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Diagnosis of Bacterial Vaginosis Using a Novel Molecular Real-Time PCR Test

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Abstract

Background: Bacterial vaginosis is commonly diagnosed using either Amsel's or Nugent's criteria. These diagnostic tests are somewhat subjective and there is a need for more objective and reliable tests for the diagnosis of bacterial vaginosis.

Methods: A longitudinal study was conducted at a hospital in Sweden during 2012-2013 involving 300 pregnant women seeking legal abortion was conducted at a hospital in Sweden. Bacterial vaginosis was determined to be absent or present on the basis of a modified Hay/Ison criteria assessment and compared with a molecular test analyzing six different bacteria associated with bacterial vaginosis (*A. vaginae*, BVAB2, *G. vaginalis*, *Leptotrichia/Sneathia* spp., *Megasphaera* spp., and *Mobiluncus* spp.) in relation to *Lactobacillus* spp. using real-time PCR. The Cohen kappa coefficient test was used to determine the measure of agreement between the two diagnostic tests.

Results: This study showed that there is an excellent agreement between the compared methods, with a kappa coefficient value of 0.87 (0.76–0.99). As compared to the modified Hay/Ison critera, the molecular test achieved a sensitivity of 91%, specificity of 97%, positive predictive value of 91% and negative predictive value of 97%.

Conclusions: The molecular test, using six different pathogens in an algorithm comparing their presence with that of lactobacilli, can accurately diagnose bacterial vaginosisin a clinical setting. The molecular test performed comparably to wet mount microscopy of vaginal swab samples. If the molecular test will be equally as effective when used as a "test of cure" needs to be investigated.

Trial registration: Clinical trial registration # NCT04067557, retrospectively registered.

Keywords: Vaginosis; bacterial; Diagnosis; Molecular Diagnostic Techniques; Polymerase Chain Reaction.

Abbreviations: BV: Bacterial vaginosisgestational hypertension; PE: preeclampsia; AII: angiotensin II; ASA: acetyl-salicylic acid; IVF: in-vitro fecundation

Key message: Diagnosis of bacterial vaginosis using a molecular test based on self-sampled vaginal swab is comparable to diagnosis by Hay/Ison criteria.

Background

Bacterial vaginosis (BV) is a disturbance in the bacterial flora of the vagina. It is prevalent in fertile women, occurring only rarely in post-menopausal women. BV is the most common cause of abnormal vaginal discharge and diffuse vaginal problems. The etiology is still unknown but is most certainly multifactorial [1]. BV decreases the presence of lactobacilli, consequently raising the pH in the vagina, often to a pH level between 5.5–6.0, beyond the normal healthy vaginal pH level range of 3.8–4.5.

There is no simple and easy test to diagnose BV as there is no single bacterial strain attributable to BV infection. The most commonly used method for diagnosis of BV are the Amsel criteria [2], where it is necessary to demonstrate three out of four of the following clinical criteria in order to accurately diagnose BV, including: typical homogeneous vaginal discharge, elevated pH level of the vaginal secretion (above pH 4.5), positive amine test (whiff test) and the presence of clue cells. The most notable disadvantages of Amsel's criteria is the requirement of a clinical examination of the woman and the unavoidable degree of subjectivity in the assessment. The results are, thus, not easy to replicate. Therefore, other diagnostic tests have been introduced, including another commonly used method, the Nugent score [3], in which a Gram stain is performed on the vaginal sample and is subsequently investigated under a microscope at a magnification of 1000x.The final score for the Nugent scoring system for Gram stained vaginal smears is acquired by adding the Lactobacillus content score to the Gardnerella content score. Lactobacilli are evaluated and scored 0, 1, 2, 3, or 4 based on the average number of lactobacilli observed across multiple fields, where by the presence of more than 30 lactobacilli gives a score of 0,5–30 lactobacilli gives a score of 1, 1–4 lactobacilli gives a score of 2, an average score of less than 1 lactobacilli gives a score of 3, and no lactobacilli observed per visual field gives a score of 4. *Gardnerella*-like bacteria are scored in a similar manner but in the reverse order, where the absence of *Gardnerella*-like bacteria gives a score of 0 and more than 30 *Gardnerella*-like bacteria gives a score of 4. If curved rods – *Mobiluncus* – are present the score is increased by a score of 1–2 depending on the number of *Mobiluncus* observed. The scores are then added together to obtain a final score. Thus, a score of 0–3 indicates normal lactobacilli flora, a score of 4–6 indicates intermediate levels of bacterial flora, and a score greater than 7 indicates a bacterial flora consistent with a BV diagnosis.

Nugent's method of BV diagnosis also suffers from some disadvantages. Firstly, the vaginal smears are scored by quantification of the different vaginal morphotypes, which requires an experienced laboratory technician and microscopist as well as considerable time and skill [4,5]. Secondly, the microscope area can differ by as much as 300% between different microscopes [6], eg 30 lactobacilli can be viewed in the field of view of a narrow-angle microscope compared to 90 in the field of view of a wide-angle microscope.

A simpler method was described by Hay *et al.* [7, 8]. The Hay/Ison criteria is based on the inspection of Gram stains, performed to estimate the ratio of the observed morphotypes rather than determine the number of bacteria present, where observations are divided into three grades: grade 1 (normal - many lactobacilli morphotypes—few *gardnerella* morphotypes), grade 2 (intermediate - equal numbers of lactobacilli and *gardnerella* morphotypes), and grade 3 (BV - few lactobacilli and many *gardnerella* morphotypes). As with Nugent's score this method is time-consuming and requires skilled personnel.

All of the currently used methods for the diagnosis of BV have their disadvantages. Thus, the need of faster, more cost-effective and objective methods still remains. Different molecular methods have been introduced in the diagnosis of BV [9-12]. Just recently Schwebke et al. published a large study comparing the molecular method BD MAX vaginal panel from Becton Dickinson to Amsel's criteria and Nugent's score [13]. The results are promising and show better diagnostic value than the aforementioned traditional clinical diagnostic tests.

Under routine procedure, in Sweden women scheduled for an abortion are screened for bacterial infections including *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Neisseria gonorrhoeae*, together with a vaginal sample examined for the presence of BV [14].

For women admitted into hospital for an abortion there are two major strategies for reducing postoperative infections. Patients are either given antibiotic prophylaxis [14] or are screened and treated for specific for bacterial infections in order to reduce post-abortion infections [15]. Our clinic also screens for bacterial vaginos is prior to abortions. Thus, the objective of the study is to compare the use of a modified Hay/Ison criteria applied to air-dried vaginal and rehydrated wet smears with a

new molecular test for diagnosis of BV in pregnant women.

Methods

Participants

Women were recruited to this study from a cohort comprised of pregnant women admitted into the out-patient clinic in the gynecological department of Skaraborgs Hospital in Skövde, Sweden, for a legal abortion procedure. The study was conducted over a period of four consecutive months over the course of 2012-2013, collecting vaginal samples from 300 women in total. A pelvic examination and an endovaginal ultrasound were performed and samples were taken for PCR analysis for *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Neisseria gonorrhoeae* together with two vaginal samples for the diagnosis of BV.

Microscopy

The first of the set of two vaginal samples obtained from the woman for the purpose of BV diagnosis was used for microscope analysis by first transferring the vaginal sample onto a glass microscope slide. The slide was allowed to air-dry and, after adding saline, examined under 400x magnification using a Double Binocular Carl Zeiss Axiostar Plus phase contrast microscope (Carl Zeiss AG, Oberkochen, Germany) [16]. The slides were evaluated using a modified Hay/Ison criteria [17]. The observations were divided into five grades: grade 0 (vaginal smears free of bacteria), grade 1 (normal - many lactobacilli morphotypes—few *Gardnerella* morphotypes), grade 2 (intermediate - equal numbers of lactobacilli and *Gardnerella* morphotypes), grade 3 (BV - few lactobacilli and many *Gardnerella* morphotypes), and grade 4 (large concentrations of Gram-positive cocci, i.e. *Streptococcus* spp. or Staphylococcus spp. morphotypes). For comparison the microscope results were divided into BV positive (grade 3) and non-BV (grade 1). The samples with grade 0, 2 and 4 are merged to the non-BV group (grade 1).

Molecular test

The second vaginal sample obtained from the women for the purpose of BV diagnosis was collected using FLOQSwabs™ (Copan, Brescia, Italy) and analyzed by quantitative (q) PCR for *A. vaginae*, BVAB2, *G. vaginalis*, *Lactobacillus* spp., *Leptotrichia/Sneathia* spp., *Megasphaera* spp., and *Mobiluncus* spp. The analysis was performed by Dynamic Code AB in Linköping, Sweden.

In short, DNA was extracted from the vaginal swabs using ZR-96 Quick-g DNA™ (Zymo Research, Irvine, CA, USA). The cellular material in the vaginal swab was lysed in 300µl Genomic Lysis Buffer. 50 µl of the sample was diluted further in 150 µl Genomic Lysis Buffer and disrupted in a TissueLyser (Qiagen, Hilden, Germany) for 2 min at 30 Hz, and 100 µl was subsequently applied on a Silicon-A™ Plate and washed according to the manufacturer's instructions. DNA was eluted in 30 µl Elution Buffer.

Primers and TaqMan MGB probes were designed to anneal to the rRNA gene of *A. vaginae*, *BVAB2*, *G. vaginalis*, *Lactobacillus spp*., *Leptotrichia/Sneathia spp.*, *Megasphaera spp*., and *Mobiluncus spp*. TaqMan probes were labelled with either 6-FAM, VIC or NED to enable multiplex PCR reactions. Oligonucleotides were purchased from Thermo Fisher (Foster City, CA, USA). The analysis was made in three reactions: two multiplex reactions, one for *A. vaginae, G. vaginalis* and *Leptotrichia/Sneathia* spp. and the other for BVAB2, *Megasphaera* spp., and *Mobiluncus* spp.; and one singleplex reaction for *Lactobacillus* spp., respectively. Each PCR reaction was performed in PerfeCTa® MultiPlex qPCR SuperMix (Quanta Biosciences, Inc, Gaithersburg, MD, USA) in a total volume of 15µl for each reaction. Template DNA volume used was 3 µl. Analysis was performed on an ABI Prism 7300 Sequence Detection System (Life Technologies Corp, Carlsbad, CA, USA).

Before further interpretation of the results, the individual results from the analyses of each of the bacteria associated with BV (*A. vaginae, G. vaginalis*, *Leptotrichia/Sneathia* spp., BVAB2, *Megasphaera* spp., and *Mobiluncus* spp.) were "normalized" by subtracting the C_t value for each species with the C_t value from the *Lactobacillus* spp. analysis. Each individual result was then combined to produce a final result according to an algorithm developed by Dynamic Code AB.

Statistical analyses

To assess the reliability of the molecular testin comparison to the modified Ison/Hay diagnostic test a kappa value (κ) and the associated 95% confidence intervals were calculated. Cohen's kappa value shows correlation between two methods and considers the possibility of coincidence. The calculations give a value between <0,00–1,00. Values of kappa were categorized based on the amount of agreement they suggest as follows: $\kappa > 0.75$ represents excellent agreement, $0.40 \leq \kappa \leq 0.75$ represents fair to good agreement, and $\kappa < 0.40$ represents poor agreement [18].The statistics were calculated using the Open Epi Software.

Ethics approval and consent to participate

Ethical approval for the study was obtained from the regional ethics committee (EPN) for Gothenburg (EPN reference number 658-09 entitled "Molecular biological methods to verify STD-agents") on 21 January 2010. Participants consented to participate through providing verbal informed consent before their inclusion in the study.

Clinical trail registration # NCT04067557, retrospectively registered.

Results

Vaginal swabs were collected from 300 women who had consented to participate in the study. Out of the twelve incomplete sets four lacked sufficient biological material in the wet smears, one contained too many red blood cells to be evaluated by wet smear, and seven lacked either the wet smear or PCR sample. This mean that the study was based on 288 samples that both had a wet smear and a PCR test.

In the wet smear analysis, the majority of the samples were scored as grade 1 (n=199), six were grade 0, three were grade 2 and none were grade 4. These 208 vaginal samples (72%) were merged to form the "non-BV" group. The remaining 80 vaginal samples (28%) were scored as grade 3, forming the "BV positive" group.

The molecular test was normal in 212women (74%) and indicated BV in 76 women (26%). Comparing the two methods gives a kappa coefficient value of 0.82 with a confidence interval of 0.71–0.94, and the molecular test attained a sensitivity of 0.89, a specificity of 0.94, a positive predictive value of 0.85 and a negative predictive value of 0.96 (Table 1).

		Molecular test		
		BV	Normal	
Modified Hay/ Ison	BV	68	12	80
	Normal	8	200	208
		76	212	288

Table 1. The kappa coefficient value was calculatedκ = 0.82 (0.71–0.94) between a modified Hay/Ison criteria for the diagnosis of BV compared with a molecular test using six different pathogens and an algorithm using the presence of lactobacilli. This gives a sensitivity of 0.89, (0.81-0.95) a specificity of 0.94 (0.90-0.98), positive predictive value of 0.85(0.76-0.91) and negative predictive value of 0.96 (0.93-0.98).

Twenty samples gave discordant results, where twelve samples gave a positive wet smear result for BV but obtained a negative PCR test result and eight samples gave a negative wet smear result but obtained a positive PCR test result. The wet smears of these twenty sample sets were re-evaluated by microscope. This second evaluation was made simultaneously by two gynecologists, of which one is a highly experienced diagnostician. The re-evaluation showed that of the twelve sample sets that were positive for BV according to the modified Hay/Ison criteria during the first evaluation but negative for the molecular test seven were still regarded as BV positive and five were re-classified as non-BV. Out of the eight sample sets positive for BV on the molecular tests but negative using the modified Hay/Ison criteria, one wet smear was reclassified as BV positive. After these corrections, a new kappa coefficient value of 0.87 was calculated with a confidence level of 0.76–0.99. For the molecular test, the re-calculated sensitivity was 0.91 and specificity 0.97. The re-calculated positive predictive value was 0.91 and the negative predictive value was 0.97 (Table 2). One interesting observation made during the re-evaluation of the slides was that of the twelve that originally tested positive for BV on wet smear but negative in PCR, seven contained sperm.

		Molecular test		
		BV	Normal	
Modified Hay/ Ison	BV	69	7	76
	Normal	7	205	212
		76	212	288

Table 2. After re-evaluation of the air-dried wet smears the kappa coefficient value was recalculated κ = 0.87 (0.76–0.99) between the modified Hay/Ison criteria for the diagnosis of BV and a molecular test using 6 different pathogens and an algorithm using the presence of lactobacilli. This gives a sensitivity of 0.91 (0.82-0.95), a specificity of 0.97(0.93-0.98) , positive predictive value of 0.91(0.82-0.95) and negative predictive value of 0.97 (0.93-0.98).

Discussion

Vaginal discomfort is a common issue experienced during a woman's menses. BV is an aggravating condition that doesn't necessarily require treatment to clear an infection in non-pregnant healthy women, treatment serves only to relieve discomfort. In pregnant women or women undergoing gynecological procedures, however, treatment for BV is likely to decrease the risk of premature delivery [19] or postoperative complications [20].

When examining the cause of vaginal discomfort the current recommendation is to use microscopy in conjunction with the Hay/Ison criteria for clinical diagnosis of BV [21].This method for diagnosis of BV, as with the Amsel's criteria and the Nugent's score, suffers from reliability on subjective judgments [4], questionable reproducibility, high economic costs and are heavily time-consuming methods requiring highly skilled technicians and physician consultation for sampling and, more importantly, these methods are seldom used to their full extent, except in research. There is therefore a great need for an easy and objective method to assess BV in out-patient care.

The results of our study indicate that it is safe to replace a clinical evaluation of an air-dried wet vaginal smear using the modified Hay/Ison criteria with the investigated molecular PCR test for the diagnosis of BV. The kappa coefficient value, sensitivity and specificity obtained in this study compares well with other molecular methods [9- 13). Another advantage of the molecular test is that sampling can be performed by the women themselves [12]. Thus, if the test is sent to the laboratory by the woman the result can be available at the time of the clinical appointment, which may shorten the time before treatment can commence. Replacing the diagnostic method may also have positive implications for the cost of handling this type of patient. The cost for each test must be compared to the benefits of a decreased demand for physician's examination and time, reduction in the number of late miscarriages and fewer complications associated with abortion. In general, however, performing a test of cure is also important to exclude other reasons for malodorous discharge other than BV such as trichomoniasis, cervical cancer, and candida infection.

In this study all women included were pregnant, which means they were not affected by the normal hormone level changes experienced during the reproductive cycle. It could be of great interest to study a non-pregnant female population and to evaluate if the reliability is as excellent as seen in this study. Furthermore, the molecular test may be a useful tool during treatment follow-up. Treatment of BV can be challenging with high risk for relapse after treatment [22].

One parameter to look further into is whether recent intercourse, identified as presence of sperm in the sample, can affect the result of the molecular test. In this study half (7/12) of the false negative molecular tests contained sperms in the wet smear.

Conclusion

The studied molecular test that quantifies the presence of six different pathogens and lactobacilli and calculates a single result in a algorithm has, compared with traditional BV diagnosis using the modified Hay/Ison criteria, a kappa value of 0.87,a fully acceptable result clinical practice. The molecular test could successfully replace the commonly used Amsel criteria as the criteria are too subjective. If the molecular test will be equally as effective when used as a "test of cure" needs to be investigated.

Abbreviations: **BV:** Bacterial vaginosis

Declarations

Ethical approval for the study was obtained from the regional ethics committee (EPN) for Gothenburg Box 401 405 30 Göteborg Sweden, University of Gothenburg, Box 100, SE-405 30 Gothenburg, SWEDEN. (EPN reference number 658-09 entitled "Molecular biological methods to verify STD-agents") on 21 January 2010. Participants consented to participate through providing verbal informed consent before their inclusion in the study.

Clinical trail registration # NCT04067557, retrospectively registered.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due individual privacy could be compromised but are available from the corresponding author on reasonable request.

Competing interests

P.G.L. has received speaking fees from Campus Pharma AB. This study was conducted in cooperation with Dynamic Code AB, a company with a commercial interest in marketing

a reliable molecular diagnostic test for bacterial vaginosis. They provided all the materials needed for collecting vaginal smears and molecular diagnostic testing. M.F. is employed as a Senior Scientist and A.H. is employed as a Product Developer at Dynamic Code AB. AS is Medical Advisor at Dynamic Code, K.B, I.V and J.L declare that they have no conflicts of interest.

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Authors' contributions

All authors have been involved in the writing of the article. PGL is the main author, KB and JL has been designing the study and IV has administered the study and AH and MF has also done the molecular analysis, AS developed the algorithm of the tet.

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