Molecular Characterization of Bacteriophages for Microbial Source Tracking in Korea[▽]

Jung Eun Lee,¹ Mi Young Lim,¹ Sei Yoon Kim,¹ Sunghee Lee,¹ Heetae Lee,¹ Hyun-Myung Oh,²
Hor-Gil Hur,³ and GwangPyo Ko^{1*}

Institute of Health and Environment, Department of Environmental Health, School of Public Health, Seoul National University, 28 Yeongeon-dong, Chongro-gu, Seoul, South Korea 110-799¹; Department of Marine Sciences, College of Natural Sciences, Inha University, Yong-Hyun-Dong, Nam-Gu, Incheon, South Korea 402-751²; and Department of Environmental Science & Engineering, Gwangju Institute of Science and Technology, Gwangju, South Korea 500-712³

Received 25 February 2009/Accepted 11 September 2009

We investigated coliphages from various fecal sources, including humans and animals, for microbial source tracking in South Korea. Both somatic and F⁺-specific coliphages were isolated from 43 fecal samples from farms, wild animal habitats, and human wastewater plants. Somatic coliphages were more prevalent and abundant than F⁺ coliphages in all of the tested fecal samples. We further characterized 311 F⁺ coliphage isolates using RNase sensitivity assays, PCR and reverse transcription-PCR, and nucleic acid sequencing. Phylogenetic analyses were performed based on the partial nucleic acid sequences of 311 F⁺ coliphages from various sources. F⁺ RNA coliphages were most prevalent among geese (95%) and were least prevalent in cows (5%). Among the genogroups of F⁺ RNA coliphages, most F⁺ coliphages isolated from animal fecal sources belonged to either group I or group IV, and most from human wastewater sources were in group II or III. Some of the group I coliphages were present in both human and animal source samples. F⁺ RNA coliphages isolated from various sources were divided into two main clusters. All F⁺ RNA coliphages isolated from human wastewater were grouped with Q β -like phages, while phages isolated from most animal sources were grouped with MS2-like phages. UniFrac significance statistical analyses revealed significant differences between human and animal bacteriophages. In the principal coordinate analysis (PCoA), F⁺ RNA coliphages isolated from human waste were distinctively separate from those isolated from other animal sources. However, F⁺ DNA coliphages were not significantly different or separate in the PCoA. These results demonstrate that proper analysis of F⁺ RNA coliphages can effectively distinguish fecal sources.

Fecal contamination of various water resources poses a serious risk to human health through consumption of microorganisms that inhabit these environments, as well as through recreational exposure (20). To correctly assess and properly manage the human health risk associated with water contamination, it is necessary to have information regarding the source of fecal contamination (12, 19, 30, 31, 33). In previous studies, several microbiological methods have been applied to distinguish fecal contamination sources, particularly between human and animal sources (20, 30). For example, different types of antibiotic resistance patterns (14), molecular markers (2), identified animal viruses (26), and whole-genome patterns based on repetitive-element PCR or ribotyping (4, 28) have been applied to microbial source tracking (MST). However, these MST methods have not yet been fully evaluated and characterized.

Coliphages are viral indicators of fecal contamination in groundwater proposed by the U.S. Environmental Protection Agency (37) and could be useful target microorganisms for MST. Based on infectivity through the host's sex pili, coliphages can be categorized into somatic and F⁺-specific coliphages. In addition, coliphages can be categorized based on the

type of nucleic acids (RNA versus DNA). At present, F⁺ coliphages consist of those from *Leviviridae* (icosahedral, single-stranded RNA phages), including *Levivirus* and *Allolevivirus*, and *Inoviridae* (filamentous, single-stranded DNA phages) (5). *Levivirus* contains both MS2-like (group I) and GA-like (group II) viruses, whereas *Allolevivirus* contains both Q β -like (group III) and SP-like (group IV) viruses (32). These subgroups of coliphages were initially classified based on serological typing (18). Different genotypes of F⁺ RNA coliphages are associated with different types of fecal sources (30, 33). A number of previous studies have reported that groups II and III are isolated mainly from human feces and that groups I and IV are associated mainly with animal feces (10, 17, 18, 27). However, the specificity of this association can vary, because group I F⁺ RNA coliphages were isolated from human-waste-dominated municipal wastewater (13). In addition, the applicability of this MST method in different geographical regions, such as Asia, is still unclear. Furthermore, the application of DNA coliphages to MST has not been much investigated.

In several previous studies, F⁺ coliphages were isolated and analyzed using culture and serological methods (7, 22, 34). Nucleic acid sequence-based analyses of F⁺ RNA-specific genes have not been performed or are very limited (40). Stewart et al. (32) performed F⁺ RNA group III sequence analysis, and MST based on direct nucleic acid sequence was found to be more reliable than nucleic acid hybridization. However, a comprehensive analysis based on nucleic acid sequence has not been applied to coliphages. Thus, the objectives of this study

* Corresponding author. Mailing address: Department of Environmental Health, School of Public Health, Seoul National University, 28 Yeongeon-dong, Chongro-gu, Seoul, South Korea 110-799. Phone: 82 2 3668 7881. Fax: 82 2 745 9105. E-mail: gko@snu.ac.kr.

[▽] Published ahead of print on 18 September 2009.

TABLE 1. Incidence of somatic and F⁺ coliphages in animal feces and human wastewater in Korea

Source	No. of samples	Somatic coliphages			F ⁺ coliphages		
		% Positive	Median ^a	Range ^a	% Positive	Median ^a	Range ^a
Human	11	91	310	30–3,830	73	5	<1–285
Cow	12	58	11	1–25	25	1	<1–4
Chicken	7	86	1,450	3–10,000	43	2	<1–200
Goose	2	100	10	7–12	100	4	1–7
Pig							
Feces	8	86	500	2–710	63	1	<1–200
Septic tank sample	3	100	680	380–13,700	100	39	11–89

^a PFU/g of stool or PFU/ml of wastewater.

were (i) to test the applicability of both RNA and DNA coliphage-based MST in South Korea and (ii) to apply nucleic acid sequence-based analyses of both RNA and DNA coliphages isolated from various animal and human fecal sources to MST.

MATERIALS AND METHODS

Collection of fecal and municipal wastewater samples. (i) **Animal feces.** Thirty-two fecal samples from pigs, cows, chickens, and wild animals were collected from different animal farms or wild animal habitats. All of the animal farms were located in rural areas near Seoul, South Korea. The sampling sites of wild animal habitats were located in the Chonnam area of the southwestern part of South Korea, where migrating birds from Siberia rest every winter. The fecal samples were collected using disposable polyvinyl bags, which were stored in polyethylene containers at 4°C until analysis. All animal fecal samples, except wild animal feces, were processed within 8 h of sampling and analyzed within 24 h. Since the sampling site for the wild animal feces was relatively far away, the wild animal feces were transported into the laboratory on dry ice and then analyzed within 24 h.

(ii) **Human wastewater.** Municipal wastewater (containing human sewage and feces) samples were collected from the influents of sewage treatment plants in the metropolitan area of Seoul, South Korea. Since human feces and animal feces are treated separately at different waste treatment plants, we collected the samples from wastewater treatment plants only for human wastes. In total, 11 samples were collected from these wastewater treatment plants. Human wastewater samples were collected using autoclaved polyethylene bottles and stored at 4°C until analysis. Samples were analyzed within 24 h of collection.

Recovery of somatic and male-specific bacteriophages. Approximately 5 g of collected fecal samples was suspended in 20 ml phosphate-buffered saline (pH 7.4) and vortexed for 5 min. The fecal suspensions were centrifuged at 5,000 × g for 20 min, and the supernatant was carefully collected for further analysis. For human wastewater samples, each sample was centrifuged at 5,000 × g for 20 min to remove large debris, and the supernatant was carefully collected for further analysis. The resulting supernatants were analyzed using single-agar-layer methods (36). Briefly, to isolate somatic and male-specific (F⁺) coliphages from the supernatant, *Escherichia coli* CN13 (ATCC 700609) and *E. coli* F_{amp} (ATCC 700891) were used, respectively. From the plaques observed on the single-agar-layer plate, approximately 20 coliphage isolates were selected using a sterile wooden toothpick, and each isolate was subjected to two-step enrichment procedures (35). The sterile toothpick used in the plaque assay was suspended in 5 ml tryptic soy broth containing 100 µl log phase of either *E. coli* CN13 or *E. coli* F_{amp}. The tube was gently vortexed for 1 min, and the phage isolated from a single plaque was incubated at 37°C for 16 to 18 h at 150 rpm. After cultivation, chloroform was added, and the tube was vigorously vortexed for 5 min. The suspension was centrifuged at 5,000 × g for 20 min, and the single phage isolate in the resulting supernatant was removed and stored at –70°C until further analysis. For each experiment, either *E. coli* MS2 (ATCC 15597-B1) or φX174 (ATCC 13706-B1) was used as a positive control of somatic or male-specific coliphages, respectively. Sterile phosphate-buffered saline was used as the negative control.

RNase sensitivity assay to distinguish F⁺ DNA and F⁺ RNA coliphages. To distinguish F⁺ DNA and F⁺ RNA coliphages, each isolated F⁺ coliphage was subjected to an RNase sensitivity assay (18). *Escherichia coli* F_{amp} was cultivated to exponential phase and poured into a 150-mm-diameter petri dish in 0.8% tryptic soy agar with and without RNase (100 µg/ml) (Sigma-Aldrich, St. Louis,

MO). Serial dilutions (5 µl; undiluted, 10^{–2}, 10^{–4}, and 10^{–6}) of the isolated coliphages were spotted onto both RNase-positive and RNase-negative plates. The plates were incubated for 12 to 18 h at 37°C. When the plaque was observed in the presence of RNase, the phage was considered to be a DNA coliphage. When a plaque was observed only on the RNase-negative plate, the bacteriophage was considered to be an RNA coliphage.

F⁺ DNA coliphage PCR and F⁺ RNA coliphage RT-PCR. F⁺ coliphage strains were amplified using primers as previously described (38). Either DNA coliphages or RNA coliphages were heat released and diluted 1:50. The released nucleic acid was amplified using either PCR or reverse transcription-PCR (RT-PCR) assays as described in a previous study (38). Amplified PCR/RT-PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced at Cosmo Gene Tech (Seoul, South Korea).

Genotyping of F⁺ coliphages and phylogenetic analysis. Chromatographs of each sample sequence were checked and edited using SeqMan software (Lagergren 6.0; DNASTar, Inc., Madison, WI). Nucleic acid sequences with unclear chromatographs or shorter fragments were excluded from further data analysis. In addition, any possible clone from the same fecal sample was excluded as well. Each sequence of analyzed samples was exported to GenBank and confirmed using BLAST. All sequences with both F⁺ RNA and F⁺ DNA coliphages were imported into the ARB library (25). In addition, all sequences of *Leviviridae* and *Inoviridae* (M13-like phages) available in GenBank were downloaded and imported into the ARB software package. Sequences were aligned, and phylogenetic analysis was performed based on the neighbor-joining method.

PCoA by UniFrac. To distinguish the nucleic acids of bacteriophages from both animal and human fecal sources, a principal coordinate analysis (PCoA) was performed using UniFrac software (23). The nucleic acid sequences of bacteriophages were aligned using Clustal W and exported to the UniFrac software for PCoA. PCoA was performed to determine whether bacteriophages from different fecal sources were distinctive using either UniFrac significance tests or the *P* test. F⁺ RNA and F⁺ DNA coliphages were analyzed separately.

Nucleotide sequence accession numbers. The nucleic acid sequences of novel F⁺ RNA coliphage strains were deposited into GenBank and assigned GenBank accession numbers GQ268004 to GQ268012.

RESULTS

Incidences of coliphages in animal feces and human wastewater. Table 1 summarizes the prevalence of somatic and F⁺ coliphages from various fecal sources in Korea. In most fecal sources, somatic coliphages occurred with a high incidence (86 to 100%). However, the prevalence of somatic coliphages in cow feces was lower than in other fecal sources (58%). In the fecal samples from a pig septic tank and from chickens, the concentrations of somatic coliphages ranged from 380 to 13,700 PFU/g (median density of 680 PFU/g) and from 3 to 10,000 PFU/g (median density of 1,450 PFU/g), respectively. On the other hand, the concentrations of somatic coliphages in cow feces ranged from 1 to 25 PFU/g (median density of 11 PFU/g).

The prevalence of F⁺ coliphages was lower than that of somatic coliphages (Table 1). F⁺ coliphages were abundantly

TABLE 2. F⁺ coliphage characterization by RNase test and PCR or RT-PCR assay

Source	No. of isolates	No. (%) confirmed by:			
		RNase test ^a		PCR or RT-PCR ^b	
		F ⁺ DNA	F ⁺ RNA	F ⁺ DNA	F ⁺ RNA
Human	128	78 (61)	50 (39)	75 (59)	53 (41)
Cow	21	17 (81)	4 (19)	20 (95)	1 (5)
Chicken	29	22 (76)	7 (24)	23 (79)	6 (21)
Goose	21	1 (5)	20 (95)	1 (5)	20 (95)
Pig					
Feces	53	15 (28)	38 (72)	19 (36)	34 (64)
Septic tank sample	59	21 (36)	38 (64)	19 (32)	40 (68)
Total	311	154 (50)	157 (50)	157 (50)	154 (50)

^a RNase test using RNase A for inhibition of RNA coliphages.

^b PCR was used for F⁺ DNA coliphage confirmation; RT-PCR was used for F⁺ RNA coliphage confirmation. Samples that could not be verified by PCR were tested by RT-PCR for F⁺ RNA coliphage detection. Samples that could not be verified by RT-PCR were tested by PCR for F⁺ DNA coliphage detection.

found (73 to 100%) in human wastewater, the pig septic tank, and goose feces. On the other hand, F⁺ coliphages had a lower prevalence in cow feces (25%). F⁺ coliphages in human wastewater ranged from <1 to 285 PFU/ml (median, 5 PFU/ml). The median number of coliphages found in pig fecal samples was slightly higher than in other fecal samples (Table 1).

Identification of F⁺ DNA and F⁺ RNA coliphages by both RNase sensitivity and PCR/RT-PCR assays. To identify the type of nucleic acids (either DNA or RNA) in isolated coliphages, the sensitivity of 311 F⁺ coliphage isolates to RNase was tested using a spot plate assay (Table 2). Among the 311 bacteriophage samples that were tested, the numbers of F⁺ DNA and F⁺ RNA coliphages were 157 and 154, respectively. F⁺ DNA coliphages were more prevalent among cow (95%) and chicken (79%) feces. In both pig feces and the pig septic tank, F⁺ RNA coliphages were more prevalent (64% and 68%) than F⁺ DNA coliphages, and most bacteriophages from goose fecal samples were F⁺ RNA coliphages (95%). In human wastewater, the prevalences of F⁺ DNA and F⁺ RNA coliphages were 59% and 41%, respectively.

Typing of isolated F⁺ coliphage sequences. In total, 311 nucleic acid sequences from isolated F⁺ coliphages were confirmed and classified using both BLAST searches and phylogenetic analyses. Based on comparisons to the standard reference strains, each of the 133 RNA coliphage isolates from various fecal sources was classified into one of four groups (groups I to IV). Over half (52%) of the RNA coliphages were categorized into group I, 10% into group II, 12% into group III, and 19% into group IV (Table 3). Most coliphages isolated from animals were categorized into groups I and IV, except one isolate from a goose fecal sample. Most RNA coliphages isolated from human wastewater samples were categorized into groups II (32%) and III (42%). Ten RNA coliphage isolates (26%) were categorized into group I. Approximately nine isolates (7%) did not share any similarity with previously identified RNA coliphages and were classified into a novel group. Among the 131 F⁺ DNA coliphages, 109 isolates (83%) were identified as f1-like phages. Eighteen (13%) and four (3%) isolates were identified as fd-like and M13-like phages, respectively. Of the 64 F⁺ DNA coliphages isolated from human wastewater samples, 47 (73%) were identified as f1-like, 14 (22%) as fd-like, and 3 (5%) as M13-like bacteriophages. Most of the F⁺ DNA coliphages were grouped as either f1-like or fd-like bacteriophages.

Phylogenetic analysis of F⁺ coliphages using ARB software. To investigate the genetic diversity of isolated F⁺ coliphage sequences, phylogenetic analyses were performed using ARB software.

(i) F⁺ RNA coliphages. Partial regions of replicase genes (266 bp [*Levivirus*] and 229 bp [*Allolevivirus*]) were PCR or RT-PCR amplified and subsequently sequenced. Phylogenetic analyses were performed with all *Leviviridae* nucleic acid sequences from GenBank. In total, 133 isolated F⁺ RNA coliphages were grouped into two major clusters (Qβ-like and MS2-like). Among the isolated F⁺ RNA coliphages, the majority of the phages from human wastewater sources belonged to either GA-like (group II) or Qβ-like (group III) phages (Fig. 1A). The RNA coliphages isolated from animal fecal sources belonged to either MS2 (group I)- or FI (group IV)-like phages. Bacteriophages isolated from pig septic tank fecal sources were identified as a novel separate group.

TABLE 3. Typing of F⁺ coliphages by BLAST search^a and phylogenetic analysis

Source	No. (%) of coliphages									
	F ⁺ RNA						F ⁺ DNA			
	Group I	Group II	Group III	Group IV	Novel sequence	Total	f1	fd	M13	Total
Human	10 (26)	12 (32)	16 (42)			38	47 (73)	14 (22)	3 (5)	64
Cow	1 (100)					1	20 (100)			20
Chicken				5 (100)		5	11 (92)	1 (8)		12
Goose	18 (90)	1 (5)		1 (5)		20				
Pig										
Feces	33 (100)					33	14 (82)	3 (18)		17
Septic tank sample	7 (19)			20 (56)	9 (25)	36	17 (94)		1 (6)	18
Total	69 (52)	13 (10)	16 (12)	26 (19)	9 (7)	133	109 (83)	18 (13)	4 (3)	131

^a The *E* value for nucleic acid sequence search was >1e-30 in this study.

FIG. 1. Neighbor-joining trees of F⁺ RNA (A) and F⁺ DNA (B) coliphage sequences using the ARB software package. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. Pig, pig feces; swine, samples from a pig septic tank (Table 1).

(ii) F⁺ DNA coliphages. To investigate the genetic variability of F⁺ DNA coliphages, the nucleic acids of the gene IV region were sequenced (38). All F⁺ DNA coliphages from various sources and reference strains were categorized into two different major clusters (fd-like and M13-like). The majority of phages from human wastewater sources were identified as fd-like phages. In addition, 1 phage from chicken and 16 phages from swine septic tank sources were included as well in the fd-like phages. The phages from the other animal fecal sources, including chicken, cow, and pig, belonged to the M13- and f1-like phage groups (Fig. 1B).

PCoA of F⁺ coliphages by UniFrac. To determine differences among F⁺ coliphages isolated from different fecal sources, we performed significance tests and PCoA based on the phylogenetic analysis. The genetic characteristics of F⁺ RNA bacteriophages isolated from human fecal samples were significantly different from those of the other samples, as determined by the *P* test (*P* value < 0.05), except the cow samples (Table 4). The significant *P* values indicated that F⁺ RNA coliphages isolated from various sources have unique genetic characteristics. Among the fecal samples from various sources, only the F⁺ RNA coliphages from humans were significantly

TABLE 4. *P* test and UniFrac significance of F⁺ RNA coliphages

Sample (source)	<i>P</i> ^a for sample:						UniFrac distance <i>P</i> ^b
	1	2	3	4	5	6	
1 (human)	1.000						≤0.001**
2 (cow)	1.000	1.000					0.05
3 (chicken)	≤0.015*	1.000	1.000				0.139
4 (goose)	≤0.015*	1.000	≤0.015*	1.000			0.728
5 (pig feces)	≤0.015*	1.000	≤0.015*	≤0.015*	1.000		0.983
6 (pig septic tank)	≤0.015*	1.000	≤0.015*	≤0.015*	≤0.015*	1.000	0.936

^a *P* test *P* values were based on comparisons of 1,000 randomized trees. *, *P* < 0.05. Pairwise comparisons indicated that the F⁺ RNA coliphages from various sources were significantly different.
^b UniFrac results, expressed as *P* values, were based on comparisons of 1,000 randomized trees. **, *P* < 0.05. Pairwise comparisons indicated that the sequences from this sample are associated with more unique branch lengths than were expected.

different from F⁺ RNA coliphages in the other sources (*P* ≤ 0.001) (Table 4). These results indicated that the sequences from this sample are associated with unique branch lengths in the tree, suggesting that F⁺ RNA coliphages from human sources are distinctive from F⁺ RNA coliphages isolated from other fecal sources. However, when F⁺ DNA coliphages were analyzed using the same significance analysis, the *P* test result was not significant among the various source samples (data not shown). F⁺ RNA coliphage significance test observations might best be illustrated using PCoA (Fig. 2A). When a scatter plot of the first two principal components (PCs) was analyzed, both PC1 and PC2 explained the major variation in the data

(50.9 and 24.2%, respectively). PC2 clearly separated all fecal source isolates from human source isolates (Fig. 2A). However, all fecal samples from animal or human sources were not clearly separated using PCoA of F⁺ DNA coliphages (Fig. 2B).

DISCUSSION

Currently, little information is available for describing the genetic characteristics of both F⁺ and somatic coliphages for MST. This study is the first to apply nucleic acid sequence-based genotyping methods for both F⁺ DNA and F⁺ RNA coliphages to MST. Bacteriophages have been considered use-

FIG. 2. PCA of F⁺ RNA (A) and F⁺ DNA (B) coliphage sequences.

ful targets in MST. However, MST is region specific and has not been studied much in Asia (11). Our study confirmed that F⁺ RNA coliphages isolated in Korea have genetic characteristics similar to those of coliphages isolated in other regions (30, 33). Genogroups I and IV were found mostly in animal fecal samples, and genogroups II and III were found mostly in human-waste-dominated wastewater samples. These results coincide with those of other, previous studies (10, 17, 27). However, 26% of F⁺ RNA coliphages isolated from human wastewater samples were identified as genogroup I. Thus, the identification of fecal sources solely on the basis of their F⁺ RNA coliphages belonging to genogroup I would be limited (33).

F⁺ coliphages were commonly detected in human and animal fecal samples in this study (Table 1). These results are consistent with those of other studies. Previous studies reported that the prevalences of F⁺ coliphages were 79%, 60%, and 100% in human waste, goose waste, and swine fecal wastewater, respectively (3, 5). Concentrations of F⁺ coliphages in pig fecal samples were slightly higher than those in other animal sources, such as chicken, cow, and goose feces. In previous studies, F⁺ RNA coliphages were rarely detected in fecal samples from adult chickens and cattle but were more often detected in pig fecal samples (17). In this study, a much lower prevalence (25%) was observed only in cow fecal samples. Another study reported that F⁺ coliphages were either not detected or not commonly detected in cow sources (16). Different microbial communities may be present in the cow gut because of different digestion mechanisms and ingested food (1). Further study should be focused on the different characteristics of microbial ecology in the guts of cows and other animals with respect to diet and other factors.

The ecological mechanism by which F⁺ coliphages became prevalent in human wastewater and septic tanks, but not in individual fecal samples, is not fully understood. In F⁺ coliphages, prolonged replication appears improbable because a minimum host density is required for replication (39). In both wastewater and septic tanks, host density could increase because feces from many different individuals are combined. The frequency of individual fecal samples containing F⁺ coliphages is not typically high (33). However, if any individual fecal sample contains F⁺ coliphages within a wastewater sample, the F⁺ coliphages may be able to effectively replicate in the wastewater, owing to the high density of the host population (33, 39).

The RNase test was applied to distinguish F⁺ RNA and F⁺ DNA coliphages. To confirm the results of RNase testing, either PCR or RT-PCR analysis for each of the bacteriophage isolates was also performed. These results indicated that the results of both the RNase test and PCR/RT-PCR assays were generally consistent with each other. However, the results were not consistent for bacteriophages isolated from some human wastewater samples and animal fecal samples. In human and animal wastewater samples, several F⁺ DNA coliphages that were identified by spot assay were then identified as F⁺ RNA coliphages using RT-PCR analysis. These results suggest that the RNase sensitivity assay could be inaccurate in some cases, resulting from its inability to completely inactivate F⁺ RNA bacteriophages. Havelaar (15) found that F⁺ RNA coliphages could become resistant to RNase, which could lead to a false-negative result. Hence, resistance to the RNase spot assay does

not necessarily indicate the presence of F⁺ DNA coliphages (40). On the other hand, F⁺ RNA coliphages were identified as F⁺ DNA coliphages by PCR analysis in animal fecal samples. In this case, F⁺ DNA coliphages may be inactivated by RNase in some environments.

This study suggested that the ratio of F⁺ DNA coliphages to F⁺ RNA coliphages indicated the sources of the fecal samples. For example, F⁺ DNA coliphages were much more prevalent (95%) than F⁺ RNA coliphages (5%) in cow feces. On the other hand, F⁺ RNA coliphages were more prevalent (95%) than F⁺ DNA coliphages (5%) in goose feces. These results are consistent with those of a previous study (5). Cole et al. (5) reported a high ratio of F⁺ DNA coliphages (82%) to F⁺ RNA coliphages (18%) in cow feces and a low ratio of F⁺ DNA (0%) to F⁺ RNA (100%) coliphages in goose feces. For human and pig fecal samples, a mixed ratio of F⁺ DNA and RNA coliphages was observed, consistent with results of a previous study (22). This study indicated that both F⁺ DNA (59%) and F⁺ RNA (41%) coliphages were present in human wastewater and that, similarly, F⁺ DNA (32%) and F⁺ RNA (68%) coliphages were found together in pig fecal samples. A previous study also showed that F⁺ DNA (65%) and F⁺ RNA (35%) coliphages were present in human wastewater and that F⁺ DNA (34%) and F⁺ RNA (66%) coliphages were prevalent in a hog lagoon (22).

In this study, F⁺ RNA coliphages were grouped based on the nucleic acid sequences of a partial replicase gene. As indicated in other studies, most F⁺ RNA coliphages from animal fecal samples belonged to either group I or group IV, and the majority of F⁺ RNA coliphages isolated from human wastewater belonged to groups I, II, and III. Group I F⁺ RNA coliphages were thus found in both human wastewater and animal fecal samples. Many previous studies have reported that group I coliphages were predominantly found in human-waste-dominated wastewater (5, 6, 33). Schaper et al. (29) found that group I coliphages were more persistent than other groups of coliphages under various environmental conditions and stresses. This persistence is an important factor in their presence in wastewater.

Few studies have investigated the prevalence and ecology of F⁺ DNA coliphages (9), and one study suggested F⁺ DNA coliphages as a potential indicator for source tracking (22). Until now, little information has been available as to whether specific subgroups of F⁺ DNA coliphages are associated with specific fecal sources (38). Unlike the previous study that used the serotypes of F⁺ DNA coliphages (22), this study investigated F⁺ DNA coliphages as a potential target microorganism in MST using DNA sequence-based genotypes. Typically, DNA is more stable than RNA, which suggests that F⁺ DNA coliphages could be better targets for MST (38). However, our results suggest that F⁺ RNA coliphages are more strongly associated with the ability to discriminate between human and animal sources than F⁺ DNA coliphages are. Further studies should be conducted to investigate new types of F⁺ DNA bacteriophages and specific genetic markers to be used in MST.

Somatic coliphages were commonly found in both animal-waste- and human-waste-dominated wastewater samples. Concentrations of somatic coliphages varied from 30 PFU/ml to 3.8×10^3 PFU/ml in human-waste-dominated wastewater and

from 1 PFU/g to 1.4×10^4 PFU/g in animal fecal samples (Table 1). These observations are similar to those found in other studies (22, 33). A previous study reported 22 PFU/ml to 3.6×10^3 PFU/ml in wastewater, 3.8×10^2 to 1.4×10^4 PFU/g in hog lagoons, 1 to 2 PFU/g from cow sources, and 1×10^4 to 4.7×10^6 PFU/g from chicken litter (33). Because somatic coliphages are a more diverse group than F⁺ coliphages, it is more difficult to identify target genetic markers for MST. However, if the right genetic marker were identified in somatic coliphages, they would be more attractive for MST because of their high prevalence in both individual fecal samples and the environment.

Phylogenetic analyses of isolated bacteriophages were performed using the ARB software program (25). These analyses showed that F⁺ RNA coliphages isolated from various sources were divided into two main clusters (Fig. 1A). All F⁺ RNA coliphages isolated from human wastewater were grouped with the Q β -like phages, while F⁺ RNA coliphages isolated from pig, chicken, and pig septic tank sources were grouped with the MS2-like phages. These results are encouraging because different phylogenetic characteristics are exhibited based on the types of human and animal fecal sources. The novel sequences were identified from collected animal fecal samples. However, when the phylogenetic analysis was performed for F⁺ DNA coliphages (Fig. 1B), there was no distinction between human and animal fecal sources.

In this study, the UniFrac method was applied to distinguish between the fecal sources based on the phylogenetic analysis. UniFrac is a bioinformatics tool to test the statistical significance of phylogenetic distances among different samples. Lozupone and Knight demonstrated that UniFrac is robust even for very similar samples when the sample size is large (24). Recently, several studies applied UniFrac to compare samples from multiple communities (8, 21, 23). However, this tool has never been applied to MST. The results of the *P* test showed significant differences ($P < 0.005$) between F⁺ RNA coliphages isolated from human and animal fecal samples. This result indicates that RNA coliphages could be applied in MST as target microorganisms. To determine whether bacteriophages isolated from various sources were different based on a unique branch length in the phylogenetic tree, the UniFrac significance test was performed (23). Among various fecal source samples, bacteriophages isolated from human wastewater were significantly different from those isolated from other samples ($P < 0.005$) (Table 4). This result suggests that F⁺ RNA coliphages isolated from human wastewater were clearly distinctive from those from other animal fecal samples. Furthermore, a PCoA confirmed these results. The PCoA whose results are shown in Fig. 2A showed that F⁺ RNA coliphages isolated from human wastewater were clearly separated from those from other animal sources. Together, these results indicate that F⁺ RNA coliphages from human sources were significantly different from those from other fecal sources. However, when F⁺ DNA coliphage was analyzed, no distinctive pattern was observed (Fig. 2B). It also needs to be acknowledged that while phylogenetic analysis and PCoA were able to distinguish F⁺ RNA coliphage isolates from known fecal sources, analysis of an environmental water sample receiving mixed fecal inputs in a real-world setting would be more complicated. In conclusion, our study demonstrated that bacterio-

phages could be very useful targets in MST. Different criteria, such as genogroup of bacteriophages and PCoA, could be applied for identifying fecal sources in environmental samples.

ACKNOWLEDGMENT

This study was supported by the Ministry of Environment of the Republic of Korea as the Eco-Technopia 21 Project (900-20080010).

REFERENCES

1. Balch, C. C. 1950. Factors affecting the utilization of food by dairy cows; the rate of passage of food through the digestive tract. *Br. J. Nutr.* **4**:361–388.
2. Byappanahalli, M. N., K. Przybyla-Kelly, D. A. Shively, and R. L. Whitman. 2008. Environmental occurrence of the enterococcal surface protein (esp) gene is an unreliable indicator of human fecal contamination. *Environ. Sci. Technol.* **42**:8014–8020.
3. Calci, K. R., W. Burkhardt III, W. D. Watkins, and S. R. Rippey. 1998. Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human-associated wastewaters. *Appl. Environ. Microbiol.* **64**:5027–5029.
4. Carson, C. A., B. L. Shear, M. R. Ellersieck, and J. D. Schnell. 2003. Comparison of ribotyping and repetitive extragenic palindromic-PCR for identification of fecal *Escherichia coli* from humans and animals. *Appl. Environ. Microbiol.* **69**:1836–1839.
5. Cole, D., S. C. Long, and M. D. Sobsey. 2003. Evaluation of F⁺ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. *Appl. Environ. Microbiol.* **69**:6507–6514.
6. Dhillon, E. K. S., and T. S. Dhillon. 1974. Synthesis of indicator strains and density of ribonucleic acid-containing coliphages in sewage. *Appl. Environ. Microbiol.* **27**:640–647.
7. Doré, W. J., K. Henshilwood, and D. N. Lees. 2000. Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. *Appl. Environ. Microbiol.* **66**:1280–1285.
8. Fierer, N., M. Hamady, C. L. Lauber, and R. Knight. 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc. Natl. Acad. Sci. USA* **105**:17994–17999.
9. Furuse, K. 1987. Distribution of coliphages in the environment: general considerations, p. 87–124. *In* S. M. Goyal, C. P. Gerba, and G. Bitton (ed.), *Phage ecology*. John Wiley & Sons, Inc., New York, NY.
10. Furuse, K., A. Ando, S. Osawa, and I. Watanabe. 1981. Distribution of ribonucleic acid coliphages in raw sewage from treatment plants in Japan. *Appl. Environ. Microbiol.* **41**:1139–1143.
11. Furuse, K., T. Sakurai, A. Hirashima, M. Katsuki, A. Ando, and I. Watanabe. 1978. Distribution of ribonucleic acid coliphages in South and East Asia. *Appl. Environ. Microbiol.* **35**:995–1002.
12. Grabow, W. O. K. 1996. Waterborne diseases: update on water quality assessment and control. *Water S. Afr.* **22**:193–202.
13. Griffin, D. W., R. Stokes, J. B. Rose, and J. H. Paul III. 2000. Bacterial indicator occurrence and the use of an F⁺ specific RNA coliphage assay to identify fecal sources in Homosassa Springs, Florida. *Microb. Ecol.* **39**:56–64.
14. Harwood, V. J., J. Whitlock, and V. Withington. 2000. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. *Appl. Environ. Microbiol.* **66**:3698–3704.
15. Havelaar, A. H. 1986. F-specific bacteriophages as model viruses in water treatment process. Ph.D. thesis. State University of Utrecht, Utrecht, The Netherlands.
16. Havelaar, A. H., K. Furuse, and W. M. Hogeboom. 1986. Bacteriophages and indicator bacteria in human and animal faeces. *J. Appl. Microbiol.* **60**:255–262.
17. Havelaar, A. H., W. M. Pot-Hogeboom, K. Furuse, R. Pot, and M. P. Horrmann. 1990. F-specific RNA bacteriophages and sensitive host strains in faeces and wastewater of human and animal origin. *J. Appl. Microbiol.* **69**:30–37.
18. Hsu, F. C., Y. S. Shieh, J. van Duin, M. J. Beekwilder, and M. D. Sobsey. 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. *Appl. Environ. Microbiol.* **61**:3960–3966.
19. Joklik, W. K. 1992. Structure, components, and classification of viruses, p. 749–782. *In* W. K. Joklik, H. P. Willett, and D. B. Amos (ed.), *Zinsser microbiology*. Appleton & Lange, Norwalk, CT.
20. Kirs, M., and D. C. Smith. 2007. Multiplex quantitative real-time reverse transcriptase PCR for F⁺-specific RNA coliphages: a method for use in microbial source tracking. *Appl. Environ. Microbiol.* **73**:808–814.
21. Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon. 2008. Evolution of mammals and their gut microbes. *Science* **320**:1647.
22. Long, S. C., S. S. El-Khoury, S. J. G. Oudejans, M. D. Sobsey, and J. Vinje. 2005. Assessment of sources and diversity of male-specific coliphages for source tracking. *Environ. Eng. Sci.* **22**:367–377.

23. Lozupone, C., M. Hamady, and R. Knight. 2006. UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* **7**:371–384.
24. Lozupone, C., and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **71**:8228–8235.
25. Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* **32**:1363–1371.
26. Noble, R. T., S. M. Allen, A. D. Blackwood, W. Chu, S. C. Jiang, G. L. Lovelace, M. D. Sobsey, J. R. Stewart, and D. A. Wait. 2003. Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial source tracking comparison study. *J. Water Health* **1**:195–207.
27. Osawa, S., K. Furuse, and I. Watanabe. 1981. Distribution of ribonucleic acid coliphages in animals. *Appl. Environ. Microbiol.* **41**:164–168.
28. Parveen, S., K. M. Portier, K. Robinson, L. Edmiston, and M. L. Tamplin. 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. *Appl. Environ. Microbiol.* **65**:3142–3147.
29. Schaper, M., A. E. Durán, and J. Jofre. 2002. Comparative resistance of phage isolates of four genotypes of F-specific RNA bacteriophages to various inactivation processes. *Appl. Environ. Microbiol.* **68**:3702–3707.
30. Schaper, M., J. Jofre, M. Uys, and W. O. K. Grabow. 2002. Distribution of genotypes of F-specific RNA bacteriophages in human and non-human sources of faecal pollution in South Africa and Spain. *J. Appl. Microbiol.* **92**:657–667.
31. Sinton, L. W., R. K. Finlay, and D. J. Hannah. 1998. Distinguishing human from animal faecal contamination in water: a review. *N. Z. J. Mar. Freshw. Res.* **32**:323–348.
32. Stewart, J. R., J. Vinjé, S. J. G. Oudejans, G. I. Scott, and M. D. Sobsey. 2006. Sequence variation among group III F-specific RNA coliphages from water samples and swine lagoons. *Appl. Environ. Microbiol.* **72**:1226–1230.
33. Stewart-Pullaro, J., J. W. Daugomah, D. E. Chestnut, D. A. Graves, M. D. Sobsey, and G. I. Scott. 2006. F(+)RNA coliphage typing for microbial source tracking in surface waters. *J. Appl. Microbiol.* **101**:1015–1026.
34. Sundram, A., N. Jumanlal, and M. M. Ehlers. 2006. Genotyping of F-RNA coliphages isolated from wastewater and river water samples. *Water S. Afr.* **32**:65–70.
35. U.S. Environmental Protection Agency. 2001. Method 1601: male-specific (F⁺) and somatic coliphage in water by two-step enrichment procedure. Office of Water, U.S. Environmental Protection Agency, Washington, DC. <http://www.epa.gov/microbes/1601ap01.pdf>.
36. U.S. Environmental Protection Agency. 2001. Method 1602: male-specific (F⁺) and somatic coliphage in water by single agar layer (SAL) procedure. Office of Water, U.S. Environmental Protection Agency, Washington, DC. <http://www.epa.gov/microbes/1602ap01.pdf>.
37. U.S. Environmental Protection Agency. 2000. Proposed ground water rule EPA 815-F-00-003. Office of Ground Water, U.S. Environmental Protection Agency, Washington, DC.
38. Vinjé, J., S. J. G. Oudejans, J. R. Stewart, M. D. Sobsey, and S. C. Long. 2004. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. *Appl. Environ. Microbiol.* **70**:5996–6004.
39. Wiggins, B. A., and M. Alexander. 1985. Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. *Appl. Environ. Microbiol.* **49**:19–23.
40. Yee, S. Y. F., N. Y. Fong, G. T. Fong, O. J. Tak, G. T. Hui, and Y. S. Ming. 2006. Male-specific RNA coliphages detected by plaque assay and RT-PCR in tropical river waters and animal fecal matter. *Int. J. Environ. Health Res.* **16**:59–68.