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Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women

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Abstract

Objectives—Some vaginal bacterial communities are thought to prevent infection by sexually transmitted organisms. Prior work demonstrated that the vaginal microbiota of reproductive-age women cluster into five types of bacterial communities; 4 dominated by *Lactobacillus* species (*L. iners, L. crispatus, L. gasseri, L. jensenii*), and one (termed community state type (CST) IV) lacking significant numbers of lactobacilli and characterized by higher proportions of *Atopobium,*

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Prevotella, Parvimonas, Sneathia, *Gardnerella*, *Mobiluncus,* and other taxa. We sought to evaluate the relationship between vaginal bacterial composition and *Trichomonas vaginalis*.

Methods—Self-collected vaginal swabs were obtained cross-sectionally from 394 women equally representing four ethnic/racial groups. *T. vaginalis* screening was performed using PCR targeting the 18S rRNA and β-tubulin genes. Vaginal bacterial composition was characterized by pyrosequencing of barcoded 16S rRNA genes. A panel of eleven microsatellite markers was used to genotype *T. vaginalis*. The association between vaginal microbiota and *T. vaginalis* was evaluated by exact logistic regression.

Results—*T. vaginalis* was detected in 2.8% of participants (11/394). Of the eleven *T. vaginalis*-positive cases, eight (72%) were categorized as CST-IV, two (18%) as communities dominated by *L. iners* and one (9%) as *L. crispatus*-dominated (p-value:0.05). CST-IV microbiota were associated with an 8-fold increased odds of detecting *T. vaginalis* compared to women in the *L. crispatus*-dominated state (OR:8.26, 95% CI:1.07–372.65). Seven of the 11 *T. vaginalis* isolates were assigned to two genotypes.

Conclusion—*T. vaginalis* was associated with vaginal microbiota consisting of low proportions of lactobacilli and high proportions of *Mycoplasma, Parvimonas, Sneathia*, and other anaerobes.

Keywords

Trichomonas vaginalis; vaginal microbiota

Introduction

The National Health and Nutrition Examination Survey found in 2001–2004 that 3.1% of U.S. women were positive for *Trichomonas vaginalis* genital infections (1), making it a more pervasive sexually transmitted infection (STI) than *Neisseria gonorrhea* and *Chlamydia trachomatis* combined (2). In 1999, Cates *et al.* estimated an incidence of five million new cases of trichomoniasis annually in the U.S.(3) Racial disparity in trichomoniasis rates are particularly striking with non-Hispanic black women having over ten-fold higher prevalence rates (13.3%) than non-Hispanic white (1.3%) or Mexican American (1.8%) women.(1) In a longitudinal study of 268 adolescents in Indianapolis, Indiana, incident cases of *T. vaginalis* were detected in 23% of participants and re-infection episodes occurred in 31% of those individuals.(4) Although trichomoniasis is a treatable STI (5), it continues to represent a critical public health issue as over 85% of cases are asymptomatic (1), and the protozoan is associated with numerous adverse outcomes, including preterm delivery of low-birth weight infants (6) and increased susceptibility to and transmission of HIV infection.(7;8) It has been estimated that 746 new HIV cases among U.S. women each year can be attributed to enhanced acquisition of HIV by underlying *T. vaginalis* infection.(9)

Lactic acid producing bacteria in the vagina, made up in large part by *Lactobacillus* sp., are thought to play a key protective role in preventing urogenital infection (10;11) by lowering the vaginal pH, generating bacteriostatic and bacteriocidal compounds, (12) and through possible competitive exclusion of non-indigenous organisms that include opportunistic and overt pathogens. Ravel *et al.* (13) recently demonstrated by high-throughput culture-independent molecular analysis that the vaginal bacterial communities of North American women can be classified into five community state types (CST); four of which are dominated by one of *Lactobacillus iners, L. crispatus, L. gasseri* or *L. jensenii*. The remaining group (termed CST-IV) comprised 27% of the women in the study and was characterized by higher vaginal pH and greater relative abundance of strict anaerobic organisms, including *Atopobium, Prevotella, Dialister, Gardnerella, Megasphaera,*

Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia, and *Mobiluncus*. Our overall hypothesis is that the five different vaginal bacterial communities differ in terms of their ability to fend off colonization by pathogens.

In the current study, we sought to compare the vaginal microbiota of *T. vaginalis*-positive and *T. vaginalis*-negative women using cultivation-independent methods based on the analysis of 16S rRNA genes. This molecular epidemiologic study, using high-throughput cultivation-independent methodologies, is able for the first time to decipher how vaginal bacterial communities differ among *T. vaginalis* cases. Prior work on the association of vaginal microbiota and STIs have focused on cultivation-based studies of lactobacilli and microscopy evaluation of Gram stained smears.(14–18) Cultivation-based approaches are hindered as many microbial species resist cultivation in the laboratory (19) and Gram stain provides only morphological information (20). In addition, we genotyped the *T. vaginalis*-positive samples using species-specific microsatellite markers, and successfully assigned seven of the eleven isolates to the two types that characterize the parasite's global population structure (21).

Materials and Methods

Clinical study design

This report utilized secondary analysis and repository samples from the study by Ravel *et al.* of 394 North American women representing four ethnic/racial groups (white, black, Hispanic and Asian) which has been previously described.(13) Briefly, from 2008–2009, non-pregnant, women aged 12 to 45 with regular menstrual cycles were recruited to an observational, cross-sectional study in one of two U.S. cities (Atlanta or Baltimore). Women were ineligible if they reported vaginal discharge in the prior 48 hours, pregnancy, use of intravaginal products or sexual activity in the prior 48 hours, use of antibiotics or antimycotics in the prior 30 days, significant medical or gynecological conditions (eg. posthysterectomy or immunocompromised status such as congenital, acquired or secondary to medication), or current menstrual bleeding or vaginal symptoms. Participants underwent a detailed interview in a private office, which was self-administered or administered by a female interviewer. The interview was focused on personal hygiene, sexual and contraceptive history and demographic information. Participants self-reported racial classification using pre-defined categories. Data on self-reported racial identification was collected because of observed disparities in STI rates between U.S. racial groups (1).

Using validated protocols (22;23), participants self-collected two mid-vaginal swabs using the Elution-swab system (Copan) that were respectively used to prepare a smear for Nugent Gram stain scoring (20) and to characterize the composition and structure of the resident bacterial communities using pyrosequencing of barcoded 16S rRNA genes. (13) The Nugent scoring criteria reflects the relative abundance of large Gram-positive rods (lactobacilli), Gram-negative and Gram-variable rods and cocci (i.e., *Gardnerella vaginalis*, *Prevotella*, *Porphyromonas*, and peptostreptococci), and curved Gram-negative rods (*Mobiluncus*).(20) This technique assesses the relative numbers of morphotypes, allowing a coarse classification of certain vaginal microbes. Nugent scores reflect the range of vaginal microbiota states. A score of 0–3 is designated as normal, 4–6 as an intermediate state and 7–10 as a high Nugent score indicative of bacterial vaginosis (BV) diagnosis. (20) Participants also self-collected and reported vaginal pH using the VpH glove (Inverness Medical). Study staff confirmed the pH measurement and also took a digital photo of the pH strip adjacent to the color scale.

The study was approved by Institutional Review Boards at the University of Maryland School of Medicine and the Emory University School of Medicine. All participants provided

written informed consent. The study was registered at clinicaltrials.gov under ID NCT00576797.

T. vaginalis screening and primers

Whole genomic DNA extraction was performed on the self-collected vaginal swabs as previously described.(13) *T. vaginalis* was detected by polymerase chain reaction (PCR)-based methods using two primer sets, TV16Sf-2/TV16Sr-2 (a 323 bp amplicon) targeting the 18S rRNA gene (24) and TVK3/TVK7 (a 300 bp amplicon) targeting a DNA repeat region of the *T. vaginalis* genome. (25) A third primer set, BTub3/bkmt (a 195 bp amplicon), targeting the β-tubulin gene was only used on four discordant samples and confirmed the absence of *T. vaginalis*.(24) All PCRs were performed as previously reported and amplicons were visualized by gel electrophoresis.

T. vaginalis genotyping

T. vaginalis-positive samples were genotyped using eleven microsatellite (MS) loci as previously described.(26) This subset of the published 21 MS panel consists of the most informative set of markers that allow distinction between type 1 and type 2 parasites, the two groups that *T. vaginalis* parasites are found to cluster into world-wide (21), and is ideal for use with clinical samples where low levels of target parasite DNA are a confounding factor. All genotyping PCR reactions were performed in duplicate and size polymorphisms were measured by capillary gel electrophoresis on an ABI 3130*xl* sequencer. *GeneMapper 4.0* (ABI, Foster City, CA) was used to score MS allele sizes, and all peaks were manually edited. Discrepant allele calls were repeated for a third time. Type assignment was then performed using the Bayesian clustering program, *STRUCTURE 2.2*.(27)

Statistical analysis

Fisher's exact test and exact logistic regression were used to determine the association between vaginal bacterial CST (13) and the detection of *T. vaginalis*. Factors collected from questionnaires that had been identified on the basis of previous literature and biologic plausibility were evaluated in univariable analyses. The limited number of observed *T. vaginalis*-positive cases (11 women) would not allow for further multivariable odds ratio analyses. To assess differences in the bacterial community structure between *T. vaginalis* cases and controls, we also utilized distance based redundancy analysis (dRDA) (28) and a permutation test to assess significance of the results. Data were analyzed using STATA/SE 10.0 for Windows (Stata Corporation, College Station, Texas) and the dRDA was performed using the Vegan package implemented in R (R Foundation for Statistical Computing, Vienna, Austria) (29;30).

Results

Of 394 women, eleven (2.78%) were positive for *T. vaginalis*. Among the *T. vaginalis*-positive cases, eight (72%) were detected among women belonging to the low-lactobacilli CST-IV group, while two (18%) and one (9%) were among women with bacterial communities dominated by *L. iners* and *L. crispatus* respectively (p-value: 0.05, Table 1), reflecting the differences in classification of bacterial community state type by *T. vaginalis* positivity. Figure 1 displays the proportions of bacterial taxa found among the 383 *T. vaginalis*-negative cases in comparison to the 11 *T. vaginalis*-positive study participants.

A MANOVA table resulting from dRDA analysis (data not shown) indicated that there were statistically significant differences in bacterial community structure within CST-IV between the *T. vaginalis*-positive and *T. vaginalis*-negative samples (permuted p-value = 0.013, number of permutations = 10000). Among women assigned to CST-IV, *Mycoplasma* and, to

a lesser degree, *Parvimonus* and *Sneathia* were associated with *T. vaginalis*-positivity and *Streptococcus*, *Prevotella*, *L. iners* and *Atopobium* were associated, although not as strongly, with *T. vaginalis*-negative controls. However, only 2.5% of the total variation between the communities of CST-IV is explained by *T. vaginalis*, indicating that *T. vaginalis* is not a robust predictor of community structure in this study or there were too few cases observed. For detailed information on the composition of vaginal communities of the *T. vaginalis*-positive cases, see supplementary information in Ravel *et al.* (13) for the following double blinded identification numbers-- S023, S070, S102, S103, S104, S109, S234, S255, S307, S329, S383.

Of the eleven *T. vaginalis* cases, ten (91%) were observed among African-American women, one from an Asian woman (9%) and none among the Hispanic or Caucasian women (Table 1, differences between case and control, p-value: 0.00002). In addition, none of the *T. vaginalis*-positive women reported hormonal contraceptive use versus 28% of controls (p-value: 0.04). The proportions in the paired levels of vaginal pH were also significantly different (p-value: 0.01), with pH higher (over 4.5) in 90% of cases compared to 46% of *T. vaginalis*-negative women. (pH is also displayed in Figure 1.)

In exact logistic regression modeling, CST-IV was associated with a significant 8-fold increased odds for detection of *T. vaginalis* compared to women in the *L. crispatus* community state type (odds ratio (OR): 8.26, 95% CI: 1.07–372.65). (Table 2) Similarly, high Nugent score was associated with a 9-fold increased odds for the presence of *T. vaginalis* compared to women with low Nugent scores (OR: 9.53, 95% CI: 1.77–95.71).

Seven of the eleven *T. vaginalis*-positive samples were successfully genotyped at six or more microsatellite loci. Three isolates were found to cluster within type 1 (S023, S102, S103), and four isolates within type 2 (S070, S104, S234, and S329), and none of the seven isolates appeared to be made up of more than one genotype. No statistically significant associations between isolate type and participant characteristics were found, including vaginal bacterial community state type, pH, Nugent Gram stain score, age, race/ethnicity, use of hormonal contraception or number of sexual partners (data not shown).

Comment

Our study revealed a disproportionate burden of *T. vaginalis* among women whose vaginal bacterial communities were determined by molecular analysis to be comprised of higher proportion of members of the genera *Mycoplasma, Parvimonas, Sneathia*, and other taxa (Figure 1). These samples concomitantly displayed relatively low abundance of lactobacilli. A limitation of our study is that we can only report on the association between vaginal bacterial communities and the presence of *T. vaginalis* infection; we cannot establish in this cross-sectional study design whether the observed vaginal community state type occurred before or after *T. vaginalis* acquisition. However, we can hypothesize that a highly plausible mechanism for a causal relationship is the lack of production of lactic acid by lactobacilli. O'Hanlon et al, demonstrated inhibition of bacteria by lactic acid and we can hypothesize that lactic acid may play a protective role against *T. vaginalis* as well.(11) Prior longitudinal studies have shown a significant association between vaginal microbiota (as determined by Amsel's clinical criteria for BV or Gram stain analysis) and *T. vaginalis* acquisition. (15;16;31) Our data provides the critical impetus that a large, longitudinal study with frequent sampling is needed to determine if certain types of vaginal bacterial communities predispose women to acquisition of STIs or if the observed community state types are the result of the STI. Clinical intervention on the former could reduce risk for STI acquisition.

In this report, we also sought to detect whether specific correlations between the vaginal microbiota and *T. vaginalis* genotype could be made; however, with the low number of *T.*

vaginalis positive samples genotyped, we failed to find any significant associations. It has been demonstrated that globally *T. vaginalis* clusters into two genetically distinct types, type 1 and type 2, which appear to be equally geographically widespread.(21) Future studies with larger sample sizes should be considered.

Our finding of an association between *T. vaginalis* and CST-IV may be due to confounding based on two observations. First, African American women have higher prevalence rates for *T. vaginalis* (1); and second, CST-IV is more commonly observed among African-American women (13). Ravel *et al.* previously reported that African American women made up 39% of women in CST-IV,(13) and among the *T. vaginalis*-positive cases in our study, 90% were African American women. However, it is also possible that the racial disparity in *T. vaginalis* observed in our study might reflect a STD transmission core. Laumann *et al.* modeled bacterial STD transmission in a nationally representative dataset and demonstrated that African Americans' higher STI rates could be partially explained by the patterns of sexual networks and segregated partner choices (assortative mating). (32) The latter would suggest that the higher *T. vaginalis* prevalence rate among African American women in the current study indicates that women with communities with relatively low-lactobacilli abundance (CST-IV) could be at increased risk for *T. vaginalis* acquisition upon exposure.

Strengths of this study include a large, racially diverse study population, validated protocols for self-sampling (22;23) and the use of molecular techniques to detect both *T. vaginalis* and vaginal bacterial community state types. Published PCR sensitivities for *T. vaginalis* range from 81%–97%, exceeding the sensitivities which have been reported for wet mount evaluation, Papanicolaou test, DNA probe, or culture. (5)

Limitations of the study include the recruitment of only women who reported no vaginal discharge and the limited sample size of 11 *T. vaginalis* cases out of a study of 394 women. Although statistically significant, the odds ratio estimate for the low-lactobacillus CST-IV should be taken cautiously owing to the wide confidence intervals likely due to the small number of observed cases. There were no *T. vaginalis* cases detected among the *L. gasseri* and *L. jensenii* community state types which may reflect protection by these *Lactobacillus* spp. or limitations of the sample size. In addition, a limitation of PCR is that viable and nonviable organisms cannot be distinguished. Participants reported they had not taken an antibiotic in the prior 30 days and one of the *T. vaginalis*-positive cases indicated on survey questioning that she had a *T. vaginalis* infection treated in the prior 60 days. Previously treated infections could potentially have resulted in a positive PCR result without viable organisms although dead cells and free DNA are unlikely to persist.

It is now recognized that the vaginal microbiota play a major role in maintaining the reproductive health of women. A recent study by Gatski *et al.* reported that high Nugent Gram stain score was associated with early failure of metronidazole single dose treatment for trichomoniasis among HIV-positive women.(33) In addition, differences in vaginal bacterial community composition constitute a significant variable that has not been emphasized in current research on risk factors for STI acquisition. Future research should focus on functional differences of the various vaginal bacterial communities, including the dominant lactobacilli species (and strains), and their ability to protect against urogenital pathogens. Longitudinal studies of vaginal microbiome and STIs are critical in order to determine temporality and causality. All women recruited to the current study did not report vaginal discharge, highlighting further that *T. vaginalis* is often an asymptomatic infection and suggests that screening could increase awareness, reduce transmission. Rapid screening tests for *T. vaginalis* (5) are available to medical practitioners and self-collected vaginal swabs can be utilized for *T. vaginalis* detection (34). In addition, use of self-collected mid-vaginal swabs for microbiome analysis has been validated (22;23) and provides feasibility

for field-based longitudinal studies of STI incidence (16). An increased understanding of the vaginal microbial ecosystem could lead to more effective and personalized strategies for the prevention of genital *T. vaginalis* and other STIs.

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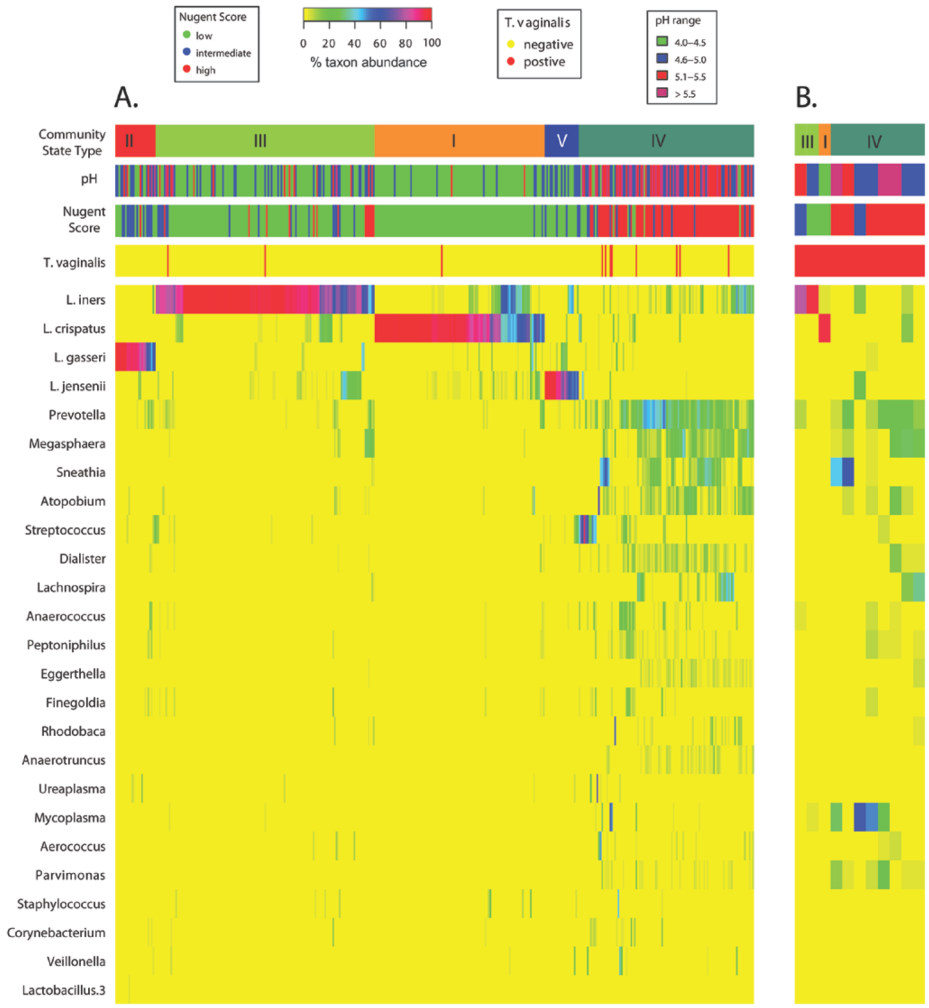

Figure 1.

Heatmap of relative percent abundance of bacterial taxa found in the vaginal bacterial communities of 394 reproductive age women (A) and of the 11 *T. vaginalis* positive cases (B). Vaginal bacterial community state type is indicated as described by Ravel *et al*[14]. Nugent Gram stain scores, pH measurements and *T. vaginalis* positivity are shown for each of the 394 samples.

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TABLE 1

Characteristics of *Trichomonas vaginalis* Positive and Negative Participants in a Cross-Sectional Study of 394 Women, Baltimore, MD and Atlanta, GA, 2008–2009

	***T. vaginalis* Negative**		***T. vaginalis* Positive**		***P****
	Number	**%**	**Number**	**%**	
Vaginal bacterial community state type (CST)					0.054
L. iners (CST-I)	133	34.7	2	18.2	
L. crispatus (CST-II)	104	27.2	1	9.1	
L. gasseri (CST-III)	25	6.5	0	0	
Low-lactobacillus (CST-IV)	100	26.1	8	72.7	
L. jensenii (CST-V)	21	5.5	0	0	
Vaginal pH					0.01
<4.5	204	53.0	1	9.1	
4.5–5.0	98	25.5	5	45.5	
>5.1	83	21.6	5	45.5	
Nugent Gram stain score					0.003
0–3	247	64.2	2	18.2	
4–6	48	12.5	2	18.2	
7–10	90	23.4	7	63.6	
Age					0.17
12–17	6	1.6	0	0.0	
18–28	163	42.3	4	36.4	
29–39	167	43.4	3	27.3	
40–45	49	12.7	4	36.4	
Race/ethnicity					0.00002
Asian	96	24.9	1	9.1	
Caucasian	98	25.5	0	0.0	
African American	94	24.4	10	90.9	
Hispanic	97	25.2	0	0.0	
Current use of hormonal contraceptive agents	95	27.8	0	0.0	0.04
Time since last routine medical checkup					0.71

	T. vaginalis Negative		*T. vaginalis* Positive		*P*[*]
	Number	**%**	**Number**	**%**	
Never	5	1.3	0	0.0	
Within the past year	283	74.9	10	90.9	
From 1 to 3 yr ago	75	19.8	1	9.1	
>3 yr ago	15	4.0	0	0.0	
Time since last Papanicolaou test					0.47
<1 yr ago	262	76.4	7	63.6	
From 1 to 3 yr ago	71	20.7	4	36.4	
>3 yr ago	10	2.9	0	0.0	
History of pregnancy	231	60.8	8	72.7	0.54
Number of sex partners in prior 6 mo					1.00
0	78	21.1	2	20.0	
1	264	71.5	8	80.0	
2	21	5.7	0	0.0	
3+	6	1.6	0	0.0	
Female sexual partner in the prior 60 d	12	3.3	0	0.0	1.00

[*] *P* value determined by Fisher exact test testing

[†] Group IV defined by Ravel et al 13 as lacking significant numbers of lactobacilli and characterized by higher proportions of strictly anaerobic organisms.

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TABLE 2

Odds of *Trichomonas vaginalis* Detection by Various Factors, Baltimore, MD and Atlanta, GA, 2008–2009 (N = 394 Women)

Characteristic	OR*	*P*	95% CI	
Vaginal bacterial community state type				
L. crispatus	REF	—	—	—
L. gasseri†	—	—	—	—
L. iners	1.56	1.00	0.08	93.14
Low-lactobacillus/CST-IV‡	8.26	0.04	1.07	372.65
L. jensenii†	—	—	—	—
Vaginal pH				
<4.5	REF	—	—	—
4.5–5.0	10.33	0.03	1.13	494.62
>5.1	12.18	0.02	1.33	583.99
Nugent Gram stain score				
0–3	REF	—	—	—
4–6	5.10	0.26	0.36	72.00
7–10	9.53	0.00	1.77	95.71
Age				
40–45	REF	—	—	—
29–39	0.22	0.11	0.03	1.36
18–28	0.30	0.20	0.05	1.69
12–17†	—	—	—	—
Ethnicity				
Asian	REF	—	—	—
Caucasian†	—	—	—	—
African American	10.13	0.01	1.39	447.72
Hispanic†	—	—	—	—

*OR, odds ratio, estimated using exact logistic regression.

†Zero *T. vaginalis* cases were detected.

‡Group IV defined by Ravel et al 13 as lacking significant numbers of lactobacilli and characterized by higher proportions of strictly anaerobic organisms.