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Novel PCR-Based Methods Enhance Characterization of Vaginal Microbiota in a Bacterial Vaginosis Patient before and after Treatment

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Deep characterization, even by next-generation sequencing, of the vaginal microbiota in healthy women or posttreatment bacterial vaginosis patients is limited by the dominance of lactobacilli. To improve detection, we offer two approaches: quantitative PCR (qPCR) using phylogenetic branch-inclusive primers and sequencing of broad-spectrum amplicons generated with oligomers that block amplification of lactobacilli.

Bacterial vaginosis (BV) is the most common vaginal infection worldwide, affecting 23% of premenopausal women (1, 2) and exhibiting high recurrence rates (3). Decades of microbiological and molecular analyses have not established an etiology (4). These difficulties result in part from the complex microbiota of the vagina, composed of hundreds of bacterial species, with titers ranging from billions to fewer than 100 cells, many of which are fastidious, unculturable, or difficult to identify (5–7).

PCR-based strategies include sequencing amplicons of the 16S rRNA gene generated by broad-spectrum primers after either cloning in *Escherichia coli* or physical separation of molecules in next-generation sequencing (NGS) (8–15). NGS has now been applied in at least 5 large-scale studies of bacterial populations in the vaginal mucosa of healthy women and asymptomatic and symptomatic BV patients (16–20). Limitations of this approach include the possibilities that broad-spectrum primers may miss whole phyla or fail to amplify some targets for unknown reasons (6, 7) and that rare species may be missed entirely in the presence of an overwhelmingly dominant species.

Blocking *Lactobacillus* amplification during broad-spectrum PCR. Our first strategy was to block the amplification of *Lactobacillus*, using oligomers (LB-blockers) that partially overlap the broad-spectrum primers but complement only (and all) *Lactobacillus* species and are phosphorylated at their 3′ ends to prevent extension (Fig. 1). These primers and blockers and their PCR programs are described in Tables S1 and S2 in the supplemental material. Methods for their design, optimization, and initial testing are detailed in File S1 in the supplemental material. When applied to 8 species of *Lactobacillus*, LB blocking resulted in >24,000-fold inhibition; in contrast, no inhibition in 32 nontarget species and relatively modest inhibition of 7 species related to *Lactobacillus* were seen (Table S3).

We used this approach on an acute BV sample (Fig. 2, top panels), comparing 204 LB-blocked versus 179 unblocked cloned sequences. LB blocking removed the *Lactobacillales* reads and 4 non-*Lactobacillales* reads seen only once without blocking, but other targets did not change significantly (Table 1). With or without blocking, the species detected are characteristic of those in acute BV samples in the literature. Chao1 prediction of actual diversity was 35 operational taxonomic units (OTUs) compared to 22 observed, with a Good's coverage of 88% and Shannon index of 1.8 (see Table S4 in the supplemental material).

The impact of application of LB-blockers to the posttreatment sample, which was dominated by *Lactobacillus*, was dramatic (Fig. 2, bottom panels). In the absence of *Lactobacillus* blocking, 100% of the reads represented *Lactobacillus*. In contrast, the presence of LB-blockers allowed detection of 21 other species in only 136 reads (Table 1), collectively representing only 0.003% of the total population. Chao1 prediction of actual diversity in the blocked sequencing was 135 OTUs compared to 21 observed, with a Good's coverage of 88.4% and Shannon index of 1.7 (see Table S4 in the supplemental material).

This pilot test of LB-blockers is limited to one pair of samples and small numbers of reads. Nevertheless, the data show that LB-blockers greatly enhance detection of non-*Lactobacillus* species without distorting the vaginal profile when *Lactobacillus* is subdominant. A more quantitative evaluation of the enhancement provided by LB blocking would require coupling it to NGS evaluation.

PB-qPCR. Our second strategy employed a complementary approach of using quantitative PCR (qPCR) with 18 phylogenetic branch-inclusive (PB) primers. Group-specific primers have been used in other applications, especially in the gut (21–24), and in one vaginal study (25). These typically are focused on one or a few target genera, whereas our approach strives for inclusivity. Each PB primer targets its own phylogenetic branch, ranging from whole phyla to family or genus, and is far more inclusive than species-specific primers. Positions of PB primers are depicted in Fig. 1 and are described in detail in Table S2 in the supplemental material. The collection was initially validated with target and nontarget single species (listed in Table S3).

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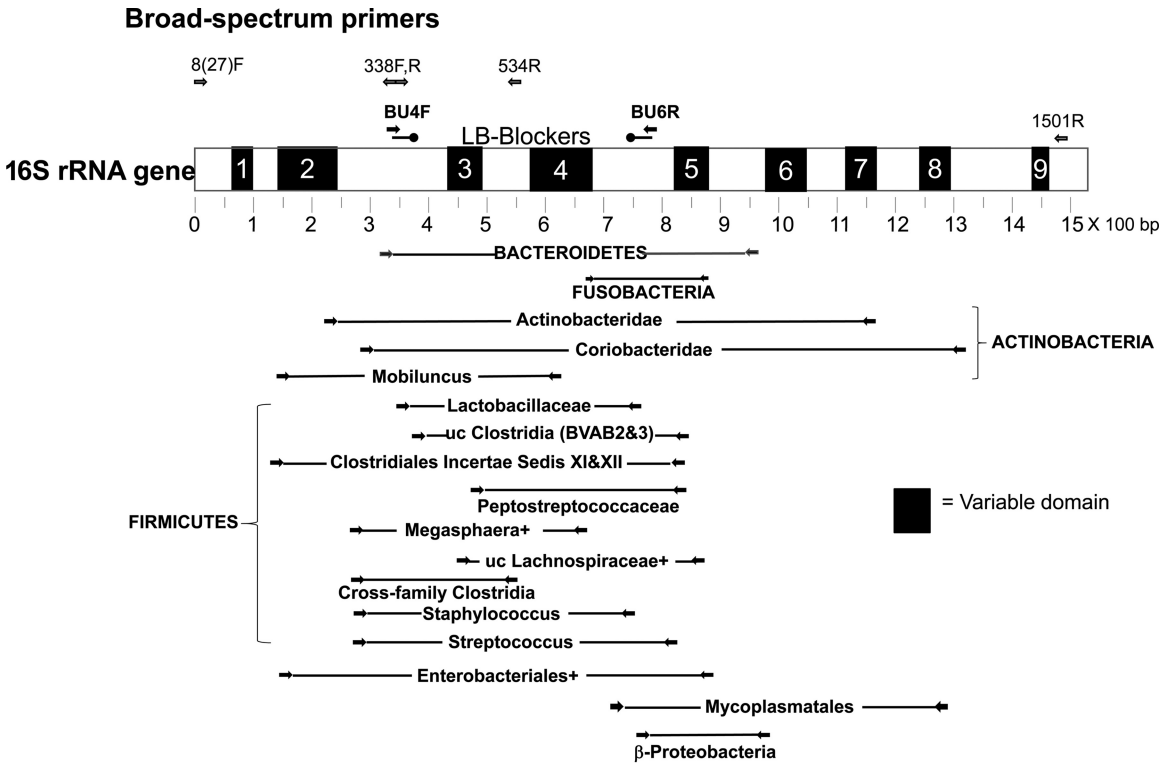

FIG 1 Relative positions of primers and LB-blockers on 16S rRNA gene. The 16S rRNA gene is depicted with its variable domains based on *E. coli*. The positions of BU4F and BU6R used in this paper as broad-spectrum primers are shown above. Also included are other primers used for this purpose in published vaginal microbiome studies (see Table S1 in the supplemental material). LB-blockers overlap BU4F and BU6R primers and complement only *Lactobacillus* species. PB primers are positioned below the 16S rRNA gene (primers are detailed in Table S2). Primers overlapping 27F and 1501R were used as first-round primers in nested PCR to detect some targets at lower titers. uc, uncultured; +, other related genera in the target as described in Table S2.

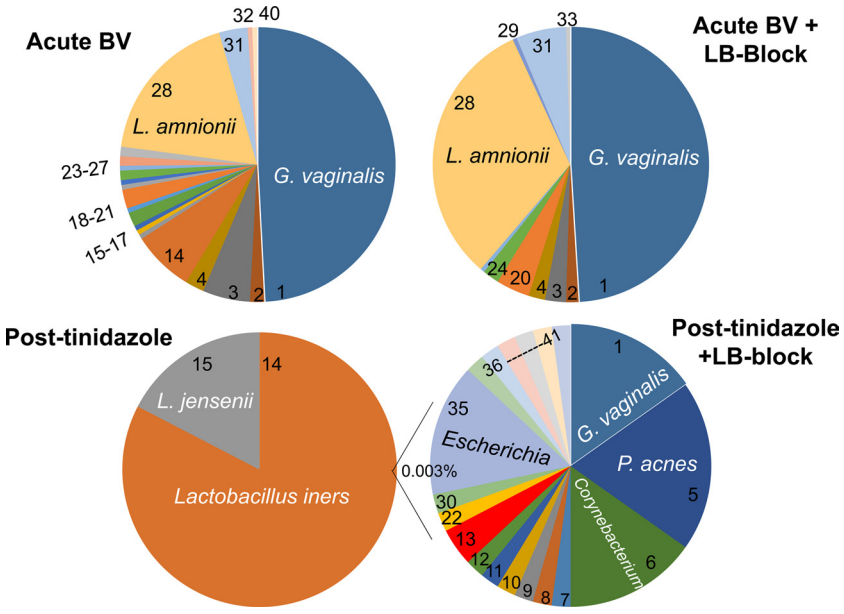

FIG 2 Compositions of vaginal microbiota from a patient with acute BV and after tinidazole treatment with and without *Lactobacillus* blocking. Amplicons from broad-spectrum primers, made with and without LB-blockers, were cloned and sequenced. The Post-tinidazole + LB-block chart represents the non-*Lactobacillus* component, collectively 0.003% of isolates. Numbers in the charts are defined in Table 1.

TABLE 1 Species identified from sequences of cloned 16S rRNA genes amplified from vaginal bacteria[a]

Species no.	SeqMatch	Phylum
1	*Gardnerella vaginalis*	A
2	~*Gardnerella*	A
3	*Atopobium vaginae*	A
4	~*Paraeggerthella*	A
5	*Propionbacteria acnes*	A
6	*Corynebacterium* (uc)	A
7	*Corynebacterium thomssenii*	A
8	*Corynebacterium amycolatum*	A
9	*Corynebacterium coyleae*	A
10	*Corynebacterium pyruviciproducens*	A
11	~*Corynebacterium ureicelerivorans*	A
12	*Brevibacterium*	A
13	*Actinomycetales* (uc)	A
14	*Lactobacillus iners*	F
15	*Lactobacillus jensenii*	F
16	*Lactobacillus* 1	F
17	*Lactobacillus* 2	F
18	*Aerococcus christensenii*	F
19	~*Aerococcus*	F
20	~*Roseburia*	F
21	*Lachnospiraceae*	F
22	~*Ruminococcus*	F
23	*Anaerococcus prevotii*	F
24	*Parvimonas*	F
25	*Peptoniphilus*	F
26	*Dialister*	F
27	~*Dialister*	F
28	*Leptotrichia amnionii*	Fu
29	*Leptotrichia*	Fu
30	*Bacteroides* (uc)	B
31	*Prevotella bivia*	B
32	*Prevotella disiens*	B
33	*Prevotella*	B
34	*Shingomonas aerolata*	B
35	*Escherichia coli*	γ-P
36	*Aggregatibacter*	γ-P
37	*Pseudomonas fluorescens*	γ-P
38	*Pseudomonas pseudoalcaligenes*	γ-P
39	*Methylobacterium aminovorans*	α-P
40	*Janthinobacterium lividum*	β-P
41	*Arcobacter cryaerophilus*	ε-P

[a] Species or taxa were identified and tabulated from sequences as the nearest RDP hits, as described in File S1 in the supplemental material. Numbers refer to numbered sectors in the Fig. 2 charts. Phyla are abbreviated as follows: A, *Actinobacteria*; F, *Firmicutes*; Fu, *Fusobacteria*; B, *Bacteroidetes*; γ-P, *Gammaproteobacteria*; α-P, *Alphaproteobacteria*; β-P, *Betaproteobacteria*; ε-P, *Epsilonproteobacteria*. ~, ≥3% divergent from the indicated, nearest RDP seqmatch hit. uc, uncultured.

PB-qPCR performed on our pair of samples generated compositions (Fig. 3; see also Table S5 in the supplemental material) similar to those in acute BV and healthy samples in the literature (16–20). Titers of the more prevalent targets (*Lactobacillaceae*, *Actinobacteria*, *Coriobacteridae*, *Bacteroidetes*, *Fusobacteria*, and *Clostridiales incertae sedis* XI clostridia) were similar in proportion to the profiles generated by unblocked and LB-blocked sequencing; the less prevalent targets could not be compared due to low numbers of sequence reads. Sequencing of uncloned amplicons, when not mixed, all verified that the PB primers were measuring their intended targets. Some amplicons, those consisting of mixtures of codominant species, were resolved by sequencing of <20 clones (Table S5) from nested PCR, confirming that intended targets were being quantified and adding to the level of diversity. PB-qPCR detected 18 targets in the acute BV sample and 14 in the posttinidazole sample; these numbers were increased to 30 and 33, respectively, by sequencing mixed amplicons.

Is there actually diversity in vaginal samples that warrants the use of PB primers instead of species-specific primers? An *in silico* comparison demonstrated that novel or atypical *Clostridium* species seen in this study had primer binding sites that had as many as 7 mismatches to the specific BVAB-1, -2, or -3 primers used in previous studies (12, 26). Consistently, these species-specific BVAB-1 primers failed to detect targets in our samples (data not shown). Furthermore, most of the variant species observed (see Table S5 in the supplemental material) have imperfect complementarity to primers specific for "expected" species in the target group and thus would be either missed or underestimated. These data support the premise that PB primers offer better profiling than species-specific primers and underscore the diversity of the vaginal microbiome. Combined with small-library sequencing, the PB-qPCR approach improves inclusiveness relative to species-specific qPCR and yet still allows rapid species identification.

Increased detection of diversity in vaginal samples. Use of LB-blockers allowed the detection of 19 subdominant species present at 6 orders of magnitude below *Lactobacillus*. PB-qPCR detected 29 targets in the two samples with estimated titers < 1/1,000 of the total (Fig. 3) which would have not been detected in 2,500-read sets by NGS. Our observed diversity with just over a hundred reads exceeds that seen in recent pyrosequencing-based NGS studies of vaginal populations. For example, one NGS study analyzed 90 samples from among Amsel- and Nugent-based healthy samples, reading an average of 2,235 sequences per sample, and detected an average of only 3 non-*Lactobacillus* sequences per sample, with 18% of samples generating only *Lactobacillus* sequences (19). Another NGS study, averaging 2,236 reads per sample, found that, among 173 asymptomatic patients with a Nugent score of between 0 and 3 and pH < 4.5, an average of only 12 reads per sample represented species other than *Lactobacillus* (17). In a study using the Illumina platform, among 79 women with a Nugent score ≤ 3 and Amsel criteria > 2, with an average depth of 41,512 reads, an average of 51 OTUs, including *Lactobacillus* species, were seen (20). Our 21 OTUs from LB-blocked sequencing of only 136 clones, or 33 OTUs, including PB-qPCR, are similar in complexity, reflecting the power of the methods even on a small scale and supporting the idea that our observation of diversity in healthy women is not artifactual.

The tools described here, pending NGS validation, potentially allow feasible, in-depth characterization of large numbers of vaginal samples, documenting sequential changes in individual patients. It could be argued that there is little value in characterizing the less dominant species; however, the etiology and ecology of BV are complex and poorly understood (27). We do not know what initiates the changes in microbial populations as they transition from healthy to abnormal compositions, what causes refractory or recurrent responses to treatment, whether there is a sexual transmission component, or whether specific compositions pose higher risks for the complications associated with BV in general (28–33). It could also be argued that effective treatment of BV

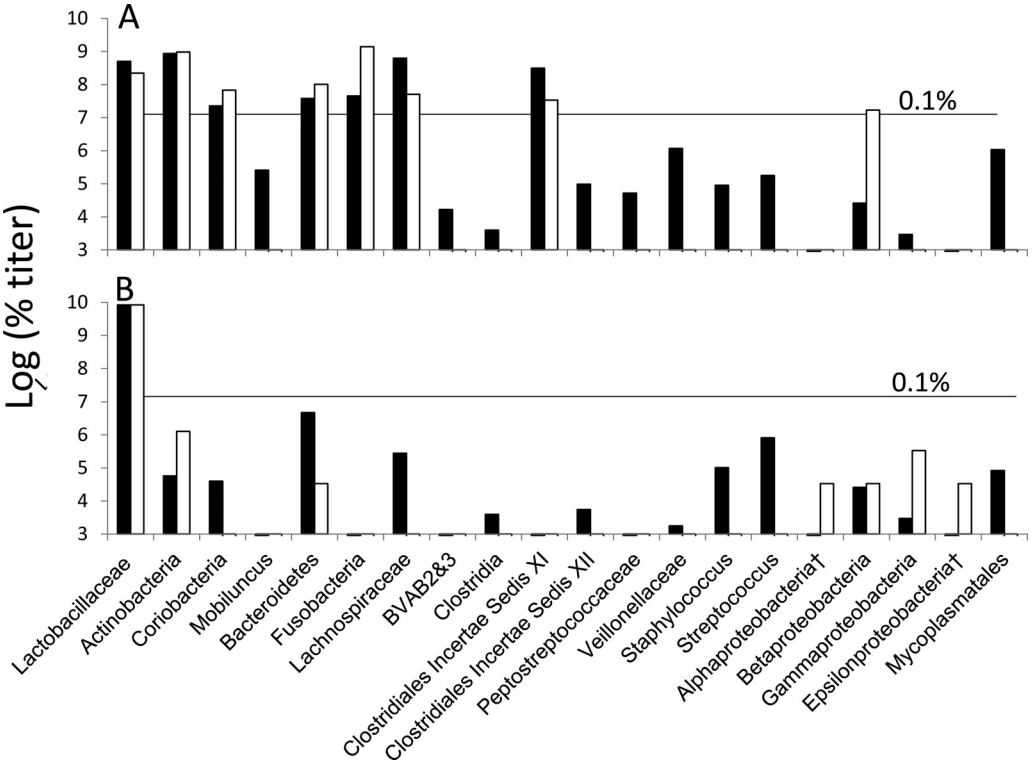

FIG 3 Compositions of acute BV (A) versus posttinidazole (B) samples by PB-qPCR versus blocked and unblocked sequencing. Titers for PB-qPCR (black bars) were calculated as described in File S1 in the supplemental material. For 16S rRNA gene clone and sequence data (white bars), percent composition values (Fig. 2; see also Table S5 in the supplemental material) were converted to titers to facilitate comparison to the qPCR data, as described in File S1. †, no PB primer was designed. Species detected at titers below the 0.1% line would have been undetected or inaccurately counted in a 2,500-read NGS project.

should have as an endpoint not merely of restoration of *Lactobacillus* species to dominance but of reduction of levels of BV-associated anaerobes below a threshold that is yet to be defined.

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