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Targeted PCR for Detection of Vaginal Bacteria Associated with Bacterial Vaginosis▽

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Several novel bacterial species have been detected in subjects with bacterial vaginosis (BV) by using broad-range PCR assays, but this approach is insensitive for detecting minority species. We developed a series of taxon-directed 16S rRNA gene PCR assays for more sensitive detection of key vaginal bacteria. We sought to determine the prevalence of each species in the vagina, its association with BV, and the utility of PCR for the microbiological diagnosis of BV. Targeted PCR assays were developed for 17 vaginal bacterial species and applied to 264 vaginal-fluid samples from 81 subjects with and 183 subjects without BV. The results were compared to those of two widely accepted methods for diagnosing BV, the use of clinical findings (Amsel criteria) and the interpretation of vaginal-fluid Gram stains (Nugent criteria). *Leptotrichia*/*Sneathia*, *Atopobium vaginae*, an *Eggerthella*-like bacterium, *Megasphaera* species, and three novel bacteria in the order *Clostridiales* are among the bacterial species significantly associated with BV. PCR detection of either a *Megasphaera* species or one of the *Clostridiales* bacteria yielded a sensitivity of 99% and a specificity of 89% for diagnosis of BV compared to the Amsel clinical criteria and a sensitivity of 95.9% and a specificity of 93.7% compared to the Nugent criteria (Gram stain). PCR detection of one or more fastidious bacterial species is a more reliable indicator of BV than detection of bacteria, such as *Gardnerella vaginalis*, previously linked to BV, highlighting the potential of PCR for the diagnosis of BV.

Bacterial vaginosis (BV) is a very common condition characterized by alterations of the vaginal flora with acquisition of diverse communities of anaerobic and facultative bacteria and depletion of the usually dominant lactobacillus flora (7, 17, 18). BV is a cause of malodorous vaginal discharge and is linked to several adverse health outcomes in women, including premature labor, pelvic inflammatory disease, and human immunodeficiency virus acquisition (8, 9, 11–13, 15). Cultivation methods have failed to unequivocally identify a specific bacterial pathogen or unique pathogenic community in subjects with BV.

Several studies have used broad-range 16S rRNA gene PCR, a cultivation-independent method, to characterize the community of vaginal bacteria (3, 4, 19). These molecular studies have discovered a large number of novel, fastidious, or uncultivated bacterial species. However, methods such as broad-range 16S rRNA gene PCR with denaturing gradient gel electrophoresis or sequence analysis of cloned amplification products are not sensitive for detecting infrequent or minority species in a bacterial community. For instance, the absence of a bacterial 16S rRNA gene clone from a particular species in a library of 100 clones does not prove that a bacterium is absent in the sample but only suggests that the bacterial 16S rRNA gene sequence is present at less than ~1% abundance in the PCR product. To prove that a bacterium is absent in a sample of vaginal fluid requires the use of more sensitive detection methods.

The diagnosis of BV is usually made clinically, most commonly when at least three out of four Amsel criteria are present (1), including a 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% potassium hydroxide is added to a slide containing vaginal fluid); and clue cells (>20% of epithelial cells with indistinct borders due to adherent bacteria) on microscopic examination of vaginal fluid. However, some patients without BV may manifest clinical findings similar to those of BV, such as those with *Trichomonas vaginalis* vaginitis, in which subjects may have vaginal discharge, elevated vaginal pH, or a positive whiff test (16). An alternative diagnostic approach is to use Gram staining of vaginal fluid to distinguish normal vaginal flora (i.e., gram-positive rods and lactobacilli) from BV flora (gram-negative and gram-variable morphotypes) using the scoring system of Nugent et al. (14) or Ison and Hay (10). Although vaginal-fluid Gram stain is the gold standard for the diagnosis of BV and is employed in many research studies, it is not commonly employed in the clinical management of BV because evaluation of vaginal-fluid Gram stains requires highly experienced personnel. Gram staining also provides very limited information regarding the identities and relative abundances of organisms present in a sample.

We developed a panel of taxon-directed 16S rRNA gene PCR assays for the detection of 17 key vaginal bacteria in a highly sensitive and specific manner. Particular species were selected for targeting based on their abundance in broad-range 16S rRNA gene clone libraries previously analyzed (4), their initial apparent specificity for BV, or their novelty. These assays were applied to vaginal-fluid samples obtained from

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women with and without BV in order to assess the prevalence of each bacterial species and to explore the potential of PCR for the microbiological diagnosis of BV. The detection of minority taxa is important to assess their utility as diagnostic markers and because they may have important functional roles despite their low abundances. We hypothesized that several of the uncultivated bacteria associated with BV by PCR would be more reliable indicators of BV than the cultivated bacteria previously linked to this condition.

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MATERIALS AND METHODS

Study population. A total of 264 baseline vaginal swabs were obtained from 81 subjects with BV and 183 subjects without BV seen between October 2001 and November 2005. In this prospective study, baseline visits consisted of the first research encounter with the subjects. Subjects were recruited from two clinic sites in Seattle, the Public Health—Seattle and King County Sexually Transmitted Disease Clinic (STD clinic; $n = 48$) and the Harborview Medical Center Women's Research Clinic (WRC; $n = 216$). The study participants attending the WRC reported engaging in same-sex behavior in the previous year, though most (77.9%) also reported a history of sexual activity with men in their lifetime. BV was defined using Amsel's clinical criteria for all subjects (with the presence of at least three of four criteria needed to establish a diagnosis of BV). In addition, Nugent criteria (Gram stain score ≥ 7) were available to evaluate BV status in all subjects attending the WRC, but not the STD clinic. The women were 17 to 55 years old, and all provided written informed consent. The study was approved by the institutional review boards at the Fred Hutchinson Cancer Research Center and the University of Washington in Seattle. We previously reported qualitative bacterium-specific PCR data for eight bacterial species in 73 subjects (4).

Sample collection and screening for other infections. All subjects underwent speculum examination with collection of vaginal fluid for saline microscopy, KOH preparation, pH measurement, and assessment for amine odor. To obtain specimens for the performance of PCR assays, a polyurethane foam swab (Catch-All; Epicenter) was brushed against the lateral vaginal wall, resheathed, and frozen until the DNA extraction step. All subjects at the STD clinic were tested routinely for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by culture, and those seen at the WRC were tested using the APTIMA-COMBO 2 assay (GenProbe) on urine.

DNA extraction. Vaginal swabs for bacterial PCR were placed in 15-ml conical vials with 2 ml of saline and vortex mixed for 1 min to dislodge cells. The sample solution was centrifuged at 14,000 rpm for 10 min, and the pellet was resuspended in 100 μl supernatant. DNA was extracted from the pellet using the Ultra Clean Soil DNA Kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. DNA was eluted from silica columns in a volume of 150 μl buffer. Sham digests using a swab without human contact were performed with each round of DNA extraction (every 10 to 25 samples) to control for contamination that might arise from kit reagents or collection swabs.

Bacterium-specific PCR assays. Bacterium-specific PCR assays were developed based on detection of species-specific regions of the 16S rRNA gene. 16S rRNA gene sequences from vaginal bacteria detected by broad-range 16S rRNA gene PCR were aligned as previously described (4). Primers were designed to target highly variable regions of the bacterial 16S rRNA gene that appeared to be unique for each species. PCR assays were developed for 17 bacterial species that were commonly detected in vaginal samples (Table 1 lists the bacteria detected and the primer sequences). Each 50-μl PCR mixture contained 1× PCR buffer II, 2 mM magnesium chloride, 0.8 mM deoxynucleoside triphosphate mixture, 1 unit AmpliTaq Gold DNA polymerase (all from Applied Biosystems), 0.2 μM each of forward and reverse primers (Table 1), and 1 μl of template DNA. The PCR conditions included a premelt at 95°C for 10 min and then 40 to 45 cycles of 95°C for 30 seconds (melt), 53 to 62°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), followed by a final extension at 72°C for 7 min. The PCR products were visualized after electrophoresis in 2% agarose gels and staining with ethidium bromide. Bacterial PCR assays were optimized so that each assay was capable of detecting ≤100 molecules of cloned 16S rRNA gene per reaction, though most assays could detect 1 to 10 molecules. Every PCR with a visible band of the expected size on gel electrophoresis was sequenced (BigDye version 3; Applied Biosystems) to confirm that the PCR product had at least 99% similarity with the expected bacterial target, thereby assuring specificity of the bacterial assay. PCRs without visible bands on gel electrophoresis or without confirmed sequence homology to the expected target were considered negative. No template PCR controls and sham digest controls were run with each PCR assay to monitor for contamination.

β-Globin PCR. Each subject's extracted DNA was subjected to a human β-globin PCR to ensure that amplifiable DNA was successfully extracted from the sample and to monitor for PCR inhibitors (5). The β-globin PCR protocol used was the same protocol listed for bacterial PCR, with the exception that the following primers were employed: GH2O, 5′-GAAGAGCCAAGGACAGGTAC-3′, and PCO4, 5′-CAACTTCATCCACGTTCACC-3′.

Statistical analysis. The prevalences of the 17 different bacteria in subjects with and without BV is reported, noting the sensitivity, specificity, and odds ratios (OR) with 95% confidence intervals (CI) for the diagnosis of BV using either Amsel criteria (entire cohort) or Nugent criteria (WRC cohort) as diagnostic standards. The number of species found in BV-positive subjects was compared to that in BV-negative subjects by using a *t* test.

RESULTS

Amplifiable DNA was recovered from all 264 samples as demonstrated by production of an amplification product with β-globin PCR. The PCR assay results for 17 bacterial taxa are presented in Tables 2 and 3, including PCR results for combinations of bacteria. Overall, the prevalence of BV was 30.7%. Table 2 displays bacterial PCR assay results for the combined group of 264 subjects using Amsel clinical criteria to define BV. Table 3 displays PCR assay results using Nugent (Gram stain) criteria to define BV rather than Amsel clinical criteria for the WRC group. The 81 subjects with BV in this study had an average of 11.1 species detected (range, 5 to 16 species) using the 17 directed assays employed. In contrast, the 183 subjects without BV had an average of only 3.6 species detected (range, 0 to 14), with *Lactobacillus crispatus*, *Lactobacillus iners*, *Peptoniphilus* species, and *Gardnerella vaginalis* the most commonly detected bacteria (significance, 11.1 versus 3.6 species; $P < 0.0001$) Overall, these results provide evidence that subjects with BV have vaginal colonization with many diverse bacterial species, including several heretofore uncultivated species that appear to be highly specific for BV, in conjunction with absence of *L. crispatus*.

Three bacteria in the order *Clostridiales* with no close phylogenetic affinities to cultivated bacteria are highly specific for BV, with specificities of >90% in all analyses. We have designated these bacteria BV-associated bacterium 1 (BVAB1), BVAB2, and BVAB3 because they are not closely related to any known cultivated bacteria. BVAB2 was detected in >80% of women with BV. BVAB1 and BVAB3 were detected in a minority of subjects with BV, though detection of these species was highly specific for BV (>96%). Use of a strategy to combine PCR assay results for BVAB1 and BVAB3 did not substantially improve assay performance for the diagnosis of BV because these bacteria tend to be detected together. In contrast, the combination of BVAB1 and BVAB2 or of BVAB2 and BVAB3 did increase sensitivity for diagnosing BV to >80% while retaining specificity (>92%).

We designed PCR assays for the detection of two bacteria distantly related to *Megasphaera* species that we previously identified in subjects with BV. *Megasphaera* phylotype 1 was detected in about 95% of subjects with BV, regardless of the diagnostic gold standard employed, and was also highly specific for BV (89 to 94%). If one combines the results from the two

TABLE 1. Bacterial targets, primers, and PCR conditions for each bacterium-specific PCR assay

Target (GenBank accession no.)	Primer[a]	Sequence	Annealing temp (°C)	Cycle no.
Atopobium sp. (AY738657, AY738658)	Atop-442F Atop-1017R	5′-GCAGGGACGAGGCCGCAA-3′ 5′-GTGTTTCCACTGCTTCACCTAA-3′	55	40
BVAB1 (AY724739)	BVAB1-1019F BVAB1-1280R	5′-GTATATTTTCTACGGAACACAGG-3′ 5′-TTTGCTCCGGATCGCTCCTT-3′	55	40
BVAB2 (AY724740)	BVAB2-619F BVAB2-1024R	5′-TTAACCTTGGGGTTCATTACAA-3′ 5′-AATTCAGTCTCCTGAATCGTCAGA-3′	55	40
BVAB3 (AY724741)	BVAB3-999F BVAB3-1278R	5′-CTTGAWCGATGTAGAGATACATAA-3′ 5′-TGCTTCGCCTCGCGACGTC-3′	55	40
BVAB-TM7 (AY738690)	TM7-641F TM7-1020R	5′-AACTGCTTGGCTCGAGATTATC-3′ 5′-TCTCCTTTCGGAGAAATTCTAGG-3′	53	45
Eggerthella-like uncultured bacterium (AY738656)	Egger-621F Egger-859R	5′-AACCTCGAGCCGGGTTCC-3′ 5′-TCGGCACGGAAGATGTAATCT-3′	55	40
G. vaginalis (AY738665-AY738668; AY738695)	G.vag 644F G.vag 851R	5′-GGGCGGGCTAGAGTGCA-3′ 5′-GAACCCGTGGAATGGGCC-3′	62	40
L. crispatus (AY738663, AY738664)	L.crisp-452F L.crisp-1023R	5′-GATAGAGGTAGTAACTGGCCTTTA-3′ 5′-CTTTGTATCTCTACAAATGGCACTA-3′	54	45
L. iners (AY738669)	L.iners-453F L.iners-1022R	5′-ACAGGGGTAGTAACTGACCTTTG-3′ 5′-ATCTAATCTCTTAGACTGGCTATG-3′	55	40
Leptotrichia/Sneathia spp. (AY724742, AY738659)	Lepto-395F Lepto-646R	5′-CAATTCTGTGTGTGTGAAGAAG-3′ 5′-ACAGTTTTGTAGGCAAGCCTAT-3′	55	40
Megasphaera phylotype 1 (AY738672)	MegaE-456F MegaE-667R	5′-GATGCCAACAGTATCCGTCCG-3′ 5′-CCTCTCCGACACTCAAGTTCGA-3′	55	40
Megasphaera phylotype 2 (AY738697)	MegaM-453F MegaM-666R	5′-AAGGTGGTAAATAGCCATCATGAG-3′ 5′-CTCTCCGACACTCAAGTCTTC-3′	57	40
M. curtisii (EF428974)	M.curt-440F M.curt-1026R	5′-TTCTCGCGAAAAAGGCACAG-3′ 5′-CTGGCCCATCTCTGGAACCA-3′	57	40
M. mulieris (AY738684)	Mobil-577F M.mulie-1026R	5′-GCTCGTAGGTGGTTCGTCGC-3′ 5′-CCACACCATCTCTGGCATG-3′	62	40
Peptoniphilus lacrimalis (AY738692)	P.lacri-999F Pepton-1184R	5′-AAGAGACGAACTTAGAGATAAGTTTT-3′ 5′-CACCTTCCTCCGATTTATCATC-3′	55	40
Peptoniphilus sp. (AY738691)	Pepton-1003F Pepton-1184R	5′-GACCGGTATAGAGATATACCCT-3′ 5′-CACCTTCCTCCGATTTATCATC-3′	55	40
Prevotella G1 (AY738676, AY738677)	PrevG1-468F PrevG1-857R	5′-GTCCCTTATTGCATGTACCATAC-3′ 5′-GCCGCTAACACTAGGTGCTA-3′	55	40

[a] Numbers on primers refer to the corresponding *E. coli* 16S rRNA gene base position of the first nucleotide.

Megasphaera phylotype assays (presence of *Megasphaera* phylotype 1 or phylotype 2), the diagnostic sensitivity for BV increases marginally (≥96%) while good specificity is maintained (89 to 94%).

The best combination of PCR results for diagnosing BV arose from detection of either BVAB2 or *Megasphaera* phylotype 1, which produced a diagnostic sensitivity of 98.8% and a specificity of 88.5% in the combined subject cohort compared to the Amsel criteria for BV (Table 2). Using Nugent scores as the diagnostic gold standard, this combination of PCR assays produced a sensitivity of 95.9% and a specificity of 93.7% for BV (Table 3).

Several additional fastidious bacteria were highly associated with BV in the combined cohort by PCR (Table 2), including *Atopobium* species (OR, 87; 95% CI, 26 to 291), an *Eggerthella*-like bacterium (OR, 67; 95% CI, 28 to 162), *Leptotrichia/Sneathia* species (OR, 39; 95% CI, 19 to 82), a BVAB in the TM7 division (BVAB-TM7) (OR, 22; 95% CI, 7.5 to 67), and two *Peptoniphilus* species (OR, 12; 95% CI, 6 to 24; and OR, 8; 95% CI, 5 to 15). We designed a PCR assay targeting one of the many *Prevotella* species clusters that were commonly detected in clone libraries from subjects with BV (4) and have designated this *Prevotella* genogroup 1 (*Prevotella* G1) (OR, 27; 95% CI, 13 to 59).

TABLE 2. PCR assay results from a combined cohort of 264 subjects analyzing the utility of each assay for the diagnosis of BV, with Amsel clinical criteria used to define BV[a]

Bacterium	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	OR (95% CI)
BVAB1	43.2 (33.0–54.1)	96.7 (93.0–98.5)	22.45 (8.90–56.6)
BVAB2	86.4 (77.3–92.2)	92.9 (88.2–95.8)	83.2 (35.6–195)
BVAB3	42.0 (31.8–52.9)	96.7 (93.0–98.5)	21.3 (8.5–53.9)
G. vaginalis	96.3 (89.7–98.7)	29.5 (23.4–36.5)	10.9 (3.3–36.0)
Atopobium spp.	96.3 (89.7–98.7)	77.05 (70.4–82.6)	87.3 (26.2–291)
Eggerthella-like bacterium	91.4 (83.2–95.8)	86.3 (80.6–90.6)	66.8 (27.65–162)
Leptotrichia spp.	82.7 (73.1–89.4)	89.1 (83.7–92.8)	39.0 (18.6–81.7)
Megasphaera phylotype 1	95.1 (88.0–98.1)	88.5 (83.1–92.4)	148.5 (49.3–448)
Megasphaera phylotype 2	18.5 (11.6–28.3)	98.9 (96.1–99.7)	20.6 (4.6–92.4)
Either *Megasphaera* sp.	97.5 (91.4–99.3)	88.5 (83.1–92.4)	305 (69.7–1,332)
BVAB-TM7	33.3 (24.0–44.2)	97.8 (94.5–99.2)	22.4 (7.5–66.8)
M. curtisii	55.6 (44.7–65.9)	92.9 (88.2–95.8)	16.3 (8.0–33.4)
M. mulieris	22.2 (14.5–32.4)	100 (97.9–100)	Infinity
Either *Mobiluncus* sp.	65.4 (54.6–74.9)	92.9 (88.2–95.8)	24.8 (12.0–51.2)
Peptoniphilus sp. type 1	83.8 (74.1–90.3)	70.0 (63.0–76.1)	12.0 (6.1–23.5)
Peptoniphilus lacrimalis	70.4 (59.7–79.2)	78.1 (71.6–83.5)	8.5 (4.7–15.4)
Either *Peptoniphilus* sp.	88.9 (80.2–94.0)	60.7 (53.4–67.4)	12.3 (5.8–26.2)
Prevotella G1	63.8 (52.8–73.4)	94.0 (89.6–96.6)	27.5 (12.9–58.9)
L. crispatus	16.1 (9.6–25.6)	10.4 (6.8–15.7)	0.022 (0.01–0.05)
L. iners	98.8 (93.3–99.8)	8.8 (5.5–13.7)	7.7 (1.0–58.8)
Either BVAB1 or BVAB2	87.7 (78.7–93.2)	92.9 (88.2–95.8)	92.8 (38.9–222)
Either BVAB1 or BVAB3	48.2 (37.6–58.9)	95.1 (90.9–97.4)	18.0 (8.1–39.9)
Either BVAB2 or BVAB3	87.7 (78.7–93.2)	92.9 (88.2–95.8)	92.8 (38.9–222)
Either BVAB1, -2, or -3	87.7 (78.7–93.2)	92.9 (88.2–95.8)	92.8 (38.9–222)
Either *Megasphaera* phylotype 1 or BVAB2	98.8 (93.3–99.8)	88.5 (83.1–92.4)	617 (81.6–4,670)

[a] The analysis consisted of 81 subjects with BV and 183 subjects without BV.

Several cultivated bacteria have been associated with BV previously, such as *G. vaginalis* and *Mobiluncus* species. Using the Amsel criteria as the gold standard for BV, we detected *G. vaginalis* by PCR in 96% of subjects with BV in the combined cohort, but it was also detected in 70% of subjects without BV, confirming the poor specificity of *G. vaginalis* on a qualitative basis as an indicator of BV. We detected *Mobiluncus curtisii* by PCR in 55.6% of subjects with BV (Table 2) with a specificity of 93% and *Mobiluncus mulieris* in 22.2% of subjects with BV with a specificity of 100%. PCR assay performance for the *Mobiluncus* species did not improve when the Nugent criteria were used to define BV (Table 3). Even combining the two

TABLE 3. PCR assay results for 216 subjects, using Nugent criteria to define BV[a]

Bacterium	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	OR (95% CI)
BVAB1	31.5 (22.0–42.9)	97.2 (93.0–98.9)	16.0 (5.3–48.5)
BVAB2	80.8 (70.3–88.2)	96.5 (92.1–98.5)	116 (40.1–338)
BVAB3	26.0 (17.3–37.1)	97.2 (93.0–98.9)	12.2 (4.0–37.6)
G. vaginalis	97.3 (90.6–99.3)	45.5 (37.5–53.6)	29.6 (7.0–125)
Atopobium spp.	95.9 (88.6–98.6)	84.6 (77.8–89.6)	128 (37.1–444)
Eggerthella-like bacterium	89.0 (79.8–94.3)	94.4 (89.4–97.1)	137 (49.3–382)
Leptotrichia spp.	73.9 (69.2–82.7)	93.7 (88.5–96.7)	42.3 (18.0–99.4)
Megasphaera phylotype 1	94.5 (86.7–97.9)	94.4 (89.4–97.1)	291 (84.7–1,000)
Megasphaera phylotype 2	6.9 (3.0–15.1)	100 (97.4–100)	Infinity
Either *Megasphaera* sp.	95.9 (88.6–98.6)	94.4 (89.4–97.1)	394 (101–1,531)
BVAB-TM7	24.7 (16.2–35.6)	99.3 (92.6–99.9)	46.5 (6.1–357)
M. curtisii	49.3 (38.2–60.5)	93.0 (87.6–96.2)	12.9 (5.9–28.5)
M. mulieris	16.4 (9.7–26.6)	98.6 (95.0–99.6)	13.9 (3.0–63.8)
Either *Mobiluncus* sp.	54.8 (43.4–65.7)	91.6 (85.9–95.1)	13.2 (6.3–28.0)
Peptoniphilus sp. type 1	78.1 (67.3–86.0)	67.1 (59.1–74.3)	7.3 (3.8–14.0)
Peptoniphilus lacrimalis	61.6 (50.2–72.0)	84.6 (77.8–89.6)	8.8 (4.6–17.0)
Either *Peptoniphilus* sp.	82.2 (71.9–89.3)	62.9 (54.8–70.4)	7.8 (3.9–15.6)
Prevotella G1	50.7 (39.5–61.8)	97.2 (93.0–98.9)	35.7 (12.0–107)
L. crispatus	8.2 (3.8–16.8)	6.3 (3.4–11.5)	0.006 (0.002–0.018)
L. iners	94.5 (86.7–97.9)	11.9 (7.6–18.2)	2.3 (0.75–7.2)
Either BVAB1 or BVAB2	80.8 (70.3–88.2)	96.5 (92.1–98.5)	116 (40.1–338)
Either BVAB1 or BVAB3	35.6 (25.6–47.1)	96.5 (92.1–98.5)	15.3 (5.6–42.0)
Either BVAB2 or BVAB3	82.2 (71.9–89.3)	96.5 (92.1–98.5)	127 (43.5–373)
Either BVAB1, -2, or -3	82.9 (71.9–89.3)	96.5 (92.1–98.5)	127 (43.5–373)
Either *Megasphaera* phylotype 1 or BVAB2	95.9 (88.6–98.6)	93.7 (88.5–96.7)	347 (91.1–1,324)

[a] The analysis consisted of 73 subjects with BV and 143 subjects without BV.

Mobiluncus species PCR assays generated a diagnostic sensitivity of only 65.4% in the combined cohort (Table 2). Accordingly, *G. vaginalis* and *Mobiluncus* species do not appear to be optimal bacterial indicators of BV when assessed qualitatively by PCR due to lack of specificity (*G. vaginalis*) or lack of sensitivity (*Mobiluncus* spp.).

L. crispatus is considered an important component of the normal vaginal flora and is depleted in subjects with BV. Our PCR assay for *L. crispatus* confirmed that the bacterium is inversely associated with BV (OR, 0.02; 95% CI, 0.01 to 0.05). *L. crispatus* was detected in 89.6% of the combined cohort without BV and in 16% of subjects with BV (Table 2). We previously detected *L. iners* in clone libraries generated from subjects with and without BV (4). Our PCR assay for *L. iners* confirmed that this bacterium is commonly found in subjects with BV (98.8%) and without BV (91.3%). *L. iners* appears to be a singularly robust bacterium capable of surviving in diverse vaginal environments and bacterial communities.

Understanding the microbiology of BV may require a viewpoint that encompasses analysis of larger taxonomic groupings of bacteria and combinations of bacterial species from different divisions. Figure 1 displays bacterial prevalence by individual species, bacterial division, and selected bacterial combinations in subjects with and without BV as defined by the Amsel criteria. Bacteria in the divisions *Firmicutes* and *Actinobacteria* were commonly found in subjects with and without BV. Bacteria in the divisions *Bacteroidetes*, *Fusobacteria*, and TM7 were more common in subjects with BV, though these results represent single species in each division. Several combinations of bacteria were seen in ≥80% of subjects with BV but less than or equal to ~10% of subjects without BV, including BVAB1, -2, or -3; BVAB1, -2, or -3 and *Coriobacteridae*; BVAB1, -2, or -3 and *Leptotrichia*; *Megasphaera* and *Coriobacteridae*; *Megasphaera*, *Coriobacteridae*, and BVAB1, -2, or -3; *Actinobacteria* and BVAB1, -2, or -3; *Megasphaera* or BVAB2; and *Megasphaera* and *G. vaginalis*. These relationships suggest the possibility of metabolic interdependencies among vaginal bacterial species or larger taxonomic groups of bacteria in subjects with BV.

DISCUSSION

The application of this panel of 17 bacterium-targeted PCR assays to vaginal-fluid samples serves several purposes. First, these data help to establish the bacterial compositions of the human vagina in subjects with and without BV and validate our earlier findings from a smaller group of subjects. The recent use of molecular microbial detection methods in well-characterized subjects has established that a large portion of the vaginal flora in subjects with BV is derived from bacteria that appear to be novel and uncultivated (2–4, 19). Methods such as broad-range 16S rRNA gene PCR with either cloning and sequence analysis of clone libraries or denaturing gradient gel electrophoresis of the PCR products have played critical roles in helping to redefine the bacteriology of BV. However, these broad-range PCR approaches are limited by their tendency to sample only the most prevalent bacteria in a community; low-abundance or minority species are likely to be missed (6). To overcome this limitation, we developed a series of highly sensitive PCR assays targeting particular bacterial species that we have previously detected in vaginal samples. Although this approach will clearly not detect new species, it is helpful in determining the true prevalences of key vaginal bacteria, which is a critical first step in understanding how vaginal bacteria interact with each other and the human host. Based on the data presented here, we conclude that the absence of bacteria such as BVAB1, BVAB2, BVAB3, *Megasphaera* species, and *Leptotrochia*/*Sneathia* species from clone libraries of subjects without BV does not arise from a limited ability to sample bacterial diversity. Rather, these BVABs are indeed uncommon in subjects without BV. Subjects with BV have complex communities of vaginal bacteria. Several subjects with BV had 16 of 17 bacterial species assayed, missing only *L. crispatus*, whereas other subjects with BV had only 5 to 7 of the species assayed.

Second, the detection of novel bacteria associated with BV through the use of targeted PCR assays creates new opportunities for the diagnosis of BV. Cultivated bacteria, such as *G. vaginalis*, are unreliable indicators of BV, as demonstrated by the high rate of detection in subjects without BV in this study. Accordingly, cultivation is not used in the diagnosis of BV. The use of clinical criteria to diagnose BV has the advantage of rapid diagnosis at the point of care but requires assessment of vaginal pH and, more importantly, performance of microscopy of vaginal fluid by a reasonably skilled practitioner or laboratory personnel. Gram stain interpretation of vaginal-fluid samples is more reliable for the diagnosis of BV but requires highly experienced personnel, is usually not performed at the point of care, and was derived using estimates of the quantity of previously cultivated vaginal-bacterial morphotypes present on Gram stain to generate a linear scale. Our data suggest that bacterial PCR methods may be employed for the diagnosis of BV based on the detection of key bacteria or bacterial combinations associated with BV, such as BVAB2 and *Megasphaera* phylotype 1. Overall, there were only minor differences in the diagnostic performances of the PCR assays when Amsel or Nugent criteria were used as gold standards. For instance, detection of BVAB1, -2, or -3 was highly specific for BV regardless of the diagnostic gold standard employed (>93%), whereas detection of BVAB1, -2, or -3 was slightly less sensitive for the diagnosis of BV when Nugent criteria were employed (BVAB2 sensitivity, 86% for Amsel, 81% for Nugent). Likewise, the combination of BVAB2 or *Megasphaera* phylotype 1 yielded the best diagnostic sensitivity and specificity regardless of the gold standard employed (sensitivity, 99%, and specificity, 89% for Amsel; sensitivity, 96%, and specificity, 94% for Nugent). Rapid PCR assays, such as real-time quantitative PCR, may allow the microbiological diagnosis of BV in the clinic, as the approach taken here to guarantee assay specificity by sequence analysis of every amplification product is not optimal in the clinical setting.

There are several limitations of our study. First, we did not attempt to assay for every known vaginal bacterium using targeted PCR assays due to practical limitations. We expect that many additional bacterial species are present in vaginal samples from subjects with and without BV. For instance, several additional *Lactobacillus* species that are members of the normal vaginal flora and numerous *Prevotella* and *Porphyromonas* species commonly found in subjects with BV were not assayed in this study. Nevertheless, these results help to build a foun-

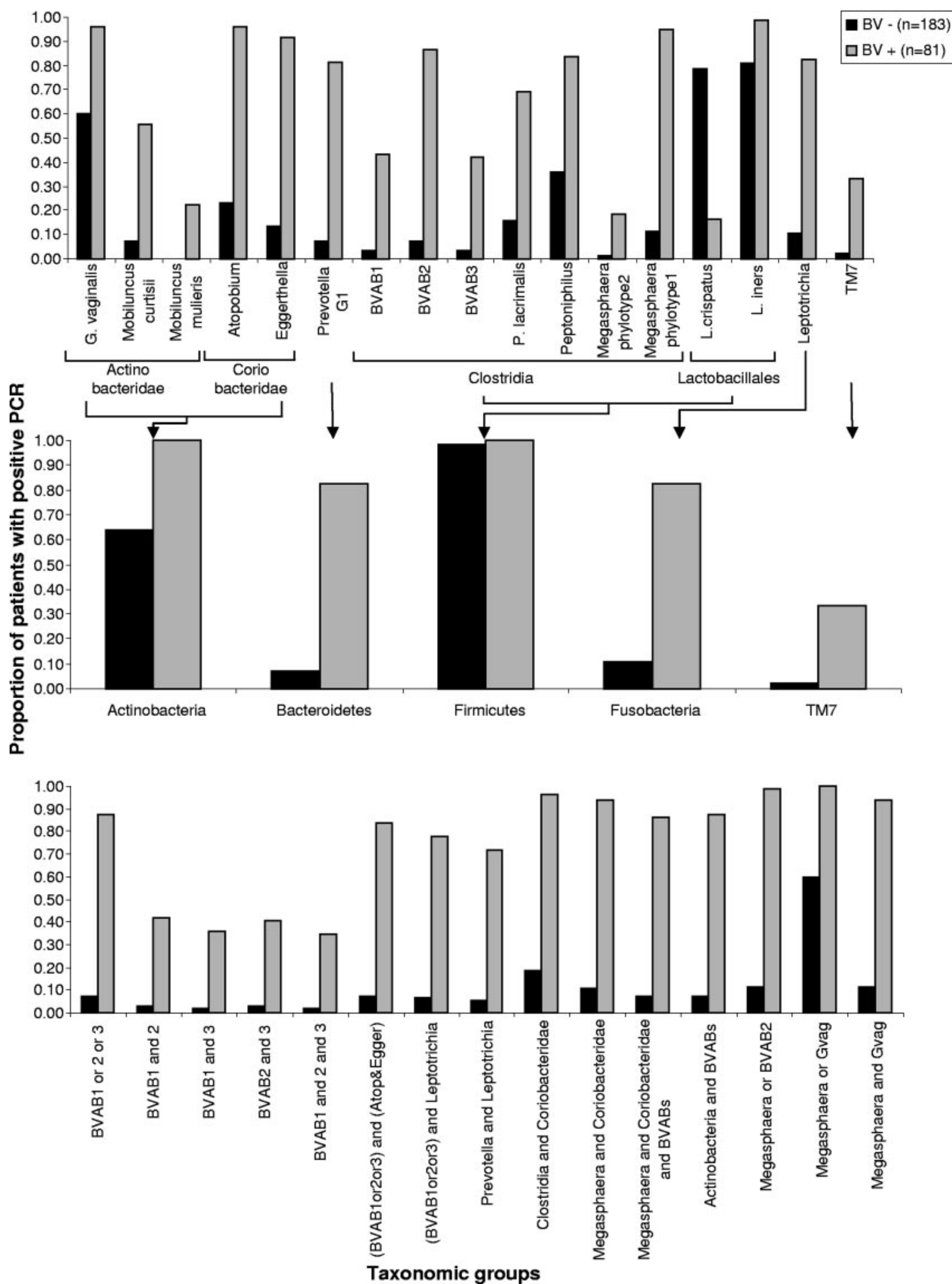

FIG. 1. Bacterium-specific PCR results summarized at multiple taxonomic hierarchies and for various taxonomic groupings. The *y* axes represent the proportion of patients with positive PCR results for each BV status as defined by the Amsel clinical criteria.

dation for understanding the bacterial diversity present in the human vagina. Second, the PCR assays developed here were qualitative and do not provide information about the quantities of vaginal bacteria present in subjects with and without BV. The quantity of bacteria may be a more important predictor of disease than qualitative presence or absence. The advantage of real-time PCR assays is the ability to quantify bacteria in vaginal fluid, but the short amplification products used limit the amount of phylogenetic information that is derived from the PCR, thus compromising specificity. By se-

quencing several hundred base pairs in our conventional assays, we are more confident that our amplification products match our intended target. Third, our research subjects were recruited from only two clinics in Seattle. The generalizability of these results will be augmented when our assays are applied to other populations of women. More detailed comparison of our subjects' characteristics, including age, race, sexual behavior, concomitant genital infections, and clinic enrollment site, as they relate to the specific PCR results presented here is under way. Fourth, Gram stain of vaginal fluid was not performed routinely on subjects recruited from the STD clinic; however, we are currently collecting these data for all newly enrolled subjects.

In conclusion, several fastidious vaginal bacteria are excellent markers of BV, either alone or in combination, including a *Megasphaera* species, three novel bacteria in the order *Clostridiales* (BVAB1 to -3), *Leptotrichia*/*Sneathia*, *Atopobium vaginae*, and an *Eggerthella*-like bacterium. PCR detection of the combination of either *Megasphaera* phylotype 1 or one of the *Clostridiales* bacteria (BVAB2) yielded a sensitivity of 99% and a specificity of 89% for diagnosis of BV. These results suggest that PCR detection of select key novel vaginal bacteria or bacterial combinations may be useful for the microbiological diagnosis or confirmation of BV. Studying how these different bacterial species interact in subjects with BV will likely help illuminate the pathophysiology of this common but poorly understood syndrome.

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