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Molecular Detection of Human Enteric Viruses in Urban Rivers in Korea

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We performed RT-nested PCR to study the distribution of human enteric viruses in urban rivers in Korea. During 2002-2003, water samples were collected from four rivers in Gyeonggi Province, South Korea. Among 58 samples, 45 (77.6%), 32 (55.2%), 12 (20.7%), 2 (3.4%), 4 (6.9%), and 4 (6.9%) showed positive results with adenoviruses (AdVs), enteroviruses (EVs), reoviruses (ReVs), hepatitis A viruses (HAVs), rotaviruses (RoVs), and sapoviruses (SVs), respectively. According to the binary logistic regression model, the occurrence of each enteric virus, except ReVs and HAVs, was not statistically correlated with the water temperature and levels of fecal coliforms ($P<0.05$). AdVs were most often detected; only 4 samples (6.9%) were negative for AdVs while positive for other enteric viruses in the studied sites. Our results indicated that monitoring human enteric viruses is necessary to improve microbial quality, and that AdVs detection by PCR can be a useful index for the presence of other enteric viruses in aquatic environments.

Keywords: Enteric virus, RT-nested PCR, adenovirus, fecal coliforms, urban river

Large numbers of human enteric viruses are excreted in human feces and urine, and these viruses have been found in a variety of aquatic environments and food [12, 13, 19, 26]. The presence of enteric viruses in aquatic environments is an emerging issue because the viral infection can be caused by ingestion, even at low concentrations [30]. Generally, microbial quality is assessed by using bacterial indicators in aquatic environments and food [6, 16, 17]. However, bacteria are thought to have limited value as indicators of enteric viruses because these viruses are generally more resistant to water treatment processes than bacteria are [27]. Therefore, it is necessary to monitor the enteric viruses for improving virological quality of water.

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Commonly studied groups of enteric viruses belong to adenovirus (AdV), enterovirus (EV), norovirus (NV), reovirus (ReV), hepatitis A virus (HAV), rotavirus (RoV), astrovirus (AstV), sapovirus (SV), and hepatitis E virus (HEV) [9, 15, 18, 31]. Traditionally, these enteric viruses have been detected in environmental samples by a cell culture assay that is based on the expression of viral cytopathic effects (CPE) in the cultured cell lines. However, it has been reported that some enteric viruses, such as RoV and AstV, grow fastidiously by cell culture assay [31] and there is a lack of efficient cell lines to isolate NV [8]. For these reasons, a nucleic acid-based method, namely reverse transcription-nested PCR (RT-nested PCR), is rightly used for such sensitive detection [5, 11].

As bacterial indicators have limited value as indicators of enteric viruses, there is a need for indicators of viral presence in order to improve the microbiological control in aquatic environments. Several investigators have proposed the detection of AdVs by PCR as a molecular index for monitoring the presence of enteric viruses because AdVs are often detected and very stable in the environment [10, 11, 26].

To evaluate the viral contamination in aquatic environments and the usefulness of AdVs detection by PCR as a molecular index of the presence of enteric viruses, we performed RT-nested PCR to study the distribution of enteric viruses in urban rivers and analyzed the occurrence of each enteric virus with water temperature and levels of fecal coliforms.

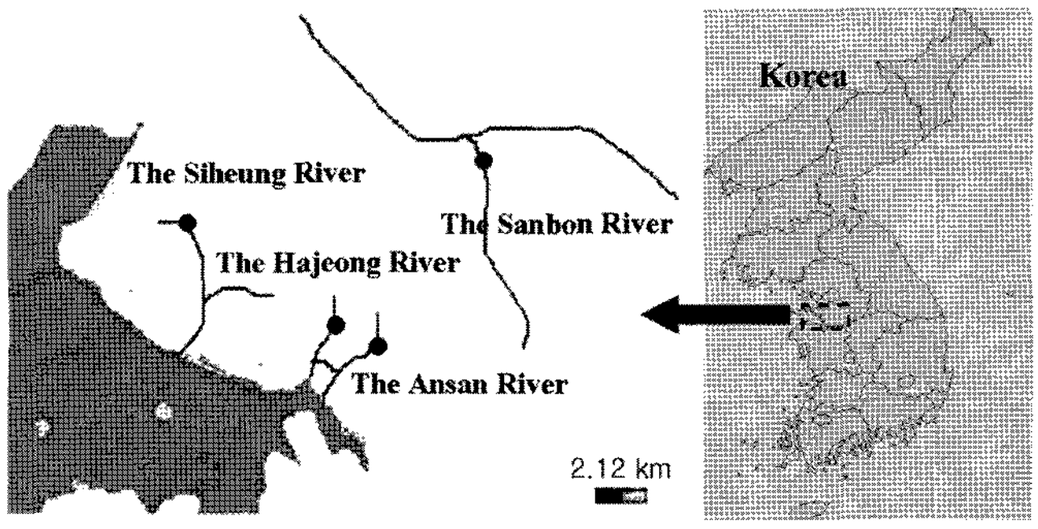

Fig. 1. Map of Korea showing the locations of the four rivers from which water samples were collected.

MATERIALS AND METHODS

Water Sample Collection

Surface water samples were collected fifteen times monthly or semimonthly between May 2002 and March 2003 at each of four river tributaries (the Sanbon, Hwajeong, Ansan, and Siheung Rivers) located in Gyeonggi Province, South Korea (Fig. 1). The Hwajeong River adjoins the Ansan River. The Sanbon River and the Siheung River are away from these rivers by about seven kilometers northeast and about six kilometers northwest, respectively. All the rivers are typical streams with low flows and run through urban areas; the pollution sources of these rivers are untreated domestic and industrial wastewaters. The water samples was obtained at 30 cm depth in the afternoon. Two liters of river water were collected aseptically in sterile polypropylene bottles to avoid contamination. Samples were stored at 4°C and immediately transported to the laboratory for processing. The water temperature was measured *in situ* using portable electrode-carrying devices (Checkmate 90; Corning, NY, U.S.A.). Fecal

Table 1. Primers used for RT nested-PCR assays.

Virus	Region	Primer	Sequence (5'→3')[f]	Polarity	Position[g]	Reference
AdV	Hexon	AV1[b]	GCCCGAGTGGTCTTACATGCACATC	Sense	18,858–18,883	[1]
		AV2[b]	CAGCACGCCGCGGATGTCAAAGT	Antisense	19,136–19,158	
		AV3[c]	GCCACCGAGACGTACTTCAGCCTG	Sense	18,937–18,960	
		AV4[c]	TTGTACGAGTACGCGGTATCCTCGCGGTC	Antisense	19,051–16,079	
EV	5NTR[a]	E1[d]	CAAGCACTTCTGTTTCCCCGG	Sense	164–184	[20]
		E2[b]	ATTGTCACCATAAGCAGCCA	Antisense	599–578	
		E3[e]	CTTGCGCGTTACGAC	Antisense	526–511	
ReV	L1 gene	L1.rv5[b]	GCATCCATTGTAAATGACGAGTCTG	Sense	1,888–1,912	[14]
		L1.rv6[b]	CTTGAGATTAGCTCTAGCATCTTCTG	Antisense	2,303–2,278	
		L1.rv7[c]	GCTAGGCCGATATCGGGAATGCAG	Sense	1,930–1,953	
		L1.rv8[c]	GTCTCACTATTCACCTTACCAGCAG	Antisense	2,273–2,249	
HAV	3A gene	HAV1[b]	AATCCTCACAATGATATG	Sense	4,811–4,835	[23]
		HAV2[b]	CAACTCCAAACTGAACCA	Antisense	5,347–5,329	
		HAV3[c]	ACATAATGTTTCATTGATGG	Sense	4,904–4,923	
		HAV4[c]	AACCCACTTGTGATTAGT	Antisense	5,123–5,106	
RoV	VP7 gene	R1[b]	GGCTTTAAAAGAGAGAATTTCCGTCTGG	Sense	1–28	[3]
		END9[b]	GGTCACATCATACAATTCTAATCTAAG	Antisense	1,062–1,036	
		R3[c]	GTATGGTATTGAATATACCAC	Sense	51–71	
		Rp[c]	TCCATTGATCCTGTTATTGG	Antisense	239–220	
AstV	Nonstructural protein gene	Ast1[b]	CCTGCCCCGAGAACAACCAAG	Sense	2,363–2,383	[4]
		Ast2[b]	GTAAGATTCCCAGATTGGT	Antisense	2,599–2,581	
		Ast3-A1[c]	CCTTGCCCCGAGCCAGAA	Sense	2,390–2,407	
		Ast4-A2[c]	TATTCACAAACTTATGGCAA	Antisense	2,577–2,558	
SV	Polyprotein gene	SV-F11[b]	GCYTGGTTYATAGGTGGTAC	Sense	5,098–5,117	[25]
		SV-R1[b]	CWGGTGAMACMCCATTKTCCAT	Antisense	5,878–5,857	
		SV-F21[c]	ANTAGTGTTTGARATGGAGGG	Sense	5,157–5,177	
		SV-R2[c]	GWGGGRTCAACMCCWGGTGG	Antisense	5,591–5,572	
HEV	Capsid gene	3156[b]	AAYTATGCWCAGTACCGGGTTG	Sense	5,687–5,708	[22]
		3157[b]	CCCTTATCCTGCTGAGCATTCTC	Antisense	6,417–6,395	
		3158[c]	GTYATGYTYTGCATACATGGCT	Sense	5,972–5,993	
		3159[c]	AGCCGACGAAATYAATTCTGTC	Antisense	6,319–6,298	

[a]5 Nontranslated region.
[b]Primers used for first PCR.
[c]Primers used for nested PCR.
[d]Primers used for first and seminested PCR.
[e]Primers used for seminested PCR.
[f]Degenerate positions: N, A or G or T or C; Y, T or C; R, A or G; M, A or C; W, A or T; K, G or T.
[g]Relative nucleotide positions of primers for adenovirus, enterovirus, reovirus, HAV, rotavirus, astrovirus, sapovirus, and human hepatitis E virus are in references to the genomes of human adenovirus type 2 (Accession No. J01917), coxsackievirus B3 (Accession No. M16572), reovirus serotype 1 (Accession No. M24734), human hepatitis A virus strain FG (Accession No. K02990), human rotavirus strain Wa (Accession No. K02033), human astrovirus type 1 (Accession No. Z25771), human sapovirus Manchester (Accession No. X86560), and hepatitis E virus strain Burmese (Accession No. D10330), respectively.

Table 2. RT-nested PCR procedures for the detection of enteric viruses.

Virus	RT condition[a]	Primer pair	Initial denaturation/ end extension	PCR condition[c]	Cycles	Product size
AdV-EV	42°C, 45 min, EV2 primer	AV1/AV2 EV1/EV2	94°C, 4 min/ 72°C, 7 min	95°C, 30 sec; 55°C, 30 sec; 72°C, 1 min	35	301 bp/ 435 bp
		AV3/AV4 EV1/EV3	94°C, 4 min/ 72°C, 7 min	95°C, 30 sec; 55°C, 30 sec; 72°C, 1 min		143 bp/ 363 bp
ReV	20°C, 10 min (42°C, 30 min)[b], random hexamer	L1.rv5/ L1.rv6 L1.rv7/ L1.rv8	94°C, 1 min/ 72°C, 10 min	94°C, 20 sec; 50°C, 30 sec; 72°C, 30 sec	35	416 bp 344 bp
HAV	42°C, 60 min, HAV2 primer	HAV1/HAV2 HAV3/HAV4	94°C, 4 min/ 70°C, 3 min	95°C, 25 sec; 37°C, 30 sec; 70°C, 1 min	30	537 bp 220 bp
RoV	42°C, 30 min, R1/END9 primer	R1/END9	94°C, 4 min/ 72°C, 2 min	94°C, 1 min; 55°C, 2 min; 72°C, 1 min	25	1,062 bp
		R3/RP		94°C, 30 sec; 50°C, 30 sec; 72°C, 30 sec	3[d]	189 bp
				94°C, 15 sec; 50°C, 15 sec; 72°C, 20 sec	27[d]	
AstV	42°C, 42 min, Ast2 primer	Ast1/Ast2	95°C, 5 min/ 72°C, 7 min	95°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec	35	243 bp/ 193 bp[e]
		Ast3-A1/Ast4-A2	95°C, 5 min/ 72°C, 7 min	95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec		143 bp
SV	42°C, 60 min, SV-R1 primer	SV-F11/SV-R1 SV-F21/SV-R2	94°C, 4 min/ 72°C, 7 min	94°C, 30 sec; 50°C, 30 sec; 72°C, 1 min	35	860 bp 416 bp
HEV	45°C, 60 min, 3,157 primer	3,156/3,157 3,158/3,159	94°C, 4 min/ 72°C, 7 min	94°C, 1 min; 42°C, 1 min; 72°C, 2 min	40	719 bp 327 bp

[a]Temperature, time, and primer(s) for RT.
[b]Reaction mixtures were incubated at 20°C for 10 min and then at 42°C for 30 min.
[c]Denaturation temperature and time; annealing temperature and time; extension temperature and time.
[d]First three round cyclings were performed, followed by second 27 round cyclings.
[e]A 243 bp product for wild-type astroviruses and a 193 bp product for astroviruses that had an adaptive deletion.

coliforms (FCs) were enumerated in triplicate using the membrane filter technique [2] and m-FC agar (Difco Laboratories, MI, U.S.A.) for each sample studied. Plates were incubated at 44.5°C and blue colonies were counted as fecal coliforms.

Sample Processing

The viruses were concentrated by standard methods [2], with a minor modification at the secondary concentration step [21]. Briefly, the 2 l of water was adjusted to pH 5.5 and 0.1 N $MgCl_2$ by adding 1.0 N HCl and 5 N $MgCl_2$ solutions, filtered through a negatively charged 0.45-μm porosity membrane filter (cellulose nitrate, 47 mm diameters; Millipore Corporation, Bedford, MA, U.S.A.), washed with 25 ml of 0.14 N NaCl solution, and eluted with 7.5 ml of 0.05 M glycine buffer (pH 9.5) containing 3.0% beef extract. The eluates were immediately adjusted to neutral pH with 1.0 N HCl; 13% (w/v) polyethylene glycol (PEG) 8,000 (plus 0.2 M NaCl) at pH 7.2 was added, and the samples were incubated for 12 h at 4°C with gentle stirring. After incubation, the eluates were centrifuged at 7,000 ×*g* for 30 min, and the resulting pellets were resuspended in 10 ml of 10 mM phosphate-buffered saline (PBS) solution. The samples were filtered through a 0.2-μm-pore-size filter and stored at −70°C until used for DNA and RNA extraction.

RNA/DNA Extraction

Viral RNA and DNA were extracted from 140 ml of each concentrate with a QIAamp Viral RNA mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and were suspended in 60 ml of RNase-free water. The samples were stored at −70°C until used for RT-nested PCR analysis.

Oligonucleotides and RT-Nested PCR

The sequences (Table 1), sensitivities, and specificities of oligonucleotide primers used were described previously [1, 3, 4, 14, 20, 22, 23, 25]. Reverse transcription was performed as described previously [3–

Table 3. Water temperatures, levels of fecal coliforms, and RT-nested PCR results for enteric viruses at sampling sites.

Sampling site and date (day/mo/yr)[a]	Temp (°C)	Fecal coliforms (log_{10} CFU/100 ml)[c]	Virus detection by RT-nested PCR							
			AdV	EV	ReV	HAV	RoV	AstV	SV	HEV
The Sanbon River										
1 (08/05/2002)	22.5	2.71±1.98	+	–	–	–	+	–	–	–
2 (21/05/2002)	26.0	2.01±1.51	+	+	–	–	–	–	–	–
3 (13/06/2002)	24.0	2.58±1.49	+	+	–	–	–	–	+	–
4 (28/06/2002)	23.6	2.20±1.89	+	–	–	–	–	–	–	–
5 (17/07/2002)	23.8	2.32±1.81	+	+	+	–	–	–	–	–
6 (30/07/2002)	19.8	2.17±1.51	+	–	–	–	–	–	–	–
7 (15/08/2002)	24.4	2.45±1.82	+	+	–	–	–	–	–	–
8 (03/09/2002)	24.7	2.63±1.79	+	+	–	–	–	–	–	–
9 (17/09/2002)	22.4	2.38±1.60	+	+	–	–	–	–	–	–
10 (01/10/2002)	22.0	2.44±1.62	+	+	–	–	–	–	–	–
11 (1/11/2002)	11.2	2.58±1.74	+	+	+	–	–	–	–	–
12 (2/12/2002)	10.7	2.53±1.93	+	–	+	–	–	–	–	–
13 (05/01/2003)	NA[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA
14 (12/02/2003)	7.0	2.10±1.51	+	+	+	–	–	–	–	–
15 (21/03/2003)	10.0	2.37±1.32	–	–	+	–	+	–	–	–
Sum		2.43±2.09[d]	13	9	5	0	2	0	1	0
The Hwajeong River										
1 (08/05/2002)	23.8	1.60±0.54	–	–	–	–	–	–	–	–
2 (21/05/2002)	24.5	2.30±1.54	+	+	–	–	+	–	–	–
3 (13/06/2002)	23.5	2.48±1.90	–	–	–	–	–	–	+	–
4 (28/06/2002)	24.0	2.07±1.40	+	–	–	–	+	–	+	–
5 (17/07/2002)	22.0	2.30±1.72	+	+	–	–	–	–	–	–
6 (30/07/2002)	23.5	2.46±1.78	+	+	–	–	–	–	–	–
7 (15/08/2002)	24.0	2.48±1.24	+	–	–	–	–	–	–	–
8 (03/09/2002)	22.1	2.27±1.49	+	+	–	–	–	–	–	–
9 (17/09/2002)	23.0	2.47±1.49	+	+	–	–	–	–	–	–
10 (01/10/2002)	22.5	2.26±1.66	+	+	–	–	–	–	–	–
11 (1/11/2002)	10.8	2.33±1.40	+	+	+	–	–	–	–	–
12 (2/12/2002)	8.5	2.37±1.18	–	–	–	–	–	–	–	–
13 (05/01/2003)	2.8	2.01±1.32	–	–	–	–	–	–	–	–
14 (12/02/2003)	3.8	2.16±1.18	+	–	+	–	–	–	–	–
15 (21/03/2003)	10.8	2.34±1.18	+	+	–	–	–	–	–	–
Sum		2.30±1.89[d]	11	8	2	0	2	0	2	0
The Ansan River										
1 (08/05/2002)	24.4	2.23±1.78	–	+	–	–	–	–	–	–
2 (21/05/2002)	24.0	2.18±1.42	+	+	–	–	–	–	–	–
3 (13/06/2002)	21.8	1.95±0.85	+	+	–	–	–	–	–	–
4 (28/06/2002)	23.2	2.48±1.86	+	+	–	–	–	–	–	–
5 (17/07/2002)	19.5	2.20±1.42	+	–	–	–	–	–	+	–
6 (30/07/2002)	23.4	2.36±1.75	+	+	–	–	–	–	–	–
7 (15/08/2002)	23.4	2.36±1.54	+	+	–	–	–	–	–	–
8 (03/09/2002)	22.7	2.23±1.64	+	+	–	–	–	–	–	–
9 (17/09/2002)	25.0	2.20±1.24	+	+	–	–	–	–	–	–
10 (01/10/2002)	22.1	2.48±1.90	–	–	–	–	–	–	–	–
11 (1/11/2002)	23.0	2.62±1.78	+	+	+	+	+	–	–	–
12 (2/12/2002)	8.4	2.57±1.89	+	+	+	+	+	–	–	–
13 (05/01/2003)	7.9	2.41±1.87	–	–	–	–	–	–	–	–
14 (12/02/2003)	0.8	1.87±0.81	+	–	+	–	–	–	–	–
15 (21/03/2003)	4.2	2.25±1.32	+	+	+	–	–	–	–	–
Sum	9.6	2.34±1.99[d]	12	11	4	2	2	0	1	0

Table 3. Continued.

Sampling site and date (day/mo/yr)[a]	Temp (°C)	Fecal coliforms (log_{10} CFU/100 ml)[c]	Virus detection by RT-nested PCR							
			AdV	EV	ReV	HAV	RoV	AstV	SV	HEV
The Siheung River										
1 (08/05/2002)	19.0	1.52±0.64	+	–	–	–	–	–	–	–
2 (21/05/2002)	24.5	1.34±0.42	+	–	–	–	–	–	–	–
3 (13/06/2002)	22.4	1.89±1.19	+	–	–	–	–	–	–	–
4 (28/06/2002)	24.5	2.12±1.18	–	–	–	–	–	–	–	–
5 (17/07/2002)	20.2	1.32±0.66	+	+	–	–	–	–	–	–
6 (30/07/2002)	23.2	2.23±1.00	+	+	–	–	–	–	–	–
7 (15/08/2002)	22.8	1.89±0.70	+	–	–	–	–	–	–	–
8 (03/09/2002)	23.5	1.34±0.30	+	–	–	–	–	–	–	–
9 (17/09/2002)	24.1	1.09±0.36	+	+	–	–	–	–	–	–
10 (01/10/2002)	22.4	1.48±0.78	–	–	–	–	–	–	–	–
11 (1/11/2002)	12.2	2.26±1.60	–	–	–	–	–	–	–	–
12 (2/12/2002)	10.8	1.26±0.42	–	–	–	–	–	–	–	–
13 (05/01/2003)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
14 (12/02/2003)	4.0	1.00±0.42	+	–	+	–	–	–	–	–
15 (21/03/2003)	9.4	1.32±0.51	–	+	–	–	–	–	–	–
Sum		1.77±1.78[d]	9	4	1	0	0	0	0	0
Total			45	32	12	2	6	0	4	0

[a]Day/month/year.
[b]Not assayed.
[c]Means±standard deviations at each sampling date.
[d]Means±standard deviations at each sampling site.

5, 14, 22, 23, 25] with minor modification. Briefly, 20 μl of the reaction mixture contained 20 pmole of the corresponding antisense primer or 75 pmole of random hexamer, 100 U of Moloney murine leukemia virus reverse transcriptase, 10 U of RNasin, and 5 μl of RNA or DNA (corresponding to 10-ml portions of water samples). RT was carried out as described in Table 2, and the tubes were then heated to 95°C for 5 min to inactivate the enzyme. The mixture was added to 80 μl of PCR mixture (final concentration: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 200 μM each of dNTP, 1.25 U of *Taq* polymerase, 0.4 μM forward and reverse primers, and 1.5 mM $MgCl_2$). The PCR protocol is described in Table 2. For nested PCR amplification, 1 μl each of RT-PCR product was added to 50 μl (final volume) of PCR reaction mixture containing 0.4 μM of primers. The protocol is also described in Table 2. Thermal cycling was carried out in a Gene Amp PCR System 9,600 (Applied Biosystems, Foster City, CA, U.S.A.). The results were analyzed by electrophoresis through a 2% agarose gel and staining with ethidium bromide. We performed PCR in duplicate with the water samples to avoid false-negative results due to nonhomologous distribution of the viral genomes. When conflicting results were produced in duplicate PCR amplifications, additional amplification was performed to confirm the results.

Quality Control of the Amplification Method

To avoid false-positive results due to contamination with DNA amplified in previous PCR assays, separate areas and sets of apparatus were used for sample preparation, reagent preparation, and sample amplification. Each cabinet was equipped with an independent batch of reagents, micropipettes, pipette tips, and sterile reagent tubes. Virus-seeded positive controls as well as negative controls were incorporated into all PCR assays to ensure the propriety of the PCR assay.

Statistical Analysis

Statistical analyses were performed with the statistical package SPSS version 12.0.1 (SPSS Inc., Chicago, IL, U.S.A.) or Origin 7.0 (Origin Lab Corporation, Northampton, MA, U.S.A.) by using a Pentium IV computer. The possible differences in levels of fecal coliforms from sampling sites were analyzed by using the one-way analysis of variance (ANOVA) test with Tukey's method at a 5% significance level. The possible correlation of water temperature and levels of fecal coliforms among sampling sites were evaluated by Pearson correlation analysis, respectively. A binary logistic regression model was utilized to determine whether water temperature and levels of fecal coliforms predicted the probability of occurrence of each enteric virus in the water samples. Each enteric virus was treated as a binary variable; that is, a score of 0 was assigned when virus was not detected, and a score of 1 was assigned when virus was detected.

Results

Water Temperature and Levels of Fecal Coliforms

At each sampling site, we measured the water temperature and the level of FCs (Table 3). The water at four of the studied sites was constantly affected by sewage effluent; the mean values for FCs ranged from 1.77 log_{10} CFU/100 ml at the Siheung River to 2.43 log_{10} CFU/100 ml at the Sanbon River. The levels of FCs fluctuated between 1 and 3 log_{10} CFU/100 ml at the studied sites (Table 3). The mean level of FCs was lower at the Siheung River than at the other sites ($P<0.05$, as determined by ANOVA test).

Table 4. Relationship with each enteric virus, water temperature and levels of fecal coliforms.

	Water temperature			Fecal coliforms		
	R^2	Odds ratio	P value	R^2	Odds ratio	P value
AdVs	0.080	1.075	0.076	0.035	1.003	0.261
EVs	0.064	1.064	0.099	0.087	1.005	0.060
ReVs	0.500	0.795	0.000*	0.037	1.003	0.240
HAVs	0.224	0.837	0.115	0.383	1.016	0.049*
RoVs	0.104	1.045	0.598	0.077	1.006	0.183
SVs	0.093	1.181	0.302	0.032	1.004	0.386

*Asterisks indicate significant correlation (P<0.05)

The levels of FCs were not correlated between sampling sites according to a Pearson correlation analysis (P>0.05). Statistically significant correlation between the water temperature and levels of FCs was not observed (P>0.05, as determined by Pearson correlation test).

Detection of Enteric Viruses by RT-Nested PCR

Fifty-eight samples were analyzed by RT-nested PCR assay for the enteric viruses. The results obtained by RT-nested PCR amplification showed that 49 water samples (84.5%) were positive for enteric viruses (Table 3). The human viruses most often detected were AdVs, which were present in 45 water samples (77.6%). EVs, ReVs, HAVs, RoVs, and SVs were also detected in 32 (55.2%), 12 (20.7%), 2 (3.4%), 6 (10.3%), and 4 (6.9%) of water samples, respectively. However, AstVs and HEVs were not detected in all water samples. Interestingly, only four (6.9%) samples were negative for AdVs, but positive for other enteric viruses. Conflicting results were produced in water samples taken from the Ansan River at Date 3 in AdVs detection and water sample taken from the Siheung River at Date 5 in EVs detection in duplicate PCR amplifications. However, these samples were confirmed to be positive for these viruses by additional RT-nested PCR amplification (data not shown).

According to a binary logistic regression model (P< 0.05), significant correlations between the occurrence of ReVs and water temperature, and between the occurrence of HAVs and levels of FCs were observed (Table 4). The occurrence of ReVs showed moderately negative correlation with water temperature (Nagelkerke's R^2=0.500; Odds ratio=0.795) and the occurrence of HAVs showed weakly positive correlation with the levels of FCs (Nagelkerke's R^2=0.383; Odds ratio=1.016). However, the occurrence of other enteric viruses was not statistically correlated with the water temperature and the levels of FCs (P>0.05).

DISCUSSION

The RT-nested PCR method described herein provided reliable information about the contamination of enteric viruses in river water. This PCR method is rapid, sensitive, and less laborious and time-consuming than cell culture assays with which enteric viruses have been traditionally detected in environmental samples [26]. Our results showed that RoVs, which grow fastidiously in cell culture [31], were sensitively detected in 10-ml portions of water samples. In addition, all sequenced products (22 AdVs-, 10 EVs-, 11 ReVs-, 2 HAVs-, 4 RoVs-, and 2 SVs-positive samples) were closely matched with reference strains from GenBank databases using the PubMed NCBI BLAST program (data not shown). Therefore, this RT-nested PCR method provides high levels of sensitivity and specificity for detecting human enteric viruses in aquatic environments, and thus allowing us to overcome the technical limitations of isolating enteric viruses in the cell culture assay.

Our data showed that viral occurrence in studied sites was very high (49/58; 84.5%) in all the sampling dates (Table 3), indicating that the river water at these studied sites was chronically exposed to viral contamination from nearby communities and that the viral occurrence in river waters reflects the circulating viruses in nearby areas. Microbial water quality is generally assessed by using bacterial indicators such as coliforms. However, bacterial indicators are not predictive of the presence of enteric viruses [2, 26, 29]. Our results also showed that the levels of FCs, which are one of the standard bacterial indicators, were not statistically correlated to the occurrence of each enteric virus, except HAVs (Table 4), confirming the fact that bacterial indicators have limited value as a parameter for viral contamination in water. In addition, only the occurrence of ReVs was significantly correlated to water temperature (Table 4). However, the occurrence of AdVs and EVs, which were frequently detected in the studied sites, did not show statistically significant correlation with water temperature. These results are consistent with previous reports that the occurrence of AdVs and EVs was not correlated with temperature in water samples [7, 28].

Table 5. Comparison of presence of AdVs with that of other enteric viruses in tested water samples (N=58).

	Other enteric viruses-positive	Other enteric viruses-negative
AdVs-positive	41 (70.7%)	4 (6.9%)
AdVs-negative	4 (6.9%)	9 (15.5%)

Therefore, it is necessary to monitor human enteric viruses in aquatic environments for improving the microbial quality.

In this study, AdVs were present in 45 (91.8%) of 49 enteric virus-positive samples; 41 (83.6%) and 4 (8.2%) of AdVs-positive samples were positive for other enteric viruses, respectively, whereas only four (6.9%) samples were negative for AdVs, but positive for these viruses (Table 5). This observation is consistent with previous reports [10, 11, 24, 26], which suggested the use of AdVs detection by PCR in environmental samples as a molecular index of viral contamination from human origin because AdVs are often detected in the environmental sample, very stable in sewage samples, excreted throughout the year with higher numbers than EVs and HAVs, and not normally detected when fecal contamination is from animal origin. Therefore, it is suggested that AdVs detection by PCR assay can be used as a molecular index of the presence of other enteric viruses in aquatic environments.

Overall, the monitoring of enteric viruses in aquatic environments is useful for the assessment of the public health risks associated with these viruses. The AdVs detection by PCR assay is very effective as a molecular index for the presence of other enteric viruses because of their high prevalence in water environments, and therefore, it could be useful for monitoring fecal contamination and improving microbial quality in aquatic environments.

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References

1. Allard, A., B. Albinsson, and G. Wadell. 1992. Detection of adenoviruses in stools from healthy persons and patients with diarrhea by two-step polymerase chain reaction. *J. Med. Virol.* **37:** 149–157.
2. American Public Health Association. 1995. *Standard Methods for the Examination of Water and Wastewater*, 19th Ed. American Public Health Association, Washington, D.C.
3. Baggi, F. and R. Peduzzi. 2000. Genotyping of rotaviruses in environmental water and stool samples in southern Switzerland by nucleotide sequence analysis of 189 base pairs at the 5' end of the VP7 gene. *J. Clin. Microbiol.* **38:** 3681–3685.
4. Chapron, C. D., N. A. Ballester, J. H. Fontaine, C. N. Frades, and A. B. Margolin. 2000. Detection of astroviruses, enteroviruses, and adenovirus type 40 and 41 in surface waters collected and evaluated by the Information Collection Rule and an integrated cell culture-nested PCR procedure. *Appl. Environ. Microbiol.* **66:** 2520–2525.
5. Cho, H.-B., S.-H. Lee, J.-C. Cho, and S.-J. Kim. 2000. Detection of adenoviruses and enteroviruses in tap water and river water by reverse transcription multiplex PCR. *Can. J. Microbiol.* **46:** 417–424.
6. Choi, J.-W., Y.-K. Kim, H.-J. Kim, W. Lee, and G. H. Seong. 2006. Lab-on-a-chip for monitoring the quality of raw milk. *J. Microbiol. Biotechnol.* **16:** 1229–1235.
7. Choi, S. and S. C. Jiang. 2005. Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. *Appl. Environ. Microbiol.* **71:** 7426–7433.
8. Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. G. Koopmans, and M. K. Estes. 2004. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* **85:** 79–87.
9. Fong, T. T. and E. K. Lipp. 2005. Enteric viruses of humans and animals in aquatic environments: Health risks, detection, and potential water quality assessment tools. *Microbiol. Mol. Biol. Rev.* **69:** 357–371.
10. Formiga-Cruz, M., A. K. Allard, A.-C. Conden-Hanson, K. Henshilwood, B. E. Hernroth, J. Cofre, *et al.* 2003. Evaluation of potential indicators of viral contamination in shellfish and their applicability to diverse geographical areas. *Appl. Environ. Microbiol.* **69:** 1556–1563.
11. Formiga-Cruz, M., A. Hundesa, P. Clemente-Casares, N. Albinana-Gimenez, A. Allard, and R. Girones. 2005. Nested multiplex PCR assay for detection of human enteric viruses in shellfish and sewage. *J. Virol. Methods* **125:** 111–118.
12. Formiga-Cruz, M., G. Tofiño-Quesada, S. Bofill-Mas, D. N. Lees, K. Henshilwood, A. K. Allard, *et al.* 2002. Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Appl. Environ. Microbiol.* **68:** 5990–5998.
13. Gerba, C. P. and J. B. Rose. 1990. Viruses in source and drinking water. *In* G. A. McFeters (ed.), *Drinking Water Microbiology.* Springer-Verlag, New York.
14. Leary, T. P., J. C. Erker, M. L. Chalmers, A. T. Cruz, J. D. Wetzel, S. M. Desai, I. K. Mushahwar, and T. S. Dermody. 2002. Detection of mammalian reovirus RNA by using reverse transcription-PCR: Sequence diversity within the λ3-encoding L1 gene. *J. Clin. Microbiol.* **40:** 1368–1375.
15. Lee, C., S.-H. Lee, E. Han, and S.-J. Kim. 2004. Use of cell culture-PCR assay based on combination of A549 and BGMK cell lines and molecular identification as a tool to monitor infectious adenoviruses and enteroviruses in river water. *Appl. Environ. Microbiol.* **70:** 6695–6705.
16. Lee, D.-G, S.-J. Kim, and S. J. Park. 2006. Effect of reservoirs on microbiological water qualities in a drinking water distribution system. *J. Microbiol. Biotechnol.* **16:** 1060–1067.
17. Lee, D.-G., S. J. Park, and S.-J. Kim. 2007. Influence of pipe materials and VBNC cells on culturable bacteria in a chlorinated drinking water model system. *J. Microbiol. Biotechnol.* **17:** 1558–1562.
18. Lee, S.-H., C. Lee, K. W. Lee, H. B. Cho, and S.-J. Kim. 2005. The simultaneous detection of both enteroviruses and adenoviruses in environmental water samples including tap water with an integrated cell culture-multiplex-nested PCR procedure. *J. Appl. Microbiol.* **98:** 1020–1029.
19. Lee, S.-H. and S.-J. Kim. Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. *Water Res.* **36:** 248–256.

20. Leparc, I., M. Aymard, and F. Fuchs. 1994. Acute, chronic and persistent enterovirus and poliovirus infection: Detection of viral genome by semi-nested PCR amplification in culture-negative samples. *Mol. Cell. Probes* **8:** 487–495.
21. Lewis, G. D. and T. G. Metcalf. 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. *Appl. Environ. Microbiol.* **54:** 1983–1988.
22. Meng. X.-J., R. H. Purcell, P. G. Halbur, J. R. Lehman, D. H. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson. 1997. A novel virus in swine is closely related to the human hepatitis E virus. *Proc. Natl. Acad. Sci. USA* **94:** 9860–9865.
23. Morace, G, F. A. Aulicino, C. Angelozzi, L. Costanzo, F. Donadio, and M. Rapicetta. 2002. Microbial quality of wastewater: Detection of hepatitis A virus by reverse transcriptase-polymerase chain reaction. *J. Appl. Microbiol.* **92:** 828–836.
24. Muniain-Mujika, I., M. Calvo, F. Lucena, and R. Girones. 2003. Comparative analysis of viral pathogens and potential indicators in shellfish. *Int. J. Food Microbiol.* **38:** 75–85.
25. Okada, M., K. Shinozaki, T. Ogawa, and I. Kaiho. 2002. Molecular epidemiology and phylogenetic analysis of Sapporo-like viruses. *Arch. Virol.* **147:** 1445–1451
26. Pina, S., M. Puig, F. Lucena, J. Jofre, and R. Girones. 1998. Viral pollution in the environment and in shellfish: Human adenovirus detection by PCR as an index of human viruses. *Appl. Environ. Microbiol.* **40:** 805–809.
27. Sellwood, J., J. Dadswell, and J. Slade. 1981. Viruses in sewage as an indicator of their presence in the community. *J. Hyg.* **86:** 217–225.
28. Tani, N., Y. Dohi, N. Kurumatani, and K. Yonemasu. 1995. Seasonal distribution of adenoviruses, enteroviruses and reoviruses in urban river water. *Microbiol. Immunol.* **39:** 577–580.
29. Taylor, M. B., N. Cox, M. A. Vrey, and W. O. Grabow. 2001. The occurrence of hepatitis A and astroviruses in selected river and dam waters in South Africa. *Water Res.* **35:** 2653–2660.
30. Ward, R. L. and E. W. Akin. 1984. Minimum infective dose of animal viruses. *CRC Crit. Rev. Environ. Contam.* **14:** 297–310.
31. Wyn-Jones, A. P. and J. Sellwood. 2001. A review: Enteric viruses in the aquatic environment. *J. Appl. Microbiol.* **91:** 945–962.