

ORIGINAL ARTICLE

Molecular Identification of Bacteria Associated with Bacterial Vaginosis

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ABSTRACT

BACKGROUND

Bacterial vaginosis affects millions of women and is associated with several serious health conditions. The cause of bacterial vaginosis remains poorly understood despite numerous studies based on cultures. Bacteria in microbial communities can be identified without cultivation by characterizing their ribosomal DNA (rDNA) sequences.

METHODS

We identified bacteria in samples of vaginal fluid with a combination of broad-range polymerase-chain-reaction (PCR) amplification of 16S rDNA with clone analysis, bacterium-specific PCR assay of 16S rDNA, and fluorescence in situ hybridization (FISH) performed directly on vaginal fluid from 27 subjects with bacterial vaginosis and 46 without the condition. Twenty-one subjects were studied with the use of broad-range PCR of 16S rDNA, and 73 subjects were studied with the use of bacterium-specific PCR.

RESULTS

Women without bacterial vaginosis had 1 to 6 vaginal bacterial species (phlotypes) in each sample (mean, 3.3), as detected by broad-range PCR of 16S rDNA, and lactobacillus species were the predominant bacteria noted (83 to 100 percent of clones). Women with bacterial vaginosis had greater bacterial diversity ($P < 0.001$), with 9 to 17 phlotypes (mean, 12.6) detected per sample and newly recognized species present in 32 to 89 percent of clones per sample library (mean, 58 percent). Thirty-five unique bacterial species were detected in the women with bacterial vaginosis, including several species with no close cultivated relatives. Bacterium-specific PCR assays showed that several bacteria that had not been previously described were highly prevalent in subjects with bacterial vaginosis but rare in healthy controls. FISH confirmed that newly recognized bacteria detected by PCR corresponded to specific bacterial morphotypes visible in vaginal fluid.

CONCLUSIONS

Women with bacterial vaginosis have complex vaginal infections with many newly recognized species, including three bacteria in the Clostridiales order that were highly specific for bacterial vaginosis.

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N Engl J Med 2005;353:1899-911.

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BACTERIAL VAGINOSIS IS A COMMON condition, affecting millions of women annually,¹ and is associated with numerous health problems including preterm labor resulting in low birth weight,^{2,3} pelvic inflammatory disease,^{4,5} and acquisition of the human immunodeficiency virus.^{6,7} Malodorous vaginal discharge may be the only symptom of bacterial vaginosis, and many affected women are asymptomatic.⁸

Studies using cultivation methods have shown that women with bacterial vaginosis have loss of vaginal lactobacilli and concomitant overgrowth of anaerobic and facultative bacteria. Several bacteria have been implicated in bacterial vaginosis, such as *Gardnerella vaginalis*⁹ and *Mobiluncus curtisii*,¹⁰ but these species are also found in subjects who do not have bacterial vaginosis and thus are not specific markers for disease.¹¹ For this reason, bacterial cultivation of vaginal fluid has not proved useful for the diagnosis of bacterial vaginosis. Rather, clinical criteria or Gram's staining of vaginal fluid is used for diagnosis.

At least three of the following four elements must be present to fulfill the clinical criteria of Amsel et al. for bacterial vaginosis¹²: thin, homogeneous, milky vaginal discharge; vaginal-fluid pH greater than 4.5; a positive whiff test (i.e., production of a fishy odor when 10 percent potassium hydroxide is added to a slide containing vaginal-fluid); and clue cells (>20 percent of epithelial cells with adherent bacteria) on microscopical examination of vaginal fluid.¹² An alternative diagnostic approach is to use Gram's staining of vaginal fluid to distinguish normal vaginal flora (i.e., gram-positive rods and lactobacilli) from bacterial vaginosis flora (gram-negative morphotypes)¹³ according to the Nugent score.¹⁴

Koch's postulates for establishing disease causation have not been fulfilled for any bacterium or group of bacteria associated with bacterial vaginosis. The disorder responds to treatment with antibiotics such as metronidazole and clindamycin, but metronidazole has a low level of activity in vitro against *G. vaginalis* and *M. curtisii*. Relapse and persistence are common.¹¹ Thus, the causes and pathogenesis of bacterial vaginosis remain poorly understood, and management can be challenging.

Only a small fraction of the bacteria present in most microbial ecosystems are amenable to propagation in the laboratory.¹⁵ Bacteria in complex microbial communities can be identified by characterizing their ribosomal RNA genes (rDNA),

an approach that has the advantage of detecting fastidious or cultivation-resistant organisms.¹⁶ We sought to detect and identify bacteria in vaginal-fluid samples with the use of molecular methods.

METHODS

STUDY POPULATION

A total of 87 women were recruited from two groups known to have a high prevalence of bacterial vaginosis.¹⁷⁻²⁰ We studied the first 73 women enrolled. Fourteen were patients at Public Health–Seattle and King County Sexually Transmitted Disease (STD) Clinic, and 59 were study participants at Harborview Medical Center Women's Research Clinic, in Seattle, who reported engaging in same-sex behavior in the previous year and most of whom were also sexually active with male partners. Women were eligible if they were 16 to 45 years of age and able to provide written informed consent.

Single baseline samples of vaginal fluid from the 73 subjects were studied with the use of bacterium-specific polymerase-chain-reaction (PCR) assays of 16S rDNA, including samples from 27 subjects with bacterial vaginosis as defined according to the clinical criteria of Amsel et al.¹² and from 46 subjects without bacterial vaginosis. Samples of vaginal fluid from 21 of these 73 subjects were studied with the use of broad-range bacterial PCR of 16S rDNA with clone analysis, including single baseline samples from 9 subjects with bacterial vaginosis and 8 subjects without bacterial vaginosis and multiple follow-up samples from 4 subjects with newly diagnosed, resolved, relapsed, or persistent bacterial vaginosis (11 samples).

At both clinics, subjects underwent examinations with a speculum to collect vaginal fluid for saline microscopy, microscopical visualization with potassium hydroxide, measurement of pH, and assessment for an amine odor. Subjects at the STD clinic were tested routinely for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, and those seen at the Women's Research Clinic were tested with the use of standard screening and diagnostic criteria.²¹ Vaginal fluid was collected for molecular studies by brushing the lateral vaginal wall with a foam swab that was subsequently frozen.

Written informed consent was obtained from all participants in this prospective study, which was approved by institutional review boards at the Fred Hutchinson Cancer Research Center and the University of Washington in Seattle. Samples

were collected between October 16, 2001, and May 5, 2004.

SAMPLE PREPARATION

Swabs for PCR assays were placed in 15-ml conical tubes with saline, and the mixture was vortexed to dislodge cells. Sham swabs (with no patient contact) were processed in parallel to monitor for contamination of reagents. Saline solution was centrifuged at 14,000×g for 10 minutes, and the supernatant was discarded. The pellet was digested with the use of the QIAmp stool kit (Qiagen) with a 95°C lysis step according to the manufacturer's instructions. Methods for broad-range PCR of 16S rDNA, bacterium-specific PCR, and fluorescence in situ hybridization (FISH) are described in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

STATISTICAL ANALYSIS

The samples of vaginal fluid were assessed as they were obtained, and the decision to stop the analysis at 73 subjects was made on the basis of data showing that there were statistically significant associations between the detection of several bacterial species and bacterial vaginosis. Differences in the number of taxa detected in bacterial vaginosis and control libraries of clones were assessed with the use of the Mann-Whitney U test. Univariate associations between the detection of individual bacteria by bacterium-specific PCR and the presence of bacterial vaginosis were measured with Fisher's exact test and SPSS software (version 10.1.4), and exact confidence intervals were calculated with the use of Stata software (version 8.2). Multivariate logistic-regression analysis was performed with the use of LogXact software (version 4.0.2), and covariates, in addition to individual bacteria and combinations of bacteria according to bacterium-specific PCR included the age of the subject, the clinical site of enrollment, the presence or absence of a report of abnormal vaginal discharge, and the presence or absence of a report of having had sex with men. All tests for significance were two-sided, and P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Of the 73 enrolled women, 27 had bacterial vaginosis at baseline and 46 did not (see Table 1 of the Supplementary Appendix). Slightly more than half

the women with bacterial vaginosis were symptomatic, and very few had another genitourinary infection in addition to bacterial vaginosis.

Broad-range bacterial PCR of 16S rDNA with analysis of cloned sequences was performed on 28 samples of vaginal fluid from 21 subjects. Table 1 shows the bacterial species (phylotypes) detected and the percentage of clones from each library derived from these bacteria. Among subjects for whom only single baseline samples were evaluated (Table 1), those without bacterial vaginosis had a mean of 3.3 bacterial phylotypes per library (range, 1 to 6). *Lactobacillus* species were the dominant bacteria detected (83 to 100 percent of clones per library; mean, 97 percent), particularly *L. crispatus* and *L. iners*. Most bacterial 16S rDNA sequences in subjects without bacterial vaginosis closely matched known bacteria.

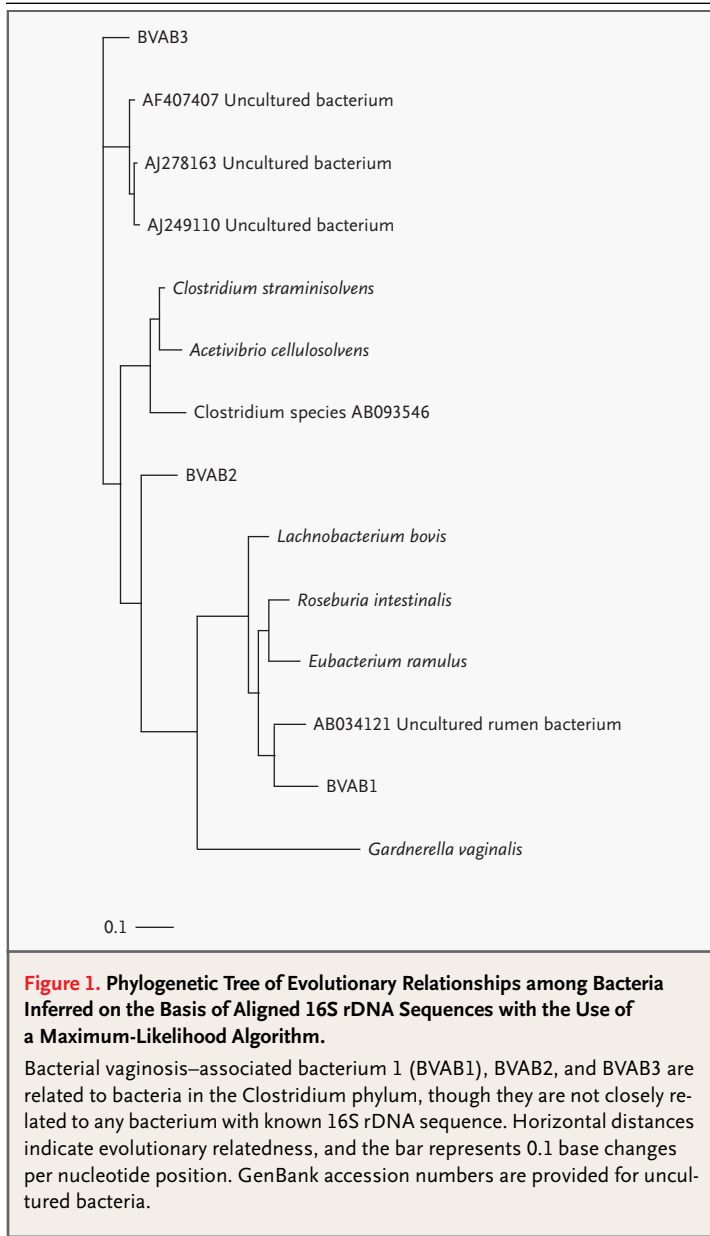
Broad-range bacterial PCR analysis of 16S rDNA from the vaginal fluid of subjects with bacterial vaginosis showed a high level of species diversity (Table 1), with a mean of 12.6 bacterial phylotypes per library of clones (range, 9 to 17), a level significantly higher than that in subjects without bacterial vaginosis ($P < 0.001$). Overall, newly recognized bacterial phylotypes (bacteria with 16S rDNA that had <98 percent similarity to known sequences) were present in 58 percent of clones per library derived from bacterial vaginosis samples (range, 32 to 89 percent).

L. crispatus 16S rDNA was not detected in libraries of clones from subjects with bacterial vaginosis, whereas *L. iners* was detected in most subjects. *G. vaginalis* was detected in all bacterial-vaginosis clone libraries and *M. mulieris* in one library. Mycoplasma species were not detected in any clone library, despite sequence homology with the broad-range PCR primers of 16S rDNA that were used. Other bacteria frequently detected in subjects with bacterial vaginosis included *Atopobium vaginae*, two megasphaera species, two distinct dialister phylotypes, *Leptotrichia amnionii* and the related bacterium *Sneathia sanguinegens*, *Porphyromonas asaccharolytica*, and a bacterium distantly related to *Eggerthella hongkongensis* (92 percent sequence similarity). Nine different bacteria related to prevotella species were detected. Three phylogenetic clusters of these bacteria were only distantly related to known prevotella species (<95 percent sequence similarity), and we have designated these clusters prevotella genogroups 1, 2, and 3 on the basis of shared sequences within each group. Less frequently detect-

Table 1. Bacteria Identified by Broad-Range 16S rDNA Polymerase Chain Reaction in Vaginal Fluid from Subjects with Bacterial Vaginosis and

Broad-Range 16S rDNA PCR Clones	Subjects with BV									Control Subjects without BV							
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8
<i>Lactobacillus crispatus</i>										49	74	99	48	60	89	40	100
<i>L. jensenii</i>													2		1		
<i>L. gallinarum</i>										13							
<i>L. gasseri</i>											9			1			
<i>L. vaginalis</i>													2				
<i>Staphylococcus epidermidis</i>											2						
<i>S. lugdunensis</i>													1				
<i>Clostridium perfringens</i> (96%) AB045286														3			
<i>Ureaplasma parvum</i>												1					
<i>L. iners</i>				7	22	3		1	5	38		1	46	36	10	60	
<i>Gardnerella vaginalis</i>	35	13	2	4	28	1	25	31	39		14						
Uncultured AB034121 (90.6%) (BVAB1)		43	66	34		36	17		26								
Uncultured AF407407 (90.9%) (BVAB2)	10	4		10	3	5	4		4								
Uncultured ULO278163 (92.9%) (BVAB3)	1	1					1										
<i>Atopobium vaginae</i>	5		3	21	1	3	3	11	11								
<i>Leptotrichia amnionii</i>	6		2	8	1	3	10	10									
<i>Megasphaera elsdenii</i> (93.8%) AY038994	4	10	7	1	18	3	2	13	6								
<i>M. micronuciformis</i> (94.6%) AF473833								1									
<i>Eggerthella hongkongensis</i> (91.8%) AY288517	2	2	2	1	4	1	3	8									
<i>Porphyromonas asaccharolytica</i>				2		5											
Dialister species (α)	1	1	2	2									1				
Dialister species (β) (94.8%) AF371693			1	2		2	2	1									
<i>Sneathia sanguinegens</i>	3		2		16	9	9										
Prevotella genogroup 1	21	24		1	7	9	12	20									
Prevotella genogroup 2	7			4		6	7		1								
Prevotella genogroup 3			7					3									
<i>P. bivia</i>																	
<i>P. buccalis</i>									6								
<i>P. dentalis</i> (93.2%) X81876						2	2										
<i>P. disiens</i>																	
<i>P. oulorum</i> (90.6%) L16472																	
<i>P. shahii</i> (90.7%) AB108825																	
Uncultured 4C28d-23 (91.2%) AB034149	1		3	1		2											
Candidate division TM7 (93.7%) AF125206			2	2		8											
<i>Mobiluncus mulieris</i>																	
<i>Peptoniphilus lacrimalis</i>								1									
Peptoniphilus species		1				2											
<i>Peptostreptococcus micros</i> (97.8%) AF542231	3			2				1									
<i>Gemella bergeriae</i> (95.8%) Y13365	1																
Aerococcus species								1	2								
<i>Anaerococcus tetradius</i>																	
Uncultured (89.8%) AF371910																	
Uncultured (88.4%) AJ400235																	
Veillonella species								1									

* For sequences with less than 98 percent similarity to known 16S rDNA sequences, the percent similarity to the closest match in GenBank is from each bacterial 16S rDNA phylotype or species (rows) is displayed for each sample library (column). Single vaginal-fluid samples were tained from four subjects in a longitudinal study and these samples are labeled L1 through L4 to designate the four subjects, and a, b, or c to designate between collection of the first sample and subsequent samples, and whether subjects had bacterial vaginosis at that visit (BV+) or did not in BV- subjects.



ed bacteria included members of the TM7 division of uncultivated bacteria and bacteria in the peptoniphilus, peptostreptococcus, gemella, aerococcus, anaerococcus, and veillonella genera.

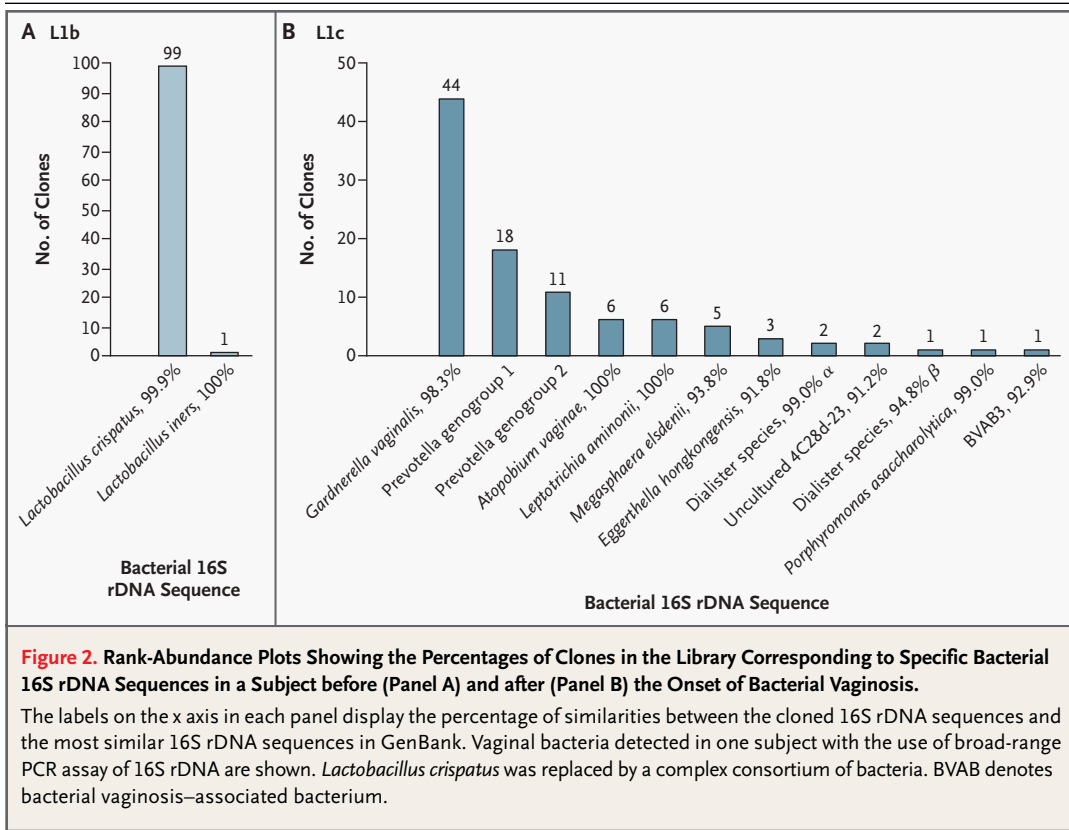
Three newly recognized bacteria were detected only in libraries of clones from subjects with bacterial vaginosis, and we have provisionally named these organisms bacterial vaginosis-associated bacteria (BVAB) 1, 2, and 3. Identical 16S rDNA sequences from these bacteria were detected in numerous bacterial vaginosis sample libraries. A phylogenetic tree depicting the evolutionary rela-

tionships among these bacteria and their closest relatives, inferred on the basis of aligned 16S rDNA sequences, is shown in Figure 1. BVAB1, BVAB2, and BVAB3 are related to bacteria in the Clostridium phylum but are not closely related to any bacteria with known 16S rDNA sequences. For instance, 16S rDNA from BVAB1 is only 90.6 percent similar to the closest sequence in GenBank (GenBank number AB034121), which is derived from an uncultivated bacterium detected in bovine rumen.

Figures 2, 3, and 4 are a series of rank-abundance plots identifying the bacterial 16S rDNA sequence types detected in vaginal fluid and their frequency in six clone libraries from Table 1. Results from sequential, prospectively obtained samples are displayed for two subjects. In one subject, lactobacilli predominated at baseline, when bacterial vaginosis was not present, but they were replaced by a diverse community of bacteria when bacterial vaginosis was detected two months later. In the second subject, who had bacterial vaginosis at baseline, the diverse community of vaginal bacteria shifted to a predominance of lactobacilli one month after successful treatment of bacterial vaginosis.

Table 2 shows the results of bacterium-specific PCR assays of 73 samples of vaginal fluid from subjects with or without bacterial vaginosis at baseline. BVAB1 was found in 41 percent of bacterial-vaginosis samples with the use of bacterium-specific PCR but was present in up to 66 percent of clones per library (Table 1). BVAB2 was found in 89 percent and BVAB3 in 41 percent of bacterial-vaginosis samples by specific PCR, but unlike BVAB1, these bacteria were not predominant in 16S rDNA clone libraries from subjects with bacterial vaginosis. BVAB1, BVAB2, and BVAB3 were highly specific indicators of bacterial vaginosis. Two subjects without bacterial vaginosis at baseline had positive PCR assays for these bacteria (BVAB1 and BVAB2 in one and BVAB2 and BVAB3 in the other), and bacterial vaginosis developed in both within a few months after these tests.

Detection of leptotrichia species was also very specific for bacterial vaginosis. Although *G. vaginalis* was present in all subjects with bacterial vaginosis according to bacterium-specific PCR, it was also found in 59 percent of subjects without bacterial vaginosis. Other bacterium-specific PCR assays showed that atopobium species, megasphaera species, and an eggerthella-like uncultured bacterium were detected in a high percentage of subjects with bacterial vaginosis, and these assays had moder-



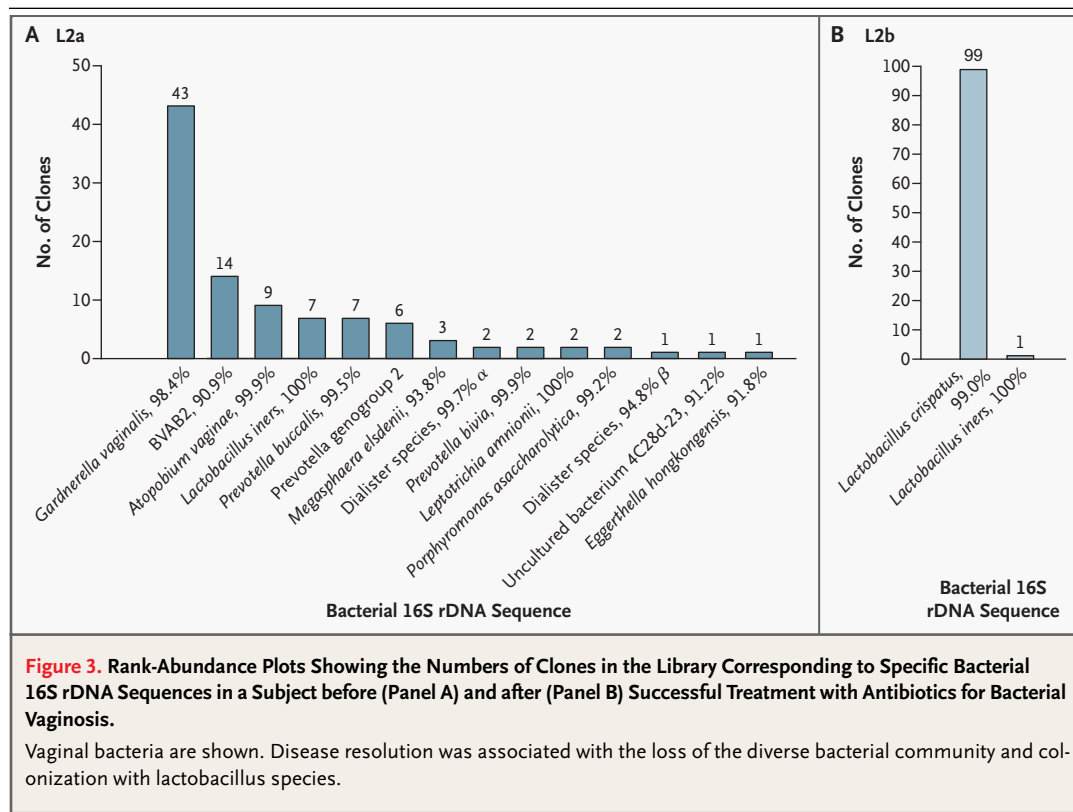
ate specificity for bacterial vaginosis. Detection in the vaginal fluid of each of the bacteria listed in Table 2 was significantly associated with bacterial vaginosis ($P < 0.001$). Combinations of bacterium-specific PCR assays did not substantially improve sensitivity or specificity, except for the combination of BVAB1 and BVAB3, which yielded 100 percent specificity, and the combination of BVAB2 and megasphaera, which yielded 100 percent sensitivity in this small series.

FISH was performed on vaginal fluid to determine whether the bacteria detected with the use of PCR were visible by fluorescence microscopy. Table 1 of the Supplementary Appendix shows the probes used, and Figure 5 shows fluorescence micrographs of vaginal fluid from subjects with bacterial vaginosis. Thin, curved bacteria hybridizing with the BVAB1 probe were found at high density in vaginal fluid from subjects with bacterial vaginosis whose 16S rDNA clone libraries had significant BVAB1 representation. The BVAB1 probe did not hybridize with cultivated relatives of BVAB1 in the Clostridium phylum, including *Lachnobacterium bovis* and *Eubacterium ramulus*, demonstrating its spec-

ificity. In contrast, under fluorescence microscopy, BVAB2 appeared to be a short, straight rod that was wider than BVAB1, whereas BVAB3 was a relatively long, wide, straight, lancet-shaped rod. Thus, BVAB1, BAVB2, and BVAB3 have distinct morphologic features; are easily distinguished from other bacteria associated with bacterial vaginosis, such as atopobium, mobiluncus, and gardnerella species; and are found attached to vaginal epithelial cells in a way that is typical of the clue cells that characterize bacterial vaginosis.

DISCUSSION

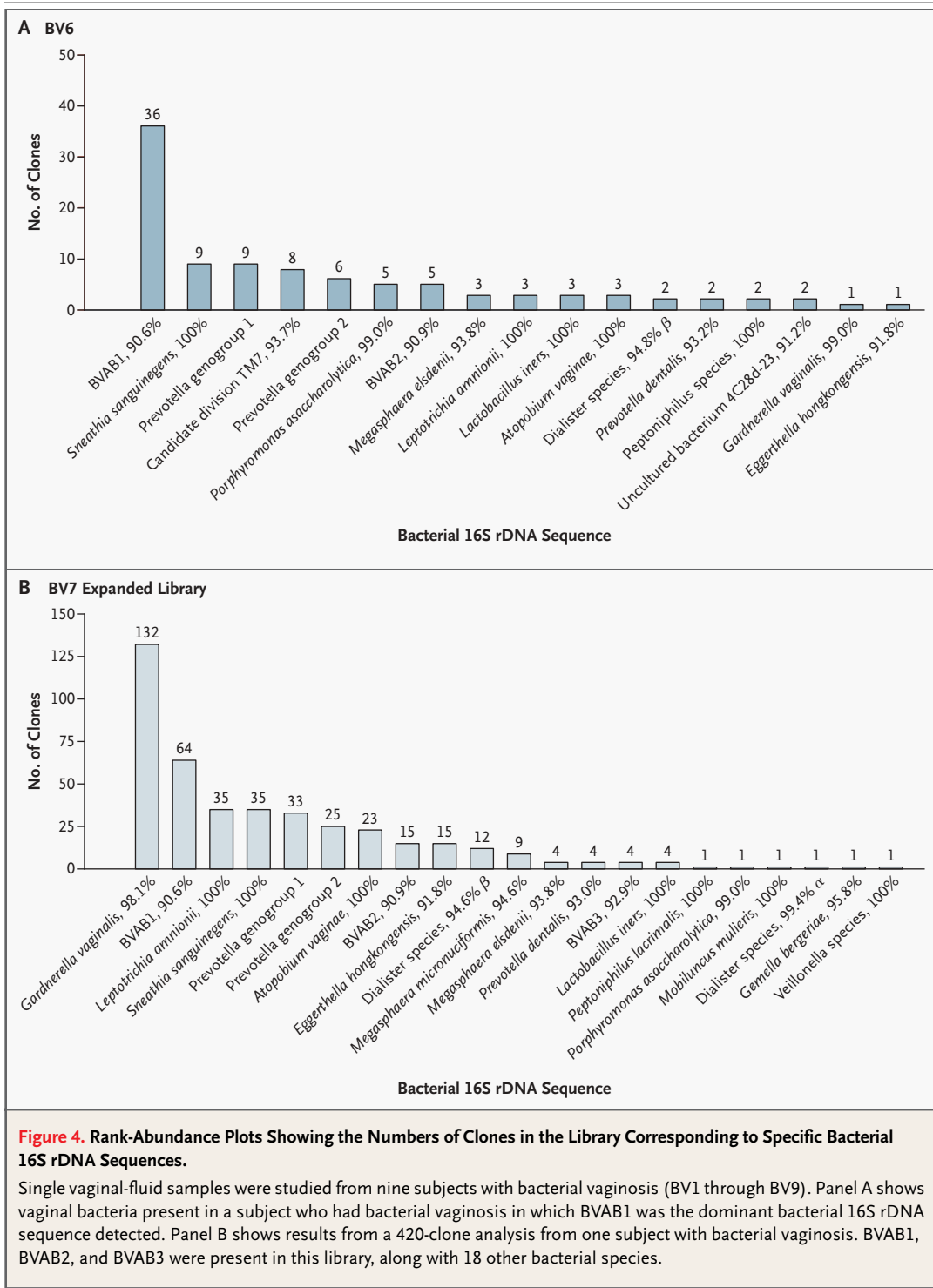
In this study, molecular analysis of the vaginal bacterial flora showed considerable bacterial diversity in subjects who had bacterial vaginosis, with 35 bacterial phylotypes detected in the 16 baseline and longitudinal samples from subjects with bacterial vaginosis. Sixteen bacterial species detected in subjects with bacterial vaginosis seem to be newly recognized on the basis of poor homology with known 16S rDNA sequences in GenBank. Numerous bacterial genera identified in this study have not, to our



knowledge, been previously detected in the vaginal milieu with the use of cultivation methods. We found no single bacterial community in all subjects with bacterial vaginosis, but common consortia of bacteria were evident. In contrast, subjects without bacterial vaginosis had relatively homogeneous vaginal flora, and bacterial 16S rDNA sequences closely matched known cultivated bacteria in the lactobacillus genus.

The cause of bacterial vaginosis remains enigmatic. No single bacterium has been shown to cause the disease, and many cultivated bacteria linked to bacterial vaginosis, such as *G. vaginalis*, frequently colonize the vagina of women without bacterial vaginosis.²² Our study identified several new bacterial species associated with bacterial vaginosis, but such associations do not prove causation. Additional molecular evidence may be required to assess causal associations with uncultivated bacteria.²³ Furthermore, the scenario of disease causation by a microbial community defies the traditional application of Koch's postulates. Bacterial vaginosis may be an example of polymicrobial disease causation due to the metabolic interdependence of several bacterial species in the vaginal niche.

The presence of BVAB1, BVAB2, or BVAB3 was highly specific for bacterial vaginosis in our subjects, and BVAB2 was found in 89 percent of subjects with bacterial vaginosis. We have not cultivated these microbes, and the 16S rDNA sequences from these bacteria are related only distantly to known bacteria; therefore, we have not suggested species or genus designations. *Atopobium*, *leptotrichia*, *megasphaera*, and *eggerthella*-like bacterial species were found in a high percentage of subjects who had bacterial vaginosis, according to bacterium-specific PCR. The presence of *atopobium* was less specific for bacterial vaginosis than was the presence of BVAB1, BVAB2, or BVAB3. Several groups have detected *A. vaginae* 16S rDNA in vaginal fluid from subjects with bacterial vaginosis and have suggested a role of this bacterium in bacterial vaginosis,²⁴⁻²⁶ although this organism has also been detected in subjects without bacterial vaginosis.²⁷ As these assays are used in larger groups of women with bacterial vaginosis and those without bacterial vaginosis, their performance characteristics should be refined, and they eventually may prove useful for the microbiologic diagnosis of bacterial vaginosis.



This study has several limitations. First, broad-range 16S rDNA primers will not amplify DNA from every known and unknown bacterial species. For instance, the Bact-338F primer used in the present

study does not amplify 16S rDNA from bacteria in the chlamydia genus.

Second, our findings may not be generalizable to all women with bacterial vaginosis, and the as-

Table 2. Associations between Particular Bacterial Species and Bacterial Vaginosis Based on Bacterium-Specific PCR Assay in 27 Subjects with Bacterial Vaginosis (BV) and 46 Subjects without Bacterial Vaginosis.*

Bacterium-Specific PCR Result	Bacterial Vaginosis		Sensitivity (95% CI)	Specificity (95% CI)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
	Present (N=27)	Absent (N=46)				
BVAB1						
Yes	11	1	40.7 (22.4–61.2)	97.8 (88.5–99.9)	30.9 (3.8–1359.9)	19.0 (2.2–910.7)
No	16	45				
BVAB2						
Yes	24	2	88.9 (70.8–97.6)	95.7 (85.2–99.5)	176.0 (22.8–1862.8)	106.1 (14.3–4755.1)
No	3	44				
BVAB3						
Yes	11	1	40.7 (22.4–61.2)	97.8 (88.5–99.9)	30.9 (3.8–1359.9)	21.9 (2.5–1056.4)
No	16	45				
Gardnerella						
Yes	27	27	100.0 (89.5–100.0)	41.3 (27.0–56.8)	(5.2–∞)†	27.2 (3.9–∞)†
No	0	19				
Atopobium species						
Yes	26	9	96.3 (81.0–99.9)	80.4 (66.1–90.6)	106.9 (12.9–4493.6)	95.0 (14.5–∞)†
No	1	37				
Eggerthella-like uncultured bacteria						
Yes	25	4	92.6 (75.7–99.1)	91.3 (79.2–97.6)	131.3 (19.0–1323.6)	103.8 (13.5–4812.8)
No	2	42				
Leptotrichia species						
Yes	23	2	85.2 (70.8–97.6)	95.7 (85.2–99.5)	126.5 (18.3–1279.6)	330.6 (23.1–∞)*
No	4	44				
Megasphaera α						
Yes	26	4	96.3 (81.0–99.9)	91.3 (79.2–97.6)	273.0 (26.6–11,428.3)	134.4 (16.6–6509.8)
No	1	42				
BVAB1 and BVAB3						
Yes	9	0	33.3 (16.5–54.0)	100.0 (93.7–100.0)	(5.7–∞)†	24.7 (3.2–∞)†
No	18	46				
BVAB2 or megasphaera α						
Yes	27	4	100.0 (89.5–100.0)	91.3 (79.2–97.6)	(57.2–∞)†	190.1 (28.3–∞)†
No	0	42				

* The values represent assays of baseline vaginal-fluid samples, with odds ratios and exact 95 percent confidence intervals unadjusted and adjusted for subject age, site of clinic enrollment, presence or absence of a report of abnormal vaginal discharge, and presence or absence of a report of having had sex with men. BVAB denotes bacterial vaginosis-associated bacterium.

† The odds ratio either could not be computed or could not be computed accurately because the value approaches infinity.

sociation between the bacteria detected in this study and bacterial vaginosis should be assessed in larger numbers of women with diverse demographic characteristics and other risk factors for the disease. However, conventional methods of cultivation have yielded remarkably similar microbiologic

profiles among different risk groups of women with bacterial vaginosis, including pregnant women, patients at gynecologic and STD clinics, and lesbians.^{20,28-30}

Third, analysis of 100 16S rDNA clones per sample library may miss certain bacterial 16S rDNA se-

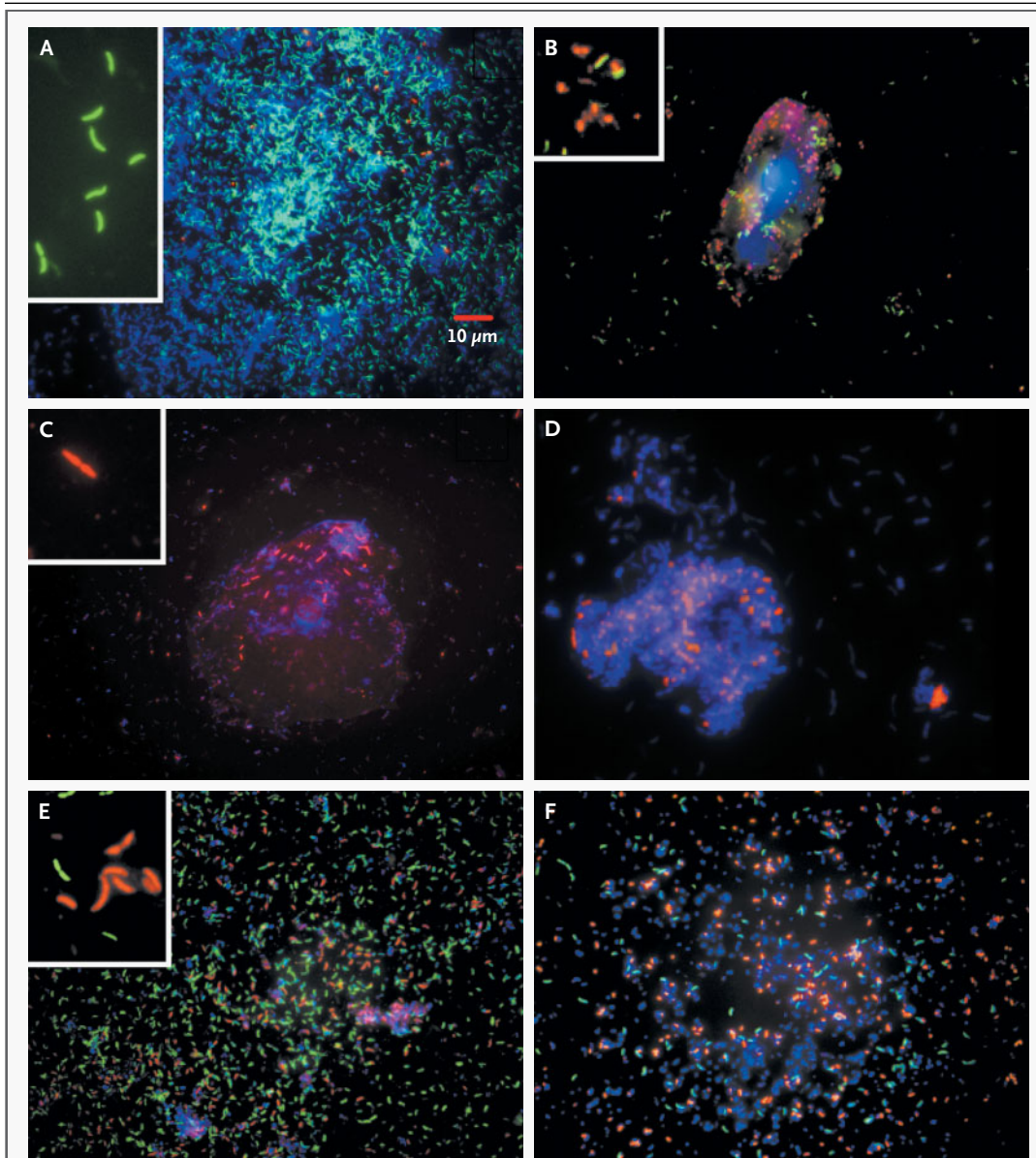


Figure 5. Fluorescence Micrographs of Vaginal-Fluid Smears Analyzed by FISH with Labeled Oligonucleotide Probes Targeting Bacterial rRNA, with Enlargements Shown in Insets.

Vaginal fluid from Subject 6 in the group of women with bacterial vaginosis (Panel A) shows a field of bacteria hybridizing with probes for BVAB1 (green), BVAB2 (red), and other bacteria (stained with 4',6-diamidino-2-phenylindole, dihydrochloride [DAPI], blue). The inset in Panel A shows that BVAB1 is a thin, curved rod. A sample from the same subject shows bacteria attached to a vaginal epithelial cell (Panel B). These bacteria include organisms hybridizing with probes for BVAB1 (green) and BVAB2 (red). The cell nucleus is stained with DAPI (blue). The inset in Panel B shows that BVAB2 is a short, wide rod (red). The bacteria attached to a vaginal epithelial cell in sample L4b (Panel C) hybridize with the probe for BVAB3 (red). Other bacteria and nuclear debris stain with DAPI (blue). The inset in Panel C shows that BVAB3 is a long, lancet-shaped rod. In Panel D, a clump of bacteria from sample L4b shows many coccoid cells hybridizing with the atropium probe (red) and other bacteria hybridizing with the broad-range bacterial probe Eub338 (blue). In Panel E, in sample L4b, bacteria hybridize with a probe for BVAB1 (green) and mobiluncus (red), but the broad-range bacterial probe Eub338 hybridizes with other bacteria (blue). The inset in Panel E shows that mobiluncus (red) is larger than BVAB1 (green) but both are small, curved rods. In Panel F, a sample from Subject 7, who had bacterial vaginosis, shows the relative proportions of BVAB1 (green), *Gardnerella vaginalis* (red), and other bacteria (DAPI, blue). *G. vaginalis* and BVAB1 were the most common clones detected in the broad-range PCR library generated from this sample.

quences present at low frequency in vaginal fluid. Although numerous statistical approaches can be used to assess species richness, we explored the effect of increasing the size of a library from 100 to 420 clones in one sample obtained from a patient with bacterial vaginosis (BV7) that was suspected to contain additional species diversity on the basis of the 5 single clones in the 100-clone library. We found that 415 of 420 clones identified (98.8 percent; see Fig. 4B) were identical to clones previously detected in the 100-clone library (Table 1, sample BV7). Five additional bacteria were present as single clones in the expanded library, increasing the total number of phylotypes detected from 16 to 21.

Fourth, different bacteria have different numbers of 16S rDNA genes per genome, and thus there is not a one-to-one relationship between the number of 16S rDNA clones detected by PCR and the number of bacteria present in a sample. Furthermore, the percentage of clones in a library should not be interpreted to indicate absolute bacterial representation, because the PCR assay is subject to amplification bias and the reaction was not stopped in the exponential phase, when it is most quanti-

tative. Quantitative PCR and FISH methods can be used to assess bacterial representation more accurately, although one needs to know the number of rRNA operons per organism to make quantitative PCR of 16S rDNA reflect absolute bacterial counts, and these data are lacking for most of the organisms in our study.

In conclusion, bacterial vaginosis in our subjects was associated with complex vaginal bacterial communities that included many newly recognized bacterial species that have not previously been detected with conventional cultivation techniques. We identified three bacterial species in the Clostridiales order that were highly specific for the presence of bacterial vaginosis and only distantly related to known bacteria.

Supported by grants (R03AI053250, to Dr. Fredricks; and R01AI052228, to Dr. Marrazzo) from the National Institute of Allergy and Infectious Diseases. Dr. Marrazzo reports receiving a research grant from Presutti Laboratories.

Presented in part at the 42nd annual meeting of the Infectious Diseases Society of America, Boston, October 2, 2004.

We are indebted to Corey Fish, Kathleen Ringwood, Katherine K. Thomas, Susan Heideke, Lauren Asaba, Nancy Dorn, and Dwyn Dithmer for their technical, organizational, statistical, and clinical contributions.

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