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Toll-Like Receptor Gene Variants Associated with Bacterial Vaginosis among HIV-1 Infected Adolescents

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Abstract

Bacterial vaginosis (BV) is a common vaginal disorder in women of reproductive age, especially among women with HIV-1 infection. Several bacterial products including lipopolysaccharides (LPS), lipoteichoic acids (LTA), and peptidoglycans (PGN) are stimulatory ligands for Toll-like receptors (TLRs), and recent evidence indicates the important role of variation in *TLR* genes for permitting overgrowth of gram negative and BV-type flora. We assessed whether genetic polymorphisms in five *TLR* genes (*TLR1*, *TLR2*, *TLR4*, *TLR6*, and *TLR9*) could be determinants of differential host immune responses to BV in 159 HIV-1-positive African American adolescents enrolled in the Reaching for Excellence in Adolescent Care and Health (REACH) study. BV was assessed biannually and diagnosed either by a Nugent Score of at least 7 of 10, or using the Amsel Criteria. Cox-proportional hazards regression models, adjusted for concurrent Chlamydia and Gonorrhea infections, douching, and absolute CD4 cell count, were used to identify host genetic factors associated with BV. Two SNPs were associated with BV as diagnosed by the Nugent Score and the combined criteria: a minor allele G of rs4986790 (frequency=0.07), which encodes a His to Tyr substitution in *TLR4* (HR=1.47, 95% CI 1.15–1.87) and rs187084 (frequency=0.24) on *TLR9*. The minor allele of rs1898830 (frequency=0.13) was associated with an increased hazard of BV defined by the Amsel criteria (HR=1.86, 95% CI 1.17–2.95). Further studies are warranted to confirm the associations of *TLR* gene variants and also to understand the underlying pathways and immunogenetic correlates in the context of HIV-1 infection.

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CONFLICT OF INTEREST

NONE

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Keywords

HIV-1; Bacterial Vaginosis; Toll Like Receptors

1. INTRODUCTION

Bacterial vaginosis (BV) is a condition characterized by the replacement of indigenous vaginal *Lactobacilli* with a variety of anaerobic bacteria and mycoplasmas. It is the most prevalent (5–30%) vaginal disorder in reproductive-age women in North America (Taha et al. 1999; Koumans et al. 2007) and is associated with increased sexually transmitted infections, genital tract infections and adverse pregnancy outcomes. Bacterial vaginosis is even more likely to occur in HIV-1 infected women; the baseline prevalence of BV was 35% among HIV-1 infected women in the HIV Epidemiology Research Study (HERS) and 42% in the Women's Interagency HIV Study (WIHS) (Greenblatt et al. 1999; Jamieson et al. 2001). The high prevalence of BV in HIV-1 infected women is concerning, since the vaginal composition of the female genital tract has been shown to be an important determinant of HIV-1 acquisition and transmission during sexual intercourse (Schwebke 2001; Atashili et al. 2008).

The composition of bacterial flora is an important and established factor for overall vaginal health. However, little is known about whether BV results as a consequence or is the cause of the shift in the indigenous flora (Schwebke 2001). Studies have shown a reduction in indigenous flora to be deleterious in HIV-1 infected women as it directly correlates with an increased HIV-1 viral load and viral shedding (Sha et al. 2005; Atashili et al. 2008). BV has been found to be much more recalcitrant to antibiotic treatment in HIV-1 infected women, and treatment is ineffective in reducing HIV-1 shedding from the vaginal tract (Wang et al. 2001; Taha et al. 2007). Further, it has been reported that antibiotic treatment reduces the diversity of vaginal tract bacteria among HIV-1 infected women and rarely results in the re-establishment of healthy lactobacilli-dominated microbiota (Hummelen et al. 2010). The biological mechanisms of differential BV-related outcomes is not fully known, but the quantity and heterogeneity of BV flora in these women have been proposed to be linked with deficiency in the local immune response (Spear et al. 2007). Also, studies have shown that the occurrence of BV is higher among HIV-1 infected women, specifically with low CD4+ T-lymphocyte cell (CD4+ T-cell) counts. However, the etiology of BV remains elusive.

Toll-like receptors (TLRs) are present on several types of cells in the lower genital tract, and their recognition of a wide variety of pathogens, including bacteria, result in the induction of an antimicrobial and inflammatory cytokine response for pathogen elimination (St John et al. 2007). Several bacterial products including lipopolysaccharides (LPS), lipoteichoic acids (LTA), and peptidoglycans (PGN), are stimulatory ligands for TLRs (Spear et al. 2007). TLR4 is a ligand for LPS and TLR2 mediates responses to LTA and PGNs. Although the role of an insufficient response to BV due to TLR inhibition has not been fully elucidated, several studies suggest the important role of host immunogenetics in a successful TLR response for clearance of BV associated bacteria (St John et al. 2007). Genetic variation, particularly single nucleotide polymorphisms (SNPs) in immunoregulatory genes such as *TLR* genes, has been shown to affect host susceptibility to numerous pathogens (Schroder and Schumann 2005). In this study, we tested the hypothesis that genetic variations in *TLR1*, *TLR2*, *TLR4*, *TLR6*, and *TLR9* genes could determine differential host immune responses to BV occurrences in HIV-1 infected adolescents.

2. MATERIALS AND METHODS

2.1. Study Population and Data Collection

This study is a retrospective analysis of 159 HIV-1 infected, African-American adolescent females enrolled in the Reaching for Excellence in Adolescent Care and Health Study (REACH) cohort. The study methodology for REACH has been described in detail elsewhere (Rogers et al. 1998; Wilson et al. 2001). Briefly, REACH consisted of HIV-1 infected and comparable at risk HIV-1 negative adolescent males and females between the ages of 13 and 18 years old enrolled at 15 locations in 13 US cities between 1996 and 2000. HIV related risk factors and clinical data were collected longitudinally at quarterly intervals and other clinical and infectious disease related variables were measured biannually (Rogers et al. 1998; Wilson et al. 2001). The REACH study consisted of 75% females and 75% African Americans. To account for the roles of HIV-1 on BV occurrence and the confounding effect of ethnicity, we only examined HIV-1 infected African-American females with BV diagnosis data in this sub-study.

2.2. Detection of Bacterial Vaginosis through Amsel Criteria and Nugent Score

Gynecological examinations along with vaginal sampling and resultant smear were tested for BV at baseline and every 6 months during follow-up on all females, as previously described (Wilson et al. 2001). The Nugent score for BV diagnosis was calculated at a central laboratory from the weighted proportions of different cellular morphologies observed in gram stained smears of vaginal samples based on the following: large gram-positive rods (*Lactobacillus* morphotypes; decrease in *Lactobacillus* scored as 0 to 4), small gram-variable rods (*G. vaginalis* morphotypes; scored as 0 to 4), and curved gram-variable rods (*Mobiluncus* spp morphotypes; scored as 0 to 2) (Nugent et al. 1991). The resulting scores ranged from 0 to 10, a score of 7 and higher was considered to be indicative of BV, whereas scores of 4–6 and 3 or less were considered intermediate and normal, respectively. For the purposes of this analysis the Nugent score was dichotomized with a BV diagnosis based on a score of 7 or above. Vaginal samples were also tested for pH and the presence of clue cells on isotonic sodium chloride solution wet mounts. BV was further diagnosed using Amsel criteria in those samples based on the established clinical criteria (Amsel et al. 1983). The presence of three of the following four criteria is considered to be consistent with Amsel criteria: vaginal pH of >4.5, clue cells on saline wet mount, release of a fish amine odor on addition of 10% KOH to a drop of vaginal discharge, and a characteristic thin, homogenous vaginal discharge (Sha et al. 2005; Spear et al. 2007). No specific study guidelines regarding treatment for BV were made in the REACH cohort. All subjects with BV outcome data, based on Nugent Score or Amsel Criteria, for at least two or more follow-up visits were included in the analyses. Other potential non-genetic confounders including various STIs (gonorrhoeae, chlamydia, trichomoniasis, and HSV-2) at each of the corresponding visits were adjusted for in the analyses.

2.3. DNA extraction and genotyping of *TLR* gene polymorphisms

Processing and storage of blood samples have been described elsewhere (Rogers et al. 1998). High-molecular-weight genomic DNA extracted from PBMCs was used for the genotyping of 38 SNPs in regions of five *TLR* genes; three non-synonymous (rs4833095, rs3923647, and rs5743612) three in the UTR (rs3924111 rs5743557 and rs5743596) seven in the intronic region (rs5743562, rs5743563, rs5743567, 5743572, rs5743592, rs5743594, and rs5743595) of *TLR1*; one in the 3'UTR (rs7656411) one in the 5'UTR (rs893629) two synonymous (rs3804099 and rs3804100) and one tagging in the intron (rs1898830) of *TLR2*; one in the 3'UTR (rs11536887) two non-synonymous (rs4986790 and rs4986791) one synonymous (rs5030710), one in 3'UTR (rs1927906) and three in the 5'UTR (rs10759930, rs1927914, and rs2737191) and two located in the intronic region (rs11536869

and rs1927912) of *TLR4*, four non-synonymous (rs5743808, rs5743810, rs5743813, and rs5743815) and one in 5'UTR (rs1039559) in *TLR6*, one synonymous (rs352140) three in the 5'UTR (rs352162, rs5743849, and rs7614535) and one in the 3'UTR (rs187084) in *TLR9*. The selection of the SNPs were based on previous reports of associations with virus and bacteria related infectious disease outcomes with known frequency in African American population. The custom Golden Gate assay was performed to genotype the SNPs using the commercial Illumina platforms (Illumina, San Diego, CA).

2.4. Statistical Analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were tested for each of the 38 SNPs. Only SNPs that had a minor allele frequency of 5% or greater in our study population were considered for allelic and genotype analyses, initially using additive models. Additionally the dominant and recessive models were also considered to assess the risk of BV infection outcome. Linkage disequilibrium (r^2) between the SNPs were assessed using the Proc Allele test in SAS Genetics© and with the software program Haploview (Barrett et al. 2005). Potential non-genetic epidemiological and clinical confounding factors for BV were selected on the basis of previous literature and biological plausibility. The variables, currently pregnant, high school dropout, AIDS status and HAART use were used as reported in the last visit; however, based on multiple follow-up-visit data, a cumulative variable “ever during follow-up” was created for BV, yeast infection, gonorrhea, Chlamydia, Herpes Simplex Virus 2 (HSV-2), douching, current oral contraceptive pills (OCP) and new partner. Cox proportional hazards models were used to assess each non-genetic covariate first to evaluate the association with BV and those significant at a p-value of <0.10 were considered for inclusion in multivariate models. Final multivariate Cox proportional hazard models included only covariates that were considered to be significant contributors to BV risk *a priori* or that had a p-value<0.05. The false discovery rate (FDR) was used to account for multiple testing by calculating the q-value for all SNPs significant at p-value<0.05. The FDR provides the expected proportion of false positives among the results, and for these SNPs with significant p-values, a q-value of 0.20 was deemed significant.

3. RESULTS

Of the 159 participants, the median age was 18 [IQR:17–18] and participants were followed for a median of 3 years [IQR:2–4]. Participants had a median of 1.00 [IQR:1–3] BV infections based on the Nugent Score and a median of 0 [IQR:0–1] BV infections based on the Amsel criteria (Table 1). Compared to the Nugent Score (89.3%), the percentage of BV diagnosed based upon the Amsel criteria was much lower in our cohort (46.2%). Beginning at baseline and throughout follow-up, the percentages of STIs diagnosed varied as 61 of the participants were diagnosed with a Trichomonas infection (38.4%), 38 participants had a Gonorrhea infection during followup (23.8%), and 56 participants were diagnosed with Chlamydia infections (35.2%) (Table 1). Variables known to be commonly associated with BV were assessed in the population (Table 1) with 79.9% of participants reporting ever douching throughout the study while 69.8% of participants reported having had a new partner throughout followup.

The genotyping success rates were >98%, and while three (rs1039559, rs4986790, rs5733810) of the 38 SNPs assessed were not in HWE at p-value<0.05, none had p-values less than 0.01, so they were considered for the analyses. There were thirteen SNPs that had a minor allele frequency of less than five percent and were excluded from the analyses. None of the remaining SNPs violated the assumption of proportional hazards and also were not in Linkage Disequilibrium (LD). In the univariate analysis, three SNPs (rs352140, rs4985790, and rs5743612) were found to be significantly (p<0.05) associated with BV, diagnosis based on the Nugent Score or the combined criteria of the Nugent Score and Amsel Criteria (Table

2). One SNP (rs187084) was found to be significantly ($p < 0.05$) associated with all three types of BV diagnosis and another (rs1898830) was found to be significantly ($p < 0.05$) associated with diagnosis by the Amsel Criteria. After adjusting for co-infection with chlamydia or gonorrhea, absolute CD4 value, and douching in the multivariable analysis, four of these SNPs (rs1898830, rs4986790, rs5743612, and rs187084) were found to be significantly ($p < 0.05$) associated with BV (Table 2). Following assessment for the FDR $q < 0.20$, three of the SNPs retained significance including rs4986790 (Table 2). It is a missense mutation (Asp→Gly) in TLR4 associated with diagnosis based on the Nugent Score (HR=1.47 95% CI 1.15–1.87; $p=0.002$; $q=0.039$) and both the Nugent Score and Amsel Criteria (HR=1.38 95% CI 1.11–1.71; $p=0.003$; $q=0.045$). The other SNP that was associated with BV in more than one diagnosis category is found on TLR-9 (rs187084) and was found to be associated with diagnosis based on the Nugent Score (HR=1.52 95% CI 1.20–1.92 $p < 0.001$; $q=0.004$) and both the Nugent Score and Amsel Criteria (HR=1.52 95% CI 1.20–1.93; $p < 0.001$; $q < 0.001$). The third SNP that retained significance following assessment for FDR was one located in TLR2, rs1898830 (frequency = 0.13) and was associated with diagnosis based on the Amsel Criteria (HR=1.86 95% CI 1.17–2.95; $p=0.004$; $q=0.099$). No combinations of SNPs in the final model were significant after assessment for the FDR.

4. DISCUSSION

In the present study, we genotyped 38 SNPs in *TLR1*, *TLR2*, *TLR4*, *TLR6* and *TLR9* and examined 25 SNPs (that met the analytical criteria) for associations with BV occurrence among HIV-1 positive African American adolescents. Although BV has been reported to be more frequently diagnosed in black women (30–50%) than whites (10–20%) (Eschenbach 1993; Hillier et al. 1995; Cauci et al. 2002), to our knowledge this is the first study to investigate genetic associations with repeated BV occurrence among African Americans.

In our study, the non-synonymous SNP rs4986790 was associated with repeated BV infections as well as with an increased hazard of BV as diagnosed by either Nugent Score (HR=1.47 95% CI 1.15–1.87) or Amsel Criteria (HR=1.38 95% CI 1.11–1.71) and retained significance after assessment for the FDR $q < 0.20$. This missense mutation in TLR4 has also been found to result in a 10-fold increase in vaginal *Gardnerella vaginalis* levels ($P < 0.001$) (Genc et al. 2004). This same SNP has also been associated with pre-term birth as well as a number of clinically relevant outcomes including higher gram negative infections and gram-negative septic shock (Agnese et al. 2002; Lorenz et al. 2002a; Lorenz et al. 2002b). Colonization of *G. vaginalis* and/or gram-negative rods are significantly higher among women with homozygote AA genotype at rs4986790 (Genc et al. 2004); on the other hand AG and GG genotypes have been associated with decreased responsiveness to inhaled LPS in humans (Arbour et al. 2000). Our findings support the hypothesis that TLR4 gene is important for the response to gram negative bacteria in the vaginal microenvironment.

The other SNP that was found to be significantly associated with BV diagnosis in more than one category is on the *TLR9* gene (rs187084) (Nugent Score: HR=1.52 95% CI 1.20–1.92 $p < 0.001$; $q=0.004$ and combined criteria: HR=1.52 95% CI 1.20–1.93; $p < 0.001$; $q < 0.001$). This SNP has been found to be associated in the literature as an important factor for an adequate response to bacterial DNA stimulation and a resulting higher sepsis morbidity rate in patients with major trauma (Chen et al. 2011). Since TLR9 can distinguish between DNA sequences containing the unmethylated dinucleotide CpG, mostly present in bacteria unlike the methylated homologue in humans, it is likely that these genes are critical for first responses to foreign bacteria (Witkin et al. 2007a). We also found that a polymorphism in the promoter region of the *TLR2* gene (rs1898830) was associated with BV based on Amsel Criteria including increased pH and presence of Clue cells (HR=1.86 95% CI 1.17–2.95),

which remained significant after assessment for multiple testing with the FDR $q < 0.20$. It is likely that this SNP could be involved in mediating the response to BV since *TLR2*, the toll-like receptor 2 gene, encodes a cell surface receptor that is a component of the innate immune system. TLR2 appears to be involved in activation of various inhibitory cytokines to protect against bacterial and viral invaders including the Lyme disease bacterium (Hajishengallis and Lambris 2011). However, the same observation was not reported in another study, with a smaller sample size (Verstraeten et al. 2009). We also evaluated the contribution of TLR1, since TLRs function as heterodimers. Although rs5743612, a SNP in *TLR1* that we investigated, did not remain significant after assessment for FDR $q < 0.20$ (rs5743612 HR=1.56 95% CI 1.02–2.37) it is possible that our findings were limited by the small sample size.

Our study is limited by its small sample size although its longitudinal nature and availability of many variables traditionally associated with BV help to strengthen the findings. Additionally there was limited treatment information for BV available in the cohort although the length of time between visits and completeness of the data for BV strengthen the findings. A lower percentage of participants were diagnosed with BV based upon the Amsel criteria (46.2%) than the Nugent Score (89.3%). This difference could be the result of many factors including both the lower specificity of the Nugent Score compared to the Amsel Criteria (83% versus 94%) as well as the lower sensitivity of the Amsel Criteria compared to the Nugent Criteria (70% versus 89%) (Schwebke et al. 1996). The hypothesis that women with healthy vaginal communities are always colonized with high numbers of lactobacilli has been disputed (Forney et al. 2006; Brotman 2011). Recent molecular studies have demonstrated that the vaginal microbiome is more diverse than previously thought among different ethnicities and races (Culhane et al. 2006; Ravel et al. 2011). Ravel et al. reported differences in species composition of vaginal bacterial communities among 396 asymptomatic North American women from four ethnic groups. Although a better understanding of normal and healthy vaginal ecosystems should be based on understanding of their true function and not simply on their composition (Ma et al. 2012), the current gold standard in BV diagnosis is still the gram stain which comprise the Nugent Criteria (Workowski and Berman 2010). We limited our genetic study to African Americans, which accounted for the diversity in vaginal microbiome and also the population stratification by potential diversity in genetic background in mixed populations.

Additionally, the prevalence of BV is even higher among HIV-1 positive women (Greenblatt et al. 1999; Jamieson et al. 2001). The Nugent Criteria has been demonstrated to be an important predictor of interaction between BV associated microbiota and HIV shedding, indicating an increased risk for transmission of HIV by women with BV (Sha et al. 2005; Coleman et al. 2007; Cohen et al. 2012). Though the clinical relationship between BV as diagnosed through the Nugent Score compared to the Amsel Criteria is becoming less well understood, clinically, the validation in HIV-1-positive adolescents is significant since BV has been shown to enhance vaginal HIV-1 replication and transmission (Schwebke 2001; Sha et al. 2005; Atashili et al. 2008; Cohen et al. 2012). A recent large prospective study (Cohen et al. 2012) of 2,236 HIV-1-seropositive women and their HIV-1 uninfected male partners in Africa found that BV diagnosed by the Nugent Score was independently associated with a greater than 3-fold increased risk of female-to male HIV-1 transmission (adjusted hazard ratio 3.17, 95% CI 1.37–7.33). A better understanding of innate immunogenetic risk factors as well as what constitutes an abnormal vaginal microbiome could result in a more targeted approach in HIV-1-positive women who have a disproportionately high burden of BV disease.

Potential pathways and the accumulation of unsaturated fatty acids in the vagina by action of BV associated bacteria could result in the blockage of local TLR activation (specifically

TLR2 and TLR4) and further hinder development of an antimicrobial immune response (Witkin et al. 2007b). Additionally, bacteria can inactivate TLRs through induction of immunosuppressive anti-inflammatory cytokines such as IL-10 and through direct inhibition of pathogen associated molecular pattern (PAMP)-TLR interaction (St John et al. 2007; Witkin et al. 2007b). In studies involving relatively healthy HIV-1 infected women, vaginal pro-inflammatory cytokine concentrations were similar to those in uninfected women, suggesting that BV, not HIV-1 infections may be the predominant factor influencing vaginal pro-inflammatory cytokine concentrations (Mitchell et al. 2008). Understanding how differences in *TLR* polymorphisms can translate to reproductive tract immune response is critical for understanding the differential effect in the occurrence and persistence of BV and other microbiota.

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Table 1

Follow-up Characteristics of 159 African American HIV-1 Positive Participants in the Study Examining the Occurrence of Bacterial Vaginosis

Study Population Characteristics		Median	(IQR)
Median Age at entry (yrs)		17.6	(16.7 – 18.3)
Median CD4 Count (cells/mm ³)		524.5	(364.0 – 712.5)
Median Log Viral Load		3.74	(3.15 – 4.30)
Median Follow-up Time (yrs)		3.0	(2.0 – 3.9)
		N	(%)
Bacterial Vaginosis from Nugent Criteria ^a	Yes	142	89.3
Bacterial Vaginosis from Amsel Criteria ^a	Yes	73	45.9
Yeast Infection ^a	Yes	71	44.6
Chlamydia ^a	Yes	56	35.2
Gonorrhea ^a	Yes	38	23.9
HSV-2 ^a	Yes	18	11.3
Douche ^a	Yes	127	79.9
Current OCP use ^a	Yes	114	71.7
New Partner ^a	Yes	111	69.8
Currently Pregnant ^b	Yes	53	33.3
High School Dropout ^b	Yes	37	23.3
AIDS ^b	Yes	4	2.5
HAART Ever ^{b,c}	Yes	78	49.4

Note: Data presented in table as number (%) of those answering yes to ever diagnosed throughout followup beginning at baseline

^a Ever reporting by subject or from diagnosis baseline through last visit

^b Reported behavior or diagnosis at last visit

^c HAART refers to those participants that are HIV-1 seropositive and began therapy

REACH, Reaching for Excellence in Adolescent Healthcare; SNP, Single Nucleotide Polymorphism; IQR, Interquartilerange; HSV, Herpes Simplex Virus; OCP, oral contraceptive pills; AIDS, Acquired Immune Deficiency Syndrome; HAART, Highly Active antiretroviral therapy.

Table 2
Single nucleotide polymorphisms (SNPs) in TLR Genes Associated with Bacterial Vaginosis (BV) using Nugent Score and Amsel Criteria

Gene/SNP	MAF ^a	Amino Acid Change	Hazard Ratio based on the Nugent Score (95% CI)		Hazard Ratio based on the Amsel Criteria (95% CI)		Hazard Ratio based on the combined ^c criteria (95% CI)	
			Unadjusted	Adjusted ^d	Unadjusted	Adjusted ^d	Unadjusted	Adjusted ^d
TLR1								
rs5743612 (C/t)	0.08	His → Tyr	1.69 (1.16–2.26)	1.56 (1.02–2.37)	1.42 (0.84–2.38)	1.21 (0.67–2.19)	1.76 (1.21–2.55)	1.21 (0.67–2.19)
TLR2								
rs1898830 ^b (A/g)	0.13	intron	1.67 (0.85–1.60)	1.16 (0.84–1.61)	1.79 (1.45–2.79)	1.86 (1.17–2.95)	1.20 (0.87–1.65)	1.19 (0.85–1.66)
TLR4								
rs4986790 ^b (A/g)	0.07	Asp → Gly	1.46 (1.12–1.91)	1.47 (1.15–1.87)	1.12 (0.72–1.75)	0.48 (0.72–2.00)	1.34 (1.08–1.65)	1.38 (1.11–1.71)
TLR9								
rs187084 ^b (C/t)	0.24	promoter	1.50 (1.20–2.90)	1.52 (1.20–1.92)	1.50 (1.04–2.16)	1.34 (0.93–1.93)	1.60 (1.26–1.96)	1.52 (1.20–1.93)
rs352140 (C/t)	0.33	Pro → Pro	1.24 (1.03–1.50)	1.94 (0.98–1.45)	1.14 (0.81–1.60)	1.04 (0.74–1.43)	1.24 (1.01–1.51)	1.19 (0.98–1.46)

^aMajor allele <minor allele; MAF, minor allele frequency

^bRetained significance after assessment for False Discovery Rate (FDR) $q < 0.20$

^cCombined criteria refers to those with both BV as diagnosed by the Nugent Score and Amsel Criteria

^dHazard Ratio after adjustment for douching, Absolute CD4 Cell Count, and having either Chlamydia or Gonorrhea as diagnosed by LCR in urine.