Comparison of the Diversity of the Vaginal Microbiota in HIV-Infected and HIV-Uninfected Women with or without Bacterial Vaginosis

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Background. Whether human immunodeficiency virus (HIV) infection is associated with a change in the diversity of genital microbiota was investigated.

Methods. Amplicon length heterogeneity polymerase chain reaction (LH-PCR) analysis and pyrosequencing of the 16S ribosomal RNA gene were used to analyze the diversity of the microbiota in HIV-positive (HIV+) and HIV-negative (HIV−) women with or without bacterial vaginosis (BV).

Results. LH-PCR analysis revealed significantly more microbiota diversity in BV-positive (BV+) women than in BV-negative (BV−) women, but no significant difference was noted between HIV+ women and HIV− women. Pyrosequencing revealed that Lactobacillus organisms constituted a median of 96% of the bacteria in BV− women. BV+ women had a significantly higher number of taxa found at ≥1% of the total genital microbiota (median, 11 taxa). Common taxa in BV+ women were Prevotella, Megasphaera, Gardnerella, Coriobacterineae, Lachnospira, and Sneathia. There was a trend (P = .07) toward the presence of a higher number of taxa in HIV+ BV+ women than in HIV− BV+ women. Propionibacterineae, Citrobacter, and Anaerococcus were the taxa found only in HIV+ women (P < .05).

Conclusions. The present study demonstrated that both LH-PCR analysis and pyrosequencing differentiated microbiota in BV+ women from that in BV− women and that pyrosequencing indicated a trend toward increased diversity in BV+ HIV+ women, suggesting that HIV infection is associated with changes in the diversity of genital microbiota.

The lower genital tract of women can be colonized by many types of bacteria. In some women, the genital microbiota consists predominantly of Lactobacillus species. In other women, there exists a common condition known as “bacterial vaginosis” (BV), in which Lactobacillus organisms are not the principal bacteria type but, instead, diverse and variable mixtures of other bacteria are present [1, 2].

The types of genital microbiota that are present in women have been associated with acquisition or expression of several sexually transmitted infections. For example, BV is associated with a higher risk of HIV infection [3–6], and HIV-infected women with BV have higher levels of HIV in genital secretions than do HIV-infected women without BV [7–9]. BV is also associated with increased susceptibility to infection with herpes simplex virus type 2, gonorrhea, Trichomonas vaginalis, and Chlamydia trachomatis [10–13].

Essentially all studies of associations between sexually transmitted infections and genital microbiota have identified BV either through the use of clinical-based methods, such as the Amsel criteria, or through the use of Gram staining of bacteria in mucosal secretions for diagnosis by use of the Nugent criteria. The Amsel crite-
ria assess vaginal pH, the presence of bacteria-coated epithelial cells (i.e., clue cells), the release of amine odor after the addition of potassium hydroxide (KOH), and the consistency of vaginal fluid, whereas the Nugent criteria evaluate the morphology and Gram stain reactivity of bacteria. Although these 2 methods are very effective for the diagnosis of BV, the Amsel criteria provide no information on the types or diversity of genital microbiota present, whereas Gram stains provide limited information in this regard.

Recent studies used cloning and sequencing of the 16S rRNA gene for the identification of genital microbiota, and they confirmed that many bacteria that were previously identified by culture, such as \textit{Gardnerella vaginalis} and \textit{Lactobacillus} species, are present at high levels in women with or without BV, respectively. However, these studies also showed that bacteria that were previously unidentified or difficult to culture represent a substantial fraction of the BV microbiota in many women [14–17]. These molecular studies also confirmed that the types and diversity of bacteria associated with BV can be very different between individuals. For example, Fredricks et al. [14] observed that, in 9 women with BV, 9 different taxa each contributed >10% of the total genital microbiota, whereas 27 different taxa were found at ≥1% of the total microbiota in at least 1 of the women. In contrast, in 7 of 8 BV-negative (BV⁻) women, only \textit{Lactobacillus} species were found to represent >10% of the microbiota. Hyman et al. [15] found at least 7 different taxa that were predominant bacteria in the genital microbiota samples obtained from 10 women in whom \textit{Lactobacillus} was not the principal microbiota taxon.

The microbiota in the lower genital tract of HIV-positive (HIV⁺) women has not been studied using molecular techniques capable of identifying the diversity and/or relative proportions of the types of bacteria present in a culture-independent manner. We hypothesized that analysis of genital microbiota by use of these types of methods could reveal previously unknown associations between HIV and microbiota, especially considering that BV involves a range of different constellations of bacteria rather than one fixed set of bacteria. The goal of the present study, therefore, was to compare the diversity of genital flora in HIV⁺ and HIV⁻ (HIV⁻) women by use of length heterogeneity polymerase chain reaction (LH-PCR) analysis and pyrosequencing, methods that are useful for assessing the diversity of microbiota in a culture-independent manner [18–22]. Because BV-positive (BV⁺) women are known to have much more diverse microbiota than BV⁻ women, both BV⁺ women and BV⁻ women were included in the HIV⁺ and HIV⁻ groups.

**MATERIALS AND METHODS**

**Patients and sample acquisition.** All patients analyzed in the present study were from the Women’s Interagency HIV Study (WIHS). WIHS is a longitudinal, multicenter cohort study of HIV-infected and -uninfected women followed at 6 clinical sites in the United States. Written informed consent was obtained from all participants. A detailed interview, physical and gynecologic examinations, and laboratory monitoring were performed at the time of sample donation.

Genital tract samples were collected by cervicovaginal lavage (CVL) that was performed by irrigation of the cervix with 10 mL of nonbacteriostatic sterile saline, followed by aspiration from the posterior fornix. CVL samples were held on ice until processing, which occurred within 6 h of collection. CVL samples were gently vortexed to evenly distribute cells before they were stored at −70°C.

CVL samples were obtained from 21 women divided into 4 groups on the basis of HIV seropositivity (HIV⁺ or HIV⁻ status) and Nugent Gram stain analysis for BV. Samples that were obtained from BV⁺ women had scores of 7–10, whereas samples obtained from BV⁻ women had scores of 0–3. The HIV⁺ BV⁺, HIV⁻ BV⁺, and HIV⁻ BV⁻ groups each consisted of 5 subjects, whereas the HIV⁺ BV⁻ group consisted of 6 subjects. The median age of the women in these groups was as follows: 39 years for the HIV⁺ BV⁺ group (range, 32–49 years), the HIV⁺ BV⁻ group (range, 33–44 years), and the HIV⁻ BV⁺ group (range, 39–45 years) and 35 years for the HIV⁻ BV⁻ group (range, 25–45 years). The median CD4 cell counts and plasma viral loads of the HIV⁺ groups were 327 CD4⁺ cells/mm³ (range, 0–555 CD4⁺ cells/mm³) and 15,000 HIV RNA copies/mL (range, 7500–210,000 HIV RNA copies/mL) for the HIV⁺ BV⁺ group and 375 CD4⁺ cells/mm³ (range, 14–726 cells/mm³) and 42,000 HIV RNA copies/mL (range, 6400–120,000 HIV RNA copies/mL) for the HIV⁺ BV⁻ group. All of the women were in good general health, none of the women were undergoing current antibiotic treatment, and none had current infection/colonization with \textit{Trichomonas} species or yeast (as was determined by the wet mount and KOH methods). Chlamydia and gonorrhea were not assessed because their prevalence in this cohort was 1% and <1%, respectively [23].

**DNA isolation.** DNA was isolated as described elsewhere [11]. In brief, bacteria from CVL samples were pelleted by centrifugation and were treated with a lysis buffer containing lysosome at room temperature for 20 min. Bacteria were further disrupted by the addition of SDS and protease K at 37°C for 30 min. Lysates were mixed with phenol/chloroform and centrifuged. DNA was precipitated by incubation with absolute ethanol, with glycogen added as a carrier.

**LH-PCR.** For LH-PCR fingerprinting of the 16S rRNA gene [18], extracted DNA (10 ng) was amplified by PCR performed using fluorescently labeled forward primer L27F (5’-6FAM]-AGAGTTTGATCCTGGCTCAG-3’) and unlabeled reverse primer L219V (5’-GCCATCGTCTGCATCG-3’). Both primers are broad-range primers for bacteria [24]. The reactions were performed using 20–μL (final-volume) mixtures containing 1 × PCR buffer, 0.01% bovine serum albumin, 2.5 mmol/L MgCl₂, 0.5 mmol/L each deoxynucleoside triphosphate, 0.2 μmol/L
each primer, and 2 U of TaqGold DNA polymerase (Applied Biosystems). The initial denaturation step was performed at 95°C for 11 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 35 min. LH-PCR samples were stored at 4°C in the dark, until they were used for fingerprinting. To ensure that these conditions would not overamplify minor components, several dilutions of the original extracted DNA were amplified and run on LH-PCR to check that amplification was in the linear range for each sample. LH-PCR of negative controls determined that there were no detectable contamination products generated from the reagents used in the process. The LH-PCR products were separated on a SCE9610 capillary fluorescent sequencer (Spectrumedix LLC) and analyzed with GenoSpectrum software (version 2.01). Negative and positive controls were analyzed for each run and gave appropriate results. The software package deconvolves the fluorescence data into electropherograms, where the peaks of the electropherograms represent PCR amplicons of different length in base pairs and identify different genus/species/strains or operational taxonomic units (OTUs) of microflora. The LH-PCR fingerprint data were then analyzed using a custom practical extraction and report language (PERL) script (Interleave, version 1.0; BioSpherex) that combines data from several runs, interleaves the various profiles, normalizes the data, calculates the averages for each amplicon size, and determines diversity indices. The normalized peak areas were calculated by dividing an individual peak area by the total peak area in that profile. LH-PCR fingerprint patterns (i.e., the presence or absence of certain amplicon peaks) were expressed as stacked histograms of the LH-PCR amplicon normalized abundances and were analyzed by visual inspection and principal coordinate analysis (PCO). PCO was used to provide a measure of the similarities between the cases directly, rather than the variables. The Multivariate Statistical Package (MVSP; Kovach Computing Services) was used for PCO.

**Multitag pyrosequencing and phylogenetic analysis.** Pyrosequencing and its use for determining the diversity of microbial communities by sequencing of the 16S rRNA gene have been described elsewhere [21, 22]. We developed and used a novel multiplexed pyrosequencing method by generating a set of 12 primers that each contained either the 27F or 355R primer (see above), which was tagged on the 5' end with a 4-base “bar code.” PCR was performed on individual patient samples by use of the unique barcode primers, and 10–12 samples then were pooled and ligated to the PCR linkers used in the emulsion step of pyrosequencing. All samples were amplified for 30 cycles, as described above for the LH-PCR. Pyrosequencing of the amplified, tagged DNA was performed by 454 Life Sciences, with the use of 10–12 separately tagged samples included in a single slot. The data from each well were “deconvoluted” by sorting the sequences into bins on the basis of the bar codes, and the taxa in the samples were normalized by the total number of reads from each bar code.

We used custom PERL scripts to automatically sort the sequences on the basis of the bar codes, search against the rRNA database, identify the reads, and exclude sequences that had multiple tags, which indicated chimeric sequences. We used the Bayesian classifier provided by the Ribosomal Database II Project (RDP 9), which uses a posteriori probability to identify query sequence on the basis of the occurrence of 7 base words in their rRNA database [25]. We then downloaded the annotations for each sequence and used a PERL script to tabulate the taxa as a percentage of the total community in each sample. Sequences were aligned using Clustal X software (version X) [26] and neighbor-joining trees were constructed using Phylogenetic Analysis Using Parsimony software (version 4; Sinauer Associates).

**RESULTS**

**LH-PCR analysis of genital microbiota in HIV+ and HIV− women.** To determine the diversity of genital microbiota in subjects in the 4 groups (HIV+ BV+, HIV+ BV−, HIV− BV+, and HIV− BV− groups), duplicate PCRs were performed for each of the samples obtained from 21 subjects, and amplified products were analyzed to determine their length. Figure 1 shows representative LH-PCR “fingerprints” of microbiota collected from subjects in each group. One to 5 peaks (median, 1 peak) were observed in samples obtained from the 11 BV− women (figure 1A and 1B). We define these peaks as OTUs, because it is unknown whether they represent a single genus, species, or strain. In contrast, analysis of microbiota in samples obtained from the 10 BV+ subjects (figure 1C and 1D) resulted in 7–14 OTUs (median, 11 OTUs; P = .002, compared with BV− subjects, as determined by the Mann-Whitney test).

The samples obtained from the 5 HIV+ BV+ women had a median of 11 OTUs (range, 2–13 OTUs), whereas the samples obtained from the 5 HIV− BV+ women had a median of 9.5 OTUs (range, 7–14 OTUs) (not significant [NS]). The 6 samples from the HIV+ BV− group had a median of 2 OTUs (range, 1–3 OTUs), whereas the 5 samples from the HIV− BV− group had a median of 1 OTU (range, 1–5 OTUs) (NS).

Twenty-three different OTUs could be differentiated in the 21 subjects, on the basis of the differential mobilities of the amplified products (figure 2). When samples from BV+ and BV− women were compared, BV+ women had much greater diversity of microbiota, with 21 different OTUs detected, compared with the 9 OTUs detected in BV− women. HIV+ women had 18 OTUs, whereas HIV− women had 20 OTUs. Two of the OTUs (362.6 bp and 372.3 bp) were found at high levels in many of the 11 BV− subjects, and several of these subjects had only 1 of the 2 OTUs present. These 2 PCR products were sequenced from several of the samples and corresponded to *Lactobacillus crispatus* and *Lactobacillus johnsonii*, according to a search of RDP 9.

The LH-PCR data were analyzed by PCO in which each point represents 1 sample and in which the data are projected onto 2 or
Figure 1. Length heterogeneity polymerase chain reaction (LH-PCR) plots of 16S rRNA–amplified products from genital microbiota. Representative LH-PCR plots of 3 subjects from each group. A, HIV positive, bacterial vaginosis (BV) negative; B, HIV negative, BV negative; C, HIV positive, BV positive; D, HIV negative, BV positive.
3 principle components of a multidimensional plot by use of classic Eigen analysis (figure 3). The PCO plot shows overlapping of subjects from the 2 BV− groups, indicating a trend toward similarity in the diversity of microbiota in samples obtained from BV− women. The plots of the 2 BV+ groups overlapped to a large extent, suggesting that the diversity of these samples was very similar. BV− and BV+ subjects had little overlap, indicating very different diversity between the BV+ and BV− groups.

**Pyrosequence analysis of genital microbiota in HIV+ and HIV− women.** We also performed pyrosequencing of the samples, to identify the bacteria present in genital microbiota, identify minor constituents of the microbiota, and determine the

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The relative abundance of operational taxonomic units (OTUs). The relative abundance of the OTUs from each sample are presented as stacked histograms and are color coded for the presence of bacterial vaginosis (BV) and HIV infection. Light blue bars denote OTUs from samples from HIV-negative (HIV−), BV-negative (BV−) women; dark blue bars, OTUs from samples from HIV-positive (HIV+), BV− women; light red bars, OTUs from samples from HIV−, BV-positive (BV+) women; dark red bars, OTUs from samples from HIV−BV+ women. *L. cripatus, Lactobacillus cripatus; L. johnsonii, Lactobacillus johnsonii.*

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Principal coordinate analysis (PCO) of length heterogeneity polymerase chain reaction (LH-PCR). Data from LH-PCR was plotted by classic Eigen analysis. Diamonds denote BV-negative, HIV-negative women; squares, BV-positive, HIV-negative women; triangles, BV-negative, HIV-positive women; circles, BV-positive, HIV-positive women.
Figure 4. Relative abundance of taxa, as determined by pyrosequencing. A, The relative abundance of taxa from one representative individual in each of the 4 groups. Taxa comprising <0.3% of the sequences are not shown for clarity. B, The relative abundance of taxa comprising ≥1% of the total microbiota averaged from all 5 women in the HIV-positive (HIV\(^+\)), bacterial vaginosis (BV)–positive (BV\(^+\)) group and the HIV-negative (HIV\(^-\)), BV\(^-\) group. The category “other” represents the proportion of sequences from taxa that averaged <1%. BV\(^-\), BV negative.
diversity of the microbiota. Pyrosequencing of samples obtained from the 21 women resulted in 36,724 sequences with a median length of 249 bp (range, 156–314 bp; mean ± SD, 250 ± 13 bp). A search for the sequences in RDP 9 revealed 137 distinct taxa of bacteria. The 10 samples obtained from BV+ women contained a total of 11 different taxa, whereas the samples from 11 BV− women had 61 taxa. The median number of taxa in samples obtained from BV+ and BV− women was 33 (range, 19–46 taxa) and 12 (range, 8–20 taxa), respectively (P = .0003, by the Mann-Whitney test). The types and relative proportions of taxa observed in representative subjects from each of the 4 groups are shown in figure 4A. The 5 samples obtained from HIV+ BV+ women contained a total of 103 different taxa, whereas the 5 samples obtained from HIV− BV+ samples had 51 taxa (median number of taxa in each group, 41 [range, 29–46] and 29 [range, 19–36] taxa, respectively [P = .1167, by the Mann-Whitney test]).

**Taxa found at >=1% of total microbiota by means of pyrosequencing.** We analyzed the taxa found at >=1% of the total microbiota community, under the a priori assumption that the most abundant taxa are the ones that significantly contribute to the functionality of the microbiota community. A total of 35 different taxa were found at >=1% of the total microbiota in at least 1 of the 21 women (table 1). The samples obtained from the 10 BV+ women contained 33 different taxa that were found at >=1% of at least 1 sample, whereas the samples obtained from the 11 BV− women contained 10 different taxa found at >=1% of the microbiota. The median number of taxa found at >=1% of microbiota in the 10 samples from BV+ women was 11 (range, 8–18 taxa), whereas the median number in the 11 samples obtained from BV− women was 2 (range, 1–4 taxa) (P < .0001, by the Mann-Whitney test). The taxa most commonly found at >=1% of the microbiota in samples from BV+ women included Prevotella, Megasphaera, Gardnerella, Coriobacteriaceae, Lachnospira, Sneathia, and Actinomycineae (table 1) (figure 5). Lactobacillus organisms were found in all 11 samples obtained from BV− women and in 5 samples obtained from BV+ women in >=1% of the total microbiota. Comparison of the Lactobacillus sequences showed that this group was highly diverse. For example, 18 different clusters, or OTUs, of Lactobacillus sequences were found in the HIV+/BV− women (figure 5), although most of the lactobacilli were restricted to 5 different clusters.

The 5 HIV+ BV+ women had a median number of 14 taxa (range, 10–18 taxa) found at >=1% of total microbiota, in contrast to a median of 10 taxa (range, 8–14 taxa) found in samples obtained from 5 HIV− BV+ women (P = .07, by the Mann-Whitney test). Relative abundances of taxa in >=1% of the total microbiota averaged from the HIV+ BV+ and HIV− BV− groups are shown in figure 4B. The 6 samples obtained from HIV− BV+ women had a median of 2 taxa (range, 1–3 taxa) found at >=1% of total microbiota, whereas the 5 samples obtained from HIV− BV− women had a median of 2 taxa (range, 1–4 taxa) (P = .005, by the Mann-Whitney test).

**Table 1. Number of subjects with taxa found at >1% of total microbiota in at least 1 subject.**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Found at &gt;1% of microbiota</th>
<th>Found at &gt;10%</th>
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<tr>
<td></td>
<td>In BV+ women</td>
<td>In BV− women</td>
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<tr>
<td>Lactobacillus</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Prevotella</td>
<td>10</td>
<td>2</td>
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<tr>
<td>Megasphaera</td>
<td>10</td>
<td>...</td>
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<tr>
<td>Gardnerella</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Coriobacterineae</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Lachnospira</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Sneathia</td>
<td>8</td>
<td>...</td>
</tr>
<tr>
<td>Actinomycineae</td>
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<td>...</td>
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<tr>
<td>Allisonella</td>
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<td>...</td>
</tr>
<tr>
<td>Micromonas</td>
<td>5</td>
<td>...</td>
</tr>
<tr>
<td>Propionibacterineae</td>
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<td>1</td>
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<tr>
<td>Dialister</td>
<td>4</td>
<td>...</td>
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<tr>
<td>Peptoniphilus</td>
<td>3</td>
<td>...</td>
</tr>
<tr>
<td>Porphyromonas</td>
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<td>...</td>
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<tr>
<td>Comamonas</td>
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<tr>
<td>Citrobacter</td>
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<td>Acinetobacter</td>
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<td>Rhodobaca</td>
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</table>

**NOTE.** Proteiniphilum, Anaerotranscru, and Corynebacterineae (predominantly Porynbacterium species) were found in >=1% of total microbiota in 2 bacterial vaginosis (BV)-positive (BV+) subjects. Acetanaerobacterium, Acetobacter, Aerococcus, Anaerococcus, Faecallibacterium, Micrococccineae, Peptostreptococcus, Pseudomonas, Staphylococcus, Stenotrophomonas, and Xylanibacter were each found in >=1% of microbiota in 2 BV− subject. Pelomonas and Sphingomonas were each found in >=1% of total microbiota in 1 BV-negative (BV−) subject.

- a Atopobium and Eggerthella species.
- b Predominantly Mobilunicus species.
- c Predominantly Propionibacterium species.

HIV− BV+ women had a median of 2 taxa (range, 1–4 taxa) (P = .93, by the Mann-Whitney test).

**Taxa found at >=10% of total microbiota by means of pyrosequencing.** A total of 10 different taxa were found at >=10% of the total microbiota in at least 1 of the 21 women (table 1). The 10 samples obtained from BV+ women contained 9 different taxa that were found at >=10% of total microbiota in at least 1 sample, whereas the samples obtained from BV− women contained 3 different taxa. Lactobacilli constituted >=80% of the taxa in all BV+ women (median, 96%). The median number of taxa found at >=10% of total microbiota in the samples obtained from 10 BV+ women was 3 (range, 1–4 taxa), whereas the median number in the samples from 11 BV− women was 1 (range, 1–2 taxa) (P < .0014, by the Mann-Whitney test). The 5 HIV+ BV+ samples contained a median of 2 taxa (range, 1–3 taxa) found at >=10% of total microbiota, whereas the 5 HIV− BV− samples had a median of 3 taxa (range, 2–4 taxa) (P = .14, by the Mann-Whitney test).
Taxa found only in HIV⁺ women by means of pyrosequencing. Four taxa were found in at least 2 of the HIV⁺ BV⁺ women but in none of the HIV⁻ BV⁻ women. Thus, Propionibacterineae organisms were found in all 5 of the HIV⁺ BV⁺ women and in 4 of these women at >1% of the flora, but it was not detected in any HIV⁻ BV⁻ women (P = .002, by χ² analysis). Interestingly, Propionibacterineae was also detected in one of the HIV⁻ BV⁻ women but in none of the HIV⁺ BV⁻ women. Anaerococcus and Citrobacter taxa were found in 3 of the HIV⁺ BV⁺ women (P = .04), whereas the Acinetobacter taxon was found in 2 of the HIV⁺ BV⁻ women (P = .11). No taxa were found only in HIV⁻ BV⁻ women, compared with HIV⁺ BV⁺ women.

DISCUSSION

The main goal of the present study was to compare the diversity of the lower genital tract microbiota in HIV⁺ and HIV⁻ women and is, to our knowledge, the first study to compare microbial diversity in these groups by use of culture-independent methods. Analysis of the sequences obtained by pyrosequencing showed that there was a trend (P = .07) toward higher microbial diversity in HIV⁺ BV⁺ women when the number of taxa found at ≥1% of the microbiota in this group was compared with that noted in HIV⁻ BV⁻ women. In addition, 3 different taxa, Propionibacterineae, Anaerococcus, and Citrobacter, were found only in HIV⁺ women, and this difference was statistically significant. Although the total number of taxa found in HIV⁺ BV⁺ women was higher than that found in HIV⁻ BV⁻ women, this difference was not significant. Taken together, sequence comparisons showed higher microbial diversity in HIV⁺ BV⁺ women than in HIV⁻ BV⁻ women. Sequence analysis did not show any differences between HIV⁺ BV⁻ women and HIV⁻ BV⁻ women. In contrast to sequence analysis, LH-PCR did not distinguish any significant differences in microbial diversity between HIV⁺ and HIV⁻ women.

Several studies have found that HIV-infected women with BV have higher levels of HIV in genital secretions than do HIV-infected women without BV [7–9], and levels of specific bacteria have been associated with HIV levels [7]. It is possible that changes in the diversity of microbiota associated with HIV infection could, in turn, be associated with changes in HIV expression in the genital tract. Changes in microbial diversity could also affect either the severity or recurrence of BV, which could, in turn, affect pregnancy in HIV-infected women.

Although we found a trend toward higher microbial diversity in HIV⁺ women, an underlying cause for this association is not yet known. Because HIV infection suppresses immunity, it is
possible that reduced immunity could play a role in the higher diversity. *Candida* vaginitis is more frequent and gynecologic diseases are more severe in HIV-infected women, suggesting that immunity in the lower genital tract that normally restricts growth of microbes is reduced in HIV-infected women [23, 27–29]. An influence of HIV on the incidence of BV was not previously noted [23, 30].

Another goal of this study was to determine the usefulness of molecular techniques, LH-PCR and pyrosequencing, in the analysis of female genital microbiota. Our data confirmed numerous studies published elsewhere [1, 14, 15, 31] that showed that the microbiota in BV+ women is much more diverse than that in BV– women, with lactobacilli constituting most of the bacteria in the latter group. Thus, both pyrosequencing and LH-PCR can differentiate these clinically important conditions, whereas only pyrosequencing was capable of showing the more subtle differences between HIV+ and HIV– women. LH-PCR has the additional limitation of not providing identification of the organisms. Pyrosequencing confirmed that BV microbiota can be highly variable between women. Thus, although some bacteria (e.g., *Prevotella, Megasphaera, Gardnerella*, and *Lachnospira* organisms) were found in essentially all BV+ women at ≥1% of microbiota, there were 11 taxa found in only 1 of the BV+ women at these levels and 8 taxa found in 2 women (table 1).

Although LH-PCR has been used to analyze the diversity of intestinal microbiota [32], there are no reports for genital microbiota. Recently, pyrosequencing was used to analyze genital microbiota in 6 samples pooled from HIV– pregnant women [33]. As in our study, high levels of lactobacilli and *Prevotella, Bifidobacterium*, and *Veillonella* organisms were found, although it was not clear how many individuals were pooled in samples. The gynecologic health of the women was not defined, in contrast to our present study, and the average read length of the amplified products was 100 bp, which is less than one-half of the size of the median read lengths in our study (249 bp). Longer read lengths and the use of 16S rRNA variable region 1 and 2 in the present study likely result in a higher accuracy of identification.

A possible limitation of our study is a potential bias introduced during PCR amplification so that the proportion of reads may not represent the actual proportions of organisms in the women. However, both methods showed high levels of diversity in individuals with BV, compared with those with no BV, providing confirmation of usefulness. In conclusion, the present study provides evidence that HIV infection is associated with increased diversity of the microbiota of the lower genital tract, which could have pathogenic consequences for HIV sexual transmission.

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