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Molecular Identification of Vaginal *Lactobacillus* spp. Isolated from Korean Women

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Abstract Indigenous lactobacilli were isolated from vaginas of Korean women for possible use in ecological treatment of bacterial vaginosis. Vaginal swab samples were obtained from a gynecological clinic and streaked on Rogosa SL agar plates to select the most predominant lactobacilli in each sample. The preliminary identification of the isolates as lactobacilli was based on microscopic observation of Gram-positive rod-shaped cell morphology. The initial characterization was performed on 108 isolates in terms of their cell surface hydrophobicity (CSH), antimicrobial activity, and hydrogen peroxide (H_2O_2) production capability, and 10 isolates were then selected for further molecular identification. For a rapid procedure to identify lactobacilli, polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses of the 16S rRNA genes were applied. The 10 selected lactobacilli and 9 different reference strains of *Lactobacillus* spp. were characterized by PCR-RFLP where the amplified 16S rDNA was digested with 7 different restriction endonucleases prior to analysis. DNA sequencing of the 16S rRNA gene of one particular isolate, KLB 46, that had been identified as *L. crispatus* by the PCR-RFLP analysis, further confirmed its identity as *L. crispatus*.

Key words: Vaginal microflora, *Lactobacillus* identification, PCR-RFLP, 16S rDNA, antimicrobial activity

Indigenous lactobacilli are considered as normal flora in vaginas and represent the primary microbiological barrier to infection by urogenital pathogens [3, 5]. Lactobacilli also produce lactic acid to help maintain acidic conditions (pH<4.0), which indicate a healthy and normal vaginal condition in the vaginal tract. A low vaginal pH and other inhibitory substances, such as hydrogen peroxide and bacteriocin, can prevent the growth of unwanted or pathogenic bacteria, including *Escherichia coli*, *Gardenerella vaginalis*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* [5-7, 13, 18].

Bacterial vaginosis (BV) is the most common vaginal infection. Although the exact cause of BV is unknown, it has been found that dominant lactobacilli disappear and anaerobic bacteria called *Gardenerella vaginalis* multiply 1,000-fold in BV patients [5]. Several possible mechanisms to explain the disappearance of vaginal lactobacilli have been proposed. These include douching, the use of spermicidal products, such as Nonoxynol-9, and treatment with antibiotics for other infections [18]. The most commonly prescribed treatment for BV is a variety of antibacterial medications (metronidazole) used orally or intravaginally in clinical medicine [2]. However, since antibiotics not only target pathogenic bacteria but also normal lactobacilli, the normal vaginal flora consequently need to be re-established after the medication. Recently, various lactobacilli have been used to restore normal vaginal flora or prevent a recurrent urinary tract [3, 15]. These products are made from dairy products, such as yogurt and acidophilus milk, however, some of them do not even contain live lactobacilli [7]. For the ecological treatment of BV, vaginal lactobacilli need to be isolated and characterized prior to the formulation of live bacterial preparations.

Recently, the PCR-RFLP method (polymerase chain reaction-restriction fragment length polymorphism) has been employed as a rapid method for the identification and phylogenic analysis of bacteria [10, 11]. In particular, 16S ribosomal DNA sequences are species-specific, and restriction fragments of 16S rDNA amplified by PCR have already been used to classify a number of bacteria [10-12, 23].

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In the present study, lactobacilli were isolated from vaginas of Korean women, and several isolates with high antimicrobial activity were chosen for molecular identification at the species level using the PCR-RFLP method. Moreover, one particular isolate was chosen for further identification by 16S rDNA sequencing.

MATERIALS AND METHODS

Isolation of Vaginal Lactobacilli

The clinical samples were obtained from Ewha Woman's University Mokdong Hospital in Seoul. All samples were taken from patients who consented to the sampling. The cotton swab (CultureSwabTM, Difco Co., Detroit, U.S.A.) containing transport medium was used to collect the vaginal sample. The samples were sealed tightly and placed in refrigerator at 4°C. To isolate the lactobacilli, the swabs were streaked onto Rogosa SL agar plates (Difco Co., Detroit, U.S.A.) which were then incubated in an anaerobic chamber of substituted CO_2 at 37°C for 48 h. Once colonies appeared, the individual colonies were re-streaked twice on a selective Rogosa SL agar plate for pure culture. Preliminary identification of the isolates as lactobacilli was based on their growth on a selective Rogosa SL agar, Gram-positive rod-shaped cell morphology, and negative catalase reaction [8, 18]. The stocks of isolates were maintained in 10% (v/v) glycerol and kept in a freezer at −70°C.

Antimicrobial Activity Test

The modified direct and deferred method and spot-on-lawn method were used to detect antimicrobial activity [1]. An overnight culture was spotted onto the surface of an MRS agar (1.5%) plate and incubated for 6 h at 37°C to allow colonies to develop. Five milliliters of an MRS soft agar (0.8%) containing 1×10^7 CFU/ml of the indicator strain (*Lactobacillus delbrueckii* subsp. *lactis* ATCC 15808) [8] was poured over the plate. After 12 h at 37°C, the plate was checked for the formation of an inhibition zone. For the spot-on-lawn method, the MRS solid agar plate was overlaid with 5 ml of an MRS soft agar containing about 10^7 CFU of the indicator strain. Ten microliters of cell-free culture supernatant was then spotted onto the overlaid surface. The plate was placed in a refrigerator at 4°C for 3 h to allow the spot to diffuse. Then, the plate was incubated at the optimum growth temperature (37°C) of the indicator strain and checked for an inhibition zone.

Detection of Hydrogen Peroxide Production

To detect hydrogen peroxide producing lactobacilli, a modified version of the method developed by Eschenbach *et al.* was used [5, 6]. The test media contained 4.3 g of Brucella agar base (Difco Co., Detroit, U.S.A.), 20 mg of bezidine (Sigma Co., St. Louis, U.S.A.), 1 mg of horseradish peroxidase (Sigma Co., St. Louis, U.S.A.), 0.5 mg of hemin (bovine crystalline; Sigma Co., St. Louis, U.S.A.), 0.1 mg of Vitamin K1 (Sigma Co., St. Louis, U.S.A.), and 1 g of starch per 100 ml. Two microliters of overnight culture was spotted on the bezidine medium and incubated under anaerobic conditions at 37°C. The benzidine is oxidized by the horseradish peroxidase in the presence of hydrogen peroxide. Therefore, the plate was exposed to ambient air after 2 to 3 days and the hydrogen peroxide-producing colony turned black. The intensity of the black color correlated with the amount of hydrogen peroxide produced by the lactobacilli.

Assay for Cell Surface Hydrophobicity (CSH)

To measure the CSH, the isolated lactobacilli were cultured in 10 ml of an MRS broth at 37°C for 12 h. The cells were harvested by centrifugation at 8,000 ×g and washed twice in 0.15 M potassium phosphate buffer. The harvested cells were then resuspended in 5 ml of the phosphate buffer and mixed with an equal volume of hexadecane (Sigma Co., St. Louis, U.S.A.) at room temperature. After standing for 1 h at room temperature, the cells were separated in the aqueous phase or hexadecane phase depending on their CSH [16]. Since cells with higher CSH partitioned into the hexadecane layer, the degree of CSH was inversely correlated with the optical density of the aqueous phase.

DNA Amplification

The genomic DNA was extracted from the lactobacilli as previously described [3]. The amplification was carried out in 50-μl thin-walled tubes using *Taq* polymerase (TaKaRa Shuzo Co., Shiga, Japan) in a Thermal cycler (Biometra, Gottingen, Germany). Eubacterial and universal primers used were: fD1 (5'AGAGTTTGATCTGGCTCAG3') and rD1 (5'AAGGAGGTGATCCAGCC3') [10, 11, 23], which supposedly yield a 1.6 kb fragment. The amplification reaction and the visualization of PCR product were carried out as previously described [17].

16S rDNA RFLP Analysis

The DNA obtained by the PCR was digested with restriction enzymes, *Alu*I, *Cfo*I, *Dde*I (Promega Co., Madison, U.S.A.), *Msp*I, *Hae*III, *Hin*fI, and *Taq*I (TaKaRa Shuzo Co., Shiga, Japan) using the appropriate reaction conditions and incubation times according to the suppliers' recommendations. The DNA fragments were size-fractionated by electrophoresis at 50 mA for 10 h through 2.0% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) [10-12].

An RFLP data analysis was performed using the program NTSYS-pc (numerical taxonomy system of multivariate statistical programs) that calculated the hereditary relativity of the isolates and reference strains.

DNA Sequencing of the 16S rDNA Fragment

To sequence the 16S rDNA of KLB 46, polymerase chain reaction (PCR) was performed using the 3 primer sets; F1/R1, F2/R2, and F3/R3 [19]. The sequence was determined using an ABI 377 automated DNA sequencer (Perkin Elmer, Foster, U.S.A.) at the Molecular Core Facility of the University of Illinois, Chicago. The 16S rDNA sequence data were compared with known sequences registered at the National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov//BLAST/). The sequence of KLB 46 was registered at the National Center for Biotechnological Information (AF243167) and strain KLB 46 was deposited in the Korean Collection for Type Cultures (KCTC 0860BP).

Results and Discussion

Isolation of Lactobacilli in Vagina

Vaginal samples were obtained from 222 women ranging from 22 to 73 years old over an 8-month period. Among the women, 144 were healthy and non-pregnant, 47 were pregnant, 26 were mildly infected, and 5 were infected and pregnant. Preliminary identification of the isolates as lactobacilli was based on three criteria: first, all the isolates were able to grow on a Rogosa SL agar plate, which is known to be highly selective for lactobacilli; secondly, all the isolates were Gram-positive and had a rod-shaped cell morphology; lastly, they were catalase-negative. Presumptive lactobacilli were detected in only 101 women, and a total of 108 isolates were obtained and the isolates from 7 women revealed 2 different colony types. Although lactobacilli are known to be the dominant microflora in the vagina, only 45% of the women appeared to carry vaginal lactobacilli.

Antimicrobial Activity of the Isolates

The isolates showing antimicrobial activity were examined by the direct and differed method, as described in the Materials and Methods [1]. Most isolates exhibited some degrees of antimicrobial activity against the indicator strain, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 15808, however, 18 isolates showed relatively higher activity than the others. To re-examine the isolates with higher antimicrobial activity, the spot and lawn method was used where 10 μl of cell-free culture supernatant was spotted on a soft agar overlay containing the indicator strain, and 10 isolates with semiquantitatively scored antimicrobial activity were selected (Table 1).

Hydrogen Peroxide Production by the Isolates

Eschenbach *et al.* [5] reported that the production of H_2O_2 by *Lactobacillus* species may represent a nonspecific antimicrobial defense mechanism of the normal vaginal ecosystem. More recently, it was reported that H_2O_2-producing *L. paracasei* and *L. crispatus* from the human vagina inhibited the growth of *S. aureus* [12, 13]. When the 108 isolates were examined, 96 (89%) of the isolates produced hydrogen peroxide. Since the amount of hydrogen peroxide produced was indicated by the color change of the colony (from white to black), the amount of hydrogen peroxide produced by an individual isolate was visually semiquantitatively scored and summarized in Table 1. Among the isolates, six exhibited hydrogen peroxide production.

Table 1. H_2O_2 production and cell surface hydrophobicity (CSH) of ten selected isolates showing high antimicrobial activity.

Isolates	Antimicrobial activity[a]	H_2O_2 production[b]	CSH[c]
KLB 12	++	White	++
KLB 39	++	Black	++
KLB 46	+++	Brown	+++
KLB 52	++	Black	+++
KLB 56	++	Black	+
KLB 57-1	++	Black	+++
KLB 66-2	++	Brown	+
KLB 82	+++	Brown	+
KLB 96	+++	Black	++
KLB 100	+++	Black	++

[a]+++, strong and ++, moderate.
[b]White, no production; Brown, weak production; Black, strong production.
[c]+++, strong; ++ , moderate; +, weak.

Cell Surface Hydrophobicity (CSH) of the Isolates

Kmet and Lucchini [9] reported that bacterial CSH is associated with aggregating activity and some *Lactobacillus*

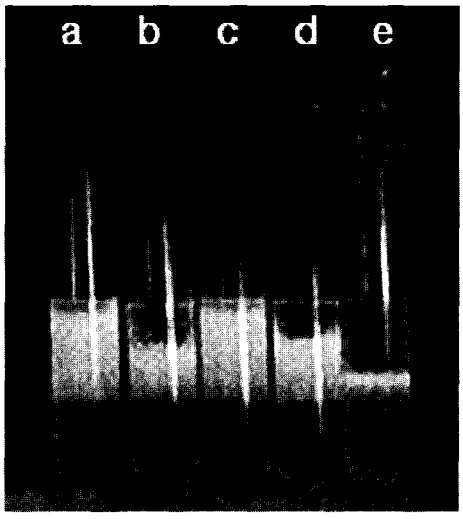

Fig. 1. Cell surface hydrophobicity (CSH) test based on partitioning *Lactobacillus* cells in upper hexadecane phase: (a) KLB 57-1, (b) KLB 39, (c) KLB 52, (d) KLB 46, and (e) *L. acidophilus* ATCC 4356.
When mixed with hexadecane, cells with higher CSH are more quickly removed from the aqueous phase compared to those with lower CSH.

sp. strain aggregate with *E. coli*. This mechanism is an important factor in the interaction of commensal microflora with strains of uropathogenic *E. coli* strains. Also, Tomeczek *et al.* [22] reported that lactobacilli with a higher CSH are more resistant to Nonoxynol-9 and vancomycin, thereby suggesting that the surface properties of lactobacilli influence their susceptibility to antimicrobial agents. The ability of lactobacilli to adhere to the vaginal epithelial cell surface is considered to be important for efficient colonization in the human vagina. Therefore, 10 selected isolates were tested for their CSH, as described previously [16]. As shown in Fig. 1 and Table 1, all were found to have a high CSH.

16S rDNA RFLP of the Isolates

Genomic DNAs prepared from 10 selected isolates and 9 ATCC reference strains (*L. gasseri* ATCC 9857, *L. fermentum* ATCC 23271, *L. plantarum* ATCC 14917, *L. casei* subsp. *Casei* ATCC 393, *L. rhamnosus* ATCC 7469, *L. jensenii* ATCC 25258, *L. crispatus* ATCC 33820, *L. delbrueckii* subsp. *Lactis* ATCC 15808, and *L. acidophilus* ATCC 4356) were used to amplify the 16S rDNA with universal primers [23]. All the strains produced a single band about 1,500 bp long [10, 11]. The amplified 16S rDNA samples were digested with various restriction endonucleases and the resulting restriction fragments were fractionated by 2.0% agarose gel electrophoresis. Based on the size distribution of the restriction fragments (Fig. 2), the 10 isolates were then divided into three groups. According to a dendrogram drawn with the RFLP patterns (Fig. 3), KLB 12 was found to be close to *L. fermentum*, while KLB 46, 66-1, 82, 96, and 100 were all close to *L. crispatus*. However, KLB 39, 52, 56, 57-1 appeared to form a separate group that did not belong to any of the reference strains. All the reference strains, except for *L. acidophilus* and *L. crispatus*, could be identified by the *Dde*I enzyme digestion (Fig. 2A), whereas the latter two strains were distinguished from each other by the *Hinf*I digestion (Fig. 2B). Reid *et al.* [20] reported that *L. jensenii* (35%) and *L. acidophilus* (24%) are most commonly found in vaginal flora, while *L. crispatus* was detected only in 3% of Canadian women. In contrast, the current results showed that 5 out of the 10 isolated lactobacilli selected randomly were *L. crispatus*. Although the sample size was small, it would appear that the predominant vaginal

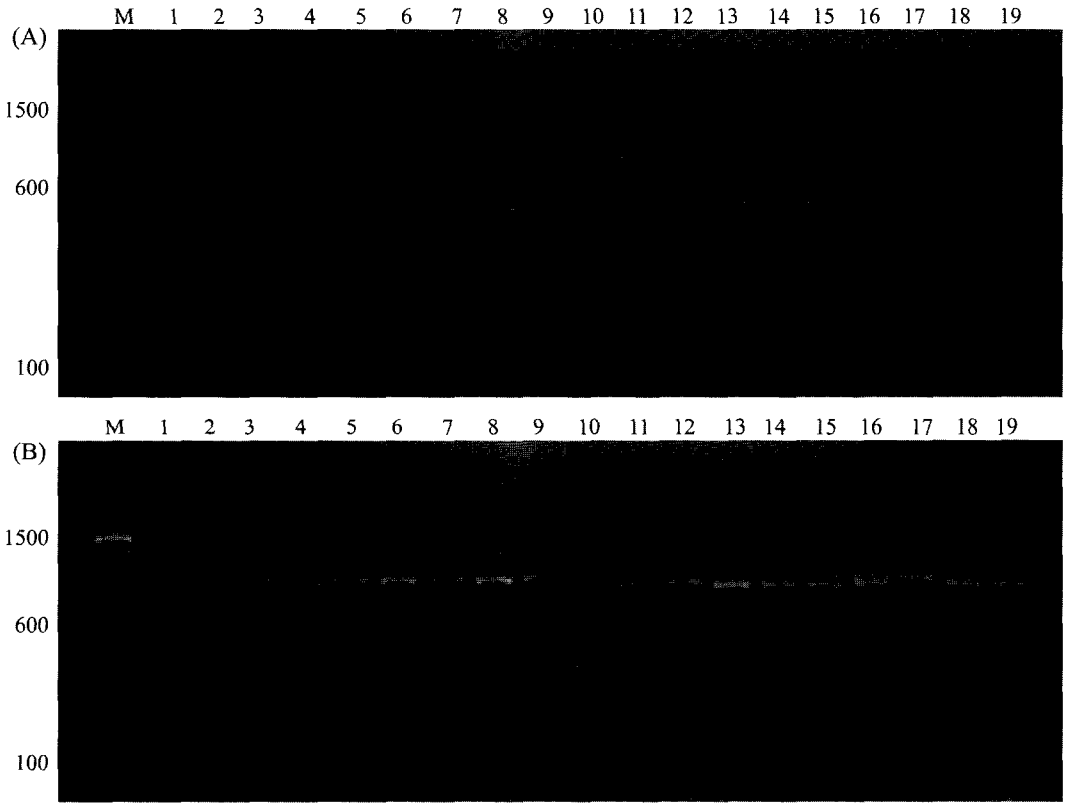

Fig. 2. Restriction pattern with (A) *Dde*I and (B) *Hinf*I of PCR-amplified product of 16S rDNA genes of ATCC type strains and Korean *Lactobacillus* isolates.

Lane M: 100 bp DNA ladder; lanes 1 to 9: *L. acidophilus* ATCC 4356, *L. fermentum* ATCC 23271, *L. gasseri* ATCC 9857, *L. crispatus* ATCC 33820, *L. delbrueckii* subsp. *Lactis* ATCC 15808, *L. jensenii* ATCC 25258, *L. plantarum* ATCC 14917, *L. rhamnosus* ATCC 7469, *L. casei* subsp. *Casei* ATCC 393; and lanes 10 to 19: KLB 12, KLB 39, KLB 46, KLB 52, KLB 56, KLB 57-1, KLB 66-2, KLB 82, KLB 96, KLB 100.

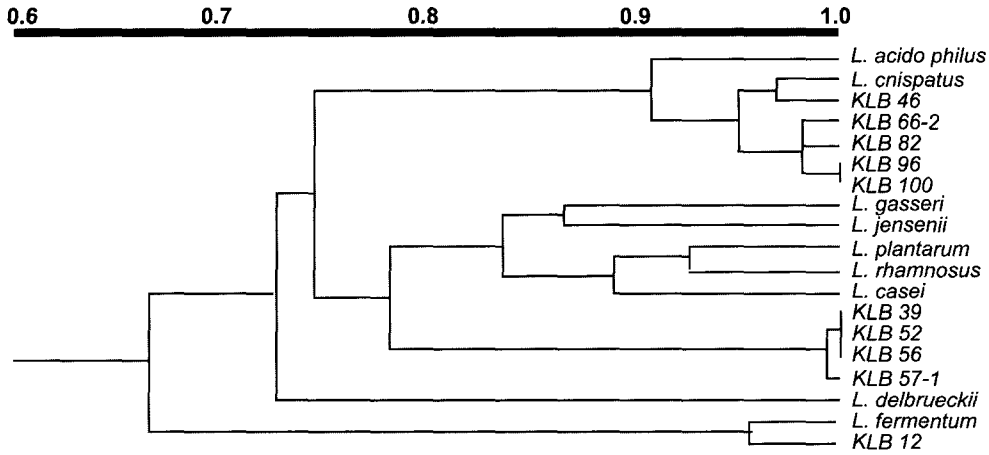

Fig. 3. Dendrogram of Korean *Lactobacillus* isolates and ATCC type strains based on analysis of 16S rDNA RFLP patterns.

lactobacilli in Korean women were *L. crispatus*. It is also noteworthy that the predominant lactobacilli in Japanese women were found to be *L. crispatus* (52.7%) and *L. gasseri* (20.8%) by DNA-DNA hybridization analysis [21]. In agreement with these reports, a recent report on worldwide studies on vaginal lactobacilli is of interest in that the three most common *Lactobacillus* species colonizing in vagina are *L. crispatus*, *L. jensenii*, and *L. gasseri* [19].

DNA Sequencing of the 16S rDNA of KLB 46

One of the 10 selected isolates, KLB 46, appeared to be the most promising in terms of its antimicrobial activity and CSH as well as cell viability. Therefore, further studies were undertaken by focusing on KLB 46. Initially, it was isolated from mildly infected non-pregnant women. Although the PCR-RFLP analysis suggested that KLB 46 was *L. crispatus*, the 16S rDNA of KLB 46 was sequenced to confirm its identity at the species level. When the sequence data were compared with known sequences of reference species, the similarity between KLB 46 and *L. crispatus* ATCC 33820 was 99%, while the similarity between KLB 46 and *L. acidophilus* ATCC 4356 was only 96%. Accordingly, when combined the PCR-RFLP and DNA sequencing data, KLB 46 was confirmed as *L. crispatus*. To utilize *L. crispatus* KLB 46 for the ecological treatment of bacterial vaginosis, further research on mass cultivation as well as formulation is in progress.

Acknowledgments

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