Assessment of Atopobium vaginae and Gardnerella vaginalis concentrations in a cohort of pregnant South African women

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Abstract

Objectives The purpose of this cross-sectional study was to assess *Atopobium vaginae* and *Gardnerella vaginalis* concentrations in pregnant women of different age groups, gestational age groups, vaginal flora categories and HIV status, and also to determine which DNA concentrations best discriminated between bacterial vaginosis (BV)-positive and non-BV categories.

Methods Self-collected vaginal swabs were obtained from 220 pregnant women attending an antenatal clinic in Pretoria, Gauteng, South Africa, from July 2012 to December 2012. BV was detected with the Nugent scoring system, and *A. vaginae* and *G. vaginalis* DNA was quantified with a multiplex quantitative real-time PCR assay.

Results Median concentrations of *A. vaginae* and *G. vaginalis* were not significantly different among various age groups (*A. vaginae* p=0.98 and *G. vaginalis* p=0.18) or different trimesters (*A. vaginae* p=0.31 and *G. vaginalis* p=0.19), but differed significantly among the vaginal flora categories (*A. vaginae* p<0.001 and *G. vaginalis* p<0.001) and HIV status (*A. vaginae* p<0.001 and *G. vaginalis* p=0.004). The presence of *A. vaginae* (OR=5.8; 95% CI 1.34 to 25.21 and p value=0.02) but not that of *G. vaginalis* (OR=1.90; 95% CI 0.81 to 4.43 and p value=0.14) was associated with HIV infection. An *A. vaginae* DNA concentration of $\geq 10^7$ copies/mL together with a positive *G. vaginalis* result ($\geq 10^0$ copies/mL) best discriminated between BV-positive (39/220) and non-BV categories (181/220) with a sensitivity of 85% (95% CI 0.70 to 0.94) and a specificity of 82% (95% CI 0.76 to 0.88).

Conclusions *A. vaginae* and *G. vaginalis* were present in high numbers and concentrations in this pregnant cohort. Threshold concentrations should be established for specific populations to ensure sensitive molecular assays for BV detection.

Introduction

Bacterial vaginosis (BV) is the most common reproductive tract infection of women of reproductive age.[1] This condition is characterised by the depletion of dominant *Lactobacillus* flora and the overgrowth of anaerobic and facultative bacteria.[2] During pregnancy, BV may reach prevalence rates of up to 55% and is associated with adverse pregnancy outcomes.[3,4]

Evidence suggests that there is a direct relationship between the depletion of *Lactobacillus* spp. and an increase in the concentrations of particularly *Atopobium vaginae* and *Gardnerella vaginalis* in BV infection.[5] These two species have been shown to be specific for BV, with some studies reporting *A. vaginae* to be more specific for BV than *G. vaginalis*.[5–7] However, these two species may be present in low concentrations in the vagina of healthy women and not cause any complications.[8] It is reported that there is no single 'core' microbiota of the human vagina.[9] The cervicovaginal microflora of women (including the presence and/or relative abundance of *A. vaginae* and *G. vaginalis*) is known to vary according to ethnicity, age and pregnancy.[10–12] In HIV-positive women, *G. vaginalis* has been identified as one of the core members of the vaginal microbiota and, together with *A. vaginae*, is significantly associated with viral loads above >40 copies/mL.[13,14]

The Nugent scoring system is the laboratory gold standard for the detection of BV where Gram-stained vaginal smears are graded according to different bacterial morphotypes.[15] The sensitivity of this method might be compromised by interobserver variability when grading vaginal smears and also by the fact that the assortment of bacterial species associated with BV are not all readily detected with microscopy.[16,17] Several studies have therefore proposed a molecular diagnosis of BV by targeting a combination of BV-related bacteria in a PCR, where some studies evaluated separately the threshold concentrations of these bacteria.[17,18]

The purpose of this study was to quantify *A. vaginae* and *G. vaginalis* in vaginal samples collected from pregnant women and assess the concentrations of these two species in women of different age groups, gestational age groups, vaginal flora categories and HIV status. In addition, we explored threshold DNA concentrations of *A. vaginae* and *G. vaginalis* that could discriminate between BV-positive and non-BV categories.

Materials and methods

Study setting, population and sample processing

Consenting pregnant women attending an antenatal clinic in Pretoria, Gauteng, South Africa, from July 2012 to December 2012 were included in this study. The antenatal clinic is situated in a tertiary academic hospital in an urban area and serves patients that are from several sub-Saharan African countries. Ethical approval for this study was obtained from the Student Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (approved protocol number S6/2012). This study made use of convenient sampling as participants were recruited in consecutive order over a 6-month period. Recruitment was done by a single investigator who explained a standard sample collection procedure to participants. Pregnant women of any gestational period and older than 18 years of age were

eligible to participate in the study.[19] Participant age, gestational age and HIV status were recorded.[19]

Two self-collected vaginal swabs were obtained from every participant and processed as previously described.[19] Gram-stained vaginal smears were graded with the Nugent scoring system, and bacterial DNA was isolated from the vaginal swabs with the ZR Fungal/Bacterial DNA extraction kit (Zymo Research, USA).[15,19] Nugent scoring was done by two independent observers; discrepant results were discussed, and a particular Nugent score was agreed on. As previously characterised, 148 (67.3%) samples had normal vaginal flora (NVF) (Nugent score 0–3), 33 (15%) samples had intermediate vaginal flora (IVF) (Nugent score 4–6) and 39 (17.7%) samples had BV flora (BVF) (Nugent score 7–10).[19]

Quantification of *A. vaginae* and *G. vaginalis* DNA

All samples were previously positive with a qualitative, internal control β-globin PCR.[19] The 16S rRNA gene of *A. vaginae* and the chaperonin 60 protein of *G. vaginalis* served as the PCR targets; primer and probe sequences for these targets were obtained from Menard *et al*.[17] A clinical *A. vaginae* isolate and *G. vaginalis* ATCC strain 14018 were used to construct standard curves by means of a 1/10 serial dilution. Real-time PCR reactions were performed with TaqMan probes using LightCycler 480 (Roche Diagnostics, Germany). The QuantiTect Multiplex PCR NoROX kit (Qiagen, Germany) was used to perform the real-time qPCR reactions according to the manufacturer's instructions. The amplification programme was run at 95°C for 15 min, followed by 45 two-step cycles at 94°C for 1 min and at 60°C for 1 min.[17] Clinical samples with unknown status for *A. vaginae* and *G. vaginalis* were subjected to duplex qPCRs. Quantification of *A. vaginae* and *G. vaginalis* in each clinical sample was done by comparing the amplified products with the respective standard curves to obtain extrapolated concentrations for both species.

Statistical analysis

Categorical data were expressed as percentages. Continuous variables were reported using measures of central tendency and dispersion; the mean and SD were used to describe normally distributed data, whereas the median and IQR were used to describe data that were not normally distributed. Statistical analyses were performed using STATA V.13.1 (StataCorp, College Station, Texas, USA). Differences in proportions in the number of *A. vaginae* and *G. vaginalis* positive samples in age groups, gestational age groups and vaginal flora categories were tested using the χ^2 test, and associations between *A. vaginae* and *G. vaginalis* positive samples with HIV status were assessed: ORs, 95% CIs for ORs and p values are presented. Different age categories, trimesters, vaginal flora categories and HIV status were used as independent variables to compare median concentrations of *A. vaginae* and *G. vaginalis* between the different categories of these variables. The Kruskal-Wallis test was used to test for significance in the difference between median concentrations in the different age categories, trimesters and vaginal flora categories. Following the Kruskal-Wallis test, the Dunn-Bonferroni post hoc test was used to test for significance in the difference between the medians of any two categories of a given variable. The Dunn-Bonferroni test controls for familywise error when conducting multiple pairwise compa-

risons.[20] The Mann-Whitney test was used to test for significance in the difference between median concentrations in HIV-positive and HIV-negative samples.

Diagnostic parameters, including sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), diagnostic OR (DOR) and receiver operating characteristic (ROC) area, were used to determine optimum *A. vaginae* and *G. vaginalis* threshold DNA concentrations to discriminate between BV-positive and non-BV categories. ORs and p values were calculated at every 10-fold concentration, and the optimum combination threshold DNA concentration was determined based on the highest OR and ROC area; all possible threshold combinations of a DNA concentration above a certain threshold for *A. vaginae* combined with a concentration above a certain threshold for *G. vaginalis* were considered. The different combination concentrations were used as independent variables while BVF served as the dependent variable. For all statistical tests conducted, $p<0.05$ was considered significant, and a 95% CI was considered significant if the 95% CI did not include 0 or 1.

Results

Study population

Two hundred and twenty pregnant women were included in this study. The mean age of the population sampled was 30±5.95 years, and the mean gestational age of current pregnancies was 26±7.97 weeks. Patient characteristics by vaginal flora category are presented in table 1.[19]

Table 1. Distribution of different vaginal flora categories, *Atopobium vaginae* and *Gardnerella vaginalis* across different age groups and each trimester of pregnancy in pregnant women attending antenatal care[19]

	Patient characteristic					
	Age (n=220)			**Gestational age (n=220)**		
Vaginal flora	**18–24 (n=49) (%)**	**25–34 (n=120) (%)**	**≥35 (n=51) (%)**	**Trimester 1 (n=13) (%)**	**Trimester 2 (n=98) (%)**	**Trimester 3 (n=109) (%)**
NVF	33 (67.4)	82 (68.3)	33 (64.7)	4 (30.8)	64 (65.3)	80 (73.4)
IVF	6 (12.2)	18 (15)	9 (17.65)	1 (7.7)	13 (13.3)	19 (17.4)
BVF	10 (20)	20 (16.7)	9 (17.65)	8 (61.5)	21 (21.4)	10 (9.2)
A. vaginae	41 (83.7)	91 (75.8)	43 (84.3)	9 (69.2)	83 (84.7)	83 (76.1)
G. vaginalis	38 (77.6)	80 (66.7)	31 (60.8)	9 (69.2)	72 (73.5)	69 (62.4)

BVF, bacterial vaginosis flora; IVF, intermediate vaginal flora; NVF, normal vaginal flora.

A. vaginae and *G. vaginalis* in different age groups, gestational age groups and vaginal flora categories

A total of 47 samples (21.4%) were positive for *A. vaginae* only, 21 samples (9.5%) were positive for *G. vaginalis* only and 128 samples (58.2%) were positive for both species. Both species were distributed in proportions that did not differ significantly across different age groups or trimesters (table 1). A lower proportion of samples in the NVF category (108 (73%)) was positive for *A. vaginae* compared with samples in the IVF (30 (90.9%); p=0.029) and BVF categories (37 (94.9%); p=0.004). *G. vaginalis* was present in a lower proportion of samples in both the NVF (90 (60.8%); p<0.001) and the IVF categories (23 (69.7%); p=0.014) compared with samples in the BVF category (36 (92.3%)). More samples in the BVF category (35 (89.7)) were positive for both species compared with samples in the IVF (21 (63.6%); p=0.008) and NVF (72 (48.6%); p<0.001) categories.

The overall median concentration of *A. vaginae* was 2.40×10^4 copies/mL (IQR=2.4×10^3 to 2.8×10^7 copies/mL), while the overall median concentration of *G. vaginalis* was 3.2×10^4 copies/mL (IQR=0 to 2.4×10^7 copies/mL). After stratifying by vaginal flora status, there was no significant difference in the median concentrations of *A. vaginae* (figure 1A) (p=0.98) and *G. vaginalis* (figure 1B) (p=0.18) among different age groups, and no significant difference in the median concentrations of *A. vaginae* (figure 1C) (p=0.31) and *G. vaginalis* (figure 1D) (p=0.19) among the different trimesters of pregnancy. As age and gestational age progressed, median concentrations of both species tended to be lower, with the lowest being in the advanced maternal age group (≥35) and third trimester of pregnancy (figures 1A–D). The difference in median concentrations of *A. vaginae* (figure 1E) (p<0.001) and *G. vaginalis* (figure 1F) (p<0.001) among the different vaginal flora categories was significant; the difference in median concentrations of both *A. vaginae* and *G. vaginalis* was significant between NVF and IVF (p<0.001) and NVF and BVF (p<0.001), but not between IVF and BVF (p=0.10 for *A. vaginae* and p=0.09 for *G. vaginalis*) (figure 1E and F).

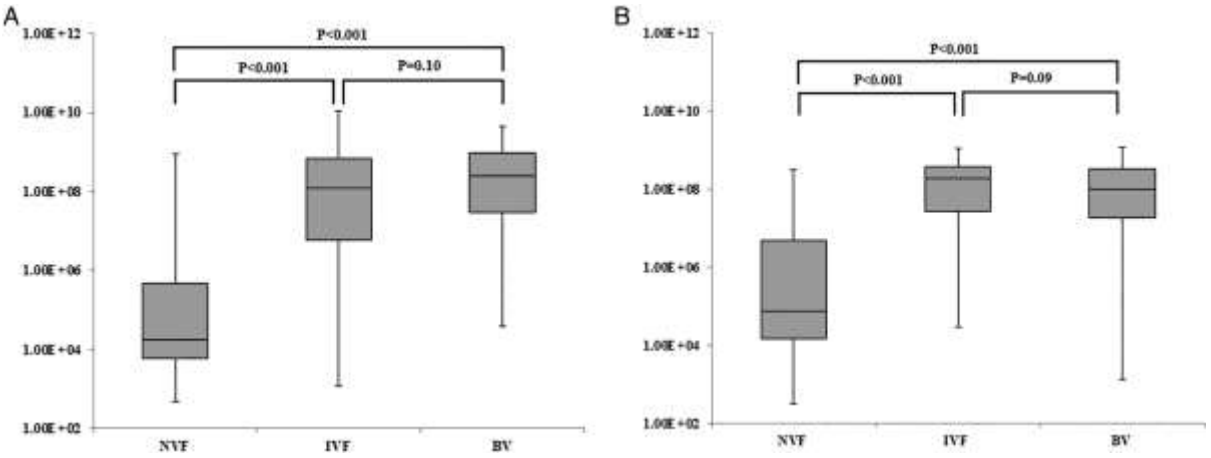

Figure 1. Box plots displaying the concentrations of *A. vaginae* (A) and *G. vaginalis* (B, D and F) in the vaginal fluid of women as stratified by vaginal flora categories (E and F). Box plots indicate the 10th percentile (bottom whisker), 25th percentile (lower box limit), median (middle line), 75th percentile (upper box limit) and the 90th percentile (top whisker) for *A. vaginae* and *G. vaginalis* concentrations. p Values for the difference in median concentrations are indicated above the box plots. BV, bacterial vaginosis; IVF, intermediate vaginal flora; NVF, normal vaginal flora.

A. vaginae and *G. vaginalis* according to HIV status

Out of 201 women with an available HIV status, 36 women (17.9%) were HIV-positive, whereas 165 women (82.1%) were HIV-negative. *A. vaginae* (OR 5.8; 95% CI 1.29 to 25.96 and p value=0.01) but not *G. vaginalis* (OR 1.90; 95% CI 0.81 to 4.46 and p=0.14) was significantly associated with a positive HIV status. However, *G. vaginalis* coexisting with *A. vaginae* (28/36 (77.8%)) was significantly associated with a positive HIV status with an OR of 3.14 (95% CI 1.35 to 7.29 and p=0.008). The HIV-positive cohort also had significantly higher median concentrations of both *A. vaginae* (p<0.001) and *G. vaginalis* (p=0.004) than the HIV-negative cohort. After stratifying by vaginal flora status (table 2), median concentrations differed significantly between HIV-positive and HIV-negative samples with NVF (p=0.02) for *A. vaginae* and samples with IVF (p=0.03) for *G. vaginalis* (figure 2).

Table 2. Distribution of *Atopobium vaginae* and *Gardnerella vaginalis* in HIV-positive and HIV-negative samples as stratified by vaginal flora category

	A. vaginae				***G. vaginalis***			
	Vaginal flora category				**Vaginal flora category**			
HIV status	**NVF (%)**	**IVF (%)**	**BVF (%)**	**Total**	**NVF (%)**	**IVF (%)**	**BVF (%)**	**Total**
HIV-positive	16 (47)	9 (26.5)	9 (26.5)	34	11 (39.3)	8 (28.6)	9 (32.1)	28
HIV-negative	80 (65)	19 (15.4)	24 (19.5)	123	70 (65.4)	13 (12.2)	24 (22.4)	107
Total	96	28	33	157	81	21	33	135

BVF, bacterial vaginosis flora; IVF, intermediate vaginal flora; NVF, normal vaginal flora.

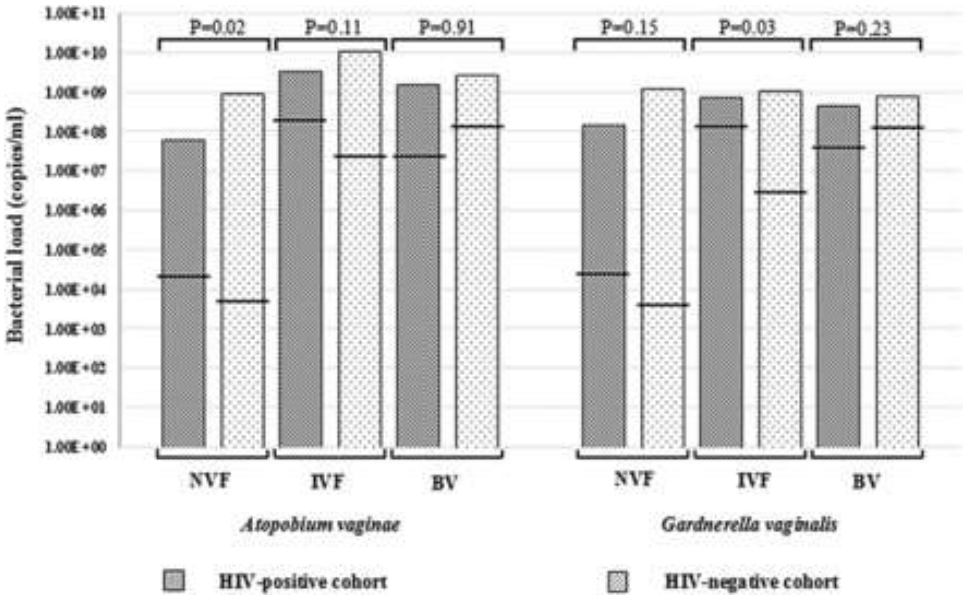

Figure 2. The range of *A. vaginae* and *G. vaginalis* concentrations in HIV-negative and HIV-positive samples as stratified by vaginal flora category. Bars depict median concentrations, and p values for the difference in median concentrations are indicated above the plotted area. BV, bacterial vaginosis; IVF, intermediate vaginal flora; NVF, normal vaginal flora.

A. vaginae and *G. vaginalis* threshold concentrations to discriminate between BV-positive and non-BV categories

Individually, *A. vaginae* and *G. vaginalis* were sensitive (95% and 92%, respectively) but not specific (24% and 38%, respectively) for BV. Nonetheless, *A. vaginae* and *G. vaginalis* DNA concentrations were considered together when discriminating between BV-positive and non-BV samples; an *A. vaginae* DNA concentration of $\geq 10^7$ copies/mL together with a positive *G. vaginalis* result ($\geq 10^0$ copies/mL) (combination AV_7GV_0) gave the highest DOR and ROC area (Suppl figure 3). Diagnostic parameters for this combination were as follows: DOR of 25.61 (95% CI of 10.11 to 65.54), sensitivity of 85%, specificity of 82%, ROC area of 0.83, PPV of 51% and NPV of 96%.

Sixty-five samples had a combination of AV_7GV_0; this combination was common among BV-positive samples (33 (84.6%); p<0.001) and IVF samples (21 (63.6%); p<0.001) compared with NVF samples (11 (7.4%)), and also in HIV-positive samples (20 (55.6%); p<0.001) compared with HIV-negative samples (39 (23.6%)).

Discussion

To our knowledge, this study is the first to assess *A. vaginae* and *G. vaginalis* concentrations in a pregnant population in an HIV-endemic setting. *A. vaginae* and *G. vaginalis* were equally distributed across age and trimester, but their microbial load was inversely related to age and gestational age. Both species were present in higher median concentrations in HIV-positive samples compared with HIV-negative samples. *G. vaginalis* was present in a higher proportion of samples in the BVF category and *A. vaginae* in a higher proportion of samples in both the IVF and BVF categories; however, *G. vaginalis* was present in the IVF category in a higher median concentration than in the BVF category. The combination criteria of an *A. vaginae* DNA concentration of $\geq 10^7$ copies/mL and a positive *G. vaginalis* result ($\geq 10^0$ copies/mL) were fairly sensitive and specific for BV.

Our results correlate with the reported inverse relationship between BV positivity (and in our study also A. vaginae and G. vaginalis concentrations) and age.[21] Decreasing concentrations of both species during pregnancy correspond to the declining BV prevalence and overall microbial diversity and richness as pregnancy progress, although we only targeted two species that might not be entirely representative of the vaginal microbiome.[11,22] Similar to the findings of Zozaya-Hincliffe *et al*,[23] *A. vaginae* and *G. vaginalis* were present in a large number of samples (50% to 85%) with NVF, confirming their roles as commensals. The concentrations of both species in vaginal fluid correspond with the reported positive association with Nugent scores.[1,18,23] High vaginal concentrations of *A. vaginae* and *G. vaginalis* might create a permissive environment for other anaerobic rods; women containing high concentrations of *G. vaginalis* and anaerobic gram-negative rods might have higher levels of proinflammatory cytokines and could be at an increased risk for spontaneous preterm delivery.[8] In women containing both species concurrently, the recurrence rates of BV are likely to be higher.[5] This could be explained by the synergism between the two species that have, in dispersed form, been associated with Nugent scores above 4; this association is stronger when either species is adherent, and strongest when both species are adherent.[24]

The higher median concentrations of *A. vaginae* and *G. vaginalis* in the IVF and BVF categories reaffirm their well-documented association with vaginal dysbiosis.[1,5,23] The IVF category with its mixed flora is largely uncharacterised and complicates clinical approaches; IVF is however equally associated with poor obstetric outcomes and HIV as BVF, and has a profile more similar to that of BV than that of NVF.[5,19,25] Although *G. vaginalis* was not significantly associated with HIV infection in our study, this species was abundant in HIV-positive women (77.8%), corresponding to previous findings in a similar population.[26] The higher median concentrations of *A. vaginae* and *G. vaginalis* in HIV-positive women might contribute substantially to the spread of HIV, as these two species have been shown to induce and/or enhance the expression of HIV in vitro.[27,28]

G. vaginalis has been reported to be less specific and *A. vaginae* to be more specific for BV.[5,7] Bradshaw *et al* [5] used threshold values of 4×10^5 copies/mL for *G. vaginalis* and 4×10^6 copies/mL for *A. vaginae* to categorise samples with high or low bacterial loads, whereas Menard *et al* [17] reported that threshold *A. vaginae* DNA levels of $\geq10^8$ copies/mL and threshold *G. vaginalis* DNA levels of $\geq10^9$ copies/mL were the best diagnostic definition of BV. Zozaya-Hincliffe *et al* [23] determined optimum threshold concentrations based on associations between these two species and BV and reported concentrations of 10^4 copies/10 ng of DNA for *A. vaginae* and 10^6 copies/10 ng of DNA for *G. vaginalis*. While most of these studies obtained threshold values by comparing with both the Nugent scoring system and Amsel's criteria, all of them looked at individual species scores and their association with BV. In our study, combinations of these two species were used to determine optimum threshold concentrations, because these bacteria in combination, rather than individually, were associated with a particular microbial profile at the time of sampling. The combination concentration of AV_7GV_0 might indicate that the mere presence of *G. vaginalis* is enough to establish a synergistic relationship with *A. vaginae* (and probably other bacterial species) and result in a dysbiotic state.

The strength of this study is that it assessed *A. vaginae* and *G. vaginalis* concentrations in a large and heterogeneous population of pregnant women with a significant number of HIV-positive participants. These women are directly at risk of adverse pregnancy outcomes and also bear the risk of enhanced HIV infection from upregulated host proteins and thereby the risk of passing the virus onto the baby.[13,28] However, infection enhancement may or may not be reduced when a woman is on highly active antiretroviral therapy.[29] The findings also contribute to the epidemiology and delineation of the roles of these two species in a population where information is limited. This study made use of a quantitative method, thereby allowing a more definitive description of the distribution of *A. vaginae* and *G. vaginalis*. The study also presents some limitations, including the fact that qPCR results were compared with a gold standard method that lacks specificity and groups a variety of morphologically similar bacteria together in a particular vaginal flora category; the exact contribution of *A. vaginae* and *G. vaginalis* to BV-positive samples is therefore unknown.[17] In addition to our approach of combining the concentrations of the two bacterial species to discriminate between BV-positive and non-BV samples, there may be alternative approaches to determine thresholds, which could lead to better performing criteria to discriminate between these samples. The performance of our threshold criteria was only evaluated within the data from which it was derived, and the sensitivity and specificity are likely to be overestimated; the performance of any potential threshold criteria should

therefore be validated in other data. Also, the performance of the qPCR should be evaluated for GV_0 at low copy numbers, as low sample volumes may indicate lower copy numbers compared with higher sample volumes. In HIV-positive cases, inferences were restricted to a qualitative level as no data were available on the treatment status and HIV viral load of participants.

Several studies have evaluated sensitive and specific PCR assays for BV detection by targeting BV-related species other than *A. vaginae* and *G. vaginalis*; PCR assays as diagnostic tools for BV detection might therefore be more advantageous if tailored for specific populations as many factors affect the type, load and relative abundance of the species involved in BV in different populations.[7,15,23] One approach of tailoring diagnostic PCR assays could be to first determine the metabolically active species in a population and then the loads and relative abundance of these species.[30] These assays would be useful if they could be developed as point-of-care tests in resource-limited settings where infrastructure and laboratory expertise are limiting factors. In our study population, syndromic management is generally used to treat symptomatic cases; a sensitive qPCR assay for the BV detection would therefore be beneficial in women with recurrent BV.

The high vaginal concentrations of *A. vaginae* and *G. vaginalis* in this pregnant population warrant the investigation of these species regarding risk of adverse pregnancy outcome, HIV proliferation and resistance to therapeutic drugs. Continued research is necessary to elucidate the aetiology of BV, and qPCR assays are sensitive techniques that may assist in such investigations.

Key messages

- *Atopobium vaginae* and *Gardnerella vaginalis* are not specific for bacterial vaginosis (BV) when considered qualitatively.
- These species are present in higher median concentrations in HIV-positive samples and the intermediate vaginal flora and BV flora categories.
- The presence of *G. vaginalis* ($\geq 10^0$ copies/mL) together with an *A. vaginae* DNA concentration of $\geq 10^7$ copies/mL best discriminated between BV-positive and non-BV vaginal flora.
- Quantitative PCR assays as a diagnostic tool to discriminate between BV-positive and non-BV samples might be more useful if tailored for specific populations.

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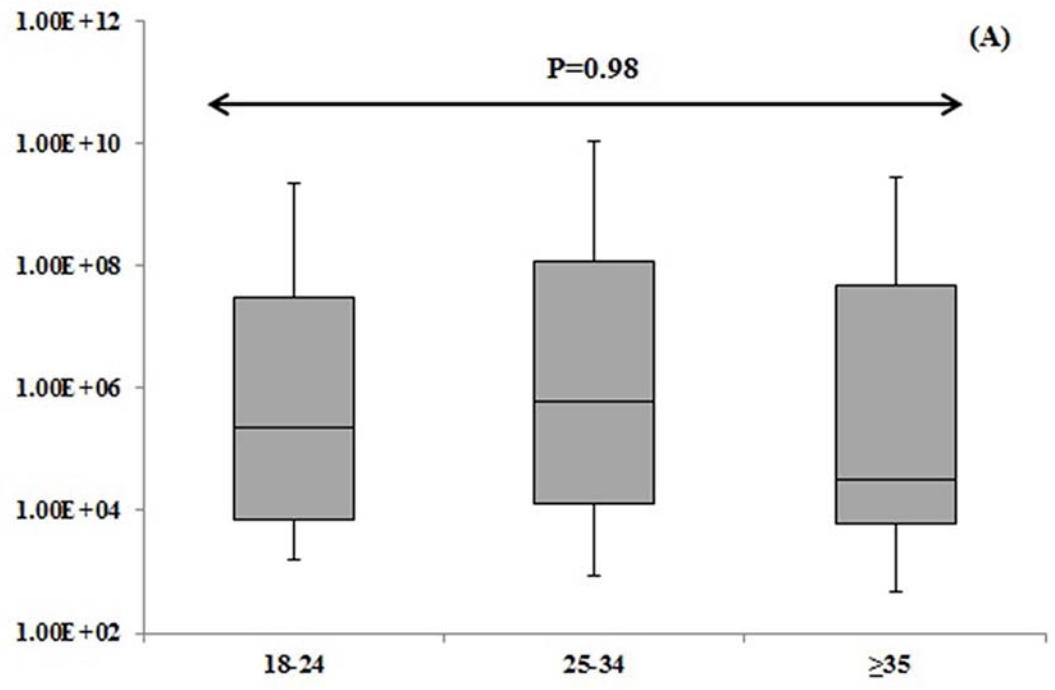
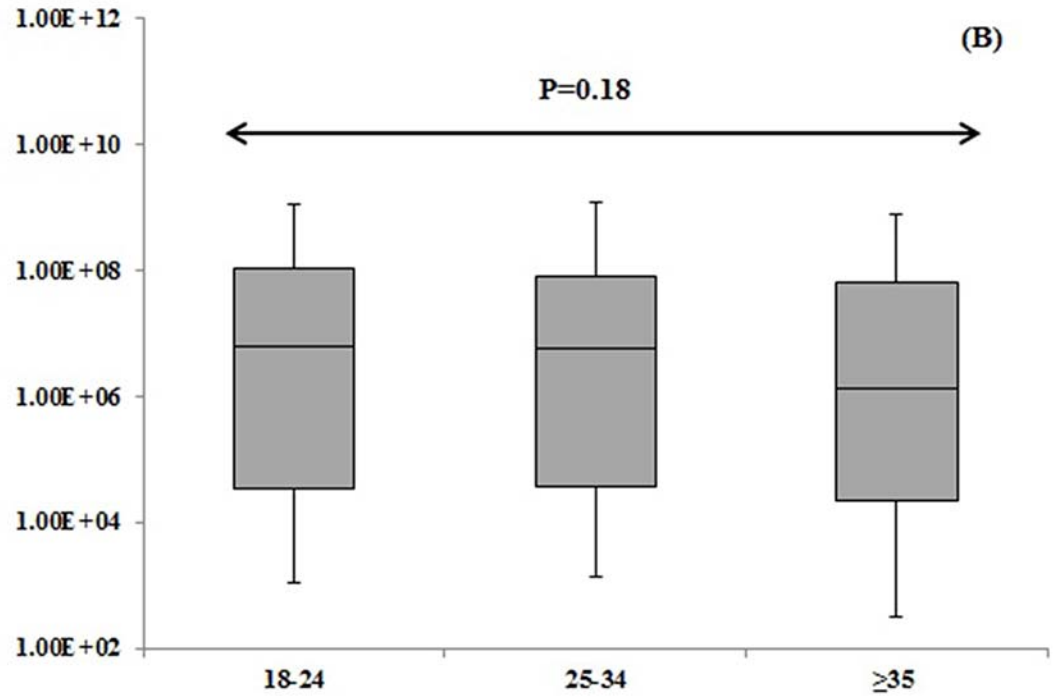
Supplementary figures

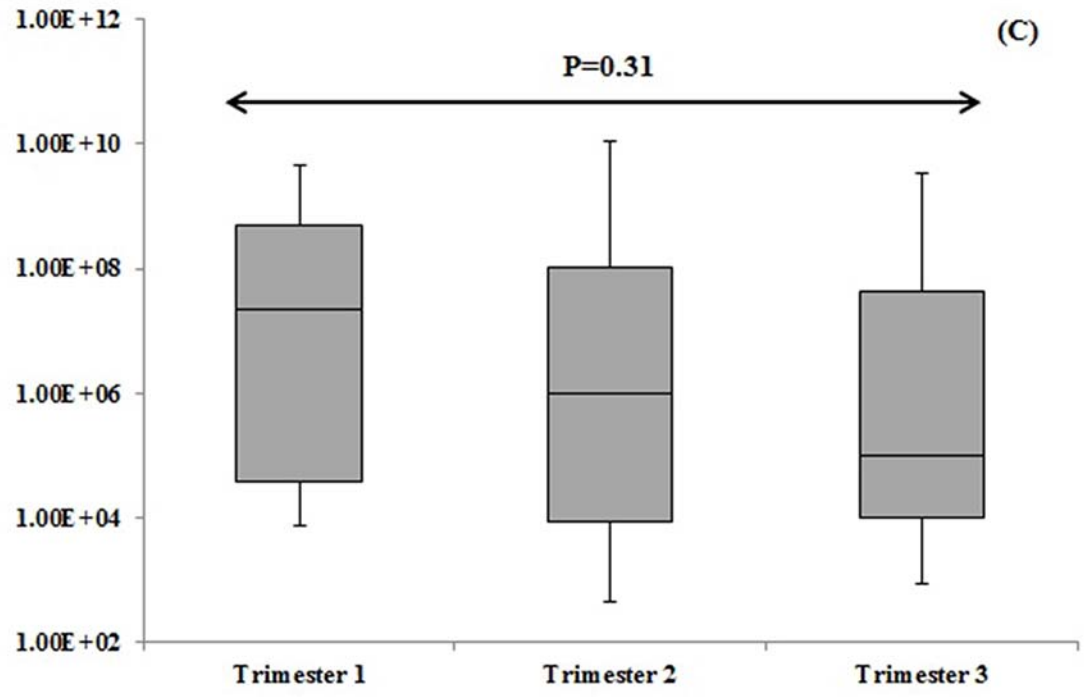
Suppl figure 1A



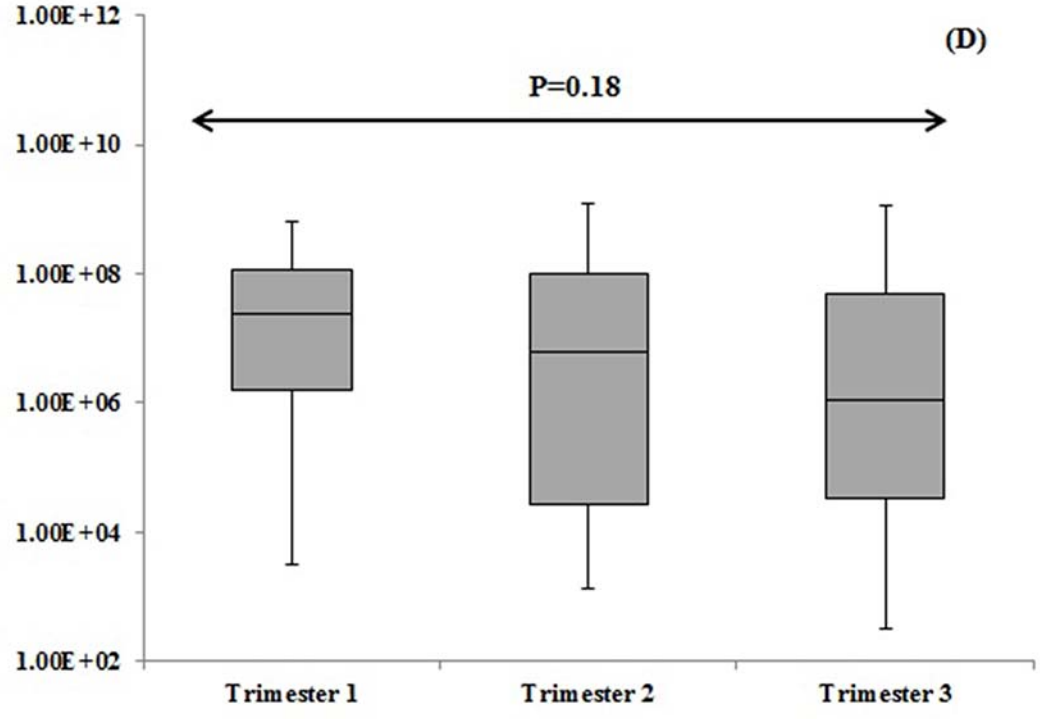
Suppl figure 1B



Suppl figure 1C



Suppl figure 1D



Suppl figure 3: Receiver operating characteristic (ROC) curve for the AV7GV0 threshold level that had the best predictive power for a positive BV result as characterised by the Nugent scoring system. The closer the area under the curve (AUC) is to 1.0, the better the predictive power for a positive BV result.