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Bacterial vaginosis and the risk of *Trichomonas vaginalis* acquisition among HIV-1 negative women

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Abstract

Background—The vaginal microbiota may play a role in mediating susceptibility to sexually transmitted infections, including *Trichomonas vaginalis* (TV).

Methods—Data were analyzed from HIV-1 seronegative women participating in HIV Prevention Trials Network Protocol 035. At quarterly visits for up to 30 months, participants completed

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structured interviews and specimens were collected for genital tract infection testing. TV was detected by saline microscopy. BV was characterized by Gram stain using the Nugent score (BV=7-10; intermediate=4-6; normal=0-3 [reference group]). Cox proportional hazards models stratified by study site were used to assess the association between Nugent score category at the prior quarterly visit and TV acquisition.

Results—In this secondary analysis, 2,920 participants from Malawi, South Africa, USA, Zambia and Zimbabwe contributed 16,259 follow-up visits. BV was detected at 5,680 (35%) visits and TV was detected at 400 (2.5%) visits. Adjusting for age, marital status, hormonal contraceptive use, unprotected sex in the last week and TV at baseline, intermediate Nugent score and BV at the prior visit were associated with an increased risk of TV (intermediate score: adjusted hazard ratio [aHR]=1.73, 95% confidence interval [CI] 1.21-2.19; BV: aHR=2.40, 95% CI 1.92-3.00). Sensitivity analyses excluding 211 participants with TV at baseline were similar to those from the full study population (intermediate score: aHR=1.54, 95% CI 1.10-2.14; BV: aHR=2.23, 95% CI 1.75-2.84)

Conclusions—Women with a Nugent score >3 were at an increased risk of acquiring TV. If this relationship is causal, interventions that improve the vaginal microbiota could contribute to reductions in TV incidence.

Keywords

Bacterial vaginosis; *Trichomonas vaginalis*; vaginal microbiota; sexually transmitted disease acquisition; prospective cohort

Introduction

Bacterial vaginosis (BV) is a common vaginal syndrome that affects hundreds of millions of women globally each year [1, 2]. The presence of BV has been associated with a number of adverse reproductive health outcomes [2], including an increased risk of sexually transmitted infections (STIs) [3-8]. *Trichomonas vaginalis* is one of the most common curable STIs [9]. It is frequently detected among women with BV [10-12] and is also associated adverse reproductive health outcomes including preterm birth, low birth weight, and HIV acquisition [13, 14]. There is mounting evidence that the vaginal microbiota may play a role in mediating susceptibility to STIs, including TV. It is hypothesized that vaginal lactobacilli, the predominant bacterial species detected among women with a healthy vaginal environment, may utilize several mechanisms to protect against genital pathogen colonization [15]. Lactic acid production contributes to maintaining a lower vaginal pH, which may inhibit pathogen colonization [16]. In addition, exposure to hydrogen peroxide (H_2O_2) producing *Lactobacillus* species *in vitro* has been shown to decrease activity of BV-associated bacteria [17] and other genital tract pathogens [18]. Findings from *in vitro* studies are supported by data from epidemiologic studies, which showed that the absence or low levels of *Lactobacillus* colonization are associated with TV infection [19, 20].

Conversely, several prospective studies have reported an increased risk of TV among women with an abnormal vaginal microbiota or BV [3, 4, 6-8]. However, published studies that have assessed the association between the vaginal microbiota and TV acquisition have

defined “normal” and “abnormal” vaginal microbiota in a variety of ways, making it challenging to compare results across studies. We sought to evaluate the association between the vaginal microbiota at the prior study visit and incident TV infection among women enrolled in a biomedical HIV prevention trial using several categorizations of the vaginal microbiota.

Materials and Methods

This is a secondary analysis of data from women enrolled in HIV Prevention Trials Network (HPTN) Protocol 035, a phase II/IIB, four-arm, multisite, randomized, controlled trial comparing BufferGel and 0.5% PRO 2000 gel against two comparator arms (HEC placebo gel and no gel) for prevention of HIV infection (Clinicaltrials.gov #NCT00074425). Detailed methods for the trial have been described previously [21]. Briefly, between February 2005 and October 2008, 3,087 HIV-1 uninfected women from five countries (Malawi, South Africa, United States of America [USA], Zambia, and Zimbabwe) were enrolled and followed for a minimum of 12 months and a maximum of 30 months, depending on the date of enrollment. Eligible women were ≥18 years of age, HIV-1 seronegative, non-pregnant and sexually active (reported vaginal intercourse at least once in the past three months). Women were not eligible to participate if they reported a history of an adverse reaction to latex, history of non-therapeutic injection drug use in the past 12 months, frequent vaginal intercourse (more than two times per day in the prior two weeks), were within six weeks of the last pregnancy outcome, or had plans to become pregnant during follow-up. All institutional review boards and relevant regulatory authorities approved the trial at each site and all participants provided written informed consent.

Participants were randomly assigned in equal proportions to one of the four study arms. Study gels were dispensed in single-use, pre-filled applicators and were similar in appearance. Participants in the three gel arms were advised to use condoms and instructed to insert a single dose of gel intravaginally up to 1 hour prior to each act of vaginal intercourse, while participants in the no gel arm were advised to use condoms. At each monthly follow-up visit, a urine pregnancy test was performed. At quarterly visits, data were collected on self-reported gel use, condom use, sexual behaviors, vaginal washing and contraceptive use. Speculum-assisted pelvic examinations were performed with collection of specimens for diagnosis of genital tract infections and blood was collected through venipuncture for HIV-1 testing. All participants received a comprehensive HIV prevention package that included ongoing HIV risk reduction counseling, free male latex condoms, and diagnosis and treatment of STIs throughout the trial.

Laboratory procedures

A Gram stain of vaginal fluid was evaluated at a central laboratory for diagnosis of BV using the Nugent score [22]. A vaginal saline wet mount was examined microscopically for the presence of motile trichomonads for diagnosis of TV. Urine specimens were tested for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* annually and at study exit using BD ProbeTec ET (Becton Dickinson; Franklin Lakes, New Jersey, USA). HIV-1 infection status was determined using a standardized algorithm. The Determine HIV 1/2 (Abbott Diagnostic

Division; Hoofddorp, Netherlands) test was used with either the OraQuick® (Orasure Technologies; Bethlehem, Pennsylvania, USA), or Uni-Gold Recombigen® HIV test (Trinity Biotech; Wicklow, Ireland). Western blot (Genetics systems HIV-1 Western Blot kit, BioRad Laboratories; Hercules, CA, USA) was performed on samples with any positive HIV-1 result. HSV-2 testing was performed at enrollment and study exit using the HerpeSelect-2 EIA (Focus Technologies; Cypress, California, USA).

Statistical analysis

The objective of this analysis was to assess the association between the vaginal microbiome at the quarterly visit prior to TV testing and first incident TV infection. The vaginal microbiome was characterized using the Nugent score, where a score of 7-10 indicated BV, 4-6 was considered intermediate and 0-3 was considered normal (reference group). In our primary analysis, we separately compared incident TV infections among women with a Nugent score of 7-10 and 4-6 versus 0-3. Secondary analyses included comparisons of incident TV among women with a Nugent score >3 versus 0-3 and a Nugent score >6 versus 0-6, as these comparisons have been utilized by others [3, 4, 6-8]. Participants were excluded from the analysis if they did not return for a follow-up visit after enrollment, did not receive TV testing during follow-up or if they were found to be HIV-1 infected at baseline. If Nugent score results were not available at the quarterly visit prior to TV testing, the TV result visit was excluded from the analysis. Log-binomial regression with generalized estimating equations using an independent correlation structure and robust standard errors was used to assess demographic, behavioral and clinical factors associated with BV. Cox proportional hazards models stratified by study site were used to assess the association between the Nugent score at the prior quarterly visit and TV acquisition. Age, marital status, unprotected sex in the past week and baseline TV status were included in the multivariable model based on *a priori* assumptions. Additional factors were considered for inclusion in the model if they were associated with BV by Nugent score and incident TV infection ($p<0.10$). Participants were censored at their first incident TV infection or if they became pregnant, HIV-1 infected or tested positive for another STI (*C. trachomatis* or *N. gonorrhoeae*). Since participants received treatment for STIs diagnosed at enrollment, we included participants diagnosed with TV at baseline in the primary analysis. However, among women with TV at baseline, subsequent infections may represent TV persistence or re-infection; therefore, we conducted a sensitivity analysis excluding participants who tested positive for TV at enrollment. All statistical tests were assessed using a 2-sided α of 0.05. Analyses were conducted using Stata version 12.0 (StataCorp, Inc., College Station, TX).

Results

Of 3,087 HIV-1-uninfected participants enrolled in HPTN 035, 71 (2%) participants did not return for follow-up, did not undergo TV testing during follow-up or did not have BV results in the interval prior to all TV testing during follow-up. In addition, 96 (3%) participants were censored for other reasons prior to having a TV test with BV results during follow-up and were excluded (81 were censored due to pregnancy, 11 were HIV-infected, and 4 were diagnosed with another STI), leaving 2,920 (95%) participants who contributed 16,259 follow-up visits for analysis. Compared to participants included in the analysis, participants

who were excluded were slightly younger (median age [interquartile range (IQR)]: 23 years (21-27) versus 25 years (22-30); p<0.001) and less likely to be married or living with a partner (58% versus 70%; p=0.007). Sexual behaviors reported at baseline, BV and STIs were similar between participants who were included versus those excluded from the analysis (data not shown).

Demographic, behavioral, and clinical characteristics at enrollment are presented in aggregate and by TV acquisition status during follow-up in Table 1. The median number of follow-up visits per participant was 4 (IQR: 3-6). BV by Nugent score was detected at 5,680 (35%) of 16,259 visits and an intermediate score was present at 2,622 (16%) visits. Being married or living with a partner was associated with a decreased likelihood of BV during follow-up (odds ratio [OR] = 0.90; 95% confidence interval [CI] 0.83, 0.98). In addition, compared to participants not using hormonal contraception, oral hormonal contraception and injectable hormonal contraception were both associated with modest decreases in the likelihood of BV during follow-up (oral contraceptive use: OR=0.90; 95% CI 0.80, 0.99; injectable use: OR=0.77; 95% CI 0.70, 0.84). Conversely, TV at baseline was associated with an increased likelihood of BV (OR=1.19; 95% CI 1.04, 1.36). Self-reported sexual behaviors (number of sex partners in the past 3 months, vaginal sex in the past week, unprotected sex in the past week), vaginal washing practices, and study arm were not associated with BV during follow-up (data not shown).

Overall, TV was detected at 400/16,259 (2.5%) visits; TV was detected at 1.5% (118/7,957) of visits with a normal Nugent score at the prior visit compared to 2.6% (67/2,622) among visits with an intermediate score at the prior visit and 3.8% (215/5,680) among women with BV at the prior visit. In univariate analyses, higher Nugent score category at the prior visit was associated with an increased risk TV infection (Table 2). An intermediate score was associated with a 1.73-fold increased likelihood of TV (95% CI 1.28, 2.33), while BV was associated with a 2.53-fold increased likelihood (95% CI 2.02, 3.17). This linear trend in the hazard ratios was statistically significant (p<0.001). After adjusting for age, marital status, hormonal contraceptive use, unprotected sex in the last week and TV at baseline, an intermediate score and BV at the prior visit were independently associated with an increased likelihood of TV (Table 2). Secondary analyses using alternative categorizations of the Nugent score showed similar results (Nugent score >3 versus 0-3: aHR = 2.15, 95% CI 1.74, 2.67; Nugent score >6 versus 0-6: aHR = 2.06, 95% CI 1.69, 2.51).

Baseline TV was also associated with an increased risk of TV in our multivariable model (aHR=2.65; 95% CI 2.03, 3.48). Although participants diagnosed with TV at baseline received treatment according to the study protocol, subsequent infections may represent TV persistence due to treatment failure or re-infection if the partner was not treated (especially among participants who tested positive for TV at their first follow-up visit). Therefore, we conducted a sensitivity analysis excluding 211 participants who had baseline TV infection (39 [18%] of which were also infected with TV at their next follow-up visit). Among 2,709 participants without TV infection at baseline, the likelihood of TV infection among women with abnormal vaginal microbiota was similar to results observed for the full study population for both intermediate Nugent score (aHR = 1.54; 95% CI 1.10, 2.14) and BV (aHR= 2.23; 95% CI 1.75, 2.84).

Discussion

Women with BV or an intermediate Nugent score were at an increased risk of acquiring TV compared to women with a normal vaginal microbiota. Results were similar when we assessed different categorizations of the Nugent score (>3 versus 0-3 and >6 versus 0-6). The findings from our analyses extend those reported by others and add to the evidence that suggests that an abnormal vaginal microbiota increases susceptibility to TV infection [3, 4, 6-8].

Although a number of studies have reported an increased risk of TV acquisition among women with BV, the precise nature of the relationship between specific vaginal bacterial species and communities and TV susceptibility is not well understood. Over the past decade, molecular techniques targeting bacterial 16S ribosomal RNA gene (rDNA) sequences have been used to characterize the vaginal microbiota [23], and hold great promise for providing insight into the relationships between vaginal health and STI acquisition, including TV. Improving our understanding of the relationship between the vaginal microbiota and TV susceptibility could lead to effective, targeted interventions that eradicate high-risk bacteria and promote vaginal colonization with bacterial species and communities that are associated with lower STI risk.

We also observed that women with TV infection at baseline had an increased likelihood of TV acquisition during follow-up, despite receiving treatment for TV at enrollment into the trial. The majority of women with TV at baseline tested negative for TV at their next follow-up visit. In addition, greater than 95% of women reported only 1 sexual partner during follow-up, suggesting that these women were re-infected with TV by their partners. Testing and treatment services were available to partners of women participating in the study; however, data on partner treatment was not systematically collected. Partner treatment services are not routinely offered in sub-Saharan Africa, where syndromic treatment is commonly used for STI treatment and microbiologic testing is not frequently performed [24]. As STI diagnostic capacity improves in resource-limited settings, additional information on the acceptability and uptake of partner treatment services in research studies and other clinical settings where pathogen specific STI testing and treatment occur as part of standard of care will be helpful for informing the scale-up of these services.

Compared to women not using hormonal contraception, we observed a decreased likelihood of TV among women using oral or injectable hormonal contraception. There is mixed evidence regarding the effect of hormonal contraception on TV acquisition. Several studies have reported a reduced risk of TV among women using oral or injectable hormonal contraception, while others have observed no association [3, 7, 25, 26]. There are a number of methodological challenges that complicate analyses assessing the effect of hormonal contraception on STI acquisition, including choice of comparison group, method switching, and inadequate control of potential time-varying confounding factors such as condom use [27]. Differences in study design and analytic approach may contribute to the heterogeneity of published results. In the present analysis, the relationship between hormonal contraception and TV acquisition was assessed as a potential confounder and was not assessed as the primary objective of the study; therefore our findings should be interpreted

in that context. Additional studies that appropriately address methodological challenges are needed to improve our understanding of the relationship between hormonal contraception and TV acquisition.

The present analysis includes several limitations that should be considered when interpreting the results. The primary limitation of this analysis is that only saline microscopy was used to detect TV infection. Although it is the most commonly used method for TV diagnosis in clinical settings, saline microscopy has considerably lower sensitivity compared with culture and nucleic acid amplification testing (NAAT) [28]. Use of a less sensitive diagnostic method for TV detection may have resulted in failure to detect TV infection in some women, which would contribute to an attenuation of the results. Furthermore, TV is more likely to be detected among women with a higher vaginal pH [13]. If organism burden differs by BV status, the low sensitivity of saline microscopy for TV detection may have resulted in greater TV detection among women with BV compared to those without. Thus, the relationship between BV and TV could be related to parasite density and its inherent impact on detection rather than differences in infection rates. Additional prospective studies using more sensitive diagnostics methods (NAAT) will help to further clarify the relationship between BV and TV acquisition. The interval between BV assessment and TV testing is also a limitation of this study. Evidence from prospective studies has shown that the vaginal microbiota fluctuates over time [29, 30]. Shorter intervals between assessment of BV and TV status, such as monthly testing, would reduce potential misclassification in the characterization of the vaginal microbiota prior to TV acquisition. In addition, our study population included women at risk for HIV infection participating in a clinical trial who may differ from women in the general population. Lastly, BV and STI acquisition are both associated with sexual activity [31]. Although the observed associations were strong and we attempted to control for confounding due to sexual behavior in our multivariable analyses, it is possible that a proportion of the observed association between BV and TV could be explained by insufficient control of important behavioral confounders.

In summary, women with an abnormal vaginal microbiota were at an increased risk of acquiring TV compared to women with a normal vaginal microbiota. We observed a dose-response relationship, whereby the risk of TV acquisition increased with higher Nugent score category. If the relationship between the vaginal microbiota and TV acquisition is causal, interventions that decrease the incidence of BV and promote a normal vaginal microbiota could potentially contribute to reductions in TV incidence. However, current BV treatments fail to produce sustained changes in the vaginal microbiota [32-34]. Alternative regimens that improve cure rates and produce sustained changes in the vaginal microbiota are needed. Such interventions that successfully cure and control BV could be evaluated in clinical trials as part of a vaginal health approach to reducing susceptibility to TV infection.

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Table 1

Participant characteristics at enrollment into HPTN 035*

	All participants N=2,920	Participants who acquired TV infection during follow-up N=400	Participants who did not acquire TV infection during follow-up N=2,520
Demographic characteristics			
Age			
<25 years	1,303 (45)	174 (44)	1,129 (45)
25-29 years	840 (29)	94 (23)	746 (30)
30-34 years	532 (18)	68 (17)	464 (18)
35-39 years	112 (4)	32 (8)	80 (3)
>40 years	133 (5)	32 (8)	101 (4)
Married or co-habiting	2,041 (70)	247 (62)	1,794 (71)
Some secondary school	1,832 (63)	207 (52)	1,625 (64)
Site			
Malawi – Blantyre	404 (14)	42 (11)	362 (14)
Malawi – Lilongwe	574 (20)	115 (29)	459 (18)
South Africa – Durban	666 (23)	48 (12)	618 (25)
South Africa – Hlabisa	317 (11)	96 (24)	221 (9)
USA – Philadelphia	193 (7)	46 (12)	147 (6)
Zambia – Lusaka	303 (10)	23 (6)	280 (11)
Zimbabwe – Chitungwiza	248 (8)	16 (4)	232 (9)
Zimbabwe – Harare	215 (7)	14 (3)	201 (8)
Study arm			
No Gel	728 (25)	97 (25)	629 (25)
Placebo	728 (25)	99 (25)	631 (25)
PRO2000	733 (25)	117 (27)	616 (24)
BufferGel	731 (25)	87 (22)	644 (26)
Behaviors			
Vaginal sex in the past 7 days	2,504 (86)	312 (78)	2,192 (87)
Condom use at last sex (%)	1,988 (68)	254 (64)	1,734 (69)
Vaginal washing[1]			
Nothing	1,800 (62)	275 (69)	1,525 (61)
Water	776 (27)	68 (17)	708 (28)
Soap & water	326 (11)	49 (12)	277 (11)
Vinegar & water	73 (3)	19 (5)	54 (2)
Paper, cotton, cloth, wool	294 (10)	39 (10)	255 (10)
Hormonal contraception			
Oral contraceptives	567 (19)	53 (13)	514 (20)
Injectables	1,419 (49)	176 (44)	1,243 (49)
Implants	37 (1)	5 (1)	32 (1)
Laboratory characteristics			
Nugent score 7-10 (BV)	1,082 (37)	197 (49)	885 (35)

	All participants N=2,920	Participants who acquired TV infection during follow-up N=400	Participants who did not acquire TV infection during follow-up N=2,520
Nugent score 4-6 (intermediate)	506 (17)	77 (19)	429 (17)
Trichomonas vaginalis	211 (7)	71 (18)	140 (6)
Chlamydia trachomatis[2]	100 (3)	17 (4)	83 (3)
Neisseria gonorrhoeae[2]	20 (1)	1(<1)	19 (1)
HSV-2 seropositive	1,265 (43)	217 (54)	1,048 (42)

[*] Data presented as N (%)

[1] At enrollment, participants were asked about vaginal washing practices in the past month. Total >100% as participants could choose more than one option.

[2] Chylamidia and gonorrhea results missing for 1 participant

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Table 2

Demographic and behavioral factors associated with incident TV

			Unadjusted			Adjusted*		
	Visits with TV N=400	**Visits without TV N=15, 859**	**HR**	**(95% CI)**	**p-value**	**HR**	**(95% CI)**	**p-value**
Nugent score at prior study visit								
0-3 (reference)	118 (30)	7,839 (49)	1.00	---	---	1.00	---	---
4-6 (intermediate)	67 (17)	2,555 (16)	1.73	(1.28, 2.33)	<0.001	1.63	(1.21, 2.19)	0.001
7-10 (BV)	215 (54)	5,465 (35)	2.53	(2.02, 3.17)	<0.001	2.40	(1.92, 3.00)	<0.001
Enrollment characteristics								
Age								
<25 years	174 (44)	6,166 (39)	1.00	---	---	1.00	---	---
25-29 years	94 (23)	4,595 (29)	0.77	(0.60, 0.99)	0.04	0.82	(0.64, 1.05)	0.12
30-34 years	68 (17)	3,284 (21)	0.83	(0.62, 1.10)	0.19	0.79	(0.58, 1.05)	0.10
35-39 years	32 (8)	710 (4)	1.97	(1.34, 2.90)	0.001	0.99	(0.65, 1.50)	0.96
>40 years	32 (8)	1,104 (7)	1.47	(0.99, 2.18)	0.06	0.74	(0.45, 1.21)	0.23
Married or co-habiting	247 (62)	11,359 (72)	0.64	(0.52, 0.78)	<0.001	0.88	(0.64, 1.22)	0.45
At least some secondary school	207 (52)	9,864 (62)	0.64	(0.53, 0.78)	<0.001			
Behaviors at each visit								
Sex partners in the past 3 months								
none	4 (1)	194 (1)	1.01	(0.38, 2.69)	0.98			
1 (reference)	385 (96)	15,336 (97)	1.00	---	---			
>1	11 (3)	329 (2)	1.48	(0.82, 2.68)	0.20			
Vaginal sex in the past week	307 (77)	12,979 (82)	0.72	(0.57, 0.91)	0.006			
Unprotected sex in the past week[1]	104 (26)	3,951 (25)	1.07	(0.86, 1.34)	0.53	1.02	(0.81, 1.28)	0.86
Hormonal contraception								
None/non-hormonal methods	145 (36)	3,966 (25)	1.00	---	---	1.00	---	---
Oral contraceptive use	63 (16)	3,285 (21)	0.44	(0.33, 0.59)	<0.001	0.64	(0.47, 0.89)	0.007
Injectable contraceptive use	188 (47)	8,384 (53)	0.52	(0.42, 0.64)	<0.001	0.60	(0.47, 0.78)	<0.001
Implant	4 (1)	224 (1.41)	0.39	(0.15, 1.03)	0.06	0.57	(0.20, 1.60)	0.29
TV at baseline	71 (18)	886 (6)	3.40	(2.62, 4.42)	<0.001	2.65	(2.03, 3.48)	<0.001

[1] Versus sex with a condom or no sex

* Stratified by site and adjusted for age (category), marital status, hormonal contraceptive use (time-varying characteristic), unprotected sex (time-varying characteristic) and TV at baseline.