Comparative Study of Vaginal Lactobacillus Phages Isolated from Women in the United States and Turkey: Prevalence, Morphology, Host Range, and DNA Homology

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Lactobacilli play an important role in maintaining vaginal health. However, during bacterial vaginosis lactobacilli decrease for unknown reasons. Our preliminary study showed that phages could infect vaginal lactobacilli. Therefore, the aim of this study was to analyze the distribution, virulence, and types of vaginal Lactobacillus phages isolated from women of two countries: the United States and Turkey. A total of 209 vaginal lactobacilli were isolated from reproductive-aged women in the United States (n = 107) and Turkey (n = 102). By analysis of 16S rRNA gene sequence and by comparison of protein profiles, most lactobacilli were identified as L. crispatus, L. gasseri, and L. jensenii. After mitomycin C induction, 28% of American lactobacilli and 36% of Turkish lactobacilli released phages. A total of 67 phages were isolated and further characterized by their host range, electron microscopy, and DNA homology. All 67 phages were infective against lactobacilli from both collections. The host ranges of most phages were broad, including multiple Lactobacillus species. Even though the phages were all temperate, they were able to cause lytic infection in various strains. The electron micrographs of these phages showed a hexagon-shaped head and a long tail with or without a contractile tail sheath. Based on their morphology, these phages belonged to Bradley’s phage groups A and B, and could be further classified into four morphotypes. All four types were found among American phages, but only three were found among Turkish isolates. DNA hybridization with labeled probes of the four types of phages revealed that additional genetic types existed within each morphotype among these phages. The phage genomic sizes ranged between 34 and 55 kb. Many of the lysogenic Lactobacillus strains released phages spontaneously at a high frequency of 10⁻³ to 10⁻⁴ PFU/cell. In conclusion, lysogeny in vaginal lactobacilli is widely spread. Some lysogenic lactobacilli spontaneously release phages with a broad host range, which can be lytic against other vaginal lactobacilli regardless of their geographic origin.

Lactobacilli indigenous to the human vagina are beneficial to women’s health (35). These bacteria can inhibit other potentially harmful microorganisms by producing lactic acid, hydrogen peroxide (H₂O₂), and antimicrobial substances (12, 23). In most healthy women, lactobacilli are the dominant species in the vagina. Theoretically, anaerobic bacteria are suppressed by lactobacilli (12, 23) and cannot replace lactobacilli unless the latter is first diminished. However, the group of anaerobic bacteria commonly outnumber lactobacilli, causing a microbial imbalance called bacterial vaginosis (BV) (3, 9, 10, 15, 38, 40).

BV is a clinical condition that is characterized by decreased lactobacilli and an increased number of anaerobic gram-negative rods, Gardnerella species, and genital mycoplasmas (10, 38, 40). Women who suffer from BV may have an increased discharge that often has an unpleasant fishy odor. BV has been associated with many health risks, including preterm birth of low-birth-weight infants, midtrimester pregnancy loss, amniotic fluid infection, postpartum endometritis, pelvic inflammatory disease, and gynecologic postoperative infections (14, 16, 17, 28, 29). Recently, a lack of vaginal lactobacilli or the presence of BV was found to promote human immunodeficiency virus transmission (8, 27, 37).

The cause of BV is currently unknown, and it is unclear what causes the decrease of vaginal lactobacilli. Several possible mechanisms by which vaginal lactobacilli decrease have been proposed. These include douching (13); the use of spermicide, such as nonoxynol-9 (18); and treatment with antibiotics for other infections. It is important to examine the possibility that vaginal lactobacilli may decrease due to natural causes, such as phages or viruses.

Lactobacillus phages have been isolated from various sources, including dairy products (22), sausage (30), human intestines (34), and sewage (24). Recently, we reported the isolation of phages from human vaginal lactobacilli and documented their infectivity in vitro against lactobacilli isolated from the same and/or different women (32, 41). This suggested that reduction of vaginal lactobacilli may be caused by phages. It is important to further study and characterize these phages. In this study, we analyzed 67 vaginal Lactobacillus phages isolated from women in the United States and in Turkey based on their morphology, host range, spontaneous induction rate, DNA homology, and prevalence.
MATERIALS AND METHODS

Bacterial strains and growth media. Vaginal samples were obtained from reproductive-aged women visiting obstetrics and gynecology clinics at the Truman Medical Center in Kansas City, Mo., and at the medical schools of Ka- dence Technical University, Trabzon, Turkey, and Firat University, Elazig, Turk- ey. These included healthy women and women with vaginal infections, such as BV and candidiasis. Both the Amel criteria (3) and Nugent scoring system (31) were used for diagnosis of vaginosis. Vaginal pH was measured with pH paper (Fisher Scientific). Microscopic examination of the Gram-stained vaginal sample slide was used to confirm the initial clinical diagnosis. During sampling, two sterile cotton swabs were inserted into the vagina, rotated a few turns along the vaginal sidewall, and allowed to absorb for a few seconds before being withdrawn. One swab was used for Gram staining. The other swab was placed into a test tube containing the RTF-glycerol transport buffer and sent to the laboratory sterile cotton swabs were inserted into the vagina. Vaginal pH was measured with pH paper (Fisher Scientific). Microscopic examination of the Gram-stained vaginal sample slide was used to confirm the initial clinical diagnosis. During sampling, two sterile cotton swabs were inserted into the vagina, rotated a few turns along the vaginal sidewall, and allowed to absorb for a few seconds before being withdrawn. One swab was used for Gram staining. The other swab was placed into a test tube containing the RTF-glycerol transport buffer and sent to the laboratory.

Phage infectivity assay. Phage infectivity was determined by the agar spot method as previously described (32). All of the 67 phages were used to infect the two collections of vaginal Lactobacillus strains of a total of 209 isolates. The positive results were verified by single plaque isolation.

Electron microscopy. One drop of the purified phage in 0.1 M ammonium acetate (pH 7.0) was spotted on grids with a carbon-coated Formvar film (Ladd Research Industry, Burlington, Vt.). After drying for 30 s, the sample was negatively stained with 2% uranyl acetate (pH 4.2). Electron microscopy was performed with the CM12 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.) at 80 kV.

Phage genomic DNA hybridization. The genomic DNA from representative phages was isolated and labeled with the nonradioactive biotinylated labeling kit from Gibco-BRL as probes (Life Technologies, Inc., Rockville, Md.). The DNA from target phages was processed by two methods. The first method was to digest the DNA with restriction enzymes. The digested DNA was then subjected to agarose gel electrophoresis on a 0.8% agarose gel at 40 V for 3 h. The gel was stained with ethidium bromide and photographed under a UV light.

Phage classification by PCR. To obtain sequence data for the PCR analysis, the genomic DNA of four phages representing each morphotype was digested with SmaI. The digested DNA fragments were cloned into the pUC18 plasmid. A pUC18 plasmid that carries a random insert of about 1 to 2 kb was selected for each phage. The sequence of the cloned DNA was determined by the automated sequencing facility at the University of Missouri—Kansas City. The sequence data were analyzed by the BLAST program and used to design PCR primers. The primers used are listed Table 1. The DNA of target phages was isolated and used as template DNA. PCR was performed by using a thermal cycler (Techne, Princeton, N.J.). The reaction mixture (final volume of 50 μl) contained 100 ng of template DNA: 1 U of Taq DNA polymerase (Biolase; Bioline, Reno, Nev.); 1× reaction buffer (buffer 1; pH 9.5, from the Invitrogen PCR optimizer kit; Invitrogen, Carlsbad, Calif.); 2 mM MgCl2; deoxynucleoside triphosphates, 0.1 mM each; primers, 50 pmol each; and bovine serum albumin, 2 μg. The thermal cycling program was as follows: initial denaturation at 94°C for 2 min and 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. Finally, there was an extension step at 72°C for 7 min. The PCR DNA products were analyzed for correct sizes and for purity by agarose gel electrophoresis.

RESULTS

Isolation and identification of vaginal lactobacilli. About 200 vaginal samples were obtained from reproductive-aged women visiting obstetrics and gynecology clinics at the Truman Medical Center in Kansas City, Mo., and at the medical schools of Kardem Technical University, Trabzon, Turkey, and Firat University, Elazig, Turkey. These included healthy women and women with vaginal infections, such as BV and candidiasis. Both the Amel criteria (3) and Nugent scoring system (31) were used for diagnosis of vaginosis. Vaginal pH was measured with pH paper (Fisher Scientific). Microscopic examination of the Gram-stained vaginal sample slide was used to confirm the initial clinical diagnosis. During sampling, two sterile cotton swabs were inserted into the vagina, rotated a few turns along the vaginal sidewall, and allowed to absorb for a few seconds before being withdrawn. One swab was used for Gram staining. The other swab was placed into a test tube containing the RTF-glycerol transport buffer and sent to the laboratory.

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RESULTS

Isolation and identification of vaginal lactobacilli. About 200 vaginal samples were obtained from reproductive-aged did not hallucinate.
Some Turkish isolates did not survive the oversea shipping, so Hispanic (3%), and Native American (2%) women. While the Turkish women were all Caucasian, women in Turkey and about 100 were obtained from the United States. While the Turkish women were all Caucasian, the American group included black (55%), white (35%), Asian (5%), and Native American (2%) women. Some Turkish isolates did not survive the oversea shipping, so only 102 Lactobacillus strains were obtained. From American women, 107 strains were obtained. Among the Turkish women, 43 cases of BV were diagnosed, but only 22 had culturable lactobacilli. Among the American women, 14 cases of BV were diagnosed, but only 4 had culturable lactobacilli. Of samples in the RTF-glycerol buffer at 20 to 70°C did not result in loss of Lactobacillus viability. Each collection had 10 obligate anaerobic strains (about 10%). All of the remaining strains were facultative anaerobes (Table 2).

Species identification of lactobacilli. Since the traditional biochemical and physiological methods could not effectively classify these lactobacilli to the species level, we applied genetic and molecular methods. First, we grouped these strains based on their sugar fermentation pattern and whole-cell protein profiles. Then, we determined the sequence of the 16S rDNA of some representative strains from each group. Based on the sequence data, we identified their species. Finally, the whole cell protein profiles were analyzed among all of the remaining strains. Several representative strains from each group that shared the same cell morphology, sugar fermentation pattern and whole cell protein profile were selected to analyze their 16S rDNA sequences. The sequence data of 23 strains (9 from Turkey and 14 from the United States) have been deposited into GenBank with accession numbers from AF243150 to AF243166 and from AF243170 to AF143175. These data were compared to those for Lactobacillus type strains already in GenBank using the BLAST program (2). Once the species of the representative strains were identified, the identification for the remaining strains was achieved by comparison of their total protein profiles with those of the representative strains. The results of species designation of these strains are listed in Table 2. Figure 1 shows the result of one of the SDS-PAGE gels. Based on the 16S rDNA analysis and the protein profile comparison, most clinical vaginal strains belonged to three Lactobacillus species, L. crispatus, L. gasseri, and L. jensenii. The protein profiles of L. gasseri and L. jensenii were highly consistent among all isolates tested. Although a major band of L. crispatus was variable among different isolates (between 40 and 60 kDa on the SDS-PAGE gel), all of the other bands were consistent within the same species. Additional species included L. fermentum, L. vaginalis, and several unknown species. Interestingly, the fourth largest species among American isolates was L. fermentum (9%), while the Turkish isolates did not have any L. fermentum strains. As shown in Table 2, the majority of vaginal lactobacilli were facultative anaerobes.

Phage isolation. Phage induction was performed by the mitomycin C method for 209 clinically isolated vaginal strains. The lysates were used to interact with these Lactobacillus strains to screen for phage-sensitive indicator strains. Sixty-seven lysates were confirmed to contain phages, because they formed single plaques on the agar plates of sensitive strains. Additionally, these phages were confirmed by DNA hybridization with labeled phage DNA probes and observation under an electron microscope to rule out possible bacterial inhibition effects due to bacteriocins, H2O2, and organic acids. Among the 67 phages, 30 were isolated from the American collection, while 37 were isolated from the Turkish collection.

Table 2 shows that the obligate anaerobes were more likely to carry a phage (65%) than the facultative anaerobic lactobacilli (29%). The difference was significant (P < 0.01). About 36% of vaginal lactobacilli from Turkish women released phages, while about 28% of lactobacilli from American women released phages. The difference was not statistically significant between the two groups (P > 0.05). Among six Lactobacillus strains isolated from the four American women with BV, 11 were infected by phages (lysogens). Overall, about 50% of lysogenic lactobacilli were isolated from women with BV, but only about 30% of lysogens were isolated from women without BV. The difference was statistically significant (P < 0.05).

Spontaneous phage induction and burst size. Phages can be spontaneously released without any inducing agent due to random errors during the host bacterial DNA replication (20). In this study, the lysogenic lactobacilli released infective phages at different rates, which were detected by observation of phage plaques on the indicator Lactobacillus plate cultures. Among American lactobacilli, 17% of lysogenic strains spontaneously released phages at a higher frequency of 10 to 10 PFU/cell, while 27% of lysogenic strains from the Turkish collection

<table>
<thead>
<tr>
<th>Species or group</th>
<th>Strain U.S. collection</th>
<th>Strain Turkish collection</th>
<th>Strain Total</th>
<th>% Lysogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. crispatus</td>
<td>27</td>
<td>34</td>
<td>61</td>
<td>19</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>30</td>
<td>33</td>
<td>63</td>
<td>19</td>
</tr>
<tr>
<td>L. jensenii</td>
<td>30</td>
<td>30</td>
<td>60</td>
<td>19</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>L. vaginalis</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Other Lactobacillus spp.</td>
<td>9</td>
<td>3</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Facultative anaerobes</td>
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<td>92</td>
<td>189</td>
<td>29</td>
</tr>
<tr>
<td>Obligate anaerobes</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>102</td>
<td>209</td>
<td>32</td>
</tr>
</tbody>
</table>

* Among Turkish lactobacilli, the obligate anaerobes were L. jensenii and L. crispatus, but among U.S. lactobacilli, the obligate anaerobes were L. gasseri and L. jensenii.
released phages at this level. About one-third of both collections released phages at an intermediate frequency (about $10^6$ PFU/cell). Approximately one-half of the culture collections from both countries spontaneously released phages at a frequency of less than $10^8$ PFU/cell. These data were repeated observations, and the frequency of phage release from each strain was highly stable. The burst sizes were between 60 and 300 phages per cell.

**Phage host ranges and infection characteristics.** All 67 temperate phages isolated from vaginal lactobacilli infected vaginal lactobacilli in vitro by forming clear plaques on agar plates. As shown in Table 3, the 30 phages from the United States and 37 phages from Turkey infected most vaginal lactobacilli from both collections, including lysogenic strains. Overall, fewer lactobacilli isolated from Turkish women resisted phage infection than lactobacilli isolated from U.S. women. A group of vaginal lactobacilli sensitive to multiple phages was identified. They were used as indicator strains to display clear single plaques after the infection and used to screen for new phages. There were no apparent differences in phage sensitivity between lactobacilli isolated from healthy women and those from women with vaginal infections.

Many phages had a broad host range and infected vaginal *Lactobacillus* strains of multiple species, including *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. fermentum*, and *L. vaginalis*. Among the obligate anaerobic lactobacilli, the American collection had mostly *L. gasseri* strains, while the Turkish collection had mostly *L. jensenii* strains. They were equally high in the rate of phage lysogeny. After infection of 100 million *Lactobacillus* cells by these phages (multiplicity of infection, 1:10), no surviving colonies or lysogens could be observed, indicating lytic infection. Nearly all temperate phages in the two collections lytically infected other sensitive lactobacilli.

**Phage morphology.** The electron micrograph (Fig. 2) showed two major morphotypes, Bradley (5) type A and B, among the 67 phages studied to date. Bradley type A is characterized by a hexagonal head and a tail with a contractile sheath. The first type, represented by φkc21T and φkc12a, belongs to Bradley phage type A (5), because both phages had a contractile tail sheath. However, there was a difference between the two phages in the head size and tail length. Additionally, φkc12a had a tail plate. Bradley type B is characterized by a hexagonal head and a tail without a contractile sheath. The second type, represented by φkc39 and φkc7a,
belonged to Bradley phage type B, because both phages were lacking a contractile tail sheath, although they differed in head size and tail length. While all four types existed in the American phage collection, only three types (all but A2) were found among the phages in the Turkish collection. All four types had hexagonal heads but were of two sizes. The smaller one, type A1 and B2, was about 45 nm in diameter, and the larger one, type A2 and B1 was about 67 nm in diameter. The length and appearance of their tails were quite different. The type A1 phages had a shorter tail, about 160 nm long, which could be completely covered by a sheath with about 50 horizontal bands. The type A2 phages had a longer tail about 260 nm long and a tail plate. The sheath was about the same size as that in type A1 phages, but it had a dotted pattern instead of horizontal bands. The tail of the type B1 phages was about 250 nm in length with about 60 disks. The type B2 phages had the longest tails, about 300 nm long with about 80 disks, and also a tail fiber about 40 nm long.

**Phage DNA restriction analysis.** To further characterize these phages, DNA from phages representing different morphotypes were isolated, digested with *Eco*RI, and subjected to agarose gel electrophoresis (Fig. 3). The phage genomes ranged from 34 to 55 kb and were all double stranded and linear as determined by the DNA-heating agarose gel electrophoresis assay (22). The DNA fingerprints showed that most of the phages were genetically different, even among phages with the same morphotype. One identical pattern, however, was found among three phages isolated from different women. According to the protein profile analysis, the three lysogenic lactobacilli belonged to two different species. They were *L. jensenii* TL34 and TL74c and *L. gasseri* TL76.

**Phage classification by DNA hybridization and PCR.** DNA probes were made of complete genomic DNA of four phages, each representing different morphotypes as shown in Fig. 2. The PCR primers were designed according to the sequence data from the shotgun-cloned phage DNA fragments representing four morphotypes. The BLAST analysis of these sequences did not yield any homology with existing data in GenBank. By Southern hybridization, we found that the genome of *φkc5a* was homologous to those of *φkc21T*, *φTL32*, and *φTL138*, representing phage type A1, and the genome of *φTL76* was homologous to those of *φTL34*, *φTL74c*, and *φTL75a*, representing phage type B2. Several homology groups were identified by additional Southern hybridization and dot blot hybridization, as well as by PCR. The results are shown in Table 4. No correlations were found between these phage types and the vaginal health status of these women, because most women who suffered from BV had no detectable lactobacilli in their vaginal samples.

**DISCUSSION**

BV is the most common vaginal disorder affecting women worldwide (38, 40). Since it can increase the risk of preterm delivery of low-birth-weight infants (14, 16, 17) and the risk of contracting human immunodeficiency virus in women (8, 27, 37), treatment and prevention of BV become an important issue (29). Unfortunately, the exact cause of BV is unknown. It has been well documented, however, that during BV, the normally predominant *Lactobacillus* vaginal flora is replaced with anaerobic bacteria (3, 9, 10 38, 40). Therefore, the question was raised of whether bacteriophages could inhibit lactobacilli in the vagina. We have previously reported the identification of phages in vaginal lactobacilli (32, 41). In this work, we report the study on the prevalence, genetic diversity, and infectivity of these phages from women in two geographically distant countries: the United States and Turkey.

To study whether the phage infection in vaginal lactobacilli was species specific, we first classified the species of these lactobacilli. By comparing the protein profiles of the strains of unknown species with those of known species and *Lactobacillus* type strains, most of the strains were characterized to the species level. The majority of strains from both countries belonged to three species, *L. gasseri*, *L. jensenii*, and *L. crispatus*, ...
with almost equal proportions. These data largely agreed with previous studies performed by DNA-DNA hybridization (4, 11, 39). The protein patterns for *L. gasseri* and *L. jensenii* were mostly consistent and reliable. *L. crispatus* was distinguished from *L. gasseri* and *L. jensenii* by having a thick band, with sizes between 40 and 60 kDa among different isolates (Fig. 1). This thick band appeared to represent its S-layer protein (19). Not only could it serve as a potential marker to differentiate *L. crispatus* from *L. gasseri* and *L. jensenii*, but it may also be used to identify different strains within the species of *L. crispatus* due to its size variability. The overall correlation between 16S rDNA data and the protein profiles was strong. The combination of these two methods offered a reliable approach to identify species of a large number of lactobacilli.

By analyzing phage host ranges and *Lactobacillus* species data, we found that many phages infected multiple *Lactobacillus* species. However, some strains remained uninfected. This implied that the phage host range in vaginal lactobacilli might not be determined by species-specific markers. Instead, certain characteristic receptors on the cell surface might determine phage host ranges. Although we do not know what may be the phage receptor on these vaginal lactobacilli, our study (data not shown) revealed that it was not the rhamnose residue of the polysaccharide on the cell surface as in the case of *L. casei* (42). Further studies are needed to identify these phage receptor molecules. Normally, a lysogenic strain is immune from infection by the same phage or the same type of phages. This is called superinfection immunity (20). However, in this study, we found that many lysogenic strains were superinfected by different phages, and some were even infected by the same phage. This suggested that the superinfection immunity might not always function in the group of vaginal *Lactobacillus* lysogens.

Our phage classification studies included electron microscopy and DNA analysis. Based on current knowledge about phage taxonomy (1), phages with similar morphology may be genetically different, but phages with different morphology are usually different in their genomics. The differences in genomic sizes and restriction patterns among the four phages (Fig. 3: lane 3, \( \phi k c7a \), 34.5 kb; lane 4, \( \phi k c12a \), 47 kb; lane 5, \( \phi k c21T \), 38 kb; and lane 8, \( \phi k c39 \), 41 kb) further indicate that these four phages may be genetically different species. Although only four phage morphotypes were noticed among the 67 phages studied, additional genetic types may exist within each morphotype, because many phages did not hybridize with the probes made of the genomic DNA of these four phages. Clearly, none of these phages displayed a prolate-shaped head like that of the dairy *Lactobacillus* phage \( \phi y 8 \), which was released by a *Lactobacillus* starter strain in one of the name brand American yogurts (22). The most prevalent phage morphotype was type B. Three phages showed an identical DNA fingerprinting pattern (Fig. 3), suggesting that a prevalent phage might be trans-
mitting among different women. Further studies will be needed to study phage transmissions.

Normally, a bacteriophage may be spontaneously released at a frequency of 10⁻⁶ per cell (20). A high-frequency spontaneous phage release by many lysogenic vaginal lactobacilli (about 10⁻³ to 10⁻⁴ per cell) is of particular interest. It suggested that a large number of free phages can be spontaneously released from these strains and found present in the vaginal secretion. This characteristic may be clinically significant, because free phages can infect other lactobacilli in the same woman or be transmitted to different women to infect their lactobacilli. This matched the clinical observation that BV, or the lack of vaginal lactobacilli, is associated with sexual transmission (38, 40).

Since many vaginal lactobacilli spontaneously released phages, it suggests that lysogenic Lactobacillus strains may be a source of potentially infectious phages.

Among lysogenic lactobacilli that had a low spontaneous induction frequency, phages were induced by mitomycin C. Some of these phages infected other Lactobacillus strains under in vitro conditions. These lysogenic strains might coexist with other phage-sensitive Lactobacillus strains in the same vaginal environment, because they rarely released phages. However, this condition could change when the vaginal environment encounters a phage-inducing agent. We have recently reported that trace amounts of cigarette smoke chemical benzo[a]pyrene diol epoxide promoted phage release from lysogenic vaginal lactobacilli (33). Among women who smoke, the cigarette-associated mutagenic chemicals could reach their vaginal secretions and cause phage induction in lysogenic lactobacilli.

All phages in the present study were temperate phages released from lysogenic strains. We have so far not been able to isolate lytic phages directly from women. Truly lytic or virulent phages are usually short lived. Once they appear, the virulent phages can rapidly eliminate their host bacteria; as a result, they lose their living shelter for self-reproduction. Therefore, phages that are temperate to some bacteria but lytic to others are of concern. It is well known that some temperate phages

### TABLE 4. Phage classification by electron microscopic (EM) morphology, DNA hybridization, and PCR

<table>
<thead>
<tr>
<th>Phage</th>
<th>EM</th>
<th>DNA hybridization</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A1</td>
<td>A2</td>
</tr>
<tr>
<td>U.S. phages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>φkc5a</td>
<td>A1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>φkc6a, -b</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φkc7a, -b, -cφ</td>
<td>B2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φkc12a</td>
<td>A2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>φkc13φ</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φkc19φ</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>φkc31</td>
<td>B1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φkc39</td>
<td>B1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φkc58aφ</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>φkc59aφ</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>φkc60aφ</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>φkc102bφ</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>φkc110aφ</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φkc148φ</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>φkc149φ</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

| Turkish phages |
| φkc21Tφ | A1  | +     | -     | -     | -     | +     | -     | -     | -     |
| φkc23Tφ | B1  | -     | -     | -     | -     | -     | -     | +     | -     |
| φkc26Tφ |     | +     | -     | -     | -     | -     | +     | -     | -     |
| φTL32φ |     | +     | -     | -     | -     | +     | -     | -     | -     |
| φTL33aφ | A1  | +     | -     | -     | -     | +     | -     | -     | -     |
| φTL34φ | B2  | -     | -     | +     | -     | +     | -     | +     | +     |
| φTL56bφ | A1  | +     | -     | -     | -     | +     | -     | +     | -     |
| φTL74cφ | B2  | -     | -     | +     | -     | -     | +     | -     | -     |
| φTL75aφ |     | -     | -     | +     | -     | -     | +     | -     | -     |
| φTL76φ |     | +     | -     | -     | +     | -     | +     | +     | -     |
| φTL87φ |     | +     | -     | -     | +     | -     | +     | +     | -     |
| φTL102bφ | A1  | +     | -     | -     | +     | -     | +     | -     | -     |
| φTL109a, -cφ |     | -     | -     | +     | -     | -     | +     | -     | -     |
| φTL110bφ |     | +     | -     | -     | +     | -     | +     | -     | -     |
| φTL122bφ | B1   | -     | -     | +     | -     | -     | +     | -     | -     |
| φTL125φ | B2   | -     | -     | -     | +     | -     | +     | -     | -     |
| φTL138φ |     | +     | -     | -     | +     | -     | +     | -     | -     |
| φTL139a, -cφ |     | +     | -     | -     | +     | -     | +     | -     | -     |
| φTL141φ | B2   | -     | -     | -     | +     | -     | +     | -     | -     |

- Phages that showed negative results included φkc36b, φkc38, φkc48, φkc55a, φkc58b, φkc72, φkc74, φTL25a, φTL25b, φTL35, φTL39b, φTL56c, φTL59c, φTL61a, φTL61b, φTL65, φTL72, φTL109c, φTL113, and φTL134.
- Hybridization was performed with the lysogenic Lactobacillus chromosomal DNA.
can become virulent due to genetic mutations (36), but it is
unknown why so many temperate phages from vaginal lactobacilli can become lytic against other vaginal Lactobacillus strains. Probably, certain differences in the bacterial host back-
ground prohibit these phages from integrating their DNA into the chromosome of their new hosts to form lysogens (7).

In conclusion, we studied phages from vaginal lactobacilli of women in Turkey and the United States. We have determined that most of these Lactobacillus strains belonged to three spe-
cies, L. crispatus, L. gasseri, and L. jensenii. Phages isolated from vaginal lactobacilli of some women lytically infected vaginal lactobacilli of other women regardless of their countries of
origin. Four morphotypes were identified among these phages, and their host range was broad and beyond any particular Lactobacillus species. Most lysogenic lactobacilli spontane-
ously released phages into the environment at varied frequen-
cies. This suggested that lysogenic lactobacilli could be a
source of infective phages. Although the phage infection ob-
served in vitro may not necessarily indicate that the same
situation could happen in vivo, the results imply that vaginal lactobacilli may be eliminated or repressed by phages. This
implication may be important for studying the etiology of BV
due to its association with a decrease in vaginal lactobacilli.
Apparently, further studies with an increased number of clinical samples will be needed to associate phage infections in vaginal lactobacilli with women’s vaginal health.

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REFERENCES

Manifoot, J. Rocourt, R. S. Safermann, J. Schneider, L. Seldin, T. Sozzi, P. R.


3. Amel, R., P. A. Totten, C. A. Spiegel, K. S. Chen, D. A. Eschenbach,

nal Lactobacillus species and the demographic and microbiologic charac-


8. Eschenbach, D. A., P. R. Davick, B. L. Williams, S. J. Klebanoff, K. Young-
peroxide-producing Lactobacillus species in normal women and women with

Identification of vaginal lactobacilli from asymptomatic women. Microbio-


Patterns of vaginal douching and their association with vaginal bacteriosis.

J. Pearson. 1994. Abnormal bacterial colonization of the genital tract and sub-

Gynecol. 169:450–454.

Eschenbach. 1995. The role of bacterial vaginosis and vaginal bacteria in
amniotic fluid infection in women in preterm labor with intact fetal mem-

373:1737–1742.

nol-9: differential antibacterial activity and enhancement of bacterial adher-

17. Johnson, M. C., B. Ray, and T. Bhowmik. 1991. Control of the microbial flora of the vagina by H2O2-generating lac-


a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor,
NY.

21. Martin, H. L., B. A. Richardson, P. M. Nyange, L. Lavreys, S. L. Hillier, B.
Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency
virus type 1 and sexually transmitted disease acquisition. J. Infect. Dis.
179:1863–1868.

22. Martius, J., M. A. Krohn, S. L. Hillier, W. E. Stamm, K. K. Holmes, and
D. A. Eschenbach. 1988. Relationship of vaginal Lactobacillus species, cer-

central Chlamydia trachomatis, and vaginal preterm birth. Obstet. Gy-

23. McGovern, J. A., J. I. French, R. Parker, D. Draper, E. Patterson, W. Jones,
K. Thorsgard, and J. McFee. 1995. Prevention of premature birth by screen-
ing and treatment for common genital tract infections. Results of a prospect-


bacterial vaginosis is improved by a standardized method of Gram stain


tobacilli in the control and maintenance of the vaginal bacterial micro-

Wahwire-Mangen, D. Serwada, C. Li, N. Kiwanuka, S. L. Hillier, L. Rabe,


