gold) standards for vaginal infections.

One possible way of looking for the best microscopic criteria would be the application of Q-PCR which would in an objective way measure the presence and ration of lactobacilli and BVAB [8, 10, 25, 27–31]. Our method offers semi-quantitative assessment of bacterial forms which may be useful for comparative studies with Q-PCR. The Menard's et al. [8] study is very interesting in this regard. Q-PCR showed good agreement ($\kappa = 0.81$) and high sensitivity (100%) and specificity (93%) in relation to the Nugent's and Amsel's criteria. Yet, 10 (40%) women in this study had discordant results for the Amsel criteria and the Nugent score. The Nugent scoring system is excellent in diagnosing samples as either normal or BV, but the intermediate flora presents problems [20, 32, 33]. Vaginal smears with intermediate flora may be considered as heterogeneous flora that may include both normal and BV flora. The molecular criteria's lower positive predictive value of 73% suggests that may represent true-positives for the molecular condition of BV that were missed by traditional diagnostic tools (Nugent's and/or Amsel's criteria). The false-negatives of both standard methods reported above may support this explanation. The PCR quantification of *Gardnerella vaginalis* and *Atopobium vaginae* clearly defines a reproducible and standardized molecularly defined BV, irrespective of the clinical and microscopic characteristics of vaginal flora. As may be seen from our results (Figure 5) most women classified by Nugent into the intermediate group belong to our poor-cell ("empty") samples, both normal and BV.

It is the semiquantitive aspect of our classification of Gram-stained samples that may be more useful for better assignment of the results of Q-PCR. In particular as Q-PCR analysis has the goal to set cut-off values for densities of lactobacilli and/or BV-associated bacteria (*Garduerella vaginalis*, *Atopobium vaginae*, *Eggerthella, Prevotella*, BVAB2 and *Megasphaera* type 1) that would enable more objective and precise distinction between normal and pathological vaginal flora. It is logical that cut-off values of individual microorganisms differ in patients with different cellularity (our classes "full", "mid" and "empty"): thus prior microscopical classification may greatly assist in defining the cut-off values. If women with "hypercellular" and "hypocellular" BV are merged into one group it would be much difficult to define a reliable cut-off value distinguishing BV and non-BV flora.

Apart from bacterial morphotypes defining BV, other (*coccae*, bifido and lepto forms) probably deserve further attention. Although both groups of Ison and Hay [2] and Verhelst et al. [21] reserved a special class for *coccae*, there is not a single published study dealing specifically with these bacteria in vaginal fluid. Further, Verhelst et al. [21] emphasized the importance of atypical Gram-positive rods classified into the I-like group (bifido or corynebacterium form). Women in

this group ran a higher risk for preterm labor. Others have not studied these patients.

In our study the long (lepto) forms were defined as longer than 20 μm; they may correspond to genera *Leptotrichai amnionii, Sneathia sanguinegens, Leptothrix*, *Actinomyces* or even others? In the Bergey's manual the length of lactobacilli is cited as up to 10 μm. If we accept the fact than the percentage of lepto forms in our women would be even higher. Their potential importance is being realized only recently: Leptotrichia was found to be associated with BV and its presence correlate with clinical symptoms of BV [6]. Moreover, it produces lactic acid [6].

As we suppose that elevated PMN reflect a state of inflammation, it is of note that, although all of our patients were asymptomatic, more than 50% of women whose Gram-stained samples contained *coccae*, bifido and lepto forms had also elevated PMN numbers (Figure 6). This fact reinforces our hypothesis that studying these forms may also give us important data about vaginal microflora and its health status.

Despite considerable body of research and recent advances, BV remains an enigmatic condition. Molecular techniques have revealed the complex microbiology of BV confirming that it is most likely a syndrome caused by different communities of vaginal bacteria, i.e. a dysbiotic condition. Future studies on BV and its associated adverse outcomes should determine if specific combinations of microorganisms are associated with different adverse events [25, 27, 34, 35].

Conclusion

We would hypothesize and conclude that Gram-positive rods seen on the Gram-stained samples which we generally lump into lactobacilli, differ in length and thickness, and staining intensity; this probably reflects various rods of lactobacilli or even other bacterial species. There are many types of bacterial vaginosis depending upon the predominant bacterial vaginosis-associated bacteria: e.g. in women with prevailing *Garduerella vaginalis* there are many clue cells, whereas in patients with more abundant *Atopobium vaginae* or other bacterial vaginosis-associated bacteria clue cells are very rare or absent. There is an interesting constellation (at ×200) of numerous very short and thin rod-forms (length 1.5–2.5 μm), but at ×1000 it is clear that actually predominate non-rod forms. We suppose that this finding corresponds to a high number of *Lactobacillus iners*. If coupled with elevated PMN numbers, there is a high (70–90%) probability to encounter also *Candida albicans*. This is the commonest variety of mixed infection or coinfection (*bacterial vaginas* and *Candida albicans*); in all "borderline" cases (our poor-cell or "empty" classes) we opt for the application of probiotics and/or acidification of vagina rather than the application of antibiotics.

Future studies should check whether these groups do differ on clinical grounds, too. Moreover, it may be useful to molecular biologists to devise a "molecular formula" for the diagnosis of bacterial vaginosis based on quantitative polymerase chain reaction and the ratio of lactobacilli and bacteria associated with bacterial vaginosis.

Acknowledgement

We highly appreciate kindness of Mr. Zdenko Tojčić from GALEN-FOKUS Company who lent us the Leica microscope and thus enabled much of the results published here.

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Received on June 12, 2014.
Revised on August 12, 2014.
Accepted on August 13, 2014.
OnLine-First October, 2014.