Twice-Daily Application of HIV Microbicides Alters the Vaginal Microbiota

Jacques Ravel, Pawel Gajer, Li Fu, Christine K. Mauck, Sara S. K. Koenig, Joyce Sakamoto, Alison A. Motsinger-Reif, Gustavo F. Doncel, and Steven L. Zeichner

Institute for Genome Sciences, University of Maryland, School of Medicine, Baltimore, Maryland, USA; Department of Microbiology and Immunology, School of Medicine, University of Maryland, Baltimore, Maryland, USA; CONRAD, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, Virginia, USA; Bioinformatics Research Center, Department of Statistics, North Carolina State University, Raleigh, North Carolina, USA; Departments of Pediatrics and Microbiology, Immunology, and Tropical Medicine, School of Medicine, The George Washington University, Washington, DC, USA; and Center for Cancer and Immunology Research, Children’s National Medical Center, Washington, DC, USA.

ABSTRACT Vaginal HIV microbicides offer great promise in preventing HIV transmission, but failures of phase 3 clinical trials, in which microbicide-treated subjects had an increased risk of HIV transmission, raised concerns about endpoints used to evaluate microbicidal safety. A possible explanation for the increased transmission risk is that the agents shifted the vaginal bacterial community, resulting in loss of natural protection and enhanced HIV transmission susceptibility. We characterized vaginal microbiota, using pyrosequencing of bar-coded 16S rRNA gene fragments, in samples from 35 healthy, sexually abstinent female volunteer subjects (ages 18 to 50 years) with regular menses in a repeat phase 1 study of twice-daily application over 13.5 days of 1 of 3 gel products: a hydroxyethylcellulose (HEC)-based “universal” placebo (10 subjects), 6% cellulose sulfate (CS; 13 subjects), and 4% nonoxynol-9 (N-9; 12 subjects). We used mixed effects models inferred using Bayesian Markov chain Monte Carlo methods, which showed that treatment with active agents shifted the microbiota toward a community type lacking significant numbers of Lactobacillus spp. and dominated by strict anaerobes. This state of the vaginal microbiota was associated with a low or intermediate Nugent score and was not identical to bacterial vaginosis, an HIV transmission risk factor. The placebo arm contained a higher proportion of communities dominated by Lactobacillus spp., particularly L. crispatus, throughout treatment. The data suggest that molecular evaluation of microbicidal effects on vaginal microbiota may be a critical endpoint that should be incorporated in early clinical assessment of microbicide candidates.

IMPORTANCE Despite large prevention efforts, HIV transmission and acquisition rates remain unacceptably high. In developing countries, transmission mainly occurs through heterosexual intercourse, where women are significantly more vulnerable to infection than men. Vaginal microbicides are considered to be one of the most promising female-controlled products, in that women themselves insert the microbicides into the vagina to prevent HIV transmission during sexual intercourse. The failure of several microbicides in clinical trials has raised questions concerning the low in vivo efficacy of such anti-HIV molecules. This study was designed to gain insights into the failures of two microbicides by testing the hypothesis that the microbicides negatively affect a critical line of defense against HIV, the vaginal microbiota. The results suggest that in the early assessment of candidate microbicides, culture-independent evaluation of their effect on the vaginal microbiota should be considered and may constitute a critical endpoint.

Despite the increasing availability of HIV testing and treatment, and campaigns to encourage the adoption of practices decreasing the risk of HIV transmission, HIV transmission rates remain unacceptably high (1). HIV poses a particular problem for many countries in sub-Saharan Africa, where there are about 1.8 million new infections estimated per year, of which 90% or more are believed to occur through heterosexual intercourse (1). Many sociocultural and economic factors limit the ability of women to insist on safer sexual practices to decrease HIV transmission risks. The development of vaginal HIV microbicides, the use of which would be controlled or initiated by women, has therefore attracted much interest as a strategy to help prevent HIV sexual transmission. A recent clinical trial (CAPRISA004) confirmed that a vaginal topical microbicide containing the antiretroviral agent tenofovir applied before and after sex can provide protection against HIV heterosexual transmission (2). The level of protection observed (54% in highly adherent subjects), however, was less than might have been expected given the results of preclinical studies.
Even more recently, a different clinical trial (VOICE) of the same tenofovir gel but with daily application was ended prematurely by the Data Safety Monitoring Board because of a lack of evidence of a beneficial effect (i.e., futility).

Among the first vaginal microbiode agents studied were nonoxynol-9 (N-9), a nonionic surfactant still widely used as an FDA-approved spermicide, and cellulose sulfate (CS), a high-molecular-weight sulfated carboxymethylcellulose polymer. While initial in vitro and early-phase clinical studies of these microbicides were promising, the results of larger phase 2B and 3 trials showed no protection against HIV compared to placebo treatment and an increased risk of infection when the microbicides were used very frequently (4–6). The reasons for the failure of these agents remain unknown. One hypothesis that may account for the failure of the microbicides holds that microbicide application alters the vaginal microbiota so as to yield a vaginal environment that has lost its natural protective abilities, either directly enhancing HIV transmission or indirectly acting to activate potential host cells, which would facilitate HIV transmission. This hypothesis was further supported by recent work using an in vitro vaginal microbiota colonization model system (7).

RESULTS AND DISCUSSION

Initial studies found that the microbicides CS and N-9 had limited effects on conventionally cultured organisms (8, 9) (mainly Lactobacillus sp.), which was not surprising, since cultivation-dependent methods provide biased quantitative and incomplete qualitative information on the composition of bacterial communities (10, 11). In this study, we undertook a comprehensive analysis of the effects of CS and N-9 on the vaginal microbiota in a repeat phase 1 study of those agents, using culture-independent molecular methods and Bayesian statistical modeling. Vaginal swabs were collected according to the study design outlined in Fig. 1A. To comprehensively evaluate the effects of the microbicides on the vaginal microbiota, we characterized the vaginal microbial community taxon composition and relative abundance using pyrosequencing of bar-coded 16S rRNA gene fragments (12). A total of 146 longitudinal samples from 35 subjects were successfully collected and analyzed (Table 1). The microbicides N-9 and CS, and to some extent the hydroxyethylcellulose (HEC) placebo, are major inhibitors of PCR amplification. Using the modified whole genomic DNA extraction procedure developed for this study, we generated DNA from which variable regions 1 and 2 (V1 and V2) of 16S rRNA genes were successfully amplified. Pyrosequencing of these bar-coded 16S rRNA gene amplicons produced a data set consisting of 791,295 high-quality sequence reads with an average length of 359 bp and 5,420 reads per sample. Overall, a total of 296 taxa were observed in the vaginal microbiota of these women. The depth of coverage for each community was sufficient to detect taxa that constituted ~0.1% of the community. Complete linkage hierarchical clustering methods were applied and revealed five major bacterial community state types (CSTs) (Fig. 1B). Three CSTs were often dominated by different Lactobacillus species: L. crispatus (CST I), L. iners (CST III), or L. gasseri (CST II) (Fig. 1B; see also Table S1 in the supplemental material). CST IV-A and CST IV-B were heterogeneous in composition, without significant numbers of Lactobacillus spp., but differed from each other in composition. The frequencies of each CST in this cohort were similar to those previously published (12, 13), except that CST V was not observed in the subjects enrolled in this study (Table 1), as its frequency in the general population is less than 2% (12). Vaginal bacterial communities from samples that clustered in CST IV-B were characterized by higher proportions and types of anaerobic bacteria (Fig. 1B; see also Table S1 in the supplemental material) such as Atochobium, Prevotella, Megascaphae, Sneathia, and Mobiluncus, as well as Gardnerella (the latter in proportions ranging from 0.2% to 9.9%), while CST IV-A comprises members of the genera Streptococcus, Enterococcus, and Escherichia, as well as small proportions of Lactobacillus spp. (Fig. 1B; see also Table S1 in the supplemental material). CST IV-B microbial composition is consistent with vaginal communities found in women with bacterial vaginosis (14, 15) and was associated with higher Nugent scores, while CST IV-A was associated with low and intermediate Nugent scores, as shown by modeling this interaction using a log-linear model (Fig. 2A). Further, using a mixed effect logistic regression model in which the presence or absence of G. vaginalis was the outcome variable and CST was the predictor variable, the log odds ratio of the presence of G. vaginalis in CST IV-B was shown to be significantly (P < 0.001) higher than in CST IV-A. Other CSTs did not show log odds ratios of the presence of G. vaginalis significantly different from those seen with CST IV-A. This holds true if the abundance of G. vaginalis (ranging from 0.2% to 9.9%) was modeled similarly (p < 1e−16), supporting the correlation observed between high Nugent score and CST IV-B (16). Because high Nugent scores and bacterial vaginosis have been associated with increased transmission and acquisition of HIV (17, 18), we evaluated the possibility that CST IV-B was associated with the application of N-9 or CS. However, the analysis revealed that 30.8% of the samples were assigned to CST IV-A (Table 2) and that CST IV-A, not CST IV-B, showed a statistically significant association with N-9 and CS use at visit V4, during the middle of the product application period, compared to placebo and visits V2 and V3 (baseline) (Fig. 2B and 3A). This result, while surprising, was supported by an analysis of the frequencies of Nugent score categories and product applications over the study period that showed no differences between the N-9, CS, or placebo arms (Fig. 3B). Application of the placebo, HEC, does not appear to affect the frequency of community state types, as shown by a consistent CST frequency distribution at each visit before (V3) and during (V4) the application period and immediately after placebo use ceased (V5) (Fig. 3A). Interestingly, a shift was observed between visits V5 and V6, when an increase in the proportion of CST IV-A was observed (Fig. 3A). However, that shift was not significantly associated with use of the placebo but rather reflected the normal temporal dynamics of the vaginal microbiota, often observed with the L. iners-dominated CST-III (13).

To better characterize the effect of N-9 and CS on shifting the vaginal microbial communities toward CST IV-A, we evaluated the ability of the communities to not shift to CST IV-A by modeling the distances of each community state from the center of CST IV-A (see Fig. S1 in the supplemental material). The model took into account that a subsequent serial microbial community sample in a particular subject depends on the previous microbial community (13) and was designed using mixed effects models inferred using Bayesian Markov chain Monte Carlo methods (see Materials and Methods). Figure 4 shows the interaction plot of the mean values and error bars of each sample’s community state with respect to the distance from the center of CST IV-A for each treatment arm and visit, computed using the mixed effects model.
Community state distances to the center of CST IV-A for the N-9 and CS groups were significantly different from those for the placebo group at visit V4 and showed borderline significance at visit 5 (Fig. 4). This analysis highlights the effect of twice-daily applications of N-9 or CS microbicides on the composition of vaginal bacterial communities after 7 days of use. Upon application of N-9 or CS, most communities shifted in composition from community states that were dominated by species of Lactobacillus to com-

FIG 1 (A) Schematic of the clinical study design. Twice-daily application of the gel products is indicated in green. Idealized menses are indicated in red. Vaginal swabs were collected at visits 2, 3, 4, 5, and 6. (B) Heatmap of phylotype relative abundances from 146 samples. Ward-linkage hierarchical clustering was performed on the Jensen-Shannon divergences between all pairs of community states to obtain five community state types. Nugent score categories (high, 7 to 10; intermediate [Int], 4 to 6; low, 0 to 3), treatment arms, and visits are indicated. The top 25 most abundant phylotypes are shown. The frequency of each community state type is shown in Table 2.
Community states mainly dominated by anaerobes and by members of the genera *Streptococcus*, *Enterococcus*, and *Escherichia* (Fig. 1B). Interestingly, it appears that the vaginal bacterial communities have the ability to rebound rapidly, as no statistically significant difference was observed between placebo and N-9 or CS use at visit 6, 3 days after the last use of the products (Fig. 4). Because CST IV-A contains low number of *Lactobacillus* spp., it is anticipated that, while not necessarily characterized by high Nugent scores, this state, like CST IV-B, would contribute to an increased risk of transmission or acquisition of sexually transmitted infections (STIs), including HIV. Our results are consistent with previous culture-based evaluations of N-9 (8, 9), which found that daily application of N-9 promoted the loss of non-H$_2$O$_2$-producing lactobacilli and did not affect vaginal colonization by H$_2$O$_2$-producing lactobacilli (8); however, that earlier study did not evaluate the effect on other bacteria present in the vagina. The earlier study also found that N-9 application did not produce increases in the Nugent score. A previous phase I safety study of CS did not show increases in Nugent scores either; however, it did show a concomitant reduction in levels of H$_2$O$_2$-producing lactobacilli and an increase in *Escherichia coli* numbers (19). Furthermore, in that study CS significantly reduced *Lactobacillus* sp. colonization of human cervicovaginal epithelial cells and tissues and increased their proinflammatory reaction to bacteria (7). Other polyanions similar to CS have been shown to interfere with Toll-like receptor (TLR)-mediated responses in human cervicovaginal cells (20). Effects of the candidate microbicides on vaginal cell signaling pathways may contribute to observed changes in CSTs that accompanied application of the microbicide candidates in our study.

If the N-9 and CS microbicide agents are associated with both an increased risk of HIV transmission and an alteration in the vaginal microbiota, it may be helpful to consider what may link these two phenomena. One potential plausible explanation is that a distortion in the microbiota may, for example, through interactions with pattern recognition receptors, lead to an increase in levels of inflammatory cytokines, which would activate potential HIV host cells. Activation of TLRs in human vaginal cells by microbial antigens induces innate and proinflammatory responses (21, 22). Since HIV replicates preferentially in activated cells and since the risk for HIV transmission at each episode of intercourse

| Treatment | No. of samples per subject | No. of subjects | Total no. of samples | Total no. of samples/treatment | Total no. of subjects/treatment |
|-----------|----------------------------|----------------|---------------------|------------------------------|--------------------------------|
| CS        | 2                          | 1              | 2                   | 49                           | 13                             |
|           | 3                          | 4              | 12                  |                              |                                |
|           | 4                          | 5              | 20                  |                              |                                |
|           | 5                          | 3              | 15                  |                              |                                |
| N-9       | 2                          | 0              | 2                   | 52                           | 12                             |
|           | 3                          | 1              | 3                   |                              |                                |
|           | 4                          | 6              | 24                  |                              |                                |
|           | 5                          | 5              | 25                  |                              |                                |
| Placebo   | 2                          | 0              | 2                   | 45                           | 10                             |
|           | 3                          | 0              | 0                   |                              |                                |
|           | 4                          | 5              | 20                  |                              |                                |
|           | 5                          | 5              | 25                  |                              |                                |
| All combined | 2                          | 1              | 2                   | 146                          | 35                             |
|           | 3                          | 5              | 15                  |                              |                                |
|           | 4                          | 16             | 64                  |                              |                                |
|           | 5                          | 13             | 65                  |                              |                                |

**FIG 2** (A) Interaction plot of the mean values and error bars of the log counts of the community state types versus Nugent score category contingency table (see Table S4 in the supplemental material) stratified by community state type and Nugent category, with error bars indicating 95% credible intervals derived from model 2 (described in Materials and Methods). (B) Interaction plot of the mean log(odds) values and the corresponding 95% credible interval of logistic regression model 1, evaluating the association between CSTs (community state types) at visit V4 compared to visits V2 and V3 for each product use arm. In the N-9 (V4) and CS (cellulose sulfate) (V4 and V5) arms, no communities were assigned to CST II. In addition, in the N-9 arm (V4), no communities were assigned to CST III (Fig. 4).
TABLE 2 Metadata and taxonomic composition of each sample analyzed

| Community state type | No. of samples | Frequency (%) |
|----------------------|----------------|--------------|
| I                    | 32             | 21.9         |
| II                   | 17             | 11.6         |
| III                  | 26             | 17.8         |
| IV-A                 | 45             | 30.8         |
| IV-B                 | 26             | 17.8         |

* As defined by Ravel et al. (12).

is low, increasing potential host cell activation may reasonably be expected to affect overall transmission rates.

The present study, despite the relatively small number of subjects, demonstrated using culture-independent methods that application of microbicides associated with higher rates of HIV transmission can affect the composition of the vaginal microbiota, although it is impossible to say whether these changes are specific to the active agent or to one or more other components of the microbicide vehicle gel, given that the universal placebo has a composition different from those of the vehicles of both N-9 and CS gels. These changes in the vaginal microbiota could plausibly contribute to higher rates of HIV transmission. While our observation involves candidate microbicides that have been shown to be ineffective at preventing HIV transmission in large phase 3 clinical trials, an analogous effect could produce a decrease in the effectiveness of microbicides that do not prevent HIV transmission. The data suggest that molecular, culture-independent evaluation of microbicide effects on vaginal microbiota, in addition to or combined with culture-based methods, may be an important component of the early clinical assessment of candidate microbicides. The data also suggest that the development of a maximally effective HIV microbicide delivery system may require, in addition to agents focused solely on the virus, components that help maintain or promote a healthy vaginal microbial community.

MATERIALS AND METHODS

Clinical sampling and study design. The samples were obtained in the course of a carefully monitored repeat phase 1 study of CS and N-9, conducted by CONRAD (protocol no. A04-097). Sixty healthy, sexually abstinent female (tested using Rapid Stain Identification Series [RSID]-Semen) volunteer subjects (ages 18 to 50 years) with regular menstrual cycles (27 to 35 days) not using systemic hormonal contraception, systemic corticosteroid antibiotics, antifungals, or antivirals and without urinary tract infections were recruited for the study and randomly assigned to use one of the following three gel products in a blinded fashion: 6% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptrol) gel, which includes lactic acid, sorbitol, a thickener (carbomer), water, and benzyl alcohol as a preservative; or 4% nonoxynol-9 (Conceptro
High-quality sequence reads were first dereplicated using 99% similarity and the UCLUST software package, and detection of potential chimeric sequences was performed using the UCHIME component. Chimeric sequences were removed prior to taxonomic assignments. Each processed 16S rRNA gene sequence was classified using the RDP naive Bayesian Classifier (24). RDP classifier quality score filtering was not used, and all reads were classified to the genus or species level as described previously (12). All sequence reads taxonomically assigned to the genus *Lactobacillus* were identified to the species level using the SpeciateIT software package.

![FIG 3](A) Nugent score category proportions for each treatment and visit. (B) Nugent score categories (high, 7 to 10; intermediate, 4 to 6; low, 0 to 3). There is no significant difference between the values from the baseline and visits V4, V5, and V6.
The higher frequency of CST IV-A at visits V4 and V5 for treatments with CS
was estimated using the mixed effects model (model 1). Error bars indicate 95% credible intervals.
Levels of CST IV-A from the center of CST IV-A for different treatment arms and visits, based on
the mixed effects model (model 1). Error bars indicate 95% credible intervals.

The frequencies were computed using the Manhattan distance to the center of CST IV-A (see Fig. S1 in the supplemental material). This was not entered into the model as it was not associated with product use but that CST IV-A was associated with both N-9 and CS application. To fully characterize this association, we evaluated the ability of the community to not enter CST IV-A by modeling the distances of each community from the center of CST IV-A (see Fig. S1 in the supplemental material). This was accomplished using mixed effects models inferred using Bayesian Markov chain Monte Carlo methods.

Modeling the effect of product application on the community distance from the center of CST IV-A. In order to measure the effect of microbicide treatment on community distance from the center of CST IV-A (see Fig. S1 in the supplemental material), we have used the following double exponential mixed effects model. Community distances were calculated using the Jensen-Shannon divergence:

\[ \gamma_i = \text{DExp}(\mu_i, \sigma_{\text{trmt}(i)}, \ldots) \]

where \( \mu_i = a + b_{\text{trmt}(i)} + c_{\text{visit}} + d_{\text{trmt}(i), \text{visit}} + e_{\text{subjId}(i)} \), and \( e_{\text{subjId}(i)} \) are the distances from the center of community state type IV-A, treatment arm, visit, and subject id, respectively, of the community state corresponding to the “i”th sample. The reference class for the treatment variable is the placebo group. The reference class for the community state type variable is CST IV-A. Thus, the baseline group for the model described above consists of placebo samples at CST IV-A. The coefficient \( a \) is the difference in mean log odds ratio of samples at visit V4 versus visit V2 or V3 for the baseline group of samples, and \( b \) and \( c \) are the main effects of treatment and community state type, respectively. The coefficient \( d \) captures the interaction between treatment and CST. In order to take into account the interactions between samples from the same subject, the model includes a random intercept term \( (e) \) that depends on subject id. Estimation of the coefficients of the model was done using just another Gibbs sampler and jags (29) and the jags R package (30, 31). A noninformative gamma prior with the shape parameter 1 and a scale parameter of 1,000 was used for the precision of the normal distribution, and for the other parameters, the prior was set to the normal distribution with a mean of 0 and a standard deviation of 1,000. Convergence of Markov chains in the model described above was verified using the Gelman-Rubin potential scale reduction factor test (32) at the 1.1 level and the coda R package (33). All Markov chain models were run using 100,000 iterations (with a 100,000-iteration burn-in) and a thinning value of 100. The coefficients and their 95% credible intervals are presented in Table S2 in the supplemental material. This analysis revealed that CST IV-B was not associated with product use but that CST IV-A was associated with both N-9 and CS application. To fully characterize this association, we evaluated the ability of the community to not enter CST IV-A by modeling the distances of each community from the center of CST IV-A (see Fig. S1 in the supplemental material). This was accomplished using mixed effects models inferred using Bayesian Markov chain Monte Carlo methods.

**Jensen-Shannon divergence.** Jensen-Shannon divergence is a measure of dissimilarity between probability distributions that was introduced by Lin (25) to alleviate the limitations associated with relative entropy (13). The Jensen-Shannon divergence between the two discrete probability distributions \( p = (p_1, \ldots, p_n) \) and \( q = (q_1, \ldots, q_n) \) is defined as follows:

\[ D_{\text{JS}}(p, q) = \frac{1}{2} \left( D_{\text{KL}}(p || q) + D_{\text{KL}}(q || p) \right) \]

where \( d = (p + q)/2 \) is the average of \( p \) and \( q \), and \( D_{\text{KL}}(x, y) \) is the relative entropy between \( x \) and \( y \). The values of the Jensen-Shannon divergence are normalized to lie between 0 and 1, and its square root is a metric that we refer to as the Jensen-Shannon metric.

The Jensen-Shannon divergence can be expressed using entropy with the following formula:

\[ D_{\text{JS}}(p, q) = H\left( \frac{p + q}{2} \right) - H\left( \frac{p}{2} \right) - H\left( \frac{q}{2} \right) \]

**Statistical analyses.** Using the methods described previously by Ravel et al. (12), each sample of a vaginal bacterial community, which represents a state of the community at a given time point (community state), was assigned to one of five CSTs (groups of community states with similar microbial species compositions and abundances) using complete linkage hierarchical clustering methods (Fig. 1B).

**Modeling the association between CST frequency and product use at V4.** CST IV-B, characterized by a lack of *Lactobacillus* spp. as well as the presence of a variety of strict anaerobes, is often associated with high Nugent scores (14, 16). Because high Nugent scores and bacterial vaginosis have been associated with increased risks of transmission and acquisition of several STIs (17, 26–28), including HIV (17, 18), we tested for an association between the frequency of each community state type and product use at visits V2 and V3 and at visit V4. More precisely, the following mixed effects logistic regression model was fitted to the data:

\[ y_{ij} \sim \text{Bernoulli}(p_{ij}) \]

\[ \logit(p_{ij}) = a + b_{\text{trmt}(i)} + c_{\text{visit}} + d_{\text{trmt}(i), \text{visit}} + e_{\text{subjId}(i)} + e_{\text{subjectId}} + e_{\text{subjectId}}(i) \]

where \( \text{Bernoulli}(p) \) is the Bernoulli distribution with mean \( p \), \( y \sim \text{Bernoulli}(p) \) means that \( y \) is sampled from the distribution \( \text{Bernoulli}(p) \), and \( y_{ij} \) is the indicator variable. It is set to equal to 0 if the “i”th sample was collected at visits V2 and V3 and set to equal to 1 if it was collected at visit V4. \( \text{trmt}(i) \), \( \text{stType}(i) \), and \( \text{subjId}(i) \) are the treatment arm, community state type, and subject identification number (id), respectively, of the community state corresponding to the “i”th sample. The reference class for the treatment variable is the placebo group. The reference class for the community state type variable is CST IV-A. Thus, the baseline group for the model described above consists of placebo samples at CST IV-A. The coefficient \( a \) is the mean log odds ratio of samples at visit V4 versus visit V2 or V3 for the baseline group of samples, and \( b \) and \( c \) are the main effects of treatment and community state type, respectively. The coefficient \( d \) captures the interaction between treatment and CST. In order to take into account interactions between samples from the same subject, the model includes a random intercept term \( (e) \) that depends on subject id. Estimation of the coefficients of the model was done using just another Gibbs sampler and jags (29) and the jags R package (30, 31). A noninformative gamma prior with the shape parameter 1 and a scale parameter of 1,000 was used for the precision of the normal distribution, and for the other parameters, the prior was set to the normal distribution with a mean of 0 and a standard deviation of 1,000. Convergence of Markov chains in the above-described

![Figure 4](http://speciateIT.sourceforge.net). Taxonomic assignments are shown in Table S1 in the supplemental material.

**Effect of Microbicides on Vaginal Microbiome**

November/December 2012 Volume 3 Issue 6 e00370-12
The log-linear model for the Nugent category versus community state type contingency table is as follows:

\[
\log(\lambda_i) = \alpha + b_{\text{mgCat},i} + c_{\text{stType},i} + d_{\text{mgCat},i,\text{stType},i}
\]

where \(\alpha\), \(b\), and \(d\) are the coefficients, and \(c\) are the count values. The model was run for 50,000 iterations with a 50,000-iteration burn-in and a thinning value of 100. Convergence of Markov chains in this model was verified using the Gelman-Rubin potential scale reduction factor test at the 1.1 level. The model was run for 50,000 iterations with a 50,000-iteration burn-in and a thinning value of 100. Coefficients and their 95% credible intervals are presented in Table S3 in the supplemental material.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00370-12/-/DCSupplemental.

**ACKNOWLEDGMENTS**

We thank Mitchell D. Creinin, University of Pittsburgh, and David F. Archer, Eastern Virginia Medical School, for assistance with the clinical aspect of the parent study.

The microbiome study was supported by a grant (AI1079798) from the Microbicide Innovation Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health. The clinical study was supported by CONRAD funds from a cooperative agreement between USAID and EVMS (HRN-A-00-98-00020-00). The study was also supported in part by a grant from NIAID, NIH, to the District of Columbia Developmental Center for AIDS Research (P30AI087714).

J.R., S.L.Z., G.F.D., and A.A.M.-R. designed the study. C.K.M., L.F., and K.D. performed the clinical experiments, including DNA extraction, amplification, and sequencing. P.G. developed and performed the statistical analysis. J.R., S.L.Z., G.F.D., and A.A.M.-R. contributed to the analytical aspects of the study. J.R., S.L.Z., and B.A.M. interpreted the findings and wrote the manuscript.

**REFERENCES**

1. Joint United Nations Programme on HIV/AIDS (UNAIDS). 2010. UNAIDS Report on the global AIDS epidemic 2010. UNAIDS, Geneva, Switzerland.

2. Abdool Karim Q, et al. 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 329:1168–1174.

3. Rohan IG, et al. 2010. In vitro and ex vivo testing of tenofovir gel shows it is effective as an HIV-1 microbicide. PLoS One 5:e9310.

4. Halpem V, et al. 2008. Effectiveness of cellulose sulfate vaginal gel for the prevention of HIV infection: results of a Phase III trial in Nigeria. PLoS One 3:e3784.

5. Van Damme L, et al. 2008. Lack of effectiveness of cellulose sulfate gel for the prevention of vaginal HIV transmission. N. Engl. J. Med. 359:463–472.

6. Van Damme L, et al. 2002. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet 360:971–977.

7. Fichorova RN, Yamamoto HS, Delaney ML, Onderdonk AB, Doncel GF. 2011. Novel vaginal microflora colonization model providing new insight into microbicide mechanism of action. mBio 2:e00168-11.

8. Richardson RA, et al. 1998. Use of nonoxynol-9 and changes in vaginal lactobacilli. J. Infect. Dis. 178:441–445.

9. Rosenberg IJ, et al. 1998. Effect on normal vaginal flora of three intra-vaginal microbicalid agents potentially active against human immunodeficiency virus type 1. J. Infect. Dis. 177:1386–1390.

10. Bakken LR. 1985. Separation and purification of bacteria from soil. Appl. Environ. Microbiol. 49:1404–1418.

11. Staley JT, Konopka A. 1985. Measurement of in situ activities of non-photosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. 39:321–346.

12. Ravel J, et al. 2011. Vaginal microbiome of reproductive-age women. Proc. Natl. Acad. Sci. U. S. A. 108(Suppl 1):4680–4687.

13. Gajer P, et al. 2012. Temporal dynamics of the human vaginal microbiota. Sci. Transl. Med. 4:132ra152.

14. Fedorovks DN, Fiedler TL, Birozzo JM. 2005. Molecular identification of bacteria associated with bacterial vaginosis. N. Engl.J. Med. 353:1899–1911.

15. Srinivasan S, Fredricks DN. 2008. The human vaginal bacterial biota and bacterial vaginosis. Interdiscip. Perspect. Infect. Dis. 2008:750479.

16. Nugent RP, Krohn MA, Hillier SL. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J. Clin. Microbiol. 29:297–301.

17. Martin HL, et al. 1999. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. J. Infect. Dis. 180:1863–1868.

18. Taha TE, et al. 1998. Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. AIDS 12:1699–1706.

19. Schwartz JL, et al. 2006. Fourteen-day safety and acceptability study of 6% cellulose sulfate gel: a randomized double-blind Phase I safety study. Contraception 74:133–140.

20. Trifonova RT, Doncel GF, Fichorova RN. 2009. Polyamionic microbicides modify Toll-like receptor-mediated cervicovaginal immune responses. Antimicrob. Agents Chemother. 53:1490–1500.

21. Herbst-Kralovecz MM, et al. 2008. Quantification and comparison of toll-like receptor expression and responsiveness in primary and immortalized human female lower genital tract epithelia. Am. J. Reprod. Immunol. 59:212–224.

22. Joseph T, et al. 2012. Induction of cyclooxygeenase (COX)-2 in human vaginal epithelial cells in response to TLR ligands and TNF-a. Am. J. Reprod. Immunol. 67:482–490.

23. McKenna P, et al. 2008. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog. 4:e20.

24. Carlton JM, et al. 2007. Draft genome sequence of the sexually transmitted pathogen Trichomonas vaginalis. Science 315:207–212.

25. Lin J. 1991. Divergence measure based on the Shannon entropy. IEEE Trans. Info. Theory 37:145–151.

26. Cherpes TL, Meyn LA, Krohn MA, Lurie JG, Hillier SL. 2003. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. Clin. Infect. Dis. 37:319–325.

27. Peters SE, et al. 2000. Behaviors associated with Neisseria gonorrhoeae and Chlamydia trachomatis cervical infection among young women attending antenatal clinics. Clin. Infect. Dis. 31:173–177.

28. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. 2003. Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. Clin. Infect. Dis. 36:663–668.

29. Plummer M. 2010. JAGS, version 2.2.0. User manual. http://www-fs.iarc.fr/martyn/software/jags/.

30. Plummer M. 2011. Jags: Bayesian graphical models using MCMC. R Package Version 2.1-0.

31. R Development Core Team. 2008. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

32. Gelman A, Rubin DB. 1992. Inference from iterative simulation using multiple sequences. Stat. Sci. 7:457–511.

33. Plummer M, Best N, Cowles K, Vines K. 2008. CODA: output analysis and diagnostics for MCMC. R Package Version 0.13–15.