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Molecular Detection and Characterization of Human Enteroviruses in Korean Surface Water

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In this study, the genetic epidemiology of enteroviruses (EVs) in Korean surface water was evaluated by conducting phylogenetic analyses of the nucleotide sequences of the 5' non-coding region (5' NCR), which was determined by RT-PCR analysis of total culturable virus assay-positive samples. The results showed that the nucleotide sequences of the EVs could be classified into 4 genetic clusters, and that the predominant presence of Korea EVs were very similar to echoviruses type 30. Interestingly, two nucleotide sequences were very similar to those of coxsackievirus type B1 isolated from aseptic meningitis patients in Seoul, Korea, implying the possibility of a common source for the viruses circulated in water systems and humans. In addition, 3 nucleotide sequences clustered strongly with the nucleotide sequences from China or Japan, and one fell into the same cluster as echovirus type 11 from Taiwan, which suggests that EVs in Asia may have evolved in a region-specific manner. Taken together, the results of this study revealed that EVs from Korea surface waters could be genetically classified as coxsackieviruses or echoviruses, and that they evolved in Asia in a region-specific manner.

***Keywords*:** phylogeny, enteroviruses, coxsackieviruses, echoviruses, 5' non-coding region

Enteroviruses (EVs) are non-enveloped single-stranded RNA viruses that belong to the *Picornaviridae* family. EVs are further divided into numerous serotypes, including poliovirus, coxsackievirus type A and B, echovirus, and EV 68-71 (Oberste *et al*., 2001; Papaventsis *et al*., 2005). It is well known that human EVs cause gastroenteritis, poliomyelitis, myocarditis, encephalitis, aseptic meningitis, and hand-foot and mouth diseases (Mark and Raymond, 2001). In addition, it is also known that these diseases have high morbidity and mortality, especially in infants and the elderly (Thapar and Sanderson, 2004).

Because EVs are found in aquatic environments and are transmitted by contaminated water via the fecal-oral route (Lawson *et al.*, 1991; Fout *et al*., 2003), EV-contaminated water can cause severe hygiene problems. For example, it has been reported that drinking water can be contaminated by waterborne viruses due to improper sewage treatment (Maurer and Sturchler, 2000; Brown *et al*., 2001), and that inadequate water treatment processes could lead to insufficient removal of waterborne viruses, leading to waterborne outbreaks (McCarthy *et al*., 1998; Boccia *et al.*, 2002). These outbreaks could, in turn, lead to hundreds of cases of gastroenteritis, which could then be followed by the secondary spread of viruses. The spread of viruses could then contaminate additional surface waters, thereby allowing this cycle of viral contamination to occur repeatedly (Lodder and De Roda Husman, 2005). Therefore, it is extremely important to survey water systems for viruses, as well to maintain adequate sewage or water treatment systems to prevent viral outbreaks.

Human EVs are genetically classified into two groups based on the 5' non-coding region (NCR), which is known to be the most conserved region among EVs (Oberste *et al*., 1999; Thoelen *et al*., 2004). Therefore, the NCR has been widely used as a target for the rapid diagnosis of EVs (Romero, 1999; Thoelen *et al*., 2003; Iturriza-Gómara *et al*., 2006; Lévêque *et al*., 2007) and as the basis for the classification of EVs and their epidemiological analysis (Caro *et al*., 2001; Dussart *et al*., 2005; Li *et al*., 2005; Siafakas *et al*., 2005; Richter *et al*., 2006; Hsu *et al*., 2007; Smura *et al*., 2007). For this reason, it is both reasonable and convenient to use the nucleotide sequences of the 5' NCR to survey water systems for EVs or to conduct phylogenetic analyses of them. However, to conduct a molecular biology-based epidemiological study and to track the sources of viral pollution, a number of nucleotide sequences of various EVs must be obtained and used to construct a database. Although substantial data regarding clinical strains of human EVs have been accumulated, data regarding waterborne EVs in Korea are very rare.

To investigate the genetic epidemiology of EVs and to track the sources of viral pollution, samples were collected from surface water, reverse transcription (RT) polymerase chain reaction (PCR) from total culturable viral assay (TCVA) positive samples and phylogenetic analysis were conducted.

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Materials and Methods

Source water samples

Source water samples (approximately 200 L) were collected from lakes or rivers in Korea between January and December 2004 and examined for waterborne enteric viruses using the United States Environmental Protection Agency (U.S. EPA) Information Collection Rule (ICR) microbial laboratory manual (1996). Briefly, each water sample was collected and passed through a 1-MDS filter (CUNO, USA) placed in a standard filter apparatus. Viruses were then eluted from the filters using 1 L of 1.5% beef extract (pH 9.5), after which they were concentrated by organic flocculation.

Total culturable virus assay (TCVA)

Buffalo green monkey kidney (BGMK) cells (kindly provided by Dr. Jeong, National Institute of Environment Research, Korea) of passage number between 140 and 150 were used. The BGMK cells were cultured at 37°C in a 5% CO_2 incubator in minimum essential medium (MEM; Gibco BRL^{TM}, Invitrogen Corporation, USA) supplemented with 5% fetal bovine serum (FBS; Gibco BRL^{TM}), Leibovitz's L-15 (Gibco BRL^{TM}), and penicillin (100 units/ml)-streptomycin (100 μg/ml) solution (Gibco BRL^{TM}). TCVA was based on the U.S. EPA ICR microbial laboratory manual (1996). Briefly, for cell culture, 0.7∼0.9 ml of the final concentrates from the source water samples were inoculated into monolayers of BGMK cells grown in T25 flasks (Nalge Nunc International, USA) at 37°C in a 5% CO_2 incubator. Fresh 2% FBS MEM was then added every 5∼7 days post infection. Cultures in which cytopathic effect (CPE) was observed were defined as viral positive. Sterilized 0.15 M sodium phosphate solution (pH 7.0∼7.5) in a volume equal to the sample inoculation volume was inoculated in a BGMK culture as a negative control. In addition, diluted attenuated poliovirus type 3 in 0.15 M sodium phosphate solution (pH 7.0∼7.5), which yielded a concentration of 20 plaque-forming units per inoculation volume, was used as a positive control. To reduce the cytotoxicity, the inoculum was decanted from the inoculated cell culture vessels following adsorption. Prewarmed washing solution (0.085% NaCl, 2% FBS) was then added to each vessel, and the cell monolayer was washed 2 times.

RT-PCR

RNA was extracted from TCVA-positive cell cultures using a QIAamp Viral RNA Mini Kit (QIAGEN, Germany). The extracted viral RNA was eluted in 50 μl of RNase-free water and then subjected to RT-PCR using the following primers: forward; 5'-caagcacttctgtttccccgg-3', reverse; 5'-attgtca ccataagcagcca-3'. RT was conducted at 45°C for 30 min and at 94°C for 3 min, after which the samples were subjected to 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 45 sec, followed by a final extension at 72°C for 7 min. RT-PCR was conducted using the GeneAmp PCR System 9700 (Applied Biosystems, USA). PCR products were resolved in 1.5% pre-made agarose gel (iNtRON Biotechnology, Inc., Korea) by electrophoresis. The PCR amplification fragments were then visualized and photographed using a gel image documentation and analysis system (ULTRA IMAGER BGI-6000, Ultra-Lum Inc., USA).

Nucleotide sequencing and accession numbers

The PCR-amplified fragments were purified using a QIAquick Gel Extraction Kit (QIAGEN) and then sequenced by automated DNA sequencing (ABI PRISM 377; Applied Biosystems). The obtained nucleotide sequences were then registered in NCBI GenBank under accession numbers DQ842181 to DQ842220. Reference sequences were selected from NCBI GenBank by using BLASTN 2.2.14.

Molecular phylogenetic analysis

To identify the EV genotypes, the 5' NCR sequences were compared to the corresponding EV 5' NCR reference sequences. The 5' NCR sequences were aligned using CLUSTAL W (version 1.82 at the European Bioinformatics Institute, http://www.ebi.ac.uk) and CLUSTAL X (version 1.83). Maximum likelihood (ML) trees were constructed with PHYLIP package version 3.61 using DNAML programs, and were viewed using the TreeView program. Nucleotide sequences variation was analyzed with DNADIST in PHYLIP package version 3.61.

Results and Discussion

Detection of EVs from surface water in Korea

One hundred and twenty two samples were obtained from surface water at 32 sites (24 sites from 19 lakes and 8 sites from 4 rivers) in Korea during 2004. The water samples were then concentrated and added to BGMK cell cultures

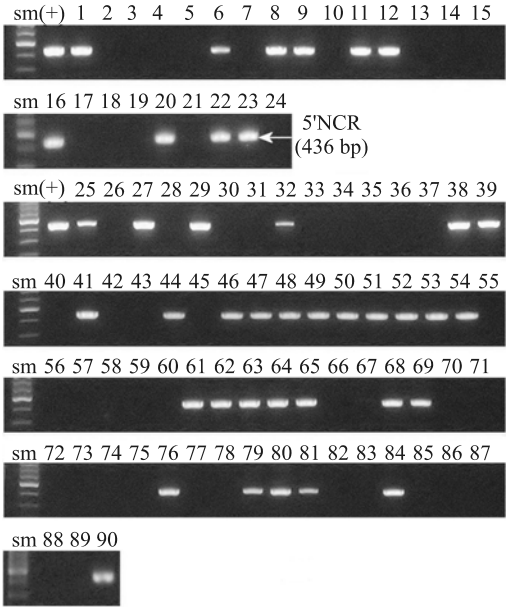

Fig. 1. Amplification of the 5' non-coding region (436 base pairs) of enterovirus by reverse transcription polymerase chain reaction of cell cultures from total culturable virus assay-positive samples. RNA was extracted from 90 TCVA-positive cell cultures. Integrated cell culture reverse transcription polymerase chain reaction was then conducted, and the products were resolved in 1.5% agarose gel and visualized using a gel image documentation system.

Table 1. Identical nucleotide sequences and their representative sequences

GenBank accession no.	Representative sequence
DQ842181, DQ842181, DQ842183, DQ842193	DQ842181
DQ842186, DQ842187, DQ842188, DQ842189, DQ842195, DQ842196, DQ842198, DQ842200, DQ842201, DQ842202, DQ842203, DQ842204, DQ8422005, DQ8422006, DQ842207	DQ842189
DQ842208, DQ842209, DQ842210, DQ842211, DQ842212, DQ842213, DQ842214	DQ842208
DQ842197, DQ842199	DQ842197
DQ842182, DQ842194	DQ842182
DQ842191, DQ842192	DQ842191

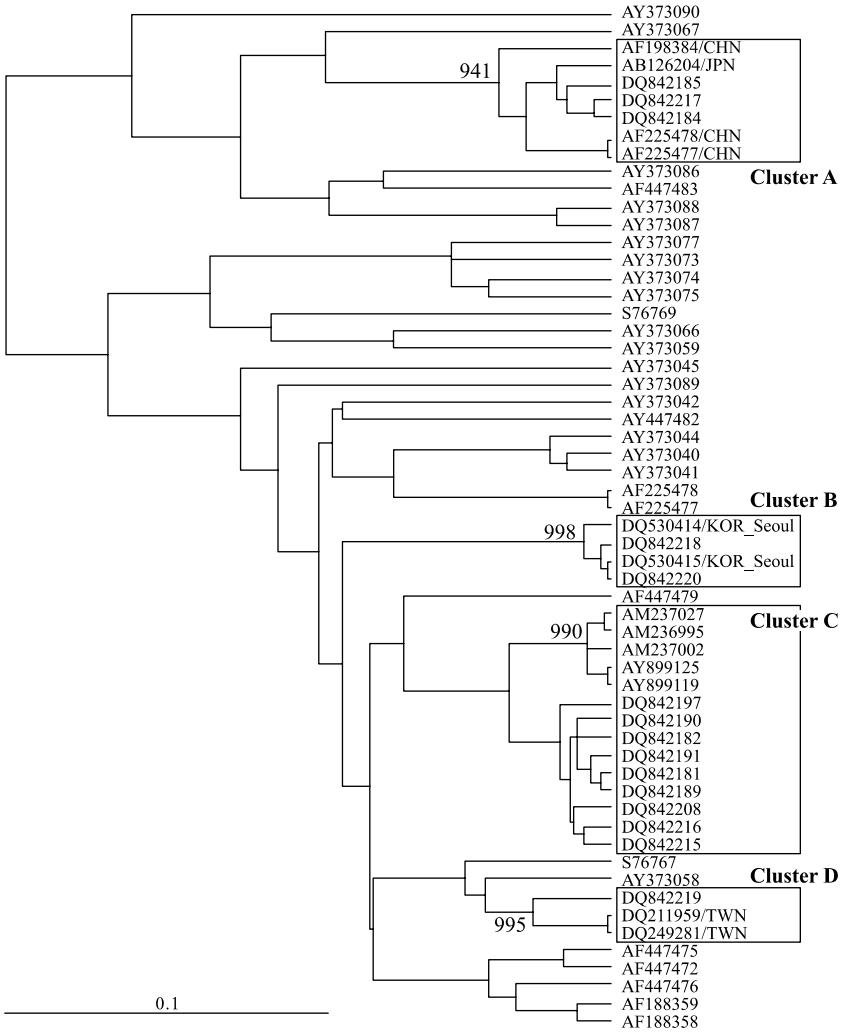

Fig. 2. Phylogenetic maximum likelihood tree of enteroviruses from Korea water samples. DQ842181 included DQ842181~DQ842183, DQ842193; DQ842189 included DQ842186~DQ842189, DQ842195~DQ842196, DQ842198, DQ842200~DQ842207; DQ842208 included DQ842208~DQ842214. CHN, China; JPN, Japan; KOR, Korea; TWN, Taiwan.

for TCVA. The overall viral occurrence was 31.1% (38 samples were viral-positive) and the average concentration of viruses was 3.2 most probable number/100 L. Overall, 90 of the 760 culture flasks (20 culture flasks per sample were analyzed by TCVA) exhibited CPE. RNA was extracted from these TCVA-positive cultures and used as a template for RT-PCR. Out of the 90 TCVA-positive cell cultures only 40 were amplified by RT-PCR using primers specific for the 5' NCR of EV (Fig. 1). EV RNA was not detected in 50 of the viral-positive cell cultures, even though they exhibited CPE in BGMK cell cultures. This finding suggests that viruses other than EVs were present or that there are dif-

ferences in viral susceptibility to BGMK cells among viruses (Chonmaitree *et al.,* 1988; Kok *et al.,* 1998; Lee *et al.,* 2004).

Phylogenetic analysis of EV

In order to investigate the genetic epidemiology of EVs present in the surface water of Korea, the nucleotide sequences of the 5' NCR of EV were determined using the 40 obtained PCR products. The sequences were then registered in GenBank under accession numbers DQ842181 to DQ842220. These sequences were then subjected to phylogenetic analysis with 43 reference sequences. The reference sequences were selected by conducting an NCBI BLASTN search using the Korean sequences determined in this study and then selecting sequences with a high homology to the sequences generated in this study. Because some of the sequences were 100% homologous (for example, DQ842181, DQ842181, DQ842183, and DQ842193), we selected a representative nucleotide sequence for phylogenetic analysis (Table 1). A phylogenetic tree was then constructed using the ML method. Our sequences formed 4 distinct clusters (A, B, C, and D; Fig. 2) with high bootstrap values (>940),

Table 2. Percentage of Asian countries as the viral sources of the nucleotide sequences of 100 blast hits in the GenBank database

Clusters	The query sequences	Countries of viral source among 100 blast hits on the query sequences					Asian countries (%)	Average percent (%)
		KOR	CHN	JPN	TWN	MAL[a]		
A	DQ842184	3	16	8	29	1	57	53.0
	DQ842185	3	16	9	31	2	61	
	DQ842217	4	12	3	22	0	41	
B	DQ842218	10	2	8	0	0	20	20.5
	DQ842220	10	2	9	0	0	21	
D	DQ842219	8	0	7	6	0	21	21.0

[a] Malaysia

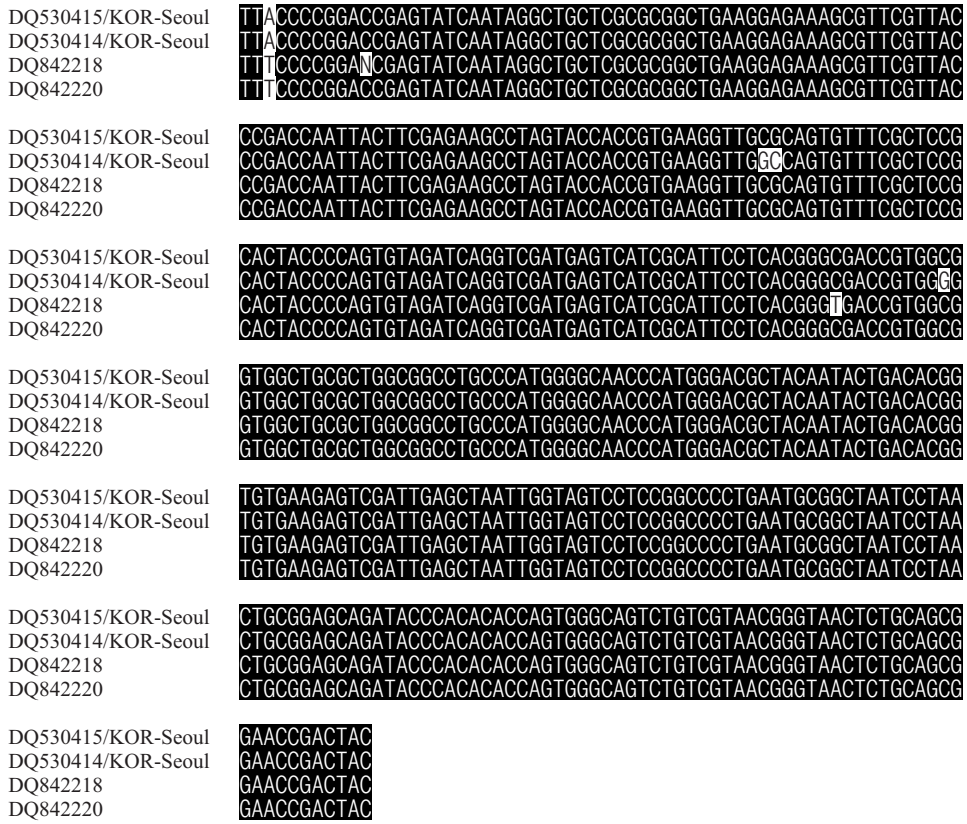

Fig. 3. Alignment of partial nucleotide sequences of the 5' non-coding region of DQ842218, DQ842220, DQ530414, and DQ530415. Four partial nucleotide sequences of the 5' non-coding region from two isolates from Seoul, Korea (DQ530414, DQ530415) and two from this study (DQ842218, DQ84222) were aligned and viewed with CLUSTAL W (version 1.82) and BOXSHADE (3.21). Light gray squares indicate nucleotide sequence variants.

Table 3. Nucleotide sequence variation among the 4 clusters (Mean±SD%)

	Cluster A	Cluster B	Cluster C	Cluster D
Cluster A	**3.3 ± 1.9**	–	–	–
Cluster B	15.2 ± 1.0	**0.5 ± 0.4**	–	–
Cluster C	13.0 ± 1.7	8.1 ± 0.8	**2.6 ± 1.9**	–
Cluster D	14.4 ± 1.4	7.2 ± 0.7	6.0 ± 0.7	**2.6 ± 2.0**

(P-value<0.0000156)

which suggests that the clusters were independent of each other.

As shown in Fig. 2, DQ842184, DQ842185, and DQ842217 formed cluster A with the sequence of coxsackievirus type B6, which was isolated from a patient in China and with the sequence of coxsackievirus type A9, which was isolated in Kanagawa, Japan, in 2003. DQ842219 was included in cluster D, which also included the nucleotide sequences of echovirus type 11, which was isolated in Taiwan. These results indirectly demonstrate that EVs in Asian countries evolved in a region-specific manner, and that there is a very close relationship among their sequences. It has been reported that viruses, including EVs such as coxsackievirus and echoviruses, evolved with other regional characteristics of the Asian region, including Korea (Cheon *et al*., 2004; Li *et al*., 2004; Dussart *et al*., 2005; Li *et al.*, 2005; Park *et al*., 2006). Therefore, a BLASTN search was conducted using the obtained nucleotide sequences, and the result showed that approximately 31.5% of the nucleotide sequences obtained in 100 blast hits were from Asian countries (Table 2).

It is interesting to note that sequences DQ842218 and DQ842220, which were in cluster B, were very similar to those of coxsackievirus type B1, which was isolated from aseptic meningitis patients in Seoul, Korea (Fig. 2). In particular, DQ842220 was identical to coxsackievirus type B1 Seoul-19 (DQ530415), with the exception of 1 base pair (Fig. 3). This result suggests that the viral sources of coxsackievirus type B1 isolated from a patient and DQ842220 in water systems possibly had the same genotype, which would indirectly demonstrate viral secondary spread into water systems.

Cluster C was comprised of 34 nucleotide sequences of the 5' NCR of EV, including those of echovirus type 30. The variation of nucleotide sequences in the cluster was analyzed to determine whether the sequences in cluster C clearly differed from those of the other clusters. The variation of the nucleotide sequences in each cluster was calculated using DNADIST in PHYLIP version 3.61. As described in Table 3, the value of the intra-group variation in the cluster C sequences was found to be 2.6±1.9, which was significantly lower than those of the inter-group variation between the sequences in cluster C and other clusters (13.0±1.7 for clusters C-A; 8.1±0.8, for clusters C-B; and 6.0±0.7 for clusters C-D). These findings imply that the sequences in cluster C clearly differ from those in the other clusters, and that these sequences possibly originated from echovirus type 30. Several studies were conducted to evaluate echovirus type 30, and a local case of aseptic meningitis caused by this virus has been reported (Joo *et al*., 2005; Faustini *et al*., 2006; Zhao *et al.*, 2006).

Water treatment systems are very important for preventing the outbreak of waterborne enteric viruses. In addition, it is extremely important to survey, prevent, and control the spread of viruses in water sources. Viral genotyping is less time consuming and can identify a wider range of genotypes than traditional methods; therefore, the results of this study indicate that conducting a molecular phylogenetic analysis and molecular evolution studies using viral genotyping can be very useful for surveying and tracking viruses in water systems. However, further studies should be conducted to obtain more nucleotide sequences of EV to generate a database for the evolutionary study of these viruses and to allow continuous monitoring, control, and prevention of the spread of viruses.

In conclusion, in this study, ICC RT-PCR was used to obtain 40 nucleotide sequences of the 5' NCR, which were subsequently identified as coxsackieviruses and echoviruses by phylogenetic analysis. In addition, the results of this study indicate that EVs in Asian countries evolved in a region-specific manner and had a very close relationship among their nucleotide sequences. Furthermore, two nucleotide sequences were very similar to those of coxsackievirus type B1, which was isolated from aseptic meningitis patients in Korea. This finding may indirectly demonstrate that viral secondary spread into water systems has occurred.

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