

## Diagnostic Evaluation of a Multiplex Quantitative Real-Time PCR Assay for Bacterial Vaginosis

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### Abstract

**Background:** Quantitative multiplex PCR assay for Bacterial Vaginosis (BV) based on the detection of the predominant contributory targets was evaluated against the conventional Nugent Score that is laborious and subjective due to morphological assessment bias of BV-associated bacteria.

**Methods:** 125 dual vaginal specimens were collected from patients aged  $\geq 18$  years at the time of presentation at the provider office to perform real time PCR and Nugent Testing. PCR assessment of BV was performed by quantitation of DNA amounts of *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp., and total amount of bacterial DNA using a multiplex RT-PCR kit (ATRiDA, Netherlands). Discordant results were resolved by the Amsel criteria or ancillary testing such as BD Affirm, when available.

**Results:** Nugent score classified 36.36% of the patients in BV and 15.45% in transitional BV categories. In contrast, the PCR method called 48.18% as BV and 12.72% as transitional BV or BV of unspecified origin categories. The overall concordance between the two methods was 81.81%. None of the BV positives by Nugent method were missed by the PCR. There were only 2 intermediates by Nugent that were called normal by PCR. PCR method was more sensitive than the Nugent and picked an additional 11% positives.

**Conclusions:** PCR based molecular BV diagnosis can standardize women health testing by removing the bias due to subjective interpretation of Nugent scoring. Our study shows that PCR method is more sensitive than conventional testing and may be a promising replacement for laborious Nugent scoring method in an era of shrinking microbiology expertise.

**Keywords:** Bacterial vaginosis; Multiplex quantitative PCR; *Gardnerella vaginalis*; *Atopobium vaginae*; *Lactobacillus* spp; Nugent graded gram stain

### Introduction

Bacterial vaginosis (BV) is a complex polymicrobial syndrome characterized by alterations of the vaginal flora with acquisition of diverse communities of anaerobic and facultative bacteria and depletion of the usually dominant *Lactobacillus* flora. BV is a cause of malodorous vaginal discharge, vulvovaginal irritation, and/or dysuria. Recurrent and/or untreated infections are associated with several obstetric and gynecologic complications, including preterm rupture of membranes, chorioamnionitis, puerperal endometritis, pelvic inflammatory disease, urinary tract infection, postoperative cellulitis, cervical dysplasia, and human immunodeficiency virus acquisition. The current diagnostic gold standard Nugent Score is based on morphological assessment of BV associated bacteria. The technique is laborious and subjective as some morphologically similar non-contributory bacteria can skew the results. As a consequence, the use of molecular techniques to detect predominant contributory targets is desirable to make the BV diagnosis. Molecular methods that solely depend on the detection of a single target such as *Gardnerella vaginalis* have limited utility due to low sensitivity and specificity [1]. Quantitative ratios of specific anaerobic microflora such as *G vaginalis* and *Atopobium vaginae* in relation to *Lactobacillus* spp. are highly sensitive and specific for the diagnosis of BV and is associated with disease recurrence. Since *A. vaginae* have advanced resistance to metronidazole, its identification can play an important role in helping to change therapy decisions [2,3].

The aim of the study was to evaluate the diagnostic value of a multiplex quantitative real-time PCR assay (AmpliSens<sup>®</sup> Florocenosis/Bacterial vaginosis-FRT PCR kit, ATRiDA, Netherlands) for Bacterial Vaginosis. A combination of Amsel criteria and Nugent Graded gram stain (used as a gold standard) was used as the reference method.

### Materials and Methods

A total of 125 patients were enrolled in a collaborative study between med fusion and 2 physician offices and Southern Methodist University (SMU), Texas. Women presenting for clinical evaluation at either the facilities between June and October 2013 were enrolled in the study. All enrollees were 18 years of age and had not received antibiotics or used vaginal medications for at least 14 days prior to enrollment. Patients were verbally consented to provide 2 additional swabs, one Aptima vaginal swab for PCR testing and second vaginal swab for preparation of smear for graded gram stain slide (NUGENT Testing). Demographic and clinical information was also collected in a de-identified manner by assigning non-traceable study number. Evaluations could not be completed for 15 enrollees; thus, results for a total of 110 patients were available for data analysis.

### Conventional diagnostic assessment

Vaginal swabs were collected and evaluated in the respective clinics according to the Amsel criteria [4]. Vaginal samples were also evaluated by Nugent Graded Gram staining at med fusion as previously described [5].

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### Molecular diagnostic assessment

DNA was extracted on an automated COBAS AmpliPrep (CAP) instrument using standard protocol for total nucleic acid using the total nucleic acid isolation kit (TNAI kit, Roche Diagnostics). Briefly, samples were lightly vortexed to dislodge the cells and 650 µl of sample was extracted and eluted in 75 µl. The amplification reaction was carried out in a total of 25 µl reaction using 10 µl of DNA extract and 15 µl of BV master mix. The BV master mix reaction mixture is prepared fresh before the test using 10 µl of PCR-mix-1-FRT Bacterial vaginosis mix and 5 µl of mixture of PCR-mix-2-FRT and polymerase. There were total of 6 controls and calibrators run with each batch. These include NTC, DNA calibrator FC1, DNA calibrator FC2, BV-NC, BV-PC and BV-SPC. The results were interpreted by the Excel-Macro created using clinical correlation with ratio coefficients (RC1, RC2 and RC3) generated for combination of targets quantified by PCR (Table 1).

Discordant results between “Nugent Score” and PCR method were resolved by the Amsel criteria or ancillary testing such as BD Affirm, when available.

### Statistical analysis

The quantitative distribution of the microorganisms in flora demonstrating BV and normal flora was analyzed using the Wilcoxon rank-sum test. Differential expression was considered to be statistically significant when  $P \leq 0.01$ . Using a dichotomous primary endpoint with 0.05% error rate and 20% incidence, we had >80% power to detect Vaginosis vs. Normal state. Further, we performed binary logistic regression analyses (SPSS statistical software, version 22) based on co-infections with other STIs such as *C trachomatis*, *N gonorrhoea* and non STIs such as yeast to evaluate the predictive value of individual targets on Vaginosis vs. Normal result. P values <0.05 after adjusting for confounders were considered significant.

### Results

Table 2 shows the demographic and clinical characteristics of the study cohort. The subjects enrolled in the study were primarily Caucasians (60.91%), followed by Hispanic (25.45%) and African Americans (9.09%). Small subsets (12.72%) of the enrollees were pregnant. Co-infections were detected in 22.72% of the enrollees, predominantly yeast followed by *Chlamydia trachomatis* and *Neisseria gonorrhoea*. Recurrent BV was recorded in 14.55% of the enrollees.

Table 3 shows the overall concordance between PCR BV assay and NUGENT Graded Gram Stain. Nugent method classified 36.36% of the patients in BV and 15.45% in transitional BV categories. In contrast, the PCR method called 48.18% as BV and 12.72% as transitional BV or BV of unspecified origin categories. The overall concordance between the two methods was 81.81%. None of the BV positives as defined by the gold standard “Nugent score” were missed by the PCR method. There were only 2 intermediates by Nugent that were called normal by PCR method. PCR based BV assay was more sensitive than the “Nugent Score” and picked an additional 10% positives. PCR based BV assay picked 7 and 5 additional cases of frank BV and 5 cases of transitional BV, which were defined as normal by NUGENT score.

Table 4 shows the overall concordance of 64.08% between Amsel criteria and NUGENT Graded Gram Stain. Amsel had low sensitivity and specificity of 61.70 and 78.57%, respectively.

Table 5 shows the predictive significance of individual targets for Normal vs. Vaginosis state after accounting for co-infections with *C. trachomatis*, *N. gonorrhoea* and yeast. The mean log copies/mL for GV,

AV, Lacto and total DNA were 2.81, 3.38, 7.22 and 7.40 for normal population vs. 7.20 ( $p < 0.0001$ ), 7.18 ( $p < 0.0001$ ), 6.52 ( $p \leq 0.01$ ) and 8.11 ( $p < 0.0001$ ), for BV population as shown in Figure 1A and Figure 1B, respectively. The values remained significant after adjusting for confounders.

### Discussion

Bacterial vaginosis (BV) is a polymicrobial disorder characterized by a shift in the vaginal flora from the dominant *Lactobacillus* spp. to a mix of curved, gram-variable anaerobes. The current gold standard for laboratory diagnosis of BV is “Nugent Score” and is based on morphological assessment of BV-associated bacteria. The technique

Log Ratios	Result	Interpretation
RC1>1	Negative	No evidence of Bacterial Vaginosis
RC1<0.5	Positive	Consistent with Bacterial Vaginosis
RC2>1 and RC3>2, RC1 can take any value	Unspecified	Consistent with vaginal flora alteration unrelated to Bacterial Vaginosis
0.5 ≤ RC1 ≤ 1	Intermediate	Consistent with transition from normal vaginal flora
Bacteria DNA is less than 10 <sup>6</sup> copies/ml	Invalid	Bacterial load insufficient for analysis. Recollect a new specimen

**Table 1:** Result interpretation by BV PCR based method. RC1 ratio coefficient is the difference between logs of concentrations *Lactobacillus* spp. (Lac) and *G vaginalis* + *A vaginae* (Gv+Av) RC1 = log (DNA Lac) – log (DNA Gv+Av) RC2 ratio coefficient is the difference between logs of concentrations of the total quantity of bacteria (Bac) and *Lactobacillus* spp. (Lac) RC2 = log (DNA Bac) – log (DNA Lac) RC3 ratio coefficient is the difference between logarithms of concentrations of bacteria (Bac) and the total quantity of anaerobic microorganisms (*G vaginalis* + *A vaginae*) RC3 = log (DNA Bac) – log (DNA Gv+Av)

Variable		N (110)	% N
Race/Ethnicity	Caucasian	67	60.91
	Hispanic	21	25.45
	African American	10	9.09
	Others	5	4.55
Pregnant		14	12.72
Recurrent BV		16	14.55
Co-infections		25	22.72
	<i>C. trachomatis</i>	4	
	<i>N. gonorrhoea</i>	1*	*Yeast co-infection
	Yeast	21	

**Table 2:** Demographic characteristics of study cohort.

Results		Molecular BV Test			Total
		Vaginosis	Intermediate / Unspecified	Normal	
Graded Gram stain	Vaginosis	40	0	0	40
	Intermediate	6	9	2	17
	Normal	7	5	41	53
	Total	53	14	43	110

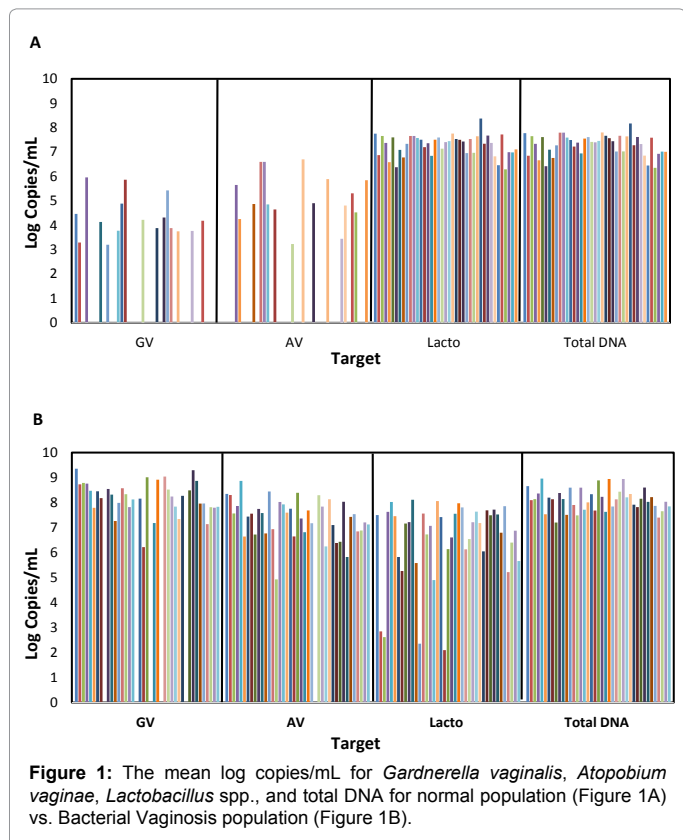
**Table 3:** Performance of BV PCR assay vs. NUGENT graded gram stain.

Results		Amsel Criteria		
		Positive	Negative	Total
Graded Gram Stain	Vaginosis/Intermediate	29	21	47
	Normal	16	44	56*
	Total	42	61	110

**Table 4:** Performance of Amsel criteria vs. NUGENT graded gram stain.

		<i>Gardnerella vaginalis</i>	<i>Atopobium vaginae</i>	<i>Lactobacillus</i> spp	Total DNA
Normal	Mean	2.81	3.38	7.22	7.40
	STDEV	2.93	3.12	0.91	0.46
Vaginosis	Mean	7.20	7.18	6.52	8.11
	STDEV	2.79	1.39	1.59	0.44
P-value		<0.001	<0.001	≤ 0.01	

**Table 5:** Predictive significance of individual targets for normal vs. vaginosis state after accounting for co-infections.



is laborious and subjective, as some morphologically similar non-contributory bacteria that can skew the results. Molecular diagnosis based on the detection of the predominant contributory targets is desirable to make a BV diagnosis.

Normal vaginal microbiome is dominated by specific *Lactobacillus* spp. and its quantification relative to *G. vaginalis* and *A. vaginae* has been shown consistently as highly specific marker for diagnosing BV [6-9]. Standard antibiotic therapy for BV offers temporary relief with about 30-40% recurrence within months. Recent literature demonstrates a close association of BV with *A. vaginae*, a metronidazole resistant anaerobe and its identification can guide changing therapy [10]. Hence, molecular techniques that can provide more objective quantitative measures of bacterial targets present in BV can aid improved diagnostics.

We demonstrate that PCR method was more sensitive in detecting BV/transitional BV as compared to NUGENT suggesting that some morphologically similar non-contributory bacteria can skew the results. The assay was specifically helpful in discriminating transitional

BV (as specified by NUGENT) from BV of unspecified origin (Vaginal imbalances contributed by other co-infections such as yeast, STDs etc.). However, we did not have sufficient N in this category to do statistical analysis.

BV is still an evolving field with much unknown about the metabolic synergies and dependencies of the bacterial communities commonly found in the vagina. We noted an overall fivefold increase in total DNA content in BV samples. This variation makes defining of stringent cut-offs for individual markers challenging. The approach of calculating clinical correlation with ratio coefficients normalizes these bacterial variations and allows better estimate of overall shift in vaginal flora. The quantitation of total DNA also served as a sample adequacy control to ensure proper sample collection.

Finally, molecular testing for BV eliminates sampling bias due to multiple collections and allows consolidation of all women health testing such as *Chlamydia*, *Gonorrhea*, *Trichomonas*, *Herpes simplex virus* and *Candida* spp. from a single convenient collection in a stable transport system.

The biggest challenge with PCR studies for BV is the inherent variations of bacterial flora by age, pregnancy, race and ethnicity. Hence, clinical correlation studies are not generalizable across all populations.

PCR based BV diagnosis can standardize women health testing by removing the bias due to subjective interpretation of Nugent scoring. Our study shows that PCR based molecular method is more sensitive than conventional testing and may be a promising replacement for laborious Nugent scoring method in an era of shrinking microbiology expertise.

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