

# Relationship of Specific Vaginal Bacteria and Bacterial Vaginosis Treatment Failure in Women Who Have Sex with Women

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**Background:** Bacterial vaginosis frequently persists after treatment. The role of newly defined bacterial vaginosis-associated bacteria (BVAB), which have a specificity for this condition of 97% or greater, has not been assessed.

**Objective:** To define risks for bacterial vaginosis persistence, including pretreatment detection of specific vaginal bacteria, among women reporting sex with women.

**Design:** Observational cohort study.

**Setting:** University-based research clinic.

**Patients:** 335 women age 16 to 29 years reporting sex with at least 1 woman in the past year. Participants were recruited through advertisements and provider referral.

**Intervention:** Bacterial vaginosis was treated with intravaginal metronidazole gel (0.75%), 37.5 mg nightly for 5 nights.

**Measurements:** Species-specific 16S recombinant DNA polymerase chain reaction assays targeting 17 bacterial species were applied to vaginal fluid obtained at baseline. Test of cure by clinical criteria and Gram stain analysis and repeated polymerase chain reaction assays of vaginal fluid were performed 1 month after treatment, and interim behaviors were assessed by using computer-assisted self-interview.

**Results:** Of 335 women, 24% of whom also reported sex with men within 3 months before enrollment, 131 (39%) had bacterial

vaginosis. In the 120 (92%) women who returned for follow-up, the incidence of persistent bacterial vaginosis was 26% and was statistically significantly higher in women with baseline detection of 3 *Clostridiales* bacteria, designated as BVAB1 (risk ratio, 2.0 [95% CI, 1.1 to 4.0]), BVAB2 (risk ratio, 8.7 [CI, 2.5 to ∞]), or BVAB3 (risk ratio, 3.1 [CI, 1.7 to 5.8]); *Peptoniphilus lacrimalis* (risk ratio, 3.5 [CI, 1.6 to 15.5]); and *Megasphaera* phylotype 2 (risk ratio, 3.4 [CI, 1.4 to 5.5]). Persistence was lower with treatment adherence (risk ratio, 0.4 [0.2 to 0.9]). Detection of these bacteria at the test-of-cure visit was associated with persistence, whereas post-treatment sexual activity was not.

**Limitations:** Findings may not be generalizable to women who have sex only with men, or to women whose bacterial vaginosis is treated with oral antibiotics. The study may be too small and may involve a population that is too highly selected to draw definitive conclusions about associations of persistent infection with posttreatment sexual behaviors.

**Conclusion:** Persistent bacterial vaginosis is associated with several bacteria in the *Clostridiales* order, *Megasphaera* phylotype 2, and *P. lacrimalis*, suggesting that vaginal microbiology at diagnosis may determine risk for antibiotic failure.

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**B**acterial vaginosis is characterized by depletion of hydrogen peroxide-producing lactobacilli that characterize normal vaginal flora and profound overgrowth of anaerobic bacteria (1). Bacterial vaginosis is the most prevalent vaginal infection in reproductive-age women, affecting 8% to 29%, and is the most common cause of vaginal symptoms prompting medical care (2). Of 3739 women enrolled during 2001 to 2004 in a nationally representative sample of the U.S. civilian noninstitutionalized population, almost 1 in 3 (29.2% [95% CI, 27.2% to 31.3%]) had bacterial vaginosis, as determined by Gram stain of vaginal fluid (3, 4). Bacterial vaginosis has been consistently associated with adverse outcomes related to the upper genital tract and with increased risk for HIV acquisition (5–7).

Treatment of bacterial vaginosis targets the abundance of anaerobes that define the condition. With oral metronidazole used for 7 days or vaginal metronidazole for 5 days, symptoms improve in 83% to 87% of women by 2 to 3 weeks (8, 9). The improvement rate is similar for women who use vaginal clindamycin regimens—both antibiotics are recommended (10)—and the restoration rate of vaginal lactobacilli at 30 days is similar (11, 12). Although short-

term treatment response is acceptable, bacterial vaginosis persists or recurs in 11% to 29% of women at 1 month (8, 13, 14) and long-term recurrence rates exceed 70% (15–17). Few studies have reported factors associated with bacterial vaginosis recurrence after successful treatment, such as black race, older age, higher Nugent score at enrollment (17), history of bacterial vaginosis, regular sex partner during study, female sex partner, and hormonal contraception (15). Even fewer studies have assessed risks associated with bacterial vaginosis persistence at 1 month after treatment. Available data suggest that condom use in the period immediately after treat-

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**Context**

Bacterial vaginosis, a condition that is particularly common among women who have sex with women, is an overgrowth of anaerobic bacteria and depletion of normally occurring lactobacilli. Bacterial vaginosis persists or recurs in 11% to 29% of women at 1 month after treatment.

**Contribution**

This study identified that the presence of specific vaginal bacteria at baseline (*Clostridia* BVAB1, BVAB2, or BVAB3; *Peptoniphilus lacrimalis*; or *Megasphaera* phylotype 2) and lower adherence to treatment were the only factors associated with persistence of bacterial vaginosis 1 month after treatment among women who have sex with women.

**Caution**

The results might not apply to women who have sex only with men.

—The Editors

ment might support maintenance of normal vaginal flora and thus increase cure rates (16, 18).

For unknown reasons, women who have sex with women have a high prevalence of bacterial vaginosis (25% to 52%) (3, 19). We hypothesized that this population might provide a unique opportunity to study the response to treatment of bacterial vaginosis because the potentially confounding exposure of unprotected vaginal intercourse was not likely to be common. In a cohort study of vaginal flora in this population, we assessed the incidence of bacterial vaginosis persisting at 1 month after treatment with vaginal metronidazole. In addition to measuring the contribution of recognized risk factors for bacterial vaginosis, including race, sexual behaviors, and douching, we assessed the contribution of specific species of bacterial vaginosis-associated bacteria (BVAB) present at initiation of bacterial vaginosis treatment. These bacteria include fastidious anaerobes detected by species-specific polymerase chain reaction (PCR) assays, some of which have not yet been cultivated and include 3 recently identified bacteria in the *Clostridiales* order (BVAB1, BVAB2, and BVAB3) that are highly specific (>97%) for bacterial vaginosis (20).

**METHODS****Participants and Clinical Definitions**

The study population comprised women age 16 to 30 years who reported sex with at least 1 woman in the previous year and who responded to recruitment through advertisements, media, and community referral between October 2004 and December 2006. Participants completed an extensive computer-assisted self-interview on demographic characteristics and medical, reproductive, and sexual history and underwent a standardized examination, in-

cluding collection of vaginal fluid for Gram stain, saline microscopy, pH measurement, potassium hydroxide evaluation, and culture of *Trichomonas vaginalis*. We asked all participants to return for 4 quarterly visits or at any time if genital symptoms developed. To obtain specimens for bacterium-specific PCR assays, we brushed a polyurethane foam swab (Catch-All, Epicentre Biotechnologies, Madison, Wisconsin) against the lateral vaginal wall, resheathed it, and froze it immediately in a  $-80^{\circ}\text{C}$  freezer until DNA extraction. We diagnosed bacterial vaginosis if 3 of 4 clinical (Amsel) criteria (vaginal pH  $>4.5$ , clue cells on saline microscopy  $>20\%$  of epithelial cells, amine odor on addition of potassium hydroxide, or homogeneous vaginal discharge) were present (21) and Gram stain of vaginal fluid confirmed abnormal flora (Nugent score  $>3$ ) (4). We treated women who had bacterial vaginosis with vaginal metronidazole gel (37.5 mg nightly for 5 days) and asked them to return in 1 month for test of cure, when we repeated examination and collected vaginal fluid for Gram stain in all women. Initially, we collected vaginal fluid samples for bacteria-specific PCR assays at test-of-cure visits for all women whose vaginal pH was greater than 4.0 and thus had suspected bacterial vaginosis or trichomoniasis. After approximately one half of the study participants were enrolled, we collected these samples routinely in all participants at the test-of-cure visit.

For the analysis, we used the first visit at which a woman was found to have bacterial vaginosis (whether at the initial enrollment visit, at a later quarterly routine visit, or at a visit self-initiated for vaginal symptoms). We used test-of-cure visits after a woman's first bacterial vaginosis-positive visit that were completed before 31 March 2007 to examine incidence of persistent bacterial vaginosis and abnormal vaginal flora. We defined persistent bacterial vaginosis by Amsel criteria. We confirmed persistent bacterial vaginosis by Nugent score of vaginal fluid greater than 6 at the 1-month follow-up visit and confirmed abnormal vaginal flora by Nugent score greater than 3. We obtained written informed consent from all participants. Conduct of the study adhered to standard guidelines for research involving human participants and was approved by the University of Washington and Fred Hutchinson Cancer Center Human Subjects Review Committees.

**Microbiological Analysis**

For DNA extraction, we placed vaginal swabs for bacterial PCR assay in 15-mL conical vials with 2 mL of saline and vortex-mixed them for 1 minute to dislodge cells. We centrifuged the solution at 14 000 rpm for 10 minutes and resuspended the pellet in 100  $\mu\text{L}$  of supernatant. We extracted DNA from the pellet by using the Ultra Clean Soil DNA Kit (MoBio, Carlsbad, California) according to the manufacturer's instructions. We eluted DNA from silica columns in a 150- $\mu\text{L}$  volume buffer. We performed sham digests by using a swab without human contact with each round of DNA extraction (every 10 to 25 samples) to con-

trol for possible contamination from kit reagents or collection swabs.

We developed bacterium-specific PCR assays to detect species-specific regions of the 16S rRNA gene. We aligned 16S rDNA sequences from vaginal bacteria detected by broad-range 16S rDNA PCR assays (22). We designed primers to target highly variable regions of the bacterial 16S rRNA gene that seem to be unique for each species. We developed PCR assays for 17 bacterial species that were commonly detected in vaginal samples (23). Each 50- $\mu$ L PCR reaction contained 1  $\times$  PCR Buffer II, 2 mmol of magnesium chloride, 0.8 mmol of deoxyribonucleotide triphosphate mix, and 1 U of AmpliTaq Gold DNA Polymerase (all from Applied Biosystems, Foster City, California), as well as 0.2  $\mu$ mol each of forward and reverse primer and 1  $\mu$ L of template DNA. The PCR conditions included a premelt at 95  $^{\circ}$ C for 10 minutes, then 40 to 45 cycles of 95  $^{\circ}$ C for 30 seconds (melt), 53  $^{\circ}$ C to 62  $^{\circ}$ C for 30 seconds (annealing), and 72  $^{\circ}$ C for 30 seconds (extension), followed by a final extension at 72  $^{\circ}$ C for 7 minutes. We visualized PCR products after electrophoresis in 2% agarose gels and stained them with ethidium bromide. We optimized bacterial PCR assays so that each could detect as few as 100 molecules of cloned 16S rDNA per reaction, although most assays could detect 1 to 10 molecules and thus had even lower detection thresholds. We sequenced every PCR assay with a visible band of the expected size on gel electrophoresis (BigDye, version 3; Applied Biosystems) to confirm that the PCR product had at least 99% similarity with the expected bacterial target, thereby assuring bacterial specificity. We considered PCR reactions to be negative if they did not have visible bands on gel electrophoresis or confirmed sequence homology. We ran no-template PCR controls (consisting of master mix, primers, and water) and sham digest controls (template consisting of water subjected to DNA extraction) with each PCR assay to monitor for contamination.

We subjected each participant's extracted DNA to a human  $\beta$ -globin PCR assay to assure that amplifiable DNA was successfully extracted from the sample and to monitor for PCR inhibitors (24). The  $\beta$ -globin PCR protocol used is the same as that listed for bacterial PCR, with the exception that the following primers were used: GH2O-5'-GAAGAGCCAAGGACAGGTAC-3' and PCO4-5'-CAACTTCATCCACGTTCCACC-3'. In addition, we performed an internal amplification control quantitative PCR assay by using DNA from each vaginal sample to detect more subtle PCR inhibitors by monitoring the amplification of an exogenously added template (jellyfish aequorin gene target) at a known concentration (25).

We tested participants for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by using the APTIMA-COMBO 2 assay (Gen-Probe, San Diego, California) on urine. We performed this test at all enrollment visits and at follow-up visits if participants reported interim risk behavior (new sex partner, more than 1 partner) or genitourinary symptoms.

## Statistical Analysis

We measured bivariate associations between participants' characteristics and detection of bacterial vaginosis at the 1-month posttreatment visit by calculating risk ratios and produced 95% CIs by using bootstrap percentiles (26). Characteristics included demographic information, sexual and genital hygiene behaviors in the interim period between diagnosis and assessment at 1 month, and PCR detection of individual bacterium in vaginal fluid at the baseline visit. We estimated adjusted risk ratios by using Poisson regression, which provides unbiased estimates of the log-relative risks and bootstrap CIs (27). We adjusted for other statistically significant covariates and for adherence to treatment. We performed these analyses among all women for whom follow-up visits were available regardless of time to follow-up and then on a limited "per-protocol" group (follow-up between 21 and 44 days after treatment). To examine whether bacteria found to be associated with persistence when present at the baseline visit were also preferentially present in vaginal fluid of women with persistent bacterial vaginosis, we applied bacterium-specific PCR assays to vaginal fluid obtained at the follow-up visits for which these specimens were available. Because the population with samples available for this analysis was enriched with bacterial vaginosis-positive women, odds ratios are presented instead of risk ratios. All tests for statistical significance were 2-sided, and we considered a *P* value less than 0.05 to be statistically significant. We performed analyses by using R, version 2.4.1 ([www.r-project.org](http://www.r-project.org)), and Stata, version 9.2 (Stata, College Station, Texas).

## Role of the Funding Source

The National Institute of Allergy and Infectious Diseases provided funding for the study. The funding source had no role in the design or conduct of the study; the collection, management, or statistical analysis of the data; or in the submission of the manuscript for publication.

## RESULTS

Table 1 summarizes the characteristics of 335 women enrolled in the year-long prospective study. Two women (0.6%) had *C. trachomatis* infection. None had *N. gonorrhoeae* infection, trichomoniasis, or clinically evident genital herpes. Ninety-six women (28.7%) had bacterial vaginosis at enrollment, and an additional 35 women had bacterial vaginosis at either a routine quarterly follow-up visit (*n* = 28) or a self-initiated visit for vaginal symptoms (*n* = 7) (Figure). Of these 131 women, 120 returned for follow-up (adherence to follow-up for all participants, 92%). Median time to follow-up was 34 days (range, 21 to 78 days); 75% returned within 36 days. Of the 120 women for whom we report test-of-cure findings, 119 returned for scheduled visits and 1 returned for a self-initiated symptom visit at 42 days after treatment. The per-protocol group, who returned between 21 and 44 days, comprised 108 women.  $\beta$ -Globin was amplified

**Table 1. Characteristics of 335 Participants, by Bacterial Vaginosis (BV) Status at Any Visit\***

Characteristic	BV Present (n = 131)	BV Absent (n = 204)
Age, y		
Median	25	25
Range	15–35	17–34
Race (self-defined), n (%)†		
White	98 (75)	156 (76)
Black	8 (6)	8 (4)
Nonwhite other than black	20 (15)	36 (18)
Declined to provide race data	5 (4)	4 (2)
Sex with women in past 3 mo, n (%)	92 (83)	164 (80)
Female sex partners in past 90 d, n		
Median	1	1
Range	0–7	0–4
Sex with men in past 3 mo, n (%)	21 (19)	453 (26)
Male sex partners in past 90 d, n‡		
Median	0	0
Range	0–5	0–4
Consistent condom use, n (%)	5 (28)	14 (31)
Current cigarette smoking, n (%)	48 (42)	57 (33)
Douching in past 1 mo, n (%)	6 (5)	8 (4)
Hormonal contraception use in past 60 d, n (%)	10 (8)	25 (12)
Vaginal symptoms present, n (%)§	42 (34)	39 (20)
Concurrent genitourinary infection, n (%)		
Vulvovaginal candidiasis	3 (2)	15 (8)
Trichomoniasis	0 (0)	0 (0)
Chlamydia trachomatis infection	2 (2)	1 (1)

\* BV was defined by the presence of Amsel criteria and confirmed by Nugent score >6.

† Participants were permitted to choose >1 category to describe their race. “White” refers to those who chose only “white” to describe their race; “black” refers to those who chose “black or African American” even if they also chose another race; and “nonwhite” refers to those who chose any other race besides white.

‡ Among women who reported vaginal intercourse with a male partner in the past 3 mo.

§ Defined as change in amount, color, or odor of vaginal discharge.

from every vaginal sample, confirming contact of swabs with a human surface. We found no evidence of PCR inhibition in any sample that used the internal amplification control PCR assay.

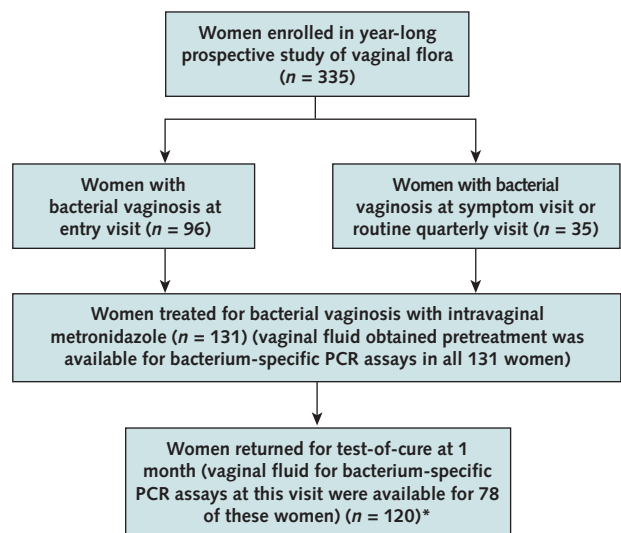
At 1-month follow-up, persistent bacterial vaginosis (defined by Amsel criteria) occurred in 31 women (25.8%); we confirmed each case by Nugent score. An additional 4 women had bacterial vaginosis by Nugent score only but were not counted as having persistent bacterial vaginosis. We observed abnormal flora (Nugent score >3) in 44 women (36.7%). Table 2 shows relationships between participants’ characteristics and the detection of either persistent bacterial vaginosis or abnormal flora. Of bacteria detected by PCR assay at initial diagnosis, *Atopobium* species, *Gardnerella vaginalis*, *Megasphaera* phylotype 1, and *Lactobacillus iners* were found in most women (>97%). Thus, we could not examine whether rates of persistence differed by presence or absence of these bacteria. However, women with BVAB1, BVAB2, or BVAB3 at baseline had statistically significantly higher rates of persistence than those without the bacterium (15 of 38 [39%]

with BVAB1 vs. 16 of 82 [19%] without, 30 of 93 [32%] with BVAB2 vs. 1 of 26 [3%] without, and 18 of 38 [47%] with BVAB3 vs. 13 of 82 [16%] without). Those with *Peptoniphilus lacrimalis* at baseline were also more likely to have persistent bacterial vaginosis (26 of 72 [36%] with vs. 5 of 48 [10%] without), as were those with *Megasphaera* phylotype 2 (4 of 5 [80%] with vs. 27 of 115 [23%] without), whereas those who adhered to metronidazole therapy were less likely to have the condition.

Of the 110 women who indicated whether they had completed treatment with vaginal metronidazole therapy, all but 8 (92%) completed the full course of treatment. Twenty-five (25%) of those completing treatment had persistent bacterial vaginosis versus 5 (63%) of the 8 who did not complete treatment. Women with *Mobiluncus curtisii* at baseline had an increased incidence of persistent bacterial vaginosis, but this did not reach statistical significance. These findings did not differ when we analyzed women in the per-protocol group separately.

Characteristics that statistically significantly predicted persistent bacterial vaginosis at 1 month also predicted detection of abnormal flora. Black race approached statistical significance. Only 5 women seen at follow-up were black; 2 had persistent bacterial vaginosis, but 4 had abnormal flora. Most women (78%) who did not complete metronidazole therapy had abnormal flora, whereas 32% of those

**Figure. Study flow diagram.**



PCR = polymerase chain reaction.

\* Vaginal fluid was not available for all 120 women because posttreatment vaginal fluid collection was not instituted until approximately one third of the way through the study. Initially, vaginal fluid was selectively collected for women with suspected bacterial vaginosis at follow-up because of limited resources. Collection of vaginal fluid at the follow-up visit was later broadened to all women. Other than having bacterial vaginosis, women who had posttreatment vaginal fluid collected did not differ from those who did not according to age, race, or posttreatment sexual behavior with male or female partners.

**Table 2. Associations between Persistent Bacterial Vaginosis (BV) or Abnormal Vaginal Flora and Participant Characteristics\***

Characteristic	Prevalence in All Participants, %	Persistent BV (n = 31), n (%) <sup>*</sup>	BV Cured (n = 89), n (%)	Risk Ratio (95% CI) <sup>†</sup>	Abnormal Flora (n = 44), n (%) <sup>‡</sup>	Normal Flora (n = 76), n (%)	Risk Ratio (95% CI) <sup>†</sup>
<b>Demographic</b>							
Age >25 y	48	25 (48)	42 (48)	1.0 (0.5–1.8)	21 (48)	36 (48)	1.0 (0.6–1.6)
Black race	4	2 (6)	3 (3)	1.6 (0.0–4.2)	4 (9)	1 (1)	2.3 (1.0–3.7)
Vaginal symptoms <sup>§</sup>	34	9 (31)	29 (35)	0.9 (0.6–2.5)	14 (34)	24 (33)	1.0 (0.6–1.7)
<b>Results of bacterium-specific PCR assays at baseline visit<sup>  </sup></b>							
BVAB1	32	15 (48)	23 (26)	2.0 (1.1–4.0)	20 (45)	18 (24)	1.8 (1.1–2.9)
BVAB2	78	30 (97)	63 (71)	8.7 (2.5–∞)	40 (91)	53 (70)	2.9 (1.4–13.2)
BVAB3	31	18 (58)	19 (21)	3.1 (1.7–5.8)	22 (50)	15 (20)	2.2 (1.4–3.8)
<i>Peptoniphilus</i> sp.	80	27 (87)	69 (78)	1.7 (0.8–8.3)	38 (86)	58 (76)	1.6 (0.8–4.7)
<i>Peptoniphilus lacrimalis</i>	60	26 (84)	46 (52)	3.5 (1.6–15.5)	33 (75)	39 (51)	2.0 (1.2–4.0)
<i>Mobiluncus curtisii</i>	49	20 (65)	39 (44)	1.9 (1.0–3.7)	26 (59)	33 (43)	1.5 (0.9–2.4)
<i>Megasphaera</i> phylotype 2	4	4 (13)	1 (2)	3.4 (1.4–5.5)	4 (9)	1 (1)	2.3 (1.0–3.6)
<i>Atopobium</i> sp.	96	13 (100)	59 (95)	–	23 (96)	49 (96)	1.0 (0.3–∞)
<i>Megasphaera</i> phylotype 1	96	13 (100)	59 (95)	–	22 (92)	50 (98)	0.5 (0.2–∞)
<i>Leptotrichia</i> sp.	79	10 (77)	49 (79)	0.9 (0.3–∞)	19 (79)	40 (78)	1.0 (0.5–3.8)
BVAB-TM7	24	4 (31)	14 (23)	1.4 (0.3–4.0)	5 (21)	13 (25)	0.8 (0.2–1.8)
<i>Eggerthella</i> sp.	89	12 (92)	55 (89)	1.4 (0.4–∞)	22 (92)	45 (88)	1.3 (0.5–∞)
<i>Lactobacillus iners</i>	97	13 (100)	60 (97)	–	23 (96)	50 (98)	∞ (0.2–∞)
<i>Lactobacillus crispatus</i>	16	2 (15)	10 (17)	1.0 (0.0–3.2)	4 (19)	8 (16)	1.1 (0.2–2.2)
<i>Gardnerella vaginalis</i>	96	13 (100)	59 (95)	–	24 (100)	48 (94)	–
<i>Mobiluncus mulieris</i>	15	4 (31)	7 (11)	2.6 (0.4–3.9)	6 (25)	5 (10)	1.9 (0.8–3.0)
<i>Prevotella</i> genogroup 1	94	11 (85)	47 (76)	1.6 (0.5–∞)	17 (71)	41 (80)	0.7 (0.4–1.9)
BVAB 1 or BVAB3	47	22 (71)	34 (38)	2.8 (1.5–6.6)	28 (64)	28 (37)	2.0 (1.2–3.9)
BVAB1, BVAB2, or BVAB3	82	31 (100)	67 (75)	–	41 (93)	57 (78)	3.1 (1.3–∞)
<b>Interim behaviors, enrollment to 1-mo follow-up visit</b>							
Adherence to treatment	93	25 (89)	77 (96)	0.4 (0.2–0.9)	33 (83)	69 (99)	0.4 (0.3–0.6)
Sex partner with BV <sup>¶</sup>	17	5 (16)	15 (17)	0.9 (0.2–2.0)	7 (16)	13 (17)	0.9 (0.4–1.8)
Any sexual activity	78	26 (84)	68 (76)	1.4 (0.7–5.2)	38 (86)	56 (74)	1.8 (0.9–5.3)
Any sex with female	68	22 (71)	60 (67)	1.1 (0.6–3.1)	31 (70)	51 (67)	1.1 (0.7–2.1)
Vaginal intercourse with male	16	6 (19)	13 (15)	1.3 (0.4–2.5)	8 (18)	11 (14)	1.2 (0.5–2.0)
Receptive oral sex <sup>**</sup>	56	18 (58)	49 (55)	1.1 (0.6–2.2)	28 (64)	39 (51)	1.4 (0.9–2.5)
Receptive anal sex <sup>††</sup>	16	3 (10)	16 (18)	0.6 (0.0–1.4)	6 (14)	13 (17)	0.8 (0.3–1.5)

BVAB = bacterial vaginosis–associated bacteria; PCR = polymerase chain reaction.

\* Defined by the presence of Amsel criteria and confirmed by Nugent score >6.

† Bootstrap resampling was used to estimate the 95% CI.

‡ Defined by Nugent score >3.

§ Defined as change in vaginal discharge, including increased or malodorous vaginal discharge.

|| n = 120 for BVAB1, BVAB2, BVAB3, *Peptoniphilus* sp., *M. curtisii*, *P. lacrimalis*, and *Megasphaera* phylotype 2 (24 with persistent BV, 35 with abnormal flora); n = 75 for *Atopobium* sp., *Megasphaera* phylotype 1, *Leptotrichia* sp., BVAB-TM7, *Eggerthella* sp., *L. iners*, *L. crispatus*, *G. vaginalis*, *M. mulieris*, and *Prevotella* genotype 1 (13 with persistent BV, 22 with abnormal flora).

¶ Assessed for 3 most recent female sex partners.

\*\* Includes sex performed by either male or female sex partners.

†† Includes any receptive anal sexual behavior (anal intercourse, oral–anal sex, digital–anal sex).

who completed metronidazole therapy did. Although most women reported sexual activity in the month after bacterial vaginosis diagnosis, persistence was not related to any specific activity, such as use of condoms or vaginal lubricants, sharing of sex toys, or genital-to-genital contact (data not shown; see Table 2 for other variables).

Although too few participants were included for us to perform extensive multivariable analysis, we assessed associations between detection of specific bacteria at baseline, adjusted for nonadherence to treatment (Table 3). Detection of either BVAB3 or *P. lacrimalis* at baseline remained statistically significantly associated with the likelihood of bacterial vaginosis persistence.

Vaginal fluid samples were available from the test-of-cure visit for 78 of the 120 women who returned. Other than having a higher likelihood of bacterial vaginosis, women for whom posttreatment vaginal fluid was collected did not differ from those for whom it was not in age, race, or posttreatment sexual behavior (data not shown). As shown in Table 4, risk for persistent bacterial vaginosis was higher among women who were positive at the test-of-cure visit for any of the bacteria associated with persistence, whether this was analyzed in all women (regardless of detection of these bacteria at baseline) (group 1) or only those who had these bacteria at baseline (group 2) (except for *Megasphaera* phylotype 2, for which numbers were too small to examine the relationship).

**Table 3. Multivariable Analysis of Factors Associated with Persistence of Bacterial Vaginosis (BV) in 113 Women, Adjusted for Nonadherence to Treatment**

BVAB Detected at Baseline	Risk Ratio (95% CI)*	Expected Risk for BV Persistence among Adherent Participants (95% CI)†
BVAB3	2.6 (1.4–5.45)	0.20 (0.04–0.44)
<i>Peptoniphilus lacrimalis</i>	2.8 (1.2–13.3)	0.22 (0.10–0.36)
Neither BVAB nor <i>P. lacrimalis</i>	Referent	0.08 (0.02–0.15)

BVAB= bacterial vaginosis–associated bacteria.  
 \* Risk ratios and 95% CIs were obtained by using Poisson regression with bootstrap CIs.  
 † Expected risks for BV persistence and 95% CIs were obtained by using Poisson regression with bootstrap CIs. In these 113 women, 34% had neither BVAB3 nor *P. lacrimalis*, 8% had only BVAB3, 37% had only *P. lacrimalis*, and 24% had both types of bacteria detected at baseline. Adherent participants with both BVAB3 and *P. lacrimalis* detected have an expected risk for BV persistence of 0.58 (CI, 0.37–0.80).

**DISCUSSION**

Among women seen 1 month after treatment of bacterial vaginosis with vaginal metronidazole, predictors of treatment failure included detection of BVAB at baseline, including the *Clostridiales* bacteria BVAB1, BVAB2, and BVAB3; detection of *P. lacrimalis* and *Megasphaera* phylotype 2 at baseline; and failure to adhere to 5 days of vaginal metronidazole therapy. Of note, a report of no specific sexual activity with either male or female partners in the month after treatment predicted either persistent bacterial vaginosis or abnormal flora. The latter finding is notable, because others have reported that unprotected vaginal intercourse is associated with recurrent bacterial vaginosis. However, our ability to detect such associations was limited by the relatively few outcomes observed and the selected population studied. The bacteria we found to be associated with persistence were detectable when treatment failure was diagnosed and were rarely detected in women whose bacterial vaginosis resolved.

Our findings have several possible explanations. First, bacterial vaginosis is a heterogeneous syndrome characterized by diverse microflora, some of which might harbor complete or relative resistance to antibiotics commonly used for treatment. Cultivable bacteria associated with bacterial vaginosis, particularly *Mobiluncus*, can be resistant to metronidazole or clindamycin (28–30). Nyirjesy and colleagues (31) assessed bacterial vaginosis–associated morphotypes on Gram stain at test-of-cure visits in women treated with vaginal metronidazole or clindamycin. Women treated with metronidazole were less likely than those treated with clindamycin to clear *Mobiluncus* morphotypes (95.5% vs. 66.7%, respectively; *P* = 0.047), and those

**Table 4. Presence of Bacterial Vaginosis–Associated Bacteria (BVAB) in Vaginal Fluid 30 Days after Treatment, by Persistent Bacterial Vaginosis (BV) versus Cure\***

Results of Bacterium-Specific PCR Assay	BV Status at 30 Days after Treatment					
	Group 1: All Available Observations (n = 59)			Group 2: Women with BVAB-Specific PCR Assay at Baseline (Pretreatment)		
	Persistence, n (%)*	Cure, n (%)	Odds Ratio (95% CI)†	Persistence, n (%)‡	Cure, n (%)	Odds Ratio (95% CI)
<b>BVAB1</b>						
Positive	13 (46)	5 (9)	6.1 (1.7–24.5)	12 (83)	4 (30)	8.0 (1.1–66.5)
Negative	18 (54)	42 (91)	–	3 (17)	8 (70)	–
<b>BVAB2</b>						
Positive	26 (88)	6 (11)	35.5 (8.6–2159)	26 (91)	5 (16)	33.8 (7.0–182)
Negative	5 (12)	41 (89)	–	4 (9)	26 (94)	–
<b>BVAB3</b>						
Positive	14 (42)	1 (3)	37.9 (4.8–164)	14 (77)	1 (11)	35.0 (2.9–1622)
Negative	17 (58)	46 (96)	–	4 (23)	11 (89)	–
<b><i>Peptoniphilus lacrimalis</i></b>						
Positive	23 (79)	7 (17)	16.4 (4.7–60.3)	22 (86)	3 (14)	38.5 (6.4–271)
Negative	8 (21)	40 (83)	–	4 (14)	21 (82)	–
<b><i>Megasphaera</i> phylotype 2§</b>						
Positive	–	–	–	3 (75)	0 (0)	–
Negative	–	–	–	1 (25)	1 (100)	–

PCR = polymerase chain reaction.  
 \* Defined by the presence of Amsel criteria and confirmed by Gram stain at 30-day visit.  
 † Odds of persistent BV in women who are positive for bacteria by PCR at the 30-day posttreatment visit versus odds in women negative for same; 95% CIs are exact.  
 ‡ Denominator varies because not all women were positive by PCR assay for all BVAB at baseline visit.  
 § PCR assay for *Megasphaera* phylotype 2 was not performed on all women at the posttreatment visit because only 5 women were positive for this bacterium at baseline.

with *Mobiluncus* morphotypes at baseline were more likely to experience cure when treated with clindamycin than with metronidazole. More recently, detection of *M. curtisii* was reported to be associated with bacterial vaginosis recurrence (32). In our study, women with *M. curtisii* at baseline were also more likely to have persistent bacterial vaginosis, although this association did not reach statistical significance. Data from our study indicate that detection of all 3 novel bacteria of the *Clostridiales* order associated with bacterial vaginosis (BVAB1, BVAB2, and BVAB3) by specific PCR assay is positively associated with detection of *Mobiluncus* morphotypes on Gram stain, especially BVAB1 (33). *Mobiluncus* species and BVAB1 have curved-rod structures when visualized by using fluorescence in situ hybridization (20). It is possible that BVAB1 may be confused with *Mobiluncus* on Gram stain, although the Gram stain characteristics of BVAB1 are currently unknown. Other data support the theory that the choice of antibiotic for bacterial vaginosis can have measurable effects on the population of cultivatable vaginal flora. In 1 study, women with bacterial vaginosis who were treated with clindamycin (but not metronidazole) had high frequencies (80%) of clindamycin-resistant anaerobic bacteria persisting for 90 days after treatment (34). *Atopobium vaginae* has also been associated with bacterial vaginosis, and many isolates are resistant to metronidazole in vitro (35) and may predict treatment failure (36, 37). Molecular characterization of *Peptostreptococcus* has resulted in the subdivision of this genus into several different genera, including the *Peptoniphilus* genus consisting of nonsaccharolytic butyrate-producing bacteria, such as *P. lacrimalis* (38, 39); these bacteria may be more susceptible to clindamycin than to metronidazole (40, 41). Finally, persistence of a tenacious biofilm that hinders access of antibiotic to BVAB may further increase women's risk for treatment failure (42).

In our study participants, bacterial vaginosis was not associated with risk factors that others have identified, such as failure to use condoms with male partners (15–18). This may partly be explained by a low incidence of sexual contact with men among our participants in the month after treatment. However, given the high prevalence of bacterial vaginosis among lesbians, the frequency of concordant bacterial vaginosis in female sex partners, and the observation that a report of sex with another woman was associated with increased risk for recurrence in 1 prospective study (15), we were surprised to find no association between persistent bacterial vaginosis and sexual activities with female partners in the month after treatment. This could be explained by the few women in these subgroups or by an overriding role of baseline vaginal flora associated with bacterial vaginosis promoting persistence in this group—rather than persistent “re-infection” or exposure to another causative factor through sex (15–18). One hypothesis is that some *Clostridiales* bacteria associated with bacterial vaginosis may produce spores that result in rapid vaginal

recolonization after antibiotic therapy, analogous to the process underlying relapse of *Clostridium difficile* colitis.

Our study has limitations. First, our participants were selected on the basis of reporting sex with other women. Although 24% also reported sex with men in the 3 months before enrollment, they are unlikely to be representative of exclusively heterosexual women, a group that should be studied in similar fashion and to whom our findings may not apply. However, the fact that our participants infrequently reported vaginal intercourse with male partners in the month after treatment might be advantageous in helping to define the role of vaginal microbiology in determining response to treatment. Second, we have not performed these analyses with data derived from quantitative vaginal cultures obtained in our participants; this analysis is under way. Third, our findings may not apply to women whose bacterial vaginosis is treated with an oral antibiotic regimen.

Our findings raise several important points for future research. First, comparative assessments of risks for persistent bacterial vaginosis, including molecular characterization of baseline microbiology of BVAB, are needed in heterosexual women. These efforts should help to define whether the molecular epidemiology of bacterial vaginosis in heterosexual women differs from that in lesbians and whether unprotected vaginal intercourse with male partners promotes persistence. Second, our BVAB-specific assays were qualitative. Quantitative PCR assays applied to vaginal fluid samples obtained in our participants should offer additional insight into the natural history of this condition. For example, if key BVAB are resistant to antibiotic therapy, then vaginal levels of these bacteria may never decline, resulting in persistent bacterial vaginosis. Alternatively, vaginal levels of these BVAB may decline with antibiotic therapy, but eradication may not be achieved. This, along with re-inoculation with these bacteria, could set the stage for future relapse. Because prolonged therapy with twice-weekly suppressive vaginal metronidazole statistically significantly reduced recurrence rates relative to placebo over 4 months in 1 study (17), more intensive antibiotic therapy might overcome the problem of persistence. Finally, the establishment of consensus definitions for persistence and recurrence of bacterial vaginosis would greatly assist in progress toward describing the natural history of this condition. Whether risk factors that promote persistence are the same as those that promote recurrence is not clear.

In summary, our findings suggest that vaginal colonization with key BVAB at diagnosis is an independent risk factor for persistent bacterial vaginosis after standard antibiotic therapy. Because bacterial vaginosis may confer an increased risk for poor pregnancy outcome and HIV acquisition and may predict upper genital tract disease, understanding the reasons for treatment failure and success should be a priority. If vaginal microbiology at diagnosis predicts persistent bacterial vaginosis, then tailored treatment approaches based on initial vaginal microflora are a possible strategy for optimizing treatment outcomes. Our

findings support the need for more rigorous study of bacterial vaginosis treatment that should pair alternative antibiotic strategies with subsequent PCR-based monitoring of bacterial populations in the vagina. Further study is also needed to cultivate the bacteria we found to be associated with treatment failure to measure complete or partial antibiotic resistance as a possible explanation for persistent bacterial vaginosis.

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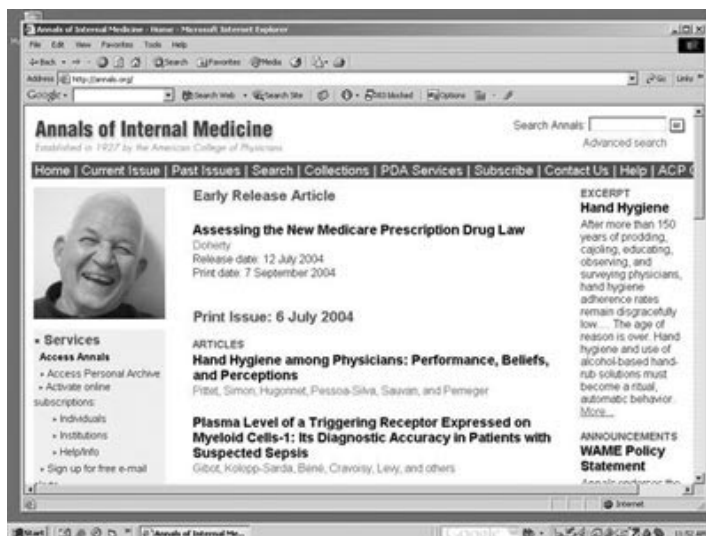
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