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Multicenter study establishing the clinical validity of a nucleic-acid amplification–based assay for the diagnosis of bacterial vaginosis

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ABSTRACT

The present study sought to validate the clinical performance of a previously described PCR-based assay for the diagnosis of bacterial vaginosis (BV). A total of 1579 patients were enrolled in 5 locations; samples were classified as BV positive (n=538) or negative (n=1,041) based on an algorithm utilizing quantitative Gram-stain analysis of vaginal discharge and clinical evaluation (Amsel criteria); a next-generation sequencing (NGS) approach to determining diversity of vaginal microbiota was used to resolve discordant results between BV-PCR and Nugent/Amsel. BV-PCR demonstrated a sensitivity of 96.0% (483/503) and a specificity of 90.2% (885/981) when measured against the conventional test standard, with 95 samples (6.0%) being classified as indeterminate. After resolution of discordant results by NGS, including elimination of the PCR indeterminate category, the resolved sensitivity, specificity, and positive and negative predictive values of the BV-PCR assay were 98.7%, 95.9%, 92.9%, and 96.9%, respectively. The results of this study conclusively demonstrate that a relatively simple, 3-biomarker, molecular amplification construct can effectively diagnose BV in symptomatic women. Results generated using this assay were congruent with those obtained using conventional and molecular reference methods.

1. Introduction

Bacterial vaginosis (BV) is reportedly the most common lower genital disorder found in reproductive-age women (Kenyon et al., 2013). This condition is characterized by a shift from a relatively homogeneous microflora dominated by lactobacilli to a highly heterogeneous state containing a complex population of anaerobic and microaerophilic organisms (Ravel et al., 2011; Srinivasan et al., 2012). Symptoms associated with presence of the characteristic BV microflora are production of a whitish-gray discharge with an unpleasant odor, accompanied by itching, burning, or pain (Eckert, 2006). Current therapeutic strategies for BV are limited and relatively ineffective; recurrence of dysbiosis and symptoms of BV occurs in up to 50% of women within 12 months (Bradshaw and Brotman, 2015). Numerous studies have reported associations between BV and clinically significant urogenital tract disorders, including upper genital tract infections, pelvic inflammatory disease, and adverse pregnancy outcomes (Leitich et al., 2003; Peipert et al., 1997; Svare et al., 2006), as well as increasing the risk of acquisition of HIV (Atashili et al., 2008), *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* (Gallo et al., 2012). It is important to note, however, that not all studies have confirmed such associations (Ness et al., 2004) and that the absence of definitive diagnostic criteria for BV continues to hamper attempts to understand the broader consequences of this poorly managed syndrome.

Diagnosis of BV has, until relatively recently, been almost entirely dependent on relatively simple and somewhat limited test methodologies. The first attempts to characterize BV were dependent on using a combination of clinical criteria and simple laboratory tests applied to vaginal samples (Amsel et al., 1983), a constellation of assessments that became known as the "Amsel criteria," of which 3 of 4 (abnormal gray discharge, pH.4.5, positive amine test, and presence of epithelial "clue" cells) are required to be positive to establish the diagnosis. This approach was superseded by the use of an assessment of the extent of microbial diversity of vaginal flora via standardized interpretation of Gram-stained preparations of vaginal samples (Nugent et al., 1991). The so-called "Nugent" score became the de facto gold standard for BV diagnosis (Schwebke et al., 1996; Workowksi and Bolan, 2015), and despite obvious limitations (Srinivasan et al., 2013), results from Nugent and Amsel scoring have remained the generally accepted standard against which novel diagnostic test methods for BV are compared (Cartwright et al., 2012; Gaydos et al., 2017).

The complexity of composition of the BV microflora has become increasingly apparent over the past decade as powerful DNA-based methods for analyzing microbial ecosystems have been applied to analyze vaginal samples from various populations (Fettweis et al., 2014; Fredricks et al., 2005; Gajer et al., 2012; Ravel et al., 2011; Srinivasan et al., 2012). These discoveries have afforded opportunities for

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developing improved methods for BV diagnosis (Cartwright et al., 2012; Fredricks et al., 2007; Gaydos et al., 2017; Shipitsyna et al., 2013; Vitali et al., 2015); one such approach, described by Cartwright et al. (2012), relies on semiquantitative, PCR-based, detection of 3 BV-associated organisms [*Atopobium vaginae*, bacterial vaginosis associated bacterium 2 (BVAB-2), and *Megasphaera*-1]. A clinical evaluation of this construct demonstrated diagnostic accuracy that was comparable to a combination of Nugent Gram-stain score and Amsel result (Cartwright et al., 2012); however, this study was conducted in a single, high–BV risk and prevalence location, with a predominantly African-American population.

The intent of the present study was to confirm the findings reported in Cartwright et al. (2012): to validate in a multicenter study, with a diverse patient enrollment, that the performance characteristics of this test support its use as an accurate and objective method for diagnosing BV in symptomatic women. A secondary goal was to assess whether determining microbiome diversity using next-generation sequencing (NGS) of vaginal swab samples could be used as an independent, unbiased reference method for assessing BV test performance.

2. Materials and methods

2.1. Study locations and subject demographics

Five locations were utilized in the present study (NS-002): 1 academic medical center [Site 001 (Alabama)], 2 primary care facilities [Site 002 (Florida) and Site 004 (Arizona)], and 2 specialist OB/GYN clinics [Site 003 (Florida) and Site 005 (Virginia)]. Based on estimated BV prevalence reported by each site prior to study commencement and assuming approximately equal subject enrollment, a BV positivity rate of 25–35% was projected. Since the sensitivity and specificity of the BV-PCR construct determined previously were 96.7% and 92.2%, respectively (Cartwright et al., 2012), to determine with 95% confidence that these parameters were within 2% of the originally reported values, an enrollment of 1200–1700 subjects was required (Buderer, 1996). A total of 1595 subjects were enrolled between August, 2016, and March, 2017, with 1579 (98.6%) successfully completing all testing. All enrollees were aged between 18 and 50, not pregnant, and presenting for evaluation of clinical symptoms consistent with vaginitis/vaginosis as determined by clinicians at individual sites. Subjects were classified as having a primary diagnosis if they had not been evaluated for vaginitis/vaginosis in the previous 28-day period and had not received antibiotics or used vaginal medications for at least 14 days prior to enrollment. Subjects were classified as having a recurrent diagnosis if they had been evaluated for symptoms consistent with vaginosis/vaginitis in the previous 28 days, received standard-of-care diagnostic evaluation and treatment, and were representing with similar or identical symptoms. Enrollment, prevalence of BV as determined by the conventional test algorithm, and key demographic data are shown in Table 1.

2.2. Sample collection and evaluation

After obtaining informed consent, a series of vaginal samples was obtained to enable comprehensive evaluation of subjects for markers of vaginosis. This sample series consisted of 2 vaginal swab samples collected in liquid Amies transport medium (Copan Diagnostics Inc., Murrieta, CA) that were utilized for Gram stain preparation and yeast culture, 1 vaginal swab sample collected in the Affirm™ VPIII transport system (Becton-Dickinson, Sparks, MD), and 2 APTIMA® vaginal swab collections (GenProbe Inc., San Diego, CA). One of the APTIMA® collections was used for performance of the BV-PCR test, plus additional NAA testing for *Trichomonas vaginalis* and *Candida* spp.; the second was retained for microbiome analysis.

Vaginal discharge was analyzed on each subject at enrollment according to Amsel criteria, with a BV-positive sample being one that had 2 of the following 3 criteria: a pH value of greater than 4.5, a positive "whiff test" ("fishy" odor upon addition of KOH), and the presence of clue cells upon microscopic examination. Assessment of the nature (color, consistency, etc.) of vaginal discharge was not included as an Amsel criterion, the subjective nature of this element of the assessment making it impossible to standardize across study locations. For reference analysis by quantitative Gram stain, duplicate smears were prepared as previously described (Cartwright et al., 2012), and 1 slide was then submitted to a single central laboratory for staining and scoring according to the Nugent criteria. In brief, this involves examining stained smears for specific bacterial morphologies and then generating a numerical Nugent score (from 0 to 10). A score of 0–3 is interpreted as normal or negative for BV; a score of 4–6 as intermediate, meaning there is some evidence of dysbiosis but insufficient to call the sample positive for BV; and a score of 7–10 as abnormal or positive for BV. If the initial slide was deemed to be of insufficient quality for analysis, according to the reference laboratory's established criteria, the second slide was stained and examined. If an accurate result could not be obtained with either slide, the subject was excluded from the study. A combined reference method using Nugent Gram stain plus Amsel was used as the comparator for the BV-PCR assay (Cartwright et al., 2012; Gaydos et al., 2017). A negative or positive Nugent score was considered definitive, samples generating intermediate scores that met the Amsel criteria for

Table 1
Study subject demographic information by sites. BV status determined by conventional testing algorithm (Nugent/Amsel).

	Site 001	Site 002	Site 003	Site 004	Site 005	Total
Enrollment	183	216	584	468	128	1579
Median age	26.7	29.1	29.6	26.4	28.8	28.4
Age range	18.4–47.7	18.2–44.9	18.1–49.3	18.2–41.0	18.0–40.8	18.0–49.3
Race/Ethnicity						
White non-Hispanic	6 (3.3%)	178 (82.4%)	113 (19.3%)	252 (53.8%)	54 (42.2%)	603 (38.2%)
White Hispanic	1 (0.5%)	14 (6.5%)	187 (32.0%)	199 (42.5%)	2 (1.6%)	403 (25.5%)
Asian	1 (0.5%)	1 (0.4%)	4 (0.7%)	7 (1.5%)	6 (4.7%)	19 (1.2%)
African-American	174 (95.2%)	21 (9.7%)	261 (44.7%)	10 (2.1%)	66 (51.5%)	532 (33.7%)
Other	1 (0.5%)	2 (0.9%)	19 (3.3%)	0 (0.0%)	0 (0.0%)	22 (1.3%)
Presentation						
Primary	168 (91.8%)	207 (95.8%)	557 (95.4%)	438 (93.6%)	105 (82.0%)	1475 (93.4%)
Recurrent	15 (8.2%)	9 (4.2%)	27 (4.6%)	30 (6.4%)	23 (18.0%)	104 (6.6%)
BV status[a]						
Positive	136	59	208	75	60	538
Negative	47	157	376	393	68	1041
BV prevalence	74.3%	27.3%	35.6%	16.0%	50.8%	34.1%

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BV were considered BV positive, and samples that failed to meet the Amsel criteria for BV were considered BV negative.

2.3. BV-PCR

BV-PCR was performed on all study samples in a single clinical laboratory, with testing completely integrated into the routine operational workflow, and results were validated according to standard operating procedures. All staff involved in testing were blinded to the identity of the study samples and to any results obtained from alternate testing methodologies. The BV-PCR assay is a semiquantitative, multiplexed construct containing real-time PCR assays specific for *Atopobium vaginae*, BVAB-2, and *Megasphaera*-1 (Cartwright et al., 2012). A numerical score is determined for each organism based on the quantity of the respective analyte in the sample; for *A. vaginae* and BVAB-2, that score can be 0, 1, or 2: for *Megasphaera*-1, only scores of 0 or 2 are possible. The sum of all 3 scores is then utilized to provide the categorical designation of the sample with respect to BV. A score of 0–1 indicates the absence of BV, a score of 3–6 indicates the presence of BV, and a score of 2 is considered indeterminate for BV.

2.4. Sequencing

NGS of the V3–V4 hypervariable region of the bacterial 16S rRNA gene was performed on selected samples to facilitate resolution of discrepancies in BV designation between BV-PCR and Nugent/Amsel. Nucleic acid was extracted from APTIMA vaginal swab collections as described previously (Cartwright et al., 2012), and an approximately 500-bp fragment of the 16S rDNA was amplified in PCR using primers 16S-314F (5'-CCTACGGGNGGCWGCAG-3') and 16S-805R (5'-GACT ACHVGGGTATCTAATCC-3'). Amplification reactions were performed under the following conditions: initial denaturation at 95 °C for 2 min, 30 cycles of amplification (95 °C for 30 s, 55 °C for 40 s, 72 °C for 60s), and a final elongation of 10 min at 72 °C. Amplification products were then prepared for paired-end Illumina sequencing using a modified procedure of a commercially available library preparation kit (KAPA Biosystems, Wilmington, MA) and custom barcodes (IDT, Coralville, IA). Libraries were purified with AMPure XP (Beckman Coulter, Brea, CA) with a bead:sample ratio of 0.8:1. Purified libraries were quantified using the KAPA Library Quant Illumina Kit (KAPA Biosystems) on a QuantStudio 7 Flex (Thermo-Fisher, Waltham, MA). Normalized and pooled libraries were then subjected to 2×300-bp sequencing on Illumina MiSeq NGS systems (Illumina Inc., San Diego, CA). Resulting FASTQ reads were trimmed and quality filtered using CLC Genomics Workbench v10.1.1 (Qiagen Inc., Germantown, MD). Minimum read filtering was set at 10,000; this resulted in 1 sample being excluded from analysis. The mean read number per sample was 74,962. OTU clustering at 97% sequence similarity, followed by taxonomy assignment using a modified Greengenes database v13.8 (McDonald et al., 2012), enhanced for discrimination of taxa present in the vaginal microflora by addition of specific accessions, was performed with the Microbial Genomics Module v2.5 (Qiagen) in CLC Genomics Workbench.

3. Results

3.1. Population-based analysis of conventional testing results

The prevalence of BV in the study population, as determined by Nugent gram stain with Amsel criteria used to resolve Nugent intermediate samples, was 34.1% (Table 1). Abnormal (BV) Nugent scores (≥7) were obtained on samples from 487 (30.8%) subjects, intermediate Nugent scores (4–6) on samples from 113 (7.2%) subjects, and normal (non-BV) Nugent scores (0–3) on samples from 979 (62.0%) subjects. Of the 113 subjects whose samples were determined to be intermediate by Nugent gram stain, 51 (45.1%) resolved as positive and 62 (54.9%) as negative based on Amsel criteria. Site-specific BV prevalence varied from a low of 16% (Site 004) to a high of 74.3% (Site 001). Analysis of the demography of enrolled subjects revealed significant differences in BV prevalence and resolution of Nugent intermediate status when cohorts were separated by race/ethnicity (Tables 2 and 3). The prevalence of BV among African-American women was significantly higher than among non-African American participants (56.2% versus 22.8%; p <0.001; OR 4.34; Table 2). In addition, both the frequency of samples with intermediate Nugent scores (10.5% versus 5.4%; Table 3) and the frequency with which positive Amsel scores were recorded on the subset of participants with intermediate Nugent score samples (58.9% versus 31.6%, Table 3) were significantly higher in African-American subjects. Eliminating subjects enrolled at Site 1 (95% African-American; 74.3% BV prevalence) from the analysis did not eradicate these differences (Table 2), confirming that site-specific bias in enrollment or evaluation was not the primary reason for observed differences in BV results between ethnic groups.

Table 2
BV status by race/ethnicity. BV status determined by conventional testing algorithm (Nugent/Amsel).

Race/ethnicity	BV status: all sites (n=1579)		BV status: excluding Site 001 (n=1396)	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
African-American	299 (56.2)	233 (43.8)	170 (47.5)	188 (52.5)
White-Hispanic	94 (23.3)	309 (66.4)	93 (23.2)	308 (76.8)
White non-Hispanic	129 (21.4)	474 (78.6)	125 (20.9)	473 (79.1)
Other	16 (39.0)	25 (61.0)	15 (38.5)	24 (61.5)
Total	538 (34.1)	1041 (65.9)	403 (28.9)	993 (71.1)

3.2. Results of BV-PCR testing

Summary results obtained using the BV-PCR test construct as compared with the conventional test algorithm are shown in Table 4. Of the 1579 samples tested, 579 (36.7%) were determined to be BV positive, 905 (57.3%) BV negative, and 95 (6.0%) BV indeterminate by PCR. Overall concordance between BV-PCR and the conventional Nugent/Amsel algorithm, after exclusion of BV-PCR indeterminate samples, was 92.2% (1368/1484). Using the Nugent/Amsel algorithm as the reference standard, the BV-PCR assay had a sensitivity of 96.0% (483/503; 95% CI: 93.9–97.6%), a specificity of 90.2% (885/981; 95% CI; 88.2–92.0%), a positive predictive value of 83.4% (95% CI: 80.6%–85.9%), and a negative predictive value of 97.8% (96.6%–98.6%). None of these parameters of assay performance were significantly different from those derived from data obtained in the primary validation study of the BV-PCR assay (NS-001; Table 4). Of samples resulted as indeterminate by BV-PCR, 60/95 (63.2%) were negative and 35/95 (36.8%) positive according to the Nugent/Amsel criteria. In the previous BV-PCR study, 21 samples (5.3%) were classified as indeterminate, of which 12 (57.1%) were negative and 9 (42.9%) positive by Nugent/Amsel. To enable comparison of these data with those generated in other studies of molecular diagnostic test algorithms for BV, results were also analyzed after elimination of the intermediate category, with samples generating a composite PCR score of 2 considered to be negative for BV. The results of that analysis are shown in Table 5.

Table 3
Frequency and resolution of Nugent GS intermediate results by race/ethnicity.

Race/ethnicity (n)	Nugent GS intermediate frequency		Resolution of Nugent GS intermediate samples[a]	
	Intermediate (%)	Neg/pos (%)	Positive (%)	Negative (%)
African-American	56 (10.5)	476 (89.5)	33 (58.9)	23 (41.1)
White-Hispanic	26 (6.5)	377 (93.5)	10 (38.5)	16 (61.5)
White non-Hispanic	28 (4.6)	575 (95.4)	6 (21.4)	22 (78.6)
Other	3 (7.3)	38 (92.7)	2 (66.7)	1(33.3)
Total	113 (7.2)	1466 (92.8)	51 (45.1)	62 (54.9)

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Table 4
Correlation of interpretive results generated by BV PCR assay analysis of study samples with consensus conventional test (Nugent/Amsel) results.

Dataset[a]	BV PCR result	BV status			Sensitivity (95% CI)	Specificity (95% CI)
		Positive	Negative	TOTAL		
NS-002	Positive	483	96	579	96.0%	90.2%
(n=1579)	Indeterminate	35	60	95	(93.9–97.6)	(88.2–92.0)
	Negative	20	885	905		
	Total	563	1016	1579		
NS-001	Positive	202	13	215	96.7%	92.2%
(n=396)	Indeterminate	9	12	21	(93.2–98.6)	(87.0–95.8)
	Negative	7	153	160		
	Total	218	178	396		

[a] Present study is designated as NS-002; original validation study as NS-001.

3.3. NGS analysis of discordant or indeterminate samples

One hundred and sixteen of the 1579 samples (7.3%) generated discordant results between Nugent/Amsel and BV-PCR testing; an additional 95 samples (6.0%) were considered as indeterminate by BV-PCR. Further analysis of these samples by NGS was undertaken to better delineate the BV status of the study subjects from whom these samples were obtained. A training set (n=100), consisting of unequivocally BV-negative (Nugent score of <2; n=50) or -positive samples (Nugent score >8; n=50), was first analyzed to determine microbiome composition by 16S-rDNA NGS. The α-diversity of each sample was then estimated by calculating the Simpson Diversity Index (SDI) (Lozupone and Knight, 2008). As expected, diversity indices were related directly to BV status (Fig. 1); receiver operator characteristic curve analysis of these data showed a strong correlation (area under the curve 0.941). An optimal α-diversity cutoff point (SDI =0.82) was established using the Youden Index method (Fluss et al., 2005), resulting in a predicted sensitivity of 100% and specificity of 85.1%. Results obtained when applying this criterion to diversity indices generated following analysis of discordant and BV-PCR indeterminate samples are shown in Table 6. When the optimal cutoff point SDI value was applied to samples yielding discordant results between the Amsel/Nugent algorithm and BV-PCR, 65.6% (63/96) of BV-PCR positive samples and 65.0% (13/20) of BV-PCR negative samples resolved as BV positive and negative, respectively. In addition, of the 95 samples resulted as indeterminate by the original BV-PCR scoring system (combined biomarker score of 2), only 9 (9.5%) were scored as BV positive based on SDI value as compared with 35 (36.8%) by Nugent/Amsel algorithm. This supported the elimination of an indeterminate designation for samples generating a PCR score of "2," with such samples being designated as BV negative. Versus a combined standard of Nugent/Amsel plus NGS-derived α-diversity score (Table 6), the BV-PCR assay exhibited a sensitivity of 98.7% (95% CI: 97.4–99.5%), specificity of 95.9% (95% CI: 94.5–97.0%), and positive predictive value of 92.9% (95% CI: 90.6–94.6%). The identity of the 17 most commonly identified taxa (identified to at least genus level for at least 150 reads) in samples subjected to 16S rDNA analysis and the frequency with which they were detected in the different samples cohorts are shown in Table 7. As expected, these data showed a strong correlation between high SDI values (BV positive) and the presence of multiple genera linked to the BV microbiotic state (i.e., *Gardnerella*, *Prevotella*, *Megasphaera*, *Sneathia*, BVAB-1/2, *Atopobium*, *Dialister*, *Parvimonas*) (Ravel et al., 2011). The converse was true of samples with low SDI values (BV negative), where the frequency of these taxa was significantly decreased (Table 7), while the frequency with which *Lactobacillus* spp. other than *L. iners* was identified was increased.

Table 5
Correlation of interpretive results generated by BV PCR assay analysis of study samples with consensus conventional test (Nugent/Amsel) results after elimination of indeterminate interpretive category.

Dataset[a]	BV PCR result	BV status			Sensitivity (95% CI)	Specificity (95% CI)
		Positive	Negative	Total		
NS-002	Positive	483	96	579	89.8%	90.8%
(n=1579)	Negative	55	945	1000	(86.9–92.2)	(88.9–92.5)
	Total	538	1041	1579		
NS-001	Positive	202	13	215	92.7%	92.7%
(n=396)	Negative	16	165	181	(88.3–95.8)	(87.8–96.1)
	Total	218	178	396		

[a] Present study is designated as NS-002; original validation study as NS-001.

4. Discussion

The results presented here conclusively demonstrate that the BV-PCR construct utilized in this study, and described previously (Cartwright et al., 2012), is a sensitive and specific approach for identifying symptomatic women with a vaginal microflora consistent with BV. A total of 1579 women, from 5 independent locations, were evaluated, and the results were entirely congruent with those obtained in a previously published study in which less than 400 women were enrolled at a single location (Table 4). Combined analysis of the quantity of the 3 specific biomarkers used in the BV-PCR assay—*Atopobium vaginae*, BVAB-2, and *Megasphaera*-1—is sufficient to produce an accurate assessment of the presence or absence of BV.

The prevalence of BV among African-American enrollees was significantly higher than either Hispanic or non-Hispanic white study subjects (Table 2). This finding has been consistently reported and appears to be independent of the method used for BV diagnosis (Koumans et al., 2007; Ravel et al., 2011). Recent analyses of the vaginal microbiome of asymptomatic women have also shown that African-American women are significantly less likely than women of European ancestry to have profiles dominated by lactobacilli, questioning the longstanding dogma that absence of these organisms is always indicative of an unhealthy microbiome (Fettweis et al., 2014). In conjunction with this finding, a significantly greater diversity of bacterial species in

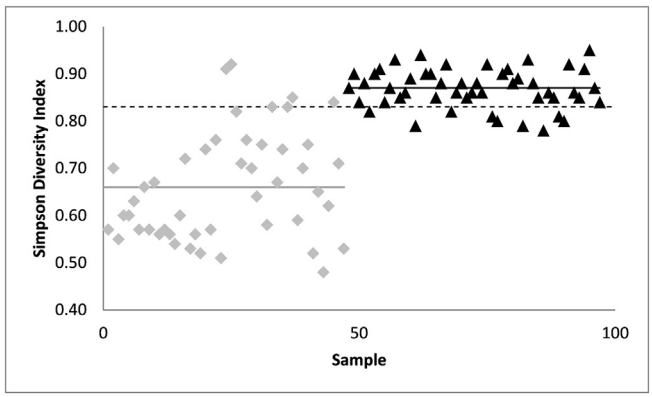

Fig. 1. Simpson Diversity Index scores generated from 16S-rDNA NGS analysis of the training sample set for BV-negative (◆) and BV-positive (▲) samples. Dashed line indicates optimal cutoff point; solid lines indicate mean SDI values for BV-negative and -positive cohorts.

Table 6
Correlation of interpretive results generated by BV PCR assay analysis of NS-002 samples after discrepancy resolution using NGS-derived α-diversity score.

BV PCR result	BV status			Sensitivity (95% CI)	Specificity (95% CI)
	Positive	Negative	Total		
Positive	546	42	579	98.7% (93.9–97.6)	95.59% (94.5–97.0)
Negative	7	984	905		
Total	562	1017	1579		

individual subjects (α-diversity), with organisms linked to BV often comprising significant proportions of the total microflora, was also reported in asymptomatic African-American women (Fettweis et al., 2014). Given this increasing evidence that ethnicity has a significant influence on composition of the vaginal microflora, there is clearly a need to confirm the performance characteristics of novel BV tests in diverse populations that mirror those encountered in routine clinical practice. In both the previous evaluation of the BV-PCR construct described here (Cartwright et al., 2012), and in a recent evaluation of a similar assay construct (Gaydos et al., 2017), populations tested were predominantly (75% and 53%) African-American; in most previous studies, subject ethnicity has not been reported (Fredricks et al., 2007; Shipitsyna et al., 2013; Vitali et al., 2015). Locations for the current investigation were selected to ensure a more ethnically balanced patient cohort than has been previously assessed and, in so doing, cover a wider spectrum of clinical settings. Study locations were also permitted to use their own standards of care for identifying patients to evaluate for vaginosis rather than being compelled to adhere to a study-specific standardized set of criteria. Finally, BV-PCR analysis was performed in a routine clinical laboratory, with study samples blinded to testing staff and incorporated into the daily workflow. The combination of the above design features made this study a significantly more rigorous examination of molecular BV assay performance than has hitherto been conducted. That the results obtained were highly consistent with those obtained in the smaller previous study (NS-001), where samples from a considerably less diverse population recruited in a single location were tested, is testament to the robustness of the BV-PCR construct and support its broad clinical applicability.

Results reported here argue strongly that addition of marker organisms beyond *Atopobium vaginae*, BVAB-2, and *Megasphaera*-1 to nucleic acid amplification assays for BV is unlikely to significantly improve the accuracy of testing while increasing the complexity and cost of the final assay construct. In the recently published study of Gaydos et al. (2017), performance characteristics of an assay including the 3 markers used in BV-PCR plus *Lactobacillus crispatus/jensenii* and *Gardnerella vaginalis* were described. The authors did not specify the relative contribution of individual markers to the overall performance of their assay; however, they reported an assay sensitivity of 90.5% (95% CI: 88.3–92.2%) and specificity of 85.8% (95% CI: 83.0–88.3%) versus an identical Nugent/Amsel reference standard to that used in the present study. The comparable parameters for the BV-PCR assay are shown in Table 5; 89.8% (95% CI: 86.9–92.2%) and 90.8% (95% CI: 88.9–92.5%), respectively. These values are not significantly different from those reported in the Gaydos et al. (2017) study and suggest that negligible probative value was added by the inclusion of *G. vaginalis* and *Lactobacillus crispatus/jensenii* to a construct containing *A. vaginae*, BVAB-2, and Megasphaera-1. A recent study by Hilbert et al. (2016) also failed to find any contribution of *Lactobacillus* spp. (they included *L. gasseri* and *L. iners* in addition to *L. crispatus* and *L. jensenii*) to the accuracy of a molecular assay for diagnosing BV. The final panel of positive markers for BV found to be most informative by Hilbert and colleagues did include both *A. vaginae* and *G. vaginalis*, however, and excluded BVAB-2 (Hilbert et al., 2016). A different gold standard for differentiating BV positive from negative samples to that used in the present study and by Gaydos et al. was used by Hilbert et al., making direct comparisons between these studies difficult, but reports such as these strongly suggest that a singular microbiome "signature" of vaginal dysbiosis consistent with BV is unlikely to exist.

The limitations of conventional test methods for diagnosing BV have been a confounder in all studies conducted thus far on nucleic acid amplification–based assays (Cartwright et al., 2012; Fettweis et al., 2014; Fredricks et al., 2005, 2007; Gajer et al., 2012; Gaydos et al., 2017; Shipitsyna et al., 2013; Srinivasan et al., 2013; Vitali et al., 2015), with the need to demonstrate concordance with Nugent Gram-stain result and Amsel score potentially leading to inaccurate assessments of the performance of constructs such as BV-PCR. In an attempt to address this problem, we used an alternate molecular approach to resolve discrepancies between the Nugent/Amsel algorithm and BV-PCR. NGS can be used to determine the relative abundance of individual microorganisms in complex microbial populations (Fettweis et al., 2014; Ravel et al., 2011; Srinivasan et al., 2012). In addition to identifying critical compositional differences between healthy and dysbiotic microflora, this method of analysis can be used to objectively determine the extent of biodiversity within a single sample (α-diversity) or between

Table 7
Frequency distribution of taxa identified in samples using 16S-rDNa sequence analysis. BV designation based on α-diversity index score.

Taxon	Frequency (% of total)				
	Training dataset		Discordant/indeterminate samples[a]		
	BV positive (*n*=50)	BV negative (*n*=43)	BV positive (*n*=74)	BV negative (*n*=137)	*P* value[b]
Gardnerella vaginalis	50 (100.0)	5 (11.6)	71 (95.9)	117 (85.4)	0.02
Megasphaera sp. 1	50 (100.0)	0 (0.0)	60 (81.1)	42 (30.7)	<0.0001
Prevotella spp.	50 (100.0)	2 (4.7)	54 (73.0)	58 (42.3)	<0.0001
Dialister spp.	50 (100.0)	2 (4.7)	43 (58.1)	46 (33.6)	0.0006
Sneathia spp.	41 (82.0)	0 (0.0)	55 (74.3)	39 (28.5)	<0.0001
BVAB1	39 (78.0)	0 (0.0)	22 (29.7)	8 (5.9)	<0.0001
BVAB2	39 (78.0)	0 (0.0)	27 (36.5)	5 (3.6)	<0.0001
Atopobium vaginae	37 (74.0)	0 (0.0)	26 (35.1)	26 (19.0)	0.01
Parvimonas spp.	36 (72.0)	0 (0.0)	24 (32.4)	10 (7.8)	<0.0001
Lactobacillus iners	23 (46.0)	32 (74.4)	60 (81.1)	84 (61.3)	0.003
Anaerococcus spp.	2 (4.0)	2 (4.7)	18 (24.3)	19 (13.9)	n/s
Lactobacillus crispatus	0 (0.0)	31 (72.1)	6 (8.1)	7 (5.1)	n/s
Lactobacillus gasseri	0 (0.0)	8 (18.6)	4 (5.4)	4 (2.9)	n/s
Lactobacillus jensenii	0 (0.0)	27 (62.8)	10 (13.5)	30 (21.9)	n/s
Lactobacillus reuteri	0 (0.0)	8 (18.6)	3 (4.1)	2 (1.5)	n/s
Lactobacillus spp.	0 (0.0)	18 (41.9)	4 (5.4)	45 (32.9)	<0.0001
Aerococcus spp.	0 (0.0)	0 (0.0)	15 (20.3)	35 (25.5)	n/s

[a] Includes samples generating discordant results between BV-PCR and Nugent/Amsel results plus samples initially assigned to indeterminate BV-PCR category.
[b] Determined for discordant/indeterminate sample set using Chi-squared test of proportions; n/s indicates not significant at 95% confidence level.

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multiple samples (β-diversity) (Lozupone and Knight, 2008). The objective of the Nugent Gram stain scoring system is to enable differentiation between the BV and non-BV state based on an assessment of the diversity and relative abundance of bacterial morphotypes found upon microscopic examination of vaginal samples. It seems reasonable to assume, therefore, that α-diversity scores generated from molecular assessments of genetic diversity within sample-specific microbiomes could be used as a less subjective and more discriminatory means of achieving the same objective (Srinivasan et al., 2013). We were able to successfully differentiate BV-positive from -negative samples using SDI as a measure of α-diversity (Fig. 1) and then to use this metric to resolve samples generating discordant results between BV-PCR and Nugent/Amsel (Table 6). The adoption of a standardized, method-agnostic, α-diversity scoring system to define dysbiotic microflora consistent with BV would appear to be a logical step forward to improve the accuracy of reference methods against which molecular diagnostic assays should be compared.

In conclusion, the present study successfully confirmed and extended the findings of our previous study describing the BV-PCR construct (Cartwright et al., 2012). In a large, diverse population of symptomatic women, the performance characteristics of this relatively simple design generated results concordant with conventional approaches to defining BV and with a novel molecular approach to identifying samples containing dysbiotic microflora. The number of genera/species identified as present as a significant fraction of the microbial community in samples from patients enrolled in this study was considerable (Table 7) and entirely expected given previous analyses of the vaginal microflora. Although it seems increasingly unlikely that there is a single definitive microbiotic "signature" in BV, we have clearly demonstrated that 3 marker organisms (*A. vaginae*, BVAB-*2*, and *Megasphaera*-1) when detected in combination above critical threshold concentrations is sufficient for an accurate determination of the presence or absence of this condition.

Disclosure

Authors C.P. Cartwright, A.J. Pherson, A.B. Harris, M.S. Clancey, and M.B. Nye are employees of Laboratory Corporation of America® Holdings, the study sponsor.

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