Comparison of nucleic acid extraction efficiency for vaginal microbiome analyses between a routine commercial kit and the bead beating method

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Abstract

To compare the efficiency of nucleic acid extraction for identifying microbiomes in cervicovaginal samples between a commercial kit (chemagic viral DNA/RNA kit,Chemagen,Germany), conventionally applied method for diagnosing human papilloma virus infection, and the standard bead beating method. Cervicovaginal samples collected from 10 participants in the Healthy Twin Study were simultaneously subjected to the two different DNA extraction methods. Extracted DNA was amplified by nested-PCR and analyzed by denaturing gradient gel electrophoresis (DGGE), DGGE profiling, and phylogenetic sequencing based on the Greengenes database. Although chemagic kit method produced slightly more diverse bands compared to bead beating method, DGGE band patterns were very similar between the two methods for 60% of samples. Multi-dimensional scaling analysis of the DGGE fingerprints revealed randomly distributed bacterial community compositions with both methods. An unweighted pair group analysis using arithmetic average clustering showed that the clustering of DGGE fingerprints was not related to the DNA extraction method. With the bead beating method, Lactobacillus sp. and Enterobacteriaceae were found most often; Lactobacillus sp. and Pseudomonas spp. were more common with the chemagic kit method. According to UniFrac distance analysis, the microbial communities by phylogenetic analysis did not differ significantly between the two methods. DNA extraction using a commercial chemagic kit did not cause significant difference in an analysis of the vaginal microbiome, compared with the bead beating method. These findings suggest that commercial nucleic acid extraction kits would be useful for analyzing the cervicovaginal microbiome, with comparable efficiency and greater convenience than the bead beating extraction method. This study will help to investigate the effect of vaginal microbiota on women' health.

keywords: *DNA; metagenome; human; vaginal smears*

Introduction

The vaginal microbiome is potentially significant in women's health care. Alterations of the cervicovaginal microbiome are known to result in vulvovaginal candidiasis and bacterial vaginosis (1; 2), which are associated with increased risk for other sexually transmitted diseases (3) and poor pregnancy outcome (4; 5). Bacterial vaginosis may also be associated with increased risk for cervical intraepithelial neoplasia (6; 7).

Colonization by Lactobacillus sp. has been suggested to play a key role in maintaining a healthy vaginal microenvironment, as indicated by in vitro studies showing that Lactobacillus strains inhibit the growth of many pathogens (2; 8). In bacterial vaginosis, hydrogen peroxide-producing Lactobacillus, the predominant organisms in a healthy vagina, are replaced by a mixed flora that includes Gardnerella vaginalis and anaerobic Gram-negative bacteria such as Prevotella bivia, Prevotella disiens, Porphyromonas sp., Mobiluncus sp., and Peptostreptococcus sp. (9; 10). The

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detection of altered cervicovaginal microbe populations could contribute to the accurate diagnosis of bacterial vaginosis.

A cervicovaginal cytological smear is highly recommended for cervical cancer screening in a clinical setting. A recently introduced liquid-based preparation system allows concomitant diagnosis of human papilloma virus (HPV) infection using cervicovaginal samples obtained for cytological screening (11). With this system, HPV infection is diagnosed based on a molecular assay that detects HPV DNA extracted from cervicovaginal samples (12). It may be possible to use this extracted DNA for identifying microbes associated with bacterial vaginosis. However, the DNA extracted using commercial kits has not been verified for microbe analysis, and differential extraction efficiencies among bacteria may adversely affect sensitivity. In this study, we compared DNA extraction efficiency between a commercial kit routinely employed for vaginal microbe analyses and the bead beating extraction method, which is the currently accepted standard practice.

Methods

Collection of cervicovaginal samples

This study was performed using cervicovaginal samples collected from 10 randomly selected adult female participants in the Healthy Twin study, a part of the Korean Genome Epidemiology Study (13). Each cervicovaginal sample was collected with a separate endocervical brush, and the cytobrush was immediately placed into liquid fixative solution according to the instructions for the liquid-based thin-layer preparation method (ThinPrep® , SurepathTM).

All participants provided written informed consent upon visiting one of the study centers. The study protocol was approved by the Korea Center for Disease Control and the institutional review boards of the three participating centers.

DNA extraction from vaginal samples

DNA was simultaneously extracted from the 10 cervicovaginal samples by both the bead beating method and a nucleic acid extraction kit method, as described below.

1) Bead beating method

Total DNA was extracted from cervicovaginal samples following the protocol described in a previous study (14). Briefly, after centrifugation of the vaginal sample at 3,000 × g for 10 min, the pellet was suspended in 200 μl of distilled water and mixed with 500 μl of extraction buffer, 210 μl of 20% SDS, 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1, pH 7.9), and 500 μl of 0.1-mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA). Microbial cells were lysed by mechanical disruption with a bead beater (BioSpec Products) set on high for 2 min at room temperature. The suspension was extracted with phenol:chloroform:isoamyl alcohol, and the DNA was precipitated with isopropanol. The DNA pellet was suspended in Tris-EDTA buffer (pH 7.5) and stored at −70°C until further analysis.

2) Nucleic acid kit method

Total DNA was extracted using a commercial nucleic acid extraction kit (chemagic viral DNA/RAN kit, Chemagen, Germany). Briefly, after centrifugation of the vaginal sample at 3,000 × g for 10 min, the pellet was suspended in 200 μl of distilled water, and the DNA was extracted according to the manufacturer's instruction. The DNA was stored at −70°C until further analysis. To detect bacterial DNA contamination of the commercial DNA extraction kit, a negative control (distilled water only) was also processed.

Amplification of the 16S rRNA gene using nested PCR

To amplify the 16S rRNA genes, nested PCR was performed as described previously (15). In the first round of PCR, part of the 16S rRNA gene was amplified using primers (27f/1492r) specific for the bacteria domain. The PCR mix consisted of 2.5 μl of 1× PCR reaction buffer, 2.5 μl of template DNA, 0.5 μM of each primer, 400 μM dNTPs, and, 2 units of G-Taq DNA polymerase (Cosmo Genetech Co., Seoul Korea). The reactions were initially denatured at 95℃ for 2 min, followed by 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 90 s, with a final extension at 72℃ for 5 min. The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The second round of PCR was performed using 2.5 μl of first-round PCR product

as template, the primers 341f and GC-clamped 534r (16), and the other reaction components as in the first round. Reactions were initially denatured at 94℃ for 45 s, followed by 25 cycles of 94℃ for 45 s, 55℃ for 45 s, and 72℃ for 50 s, with a final extension at 72℃ for 5 min. The second-round PCR products were purified using a QIAquick PCR purification kit and stored at −70°C until analysis.

Denaturing gradient gel electrophoresis (DGGE)

For all DGGE procedures, a DCode universal mutation detection system (BioRad Laboratories, Hercules, CA, USA) was used according to the manufacturer's specifications. PCR products (20 µl) were loaded onto an 8% (w/v) acrylamide gel (acrylamide/bis solution, 37.5:1; BioRad) containing a linear chemical gradient ranging from 15% to 65% denaturant, where 7 M urea in 40% (v/v) formamide was defined as 100% denaturant. Electrophoresis was performed at a constant voltage of 60 V for 900 min at 60℃ in a DGGE chamber containing approximately 7 L of 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). After electrophoresis (Mupid-21; Optima Inc., Tokyo, Japan), the gels were stained for 15 min with ethidium bromide (0.5 µg/ml) in Milli-Q water (Millipore), destained for 20 min with Milli-Q water, and photographed using a UV transilluminator (Mupid-Scope WD; Advance Co., Ltd., Tokyo, Japan).

Excision of representative DGGE bands and sequencing

DGGE bands of higher intensity and frequency were selected for excision (Fig. 1). The gel was placed on a UV transilluminator, and gel pieces were cut from the middle of the band using a sterile razor and placed into a sterile 1.5-ml microcentrifuge tube. The time the gel was exposed to UV light was minimized to protect the integrity of the DNA. After 20 µl of TE buffer were added to each tube, and the gel slices were crushed with a pipette tip and left to soak overnight at 4℃. After centrifugation at 5000 × g for 5 min, 2.5 µl of the supernatant containing the extracted DNA were used as the template for re-amplification performed according to the second-round PCR protocol described above, except using the 341f primer with the non-GC clamped 534r primer. The PCR products were purified using a QIAquick PCR purification kit (Qiagen), and the purified DNA was cloned into pGEM-T easy vector (Promega, Madison, WI, USA). Positive recombinant clones were further analyzed by nucleotide sequencing (Cosmo Genetech Co.), and the sequences were compared with sequences in the Greengenes database using Ribosomal Database Project (RDP) taxonomy annotation (17). The UniFrac program was used for UniFrac distance analysis (18).

Bacterial community composition determined by DGGE fingerprint

DGGE profiles were analyzed using BioNumerics software version 5.1 (Applied Math NV, Sint-Martens-Latem, Belgium) (19). Each lane was examined separately, and common bands were selected for normalization. The position tolerance setting was set at 0.50% for optimization and 1.00% for position tolerance for the best possible matching. Pairwise similarity of the DGGE banding patterns for the same sample extracted by the two different methods was determined by calculating the Dice coefficient and using the unweighted pair group method using arithmetic averages (UPGMA). The bacterial community compositions based on DGGE fingerprints were analyzed by multiple-dimensional scaling (MDS).

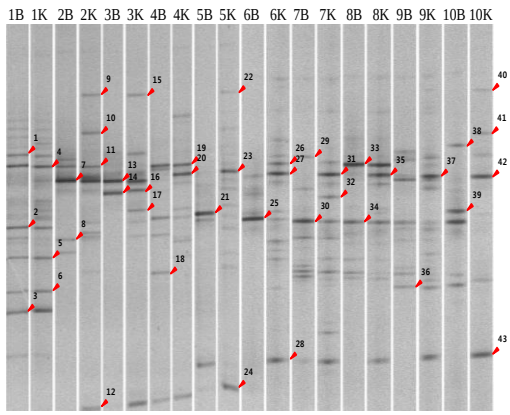

Figure 1. Denaturing gradient gel electrophoresis (DGGE) band patterns of DNA extracted from cervicovaginal samples using the bead beating method (B) or a commercially available nucleic acid extraction kit (K). The numbers correspond to the excised bands listed in Table 1.

Table 1. Microorganisms identified based on representative denaturing gradient gel electrophoresis (DGGE) bands.

Sample ID	Band	Identified microorganism	OTU	Gram (+/−)
1B	1	*Prevotella* sp.	otu_386	–
1B	2	*Niastella* sp.	otu_494	–
1B	3	*Sporomusa* sp.	otu 865	–
1K	4	*Prevotella* sp.	otu_386	–
1K	5	*Streptobacillus* sp.	otu 940	+
1K	6	*Bacillus* sp.	otu_663	+
2B	7	*Lactobacillus* sp.	otu_621	+
2B	8	*unclassified Lactobacillaceae* sp.	otu 624	+
2K	9	*Vitreoscilla* sp.	otu_1278	–
2K	10	*unclassified Lactobacillaceae* sp.	otu 624	+
2K	11	*Lactobacillus* sp.	otu_621	+
2K	12	*Propionibacterium* sp.	otu_260	+
3B	13	*unclassified Lactobacillaceae* sp.	otu 624	+
3B	14	*unclassified_Lactobacillaceae* sp.	otu_624	+
3K	15	*Vitreoscilla* sp.	otu 1278	–
3K	16	*Acinetobacter* sp.	otu_1590	–
3K	17	*Brevundimonas* sp.	otu_966	–
4B	18	*Pseudomonas* sp.	otu 1600	–
4K	19	*Lactobacillus* sp.	otu_621	+
4K	20	*Pseudomonas* sp.	otu 1600	–
5B	21	*unclassified_Enterobacteriaceae* sp.	otu_1527	–
5K	22	*Pseudomonas* sp.	otu 1600	–
5K	23	*Ralstonia* sp.	otu_1194	–
5K	24	*Propionibacterineae* sp.	otu_260	+
6B	25	*unclassified Enterobacteriaceae* sp.	otu 1527	–
6K	26	*Lactobacillus* sp.	otu_621	+
6K	27	*Staphylococcus* sp.	otu 652	+
6K	28	*Propionibacterium* sp.	otu_260	+
7B	29	*Niastella* sp.	otu_494	–
7B	30	*Propionibacterium* sp.	otu 260	+
7K	31	*Pseudomonas* sp.	otu_1600	–
7K	32	*Pseudomonas* sp.	otu 1600	–
8B	33	*Lactobacillus* sp.	otu_621	+
8B	34	*Lactobacillus* sp.	otu_621	+
8K	35	*Lactobacillus* sp.	otu 621	+
9B	36	*Streptococcus* sp.	otu_1172	+
9K	37	*Achromobacter* sp.	otu 1600	–
10B	38	*Corynebacterium* sp.	otu_128	+
10B	39	*unclassified_Enterobacteriaceae* sp.	otu_1527	–
10K	40	*Vitreoscilla* sp.	otu 1278	–
10K	41	*Pseudomonas* sp.	otu_1600	–
10K	42	*Pseudomonas* sp.	otu 1600	–
10K	43	*Pseudomonas* sp.	otu_1600	–

B: bead beating method; K: nucleic acid extraction kit method; OTU: operational taxonomic unit

Analysis of DGGE fingerprints

MDS analysis of the DGGE fingerprints from the 10 cervicovaginal samples revealed randomly distributed bacterial community compositions. When the results of the bead beating and chemagic kit methods were pooled, no significant difference in bacterial community composition was found between the two methods, and the results of one method were not distinguishable from those of the other method (Fig. 2). With UPGMA clustering

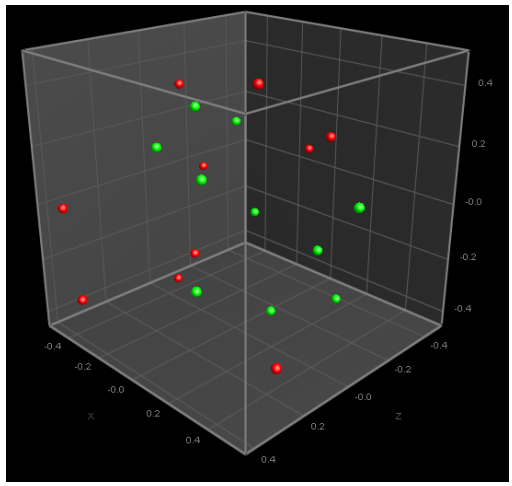

Figure 2. Multiple-dimensional scaling (MDS) analysis comparing the results for DNA extracted using a nucleic acid extraction kit (green dots) and DNA extracted using the bead beating method (red dots).

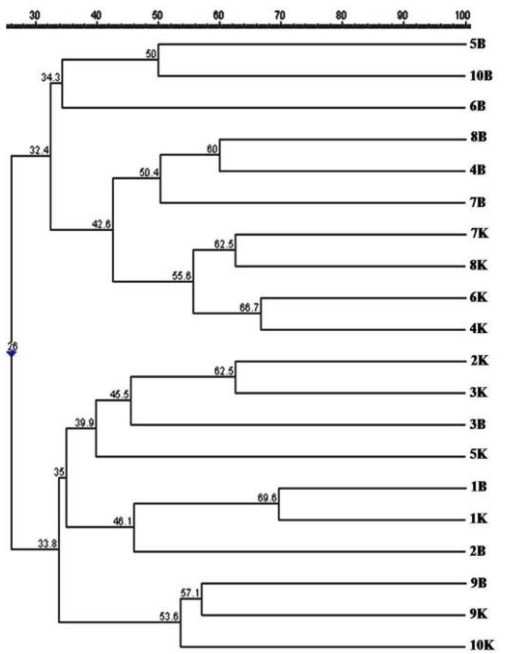

Figure 3. Clustering of DGGE fingerprints estimated using the Dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA). Similarity of banding patterns is expressed as a percentage of the Dice coefficient. B indicates bead beating method. K indicates nucleic acid extraction kit method.

Sequence analysis

Using the Greengenes database and RDP annotation, 43 sequences with 92–100% similarity to known bacterial sequences were identified from the excised and re-amplified DGGE bands. The phylogenetic affiliations of the excised DGGE bands from the bead beating extracts were dominantly represented by Lactobacillus sp. and Enterobacteriaceae. With the chemagic kit method, Lactobacillus sp. and Pseudomonas sp. were the most dominant bacterial affiliations (Table 1). Although Gram-positive bacteria tended to be observed more frequently in the DGGE bands from bead beating samples compared with the chemagic kit samples (53% vs. 42%, respectively), the difference was not significant. Based on average unweighted UniFrac distances, microbial communities by phylogenetic analysis did not differ significantly between the chemagic kit extracts and the bead beating extracts ((P > 0.5; Fig. 4).

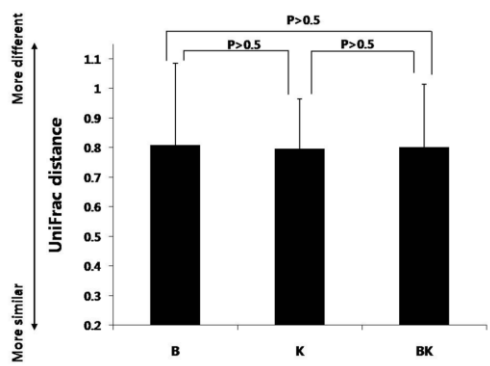

Figure 4. Average unweighted UniFrac distance for DNA extracts prepared using the bead beating method (B), a nucleic acid extraction kit (K), and unrelated (BK). Bar indicates average ± standard deviation, and p-values were assessed by t-test.

Discussion

The present study showed that the DNA extraction efficiency of chemagic kit method is comparable to that of the bead beating method in cervicovaginal samples collected during routine screening for cervical cancer and HPV infection. This finding suggests that the chemagic viral DNA/RNA extraction kit would be a useful DNA extraction method with its ease of use not only for screening cervicovaginal HPV infection but also for identifying cervicovaginal microorganisms in clinical settings.

To our knowledge, this is the first study to analyze microbial profiles of cervicovaginal samples based on molecular assays of samples prepared using two different nucleic acid extraction methods, bead beating and the chemagic nucleic acid extraction kit. We compared the two methods according to several criteria, including DGGE band patterns, MDS analysis, UPGMA clustering analysis, and average unweighted UniFrac distances. The two methods gave similar results for most samples. These findings indicate that DNA extraction with a chemagic kit would be appropriate for analysis of microbial communities in cervicovaginal samples.

In the present study, the bead beating method was used as a reference for validation of the efficiency of a commercial DNA extraction kit, because the bead beating method has been generally accepted as an efficient method for DNA extraction of microbiomes from various samples, including both clinical and environmental samples (20; 21). In general, gut microbiome studies recommend using DNA samples extracted by bead beating, which shatters the bacterial cell wall, for microbiome analysis (22).

DGGE is among the most common methods for determining microbial diversity in various samples, and DGGE band patterns have been shown to represent both the most abundant and the most scarce bacterial species in a sample (23). After PCR amplification of the 16S rRNA genes, DGGE was used to separate the PCR products in the present study. The DGGE band patterns were analyzed by both UPGMA and MDS using BioNumerics software (19). UniFrac distances, which were used for analysis of phylogenetic microbial similarity in the present study, have been validated for various samples (14; 18; 24; 25). The sequences obtained from the DGGE bands were analyzed using the RDP database, which provides a quality-controlled bacterial and archaeal small subunit rRNA alignment and analysis tool (26). We believe that the use of these validated analytical methods

enabled us to compare the efficiencies of the two different DNA extraction methods.

A previous vaginal microbe study reported that some sequences in the “unidentified” and “uncultured” GenBank categories were replaced by Pseudomonas and Eubacterium by using the RDP database, and that Lactobacillus and Pseudomonas/Enterobacteriaceae were the most common Gram-positive and Gram-negative bacteria in the vaginal epithelium (27). In agreement with these earlier results, the present study found Lactobacillus and Enterobacteriaceae to occur commonly in samples extracted by the bead beating method, while Pseudomonas and Lactobacillus were common in the chemagic kit samples.

The difference in numbers of excision band between the two DNA extraction methods could be a limitation of our study. However, findings that more Gram-positive bacteria were identified by chemagic kit and that Gram-positive bacteria identified by chemagic kit methods were mostly confirmed by the bead beating methods suggest that the chemagic kit method would be especially efficacious for detecting Gram-positive bacteria in cervicovaginal samples.

In conclusion, the efficiency of DNA extraction from cervicovaginal samples using the commercially available chemagic kit was comparable to that using the bead beating method. Given its efficiency and ease of clinical applicability, the chemagic viral DNA/RNA kit may be useful for microbiome studies of cervicovaginal samples.

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