Utility of Amsel Criteria, Nugent Score, and Quantitative PCR for *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Lactobacillus* spp. for Diagnosis of Bacterial Vaginosis in Human Immunodeficiency Virus-Infected Women

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Bacterial vaginosis (BV) is a clinical syndrome presenting with a malodorous vaginal discharge and increased vaginal pH. Diagnosis has been based on clinical Amsel criteria and direct Gram stain of vaginal secretions. Human immunodeficiency virus (HIV)-infected participants in the Women's Interagency HIV Study contributed cervicovaginal lavage (CVL) samples. Lactobacilli, *Gardnerella vaginalis*, and *Mycoplasma hominis* in cervicovaginal lavage samples were quantified by PCR. Gynecologic evaluation included Nugent score and Amsel criterion assessment. We compared the gold standard Nugent score to Amsel criteria and quantitative bacterial PCR for diagnosing BV in 203 CVL samples from women with Nugent scores of 7 to 10 (BV group) and 203 samples from women with BV Nugent scores of 0 to 3 ("No-BV" group). Only 75 of the 203 CVL samples from women with Nugent scores of 7 to 10 met positive Amsel criteria. Increasing levels of *G. vaginalis* and *M. hominis* and decreasing levels of lactobacilli were significantly associated with BV by Nugent score. Of the group with Nugent scores of 7 to 10, 83% and 81% had log₁₀ *G. vaginalis* counts and log₁₀ *M. hominis* counts greater than 6.81 and 4.82, respectively, while only 30% and 31% of the group with Nugent scores of 0 to 3 were above these thresholds, respectively. There was significant overlap in the log₁₀ lactobacillus counts between the two groups. Utilizing all three log₁₀ bacterial counts (*G. vaginalis*, *M. hominis*, and lactobacilli) in our model improved the sensitivity and specificity to 83% and 78%, respectively, in comparison with Nugent score. In this cohort, Amsel criteria were poorly predictive of BV. PCR quantification of *G. vaginalis* and *M. hominis* from CVL is significantly more sensitive than Amsel criteria for diagnosing BV.

In women of childbearing age, bacterial vaginosis (BV) is the most common cause of vaginitis. In BV, normal vaginal flora, consisting of hydrogen peroxide-producing lactobacilli, are replaced by *Gardnerella vaginalis*, *Mycoplasma hominis*, *Mobiluncus* species, and anaerobic gram-negative rods (14). BV has been associated with preterm delivery, chorioamnionitis, post-abortion infection, and pelvic inflammatory disease. In 1983, Amsel et al. established clinical criteria for diagnosing BV. The presence of three of the following four criteria is considered to be consistent with the presence of BV: 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 (1). In 1991, Nugent et al. described a Gram stain scoring system of vaginal smears to diagnose BV (10). The Nugent score is calculated by assessing for the presence of 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) and can range from 0 to 10. A score of 7 to 10 is consistent with BV.

Compared to the Amsel criteria, the Nugent score allows for assessment of alteration in vaginal flora as a continuum rather than a dichotomy. Vaginal cultures for *G. vaginalis* are sensitive but not specific, as 50 to 60% of healthy asymptomatic women will be culture positive (14). Because Amsel criteria are dependent on the acumen of the clinician, the Nugent score has been favored for diagnosing BV due to superior reproducibility and sensitivity (9). Nevertheless evaluation of smears is also subjective and therefore requires an experienced slide reader (10).

Recently we reported on a PCR method to quantify *M. hominis*, *G. vaginalis*, and lactobacilli in cervicovaginal lavage (CVL) samples obtained from human immunodeficiency virus (HIV)-infected women and were able to demonstrate that *M. hominis* and *G. vaginalis* bacterial counts were significantly increased and lactobacillus bacterial counts were significantly decreased in the presence of BV (13, 16).

To further explore the relationships between BV diagnosed by Amsel criteria and Nugent score and that diagnosed by quantitative PCR for *G. vaginalis*, *M. hominis*, and lactobacilli, we analyzed 406 CVL samples from the Women's Interagency HIV Study (WIHS) repository.

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MATERIALS AND METHODS

Patient cohort. Four hundred six CVL samples obtained from 362 HIV-infected women were selected from the WIHS repository. WIHS is a longitudinal, multicenter cohort study of HIV-infected and high-risk uninfected women followed at six clinical sites in the United States. Following institutional review board approval, 2,059 HIV-infected women were enrolled from October 1994 through November 1995. Informed consent was obtained from all subjects, and human experimentation guidelines of the U.S. Department of Health and Human Services and/or those of the investigators' institutions were followed. A detailed interview, physical and gynecologic examination, and laboratory monitoring are performed every 6 months (3). A detailed description of the women from whom the 406 CVL samples were obtained has been previously published (13).

Gynecologic examination included assessment for genital tract infections and genital tract dysplasia as previously described (6). Women were scheduled for visits when they were not menstruating and were instructed to avoid inserting any substance into the genital tract for 48 h before their examination. Samples were collected in the following order: vaginal pH; vaginal swabs for Gram stain, wet mount, and KOH with amine odor test; CVL collection; and Pap smear. Bacterial vaginosis was assessed by Amsel criteria (1) and Nugent score (central reading at the laboratory of Sharon Hillier, University of Pittsburgh-Magee Women's Hospital) (10). We required the presence of at least three of the four Amsel criteria for the diagnosis of BV by Amsel criteria.

CVL collection and processing. CVL samples were collected by irrigation of the cervix with 10 ml of nonbacteriostatic sterile saline followed by aspiration from the posterior fornix. CVL was held on ice until being processed within 6 hours of collection. CVL was gently vortexed to evenly distribute cells before aliquoting and freezing at -70°C . Two hundred three CVL samples from women with Nugent scores of 7 to 10 (bacterial vaginosis group) and 203 CVL samples from women with Nugent scores of 0 to 3 (non-bacterial vaginosis group) in 1-ml aliquots were selected for analysis. Paired CVL samples from the two groups were each matched by plasma HIV RNA levels within $0.3 \log_{10}$ and by site and visit number (visits 2 to 5).

Bacteria. *Mycoplasma hominis* and *Gardnerella vaginalis* were both obtained from the American Type Culture Collection (Manassas, Va.), and *Lactobacillus crispatus* was kindly provided by Lin Tao (University of Illinois at Chicago Dental School). Bacteria were grown as previously described (16).

DNA isolation and real-time PCR. DNA was isolated, and real-time PCR was performed as previously described (16). Briefly, bacteria obtained by centrifugation of 1 ml of CVL were disrupted by treatment with lysis buffer. DNA was purified using phenol-chloroform. DNA (50 ng) was mixed with MgCl_2 , deoxynucleoside triphosphates, primers, uracil-DNA *N*-glycosylase, and Taq polymerase (Applied Biosystems, Foster City, CA). Amplification used an ABI Prism 5700 Thermocycler (Applied Biosystems, Foster City, CA). Primer sequences were as follows: for *M. hominis*, F-RNAHI, 5'-CAATGGCTAATGCCGGATA CGC-3', and R-RNAH2, 5'-GGTACCGTCAGTCTGCAAT-3'; for *G. vaginalis*, F-GVI, 5'-TTACTGGTGTATCACTGTAAGG-3', and R-GV3, 5'-CCGTC ACAGGCTGAACAGT-3' (16). The *Lactobacillus* primer sequences were designed to detect both *L. jensenii* and *L. crispatus*, the two most common *Lactobacillus* species in the female genital tract (F-LBF, 5'-ATGGAAGAACA CCAGTGGCG-3'; R-LBR, 5'-CAGCACTGAGAGGCGGAAC-3') (7, 16). All three primer sets resulted in amplification of DNA from the appropriate type of bacteria but showed no cross-reactivity to the other two organisms or any of a panel of 16 common genital tract bacteria (16). In each thermocycler run, six standards consisting of 10^3 to 10^7 copies of DNA from the appropriate bacterium were included to generate a standard curve (16). CVL sample DNAs that resulted in values higher than the standard curve were diluted and rerun. The bacterial counts represent the total amount in 1 ml of CVL. This was obtained by determining the total DNA isolated from the CVL samples times the number of bacteria in 50 ng DNA.

Statistical methods. Descriptive statistics for the three bacterial counts were computed. SAS 8.1 (Cary, NC) software was used for all the statistical analyses. The associations between BV by Nugent score criterion and the quantitative bacterial PCR counts were analyzed by the logistic regression models (SAS PROC LOGISTIC) with the subject's BV status as the outcome and one or more quantitative bacterial PCR counts as predictors. Cut points for the bacterial counts to predict BV versus its absence ("No-BV") were determined according to whether the predictive probability obtained from the fit of the logistic model was less or greater than 0.5. The cut points were used to reclassify the observed samples based on their bacterial counts, and the sensitivity and specificity were computed. Receiver operating characteristic (ROC) curves were generated using different combinations of quantitative bacterial PCR counts to define BV. In our

FIG. 1. Box plot diagram showing the distribution of $\log_{10}$ bacterial counts for each bacterial species, *Gardnerella vaginalis* (G vag), *Mycoplasma hominis* (M hom), and *Lactobacillus* spp. (Lacto) by Nugent score. The cut points identified for *G. vaginalis*, *M. hominis*, and *Lactobacillus* spp. that differentiate the BV group from the No-BV group are denoted by a dashed line.

analyses we controlled for plasma viral load to account for possible selection bias because, in the sampling design, our CVL samples were matched between the BV group and the No-BV group by plasma viral load.

Logistic regression models with BV status by Nugent score as outcome and Amsel criteria alone or Amsel criteria plus the bacterial counts as predictors were also fitted to the data. Similarly, the plasma viral load was adjusted in the analyses. Sensitivity and specificity were estimated.

Since 362 subjects contributed the 406 CVL samples, 12% of the samples were repeated measures. Because the number of subjects contributing repeated measures was relatively small, and because the repeated measures were separated, in general, by ≥ 6 months, the possible correlations among repeated measures were ignored in the analysis for simplicity. We also performed the same analyses on data that excluded the 12% repeated measures.

RESULTS

The mean $\log_{10}$ bacterial counts/ml CVL for *G. vaginalis*, *M. hominis*, and lactobacilli are 7.6, 4.57, and 8.17, respectively, for the BV group and 5.65, 3.8, and 8.8, respectively, for the No-BV group (Fig. 1). The Pearson correlation coefficient between BV by Nugent score and levels of *G. vaginalis*, *M. hominis*, and lactobacilli are 0.38, 0.47, and -0.24 , respectively.

Using Nugent score as the gold standard for diagnosing BV, we assessed the sensitivity and specificity of the Amsel criteria. Seventy-five of the 203 CVL samples from women with Nugent scores of 7 to 10 and two of the 203 CVL samples from women with Nugent scores of 0 to 3 met positive Amsel criteria (sensitivity = 37%, specificity = 99%). Because of the poor sensitivity of Amsel criteria in this population, we analyzed the performance of each of the individual components of the Amsel criteria compared to Nugent score. Vaginal pH of >4.5 improved the sensitivity of diagnosing BV at the expense of specificity compared to the other three components of the Amsel criteria (Table 1).

For the 406 CVL samples, BV by Nugent score and BV by

TABLE 1. Sensitivities and specificities of Amsel criteria and individual components in comparison to Nugent score for the diagnosis of bacterial vaginosis

Diagnostic method	Sensitivity (%) ^a	Specificity (%) ^a
Total Amsel criteria	37	99
Vaginal pH of >4.5	83 (84)	69
Clue cells present	33 (32)	98
Positive whiff test	45 (44)	96
Characteristic vaginal discharge	35 (33)	85 (84)

^a Numbers in parentheses reflect analyses performed without the 12% repeated measures. In cases where no number in parentheses is present, the sensitivity and/or specificity was identical with or without repeated measures.

Amsel criteria were strongly associated (odds ratio = 58.9, 95% confidence interval [CI]: 14, 244). The association remained strong (odds ratio = 143, 95% CI: 21, 990) even after the bacterial counts were adjusted in the model. The odds ratios between BV by Nugent score and a 10-fold increase in *G. vaginalis*, *M. hominis*, or lactobacilli after controlling for plasma viral load are 1.45 (95% CI: 1.3, 1.62), 1.85 (95% CI: 1.6, 2.14), and 0.61 (95% CI: 0.5, 0.75), respectively. When all the other bacterial counts and Amsel criteria in addition to the plasma viral load are included in the logistic model, the odds ratios between BV by Nugent score and a 10-fold increase in *G. vaginalis*, *M. hominis*, or lactobacilli are 1.24 (95% CI: 1.04, 1.4), 1.82 (95% CI: 1.51, 2.19), and 0.54 (95% CI: 0.43, 0.68), respectively.

Cut points separating the BV group from the No-BV group were determined for each individual bacterial count based on whether the predictive probability (Fig. 1) is greater than or less than 0.5. We found that, if these cut points were used to differentiate the BV group from the No-BV group, a high agreement with BV by Nugent score was observed for *G. vaginalis* counts and for *M. hominis* counts. Log₁₀ *G. vaginalis* counts/ml CVL were greater than 6.81 in 83% of the BV group and lower than 6.81 in 70% of the No-BV group. Log₁₀ *M. hominis* counts/ml CVL were greater than 4.82 in 81% of the BV group and lower than 4.82 in 69% of the No-BV group. The cut point obtained based on the log₁₀ lactobacillus counts/ml CVL (<8.5 or >8.5) differentiated the BV from the No-BV group poorly with only 58% correct for both the BV group and the No-BV group.

We also determined cut points (using predictive probability) based on counts of pairs of bacteria or combining all three bacteria to see if this would improve the agreement with BV by Nugent score (Table 2). Using BV by Nugent score as the gold standard for diagnosing BV, we calculated sensitivities and specificities for each approach (Table 2). Combining two or more bacteria improved the specificity with only a mild loss in sensitivity of quantitative bacterial PCR for diagnosing BV when the same rule was followed to obtain the cut points.

To investigate the diagnostic characteristics of the predictor(s) obtained in the logistic regression models, we computed sensitivity and specificity for all possible cut points corresponding to the fit of each logistic regression model and plotted them as ROC curves (Fig. 2). The values of the area under the curve (AUC in Fig. 2) show clearly that the combined predictors are better for the diagnosis of BV. The analyses were repeated for

the data with the 12% repeated measures removed. The results were either unchanged or minimally changed. The changed sensitivity and specificity are provided in parentheses next to the first set of analyses in the tables. Other changes corresponding to the analyses without the repeated measures including the ROC curves were not presented due to the minimal differences seen.

DISCUSSION

At least 30% of women presenting with vaginal complaints remain without a diagnosis after a comprehensive workup (2). Thus, improved diagnostic tests are needed. BV is the most common identified cause of vaginitis and is a common disorder in women throughout the world, regardless of HIV status. Although the diagnosis of BV by Amsel criteria is simple, it is relatively insensitive. Of the 203 samples we analyzed from the WIHS cohort that had BV (Nugent score of 7 to 10), only 75 met the definition of BV by Amsel criteria. Thus, if Nugent score is considered the gold standard for diagnosing BV, the sensitivity and specificity of Amsel criteria in this cohort were 37% and 99%, respectively. Schwebke et al. reported the sensitivity and specificity of Amsel criteria compared to Nugent score to be 70% and 94%, respectively, in a cohort of non-pregnant women (12). The sensitivity of Amsel criteria in our cohort was very poor and much worse than the sensitivity of 70% reported by Schwebke et al. However, in two pregnant cohorts the sensitivity of Amsel criteria compared to Gram stain was only 35% and 46% (5, 15). While we suspect that variability in clinician interpretation was an important factor, we cannot exclude the possibility that the sensitivity of the Amsel criteria is different in women with HIV infection.

TABLE 2. Log₁₀ bacterial cut points for *M. hominis* (MH), *G. vaginalis* (GV), and lactobacilli (L) alone and in combination and sensitivities and specificities of these models using Nugent score as the gold standard method to diagnose bacterial vaginosis

Log ₁₀ bacteria	Cut points ^a identified to diagnose BV	Sensitivity (%) ^c	Specificity (%) ^c
MH	MH > 4.82 ^b	81 (80)	69 (72)
GV	GV > 6.81	83 (82)	70 (71)
L	L < 8.50	58 (56)	58
MH + GV	0.72MH + 0.28GV > 5.38 ^d	80 (79)	70 (72)
MH + L	-0.54MH + 0.46L < 1.30	79	78
GV + L	-0.44GV + 0.56L < 1.73	80	74
MH + GV + L	-0.31MH -0.29GV + 0.40L < 2.0	83 (82)	78 (79)

^a Bacterial counts are in log₁₀ scale/ml CVL. These cut points apply only to this sample set or a similarly selected population where plasma viral load has been controlled for. In a randomly selected sample set, the cut points could be different from what we determined here; however, the sensitivity and specificity of this approach would be expected to be similar.

^b Cut point(s) for each diagnostic measure based on combinations of one or more of the bacterial counts was generated by setting the predictive probability to zero in the corresponding logistic regression model. For example, if a CVL sample contained 4.83 log₁₀ of MH/ml of CVL or greater, our model would predict that the woman had BV because the probability that she has BV is greater than the probability that she does not have BV.

^c Numbers in parentheses reflect analyses performed without the 12% repeated measures. In cases where no number in parentheses is present, the sensitivity and/or specificity was identical with or without repeated measures.

^d If a CVL sample contained 5.5 log₁₀ of MH/ml CVL and 7.0 log₁₀ of GV/ml CVL, then our model would predict that this woman had BV as (0.72 × 5.5) + (0.28 × 7.0) = 5.92, and 5.92 is greater than 5.38, which is the cut point determined by our model.

FIG. 2. ROC curves for combinations of one or more bacterial counts as diagnostic criteria for BV. MH, *M. hominis*; GV, *G. vaginalis*; LA, lactobacilli; AUC, area under the curve. The closer the AUC is to 1.0, the better the bacterial counts predict BV. The bottom row of curves shows that the inclusion of all three quantitative bacterial counts in the model provides better predictive power. The addition of Amsel criteria further improves the power, and inclusion of plasma viral load does not change the power.

Our data demonstrate that quantitative bacterial PCR for *G. vaginalis*, *M. hominis*, and lactobacilli significantly correlates with the Nugent Gram stain method to diagnose BV. We found that women with BV diagnosed by Nugent score have significantly higher numbers of *G. vaginalis* ($P < 0.0001$) and *M. hominis* ($P < 0.0001$) and significantly lower numbers of lactobacilli ($P < 0.0001$) organisms in the CVL than women without BV. Using quantitative culture techniques on vaginal swabs, others have shown similar $\log_{10}$ changes in *G. vaginalis* (4, 8) and *M. hominis* (8) bacterial counts in women with BV by Nugent score. Variable results have been seen using quantitative culture techniques to assess $\log_{10}$ lactobacilli changes in the presence of BV. Hillier et al. (8) found significant loss of

total and H_2O_2 -producing lactobacilli in 39 vaginal swabs taken from women with Nugent scores of 7 to 10 compared to 47 vaginal swabs taken from women with Nugent scores of 4 to 6 and 85 vaginal swabs taken from women with Nugent scores of 0 to 3. Mean $\log_{10}$ total lactobacilli CFU/ml were 6.3 for the BV group (Nugent scores, 7 to 10), 6.6 for the intermediate group (Nugent scores, 4 to 6), and 7.0 for the No-BV group (Nugent scores, 0 to 3, $P < 0.001$) (8). In contrast, Delaney et al. found no significant change in $\log_{10}$ lactobacilli counts by Nugent score but did find that the proportion of H_2O_2 -producing strains of *Lactobacillus* markedly declined as Nugent score increased from 0 to 3 to 7 to 10 (4). He analyzed only 11 vaginal swabs from women with Nugent scores of 7 to 10 and

71 and 22 vaginal swabs from women with Nugent scores of 0 to 3 and 4 to 6, respectively. While we found significantly lower numbers of mean lactobacilli/ml of CVL in the BV group (Nugent scores, 7 to 10) than in the No-BV group (Nugent scores, 0 to 3), there was significant overlap between the two groups (Fig. 1). There were differences between our study design and those of Hillier and Delaney. Because we utilized CVL fluid and not vaginal swabs, we sampled both cervical and vaginal flora. Our PCR primers were designed to detect and quantify two species of *Lactobacillus* (*L. jensenii* and *L. crispatus*), whereas Hillier and Delaney performed culture to detect and quantify all *Lactobacillus* spp. We also did not assess for H₂O₂ production.

Others have used PCR methods to assess BV. Obata-Yasukawa et al. previously reported on a multiplex PCR method of diagnosing BV via quantifying *Mobiluncus mulieris*, *Mobiluncus curtisii*, *Bacteroides fragilis*, and *G. vaginalis* from vaginal swabs in pregnant ($n = 138$) and nonpregnant ($n = 34$) women (11). PCR was considered positive for BV when 10^3 to 10^4 CFU of any tested bacterial species was present in a vaginal swab. Compared to Gram stain, 78.4% (29/37) of the samples with Nugent scores of 7 to 10 were found to be positive for one or more bacteria by PCR compared to 4.4% (6/135) with Nugent scores of 0 to 6. Overall the sensitivity and specificity of the multiplex PCR in comparison to Nugent score were 78.4% and 95.6%, respectively.

In addition we were able to identify cut points for *G. vaginalis* and *M. hominis* that differentiated the BV group from the No-BV group. Modeling of combinations of bacteria demonstrated that the combination of the three bacteria provided the best combined sensitivity and specificity for diagnosing BV when using Nugent Gram stain as the gold standard. We did not assess for other bacteria identified as important in bacterial vaginosis such as *Prevotella* spp., *Bacteroides* spp., *Mobiluncus* spp., *Peptostreptococcus* spp., or *Ureaplasma urealyticum* (4, 8). The addition of one or more of these bacteria to our model could improve the correlation with Nugent score.

We did not include plasma HIV RNA levels as a variable in the models because the effects were insignificant. While we are unaware of any data to suggest that BV alters plasma HIV RNA levels, our results should be interpreted as being controlled for plasma HIV RNA levels because our selection of CVL samples was not random (the BV group and the No-BV group were matched by plasma HIV RNA levels). Thus, while the sensitivities and specificities of our models should be expected to be similar when applied to a randomly selected population (even HIV-negative populations), the specific cut points generated here cannot be generalized to a randomly selected population if the plasma HIV RNA level is associated with BV by Nugent score. We treated repeated measures in a single subject as independent samples. Although this can result in underestimation of the variability, it does not introduce bias into estimates of regression coefficients. The underestimation of the variability can be expected to be small in our analysis, because repeated measures accounted for only 12% of the samples, and the repeated measures were separated by significant time intervals. Further analyses excluding repeated measures confirmed this conclusion.

While a PCR method of detecting BV is more labor-intensive and costly than the Nugent Gram stain scoring method for

clinical diagnosis, it is less cumbersome than older quantitative culture techniques utilized in research settings where assessing for changes in specific bacteria could aid in understanding the pathogenesis and/or consequences of BV. For example, we recently demonstrated that BV was associated with increased HIV expression in the CVL of HIV-infected women. In multivariate analysis, this was found to be inversely associated with lactobacillus bacterial counts and positively associated with *M. hominis* bacterial counts in the CVL but not associated with *Gardnerella vaginalis* bacterial counts. This analysis could not have been done with the Nugent Gram stain method as the Nugent score cannot assess for *M. hominis* due to its lack of a cell wall nor provide quantitative information about specific bacteria associated with BV.

We conclude that quantitative bacterial PCR has potential as a tool to evaluate the bacterial microflora of the vagina. These data need to be confirmed in HIV-seronegative populations.

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