Pyrosequencing of the Genital Microbiotas of HIV-Seropositive and -Seronegative Women Reveals *Lactobacillus iners* as the Predominant *Lactobacillus* Species[▽]

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The species of vaginal lactobacilli in HIV-seropositive and -seronegative women were determined by 16S gene pyrosequencing. *Lactobacillus iners* sequences were the predominant lactobacillus sequences in 66% of HIV⁺ women and 90% of HIV[−] women. This has implications for resistance of HIV⁺ and HIV[−] women to genital colonization by pathogenic organisms.

Lactobacillus is the predominant bacterial genus in the lower genital tract of many women. Common species include *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners* (2, 13, 26, 32). Predominant vaginal colonization by lactobacilli is believed to be beneficial in inhibiting development of bacterial vaginosis (BV), which causes conditions such as preterm delivery and pelvic inflammatory disease (16, 20, 32). Colonization by lactobacilli is also associated with lower rates of infection with several sexually transmitted diseases (1, 6, 7, 19, 24, 28).

Different species of lactobacilli have been associated with differing protective effects from colonization by pathogenic microorganisms. For example, the presence of H₂O₂-producing lactobacilli was associated with a lower occurrence of BV, and *L. crispatus* and *L. jensenii* frequently produce hydrogen peroxide while *L. iners* and *L. gasseri* do not (2, 9, 14, 15, 21, 30). Thus, identification of the types of lactobacilli that colonize the genital tract can potentially help in understanding susceptibility to pathogens.

Many studies that identify the lactobacilli in genital tract samples first culture lactobacilli and then use molecular methods to determine the species. Using this approach, several studies showed that *L. crispatus* and *L. jensenii* are most frequently the predominant species (2, 23, 29). However, some culture methods can select certain types of lactobacilli over others (10, 11, 32), possibly introducing a bias into identification. To avoid culture bias, recent studies characterized vaginal microbiota by cloning and sequencing the bacterial 16S rRNA gene. These studies also showed that *L. crispatus* was frequently predominant (12, 17).

Since it is of interest to identify the species of lactobacilli present in women because of their protective ability, and since no studies have done this using culture-independent methods

in HIV-seropositive women, a group that is already at increased risk of many types of infections due to compromised T-cell immunity, we sequenced a region of the 16S gene to determine the species of vaginal lactobacilli present in HIV⁺ and HIV[−] women.

The subjects were a subset of those enrolled in the Women's Interagency HIV Study (WIHS) (4). Informed consent was obtained from all subjects. The HIV⁺ women were selected randomly from 406 HIV⁺ subjects studied previously (25), and the HIV[−] women were described previously (27). A total of 30 of the 36 HIV⁺ women and 9 out of 10 of the HIV[−] women were African-American. The median age was 39 years (range, 32 to 49) for the HIV⁺ women and 37 years (range, 25 to 45) for the HIV[−] women. None of the women were undergoing current antibiotic treatment, and none had current infection/colonization with *Trichomonas* or yeast (determined by wet mount and KOH). None of the women were on highly active antiretroviral therapy (HAART).

Methods for genital tract sample collection by cervical-vaginal lavage, DNA isolation, multitag pyrosequencing of a portion of the 16S rRNA gene spanning the V1 and V2 regions, CD4 counts, and Nugent Gram stain were previously described (4, 22, 27). *Lactobacillus* 16S rRNA gene sequences were identified using the Ribosomal Database II Project (RDP 10) classifier (8) and assembled with reference sequences into phylogenetic trees with a 96% overlap identity and 80% confidence threshold using Geneious Pro 4.6.1 software (Auckland, New Zealand). Reference sequences were AF257097 (*L. crispatus*), M58820 (*L. gasseri*), AF243176 (*L. jensenii*), AF243177 (*L. vaginalis*), and AY526083 (*L. iners*).

A total of 48,000 16S gene sequences from 46 women were analyzed. For the 36 HIV⁺ women, (Table 1) a median of 24% (range, 0 to 99%) of the sequences corresponded to lactobacilli (3 had no lactobacilli). For the 10 HIV[−] women, a median of 44% of the sequences corresponded to lactobacilli (range, 1 to 98%).

In the HIV⁺ women, *L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii* were the predominant lactobacillus sequences in 66%,

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TABLE 1. Proportions of species of *Lactobacillus* in HIV⁺ and HIV⁻ women

Subject no. and HIV status	% <i>Lactobacillus</i> ^a	% of <i>Lactobacillus</i> spp. representing ^b :					CD4 count
		<i>L. crispatus</i>	<i>L. jensenii</i>	<i>L. iners</i>	<i>L. gasseri</i>	Other ^c	
HIV ⁺							
1	99	84	0	2	0	15	401
2	99	0	0	92	0	8	133
3	99	0	0	83	6	13	4
4	98	0	1	0	88	12	14
5	98	15	4	81	0	0	303
6	97	0	53	30	0	16	479
7	97	99	0	1	0	0	453
8	96	0	0	12	88	0	202
9	96	1	10	83	0	6	6
10	95	75	0	25	0	0	726
11	94	61	12	12	0	15	519
12	86	4	1	95	0	0	451
13	81	0	0	87	0	13	193
14	79	22	13	51	1	12	8
15	67	82	0	1	0	17	324
16	35	0	0	84	0	21	245
17	33	0	0	92	0	8	208
18	26	0	0	80	0	20	333
19	22	0	0	77	0	23	395
20	20	0	1	71	0	28	0
21	18	0	0	71	0	29	86
22	10	0	0	88	0	12	157
23	5	0	0	89	0	11	73
24	5	0	0	36	64	0	327
25	5	0	0	95	0	5	809
26	3	0	0	94	0	6	ND ^d
27	3	0	0	88	0	13	ND
28	2	0	0	54	0	46	198
29	2	50	0	33	0	17	516
30	1	0	0	78	11	11	555
31	1	0	0	100	0	0	200
32	0	0	0	0	0	100	395
33	0	0	0	88	0	12	168
34	0	0	0	0	0	0	168
35	0	0	0	0	0	0	352
36	0	0	0	0	0	0	254
HIV ⁻							
1	98	5	5	89	0	3	
2	98	94	1	2	0	2	
3	96	1	0	97	0	4	
4	94	0	0	90	0	9	
5	80	0	2	87	0	10	
6	8	1	0	99	0	0	
7	6	0	0	100	0	0	
8	1	0	0	100	0	0	
9	1	11	0	89	0	0	
10	1	0	0	100	0	0	

^a Percentage of all sequences that corresponded to lactobacilli.^b Percentage of all lactobacillus sequences that were part of the designated species.^c Lactobacillus sequences that were less than 95% similar to *L. crispatus*, *L. jensenii*, *L. iners*, or *L. gasseri*.^d ND, not determined.

18%, 9%, and 3% of subjects, respectively. In the 10 HIV⁻ women, *L. iners* was predominant in 90%. *L. iners* sequences were also present at substantial levels in several of the women who did not have *L. iners* as the predominant type. The percentage of *L. iners* was significantly higher in the HIV⁻ group (mean, 85%) than in the HIV⁺ women (mean, 60%; $P = 0.004$, two-tailed Mann-Whitney test), although this difference may have been due to the relatively small number of subjects ($n = 10$) in the HIV⁻ group.

HIV⁺ women with sequences that were >50% lactobacilli

had a significantly ($P = 0.006$, two-tailed Mann-Whitney) higher proportion of *L. crispatus* sequences (median, 4%) than women with <50% lactobacilli (median, 0%). There was a trend ($P = 0.07$) toward higher levels of *L. jensenii* in HIV⁺ women with >50% lactobacillus sequences. Conversely, the proportion of *L. iners* sequences was significantly lower ($P = 0.05$) in HIV⁺ women with microbiota comprised of >50% lactobacilli (median, 30%; mean, 44%) than in women with <50% lactobacilli (median, 81%; mean, 73%).

In HIV⁻ women, *L. crispatus* levels were not significantly

higher in the five women with lactobacilli comprising >50% of sequences than in those with <50%. However, *L. iners* levels were significantly lower in the five with >50% than in those with <50% ($P = 0.05$; median, 89% versus 100%).

L. iners, compared to *L. crispatus* or *L. jensenii*, produces less H_2O_2 and relatively lower resistance to colonization by pathogens (2, 9, 14, 15, 21, 30). Our observations are in contrast to those that showed *L. crispatus* as the predominant lactobacillus species using either culture-dependent or -independent methods. A study that used culture-independent methods (12) found that in eight women with predominant lactobacilli, *L. crispatus* sequences were predominant in five and comprised 40 to 49% of sequences in three. Antonio et al. (2), using culture, reported that the largest numbers of women were colonized by *L. crispatus* (32%) and *L. jensenii* (23%) while other lactobacilli colonized at much lower frequencies. Other culture-based studies also showed that *L. crispatus* was the prevalent lactobacillus species in a majority of women (23, 29). One study using molecular methods, however, found that women were frequently colonized with *L. iners* (5).

Interestingly, we found that *L. jensenii* was absent or present at relatively low levels in all women except one. In contrast, culture-based studies found substantial levels of *L. jensenii* (2, 3). While *L. jensenii* and *L. crispatus* are easily cultured on medium commonly used for isolation, strains of *L. iners* are not (11). Thus, culture bias could alter the proportions of species reported in some studies, possibly providing an explanation for why *L. iners* was found less frequently in some previous studies.

The CD4 cell count for the HIV⁺ women ranged from 0 to 726 cells/mm³ (median, 245) (Table 1). Thirteen of the women had CD4 counts of ≤ 200 cells/mm³, and the percentage of *L. crispatus* in these women was significantly lower ($P = 0.04$, Mann-Whitney) than in the 17 women with CD4 counts of >200 cells/mm³ (median of 0% *L. crispatus* for both groups). In contrast, the amount of *L. iners* in the 17 women with CD4 counts of ≤ 200 cells/mm³ was significantly higher in women with low CD4 counts (median, 83%; $P = 0.05$) than in women with high CD4 counts (median, 32%). *L. jensenii* levels and total lactobacillus levels were not significantly different between the CD4 groups. No previous studies compared lactobacillus types with CD4 levels, although a prospective study over time (18) found that vaginosis was more prevalent and persistent among HIV⁺ women and even more pronounced in those with CD4 counts of ≤ 200 . We speculate that this could have been due to lower levels of H_2O_2 -producing *L. crispatus* and *L. jensenii* (2, 9, 14, 15, 21). Studies of the WIHS have not demonstrated an inverse relationship between BV and HIV status, but this may reflect behavioral factors such as condom use rather than biologic susceptibility (31). It should be noted that the HIV⁻ group of women analyzed here had higher levels of *L. iners* than the HIV⁺ group even though they had no known immunodeficiency. The explanation for why *L. iners* levels were lower in HIV⁺ women with high CD4 numbers but higher in women with no decrease in CD4 (HIV⁻) may have been the relatively small sample size of the HIV⁻ group.

In conclusion, we showed that *L. iners* was the predominant lactobacillus in these HIV⁺ and HIV⁻ women. Since previous studies indicate that *L. iners* is less effective at preventing vaginal colonization by pathogens than other lactobacilli (*L. crispatus* and *L. jensenii*), this study has important implications

for the resistance of women to colonization by pathogens and the effects of HIV disease on this resistance.

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