S3-5

DISTRIBUTION OF VIRUSES IN WATER ENVIRONMENT

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Summary

We investigated the viral contamination of water environment including tap water in Korea. River water used for source water was analyzed about monthly between 1997 and 1999 over a period 26 months. A total of 22 tap water samples were collected in 10 sites in 2 urban areas between 1997 and 1998 over a 11 months. All samples were examined for infectious enteroviruses and adenoviruses by a cell culture technique followed by PCR amplification. To identify the recovered viruses from tap water, sequence analysis of PCR products was performed. Infectious viral particles were detected in river water all year round, ranging from 0.93 to 17.3 Most Probable Number of Infectious Unit (MPNIU) /100L. Tap water samples also contained infectious viral particles. The frequency of enteroviruses and adenoviruses in tap water were 50.0% (11/22) and 36.7% (8/22), respectively. Both enteroviruses and adenoviruses were detected in five tap water samples (22.7%). The level of viral contamination in tap water was quite high, ranging from 0.2 to 2.9 MPNIU/100L, far above the recommended virus level in drinking water set by the U.S. EPA. Poliovirus type 1 derived from vaccine was frequently detected and the remainder comprised coxsackievirus B type or echovirus type 6, which were causative agents of aseptic meningitis in Korea in 1997 and 1998, respectively. Several types of adenovirus were detected in tap water samples and some water samples were found to contain adenoviruses which were closely related to enteric adenovirus type 40 and 41. This stusy shows that surface water and tap water in Korea may be exposed to the risk of viral contamination, especially from recently recognized viruses and this constitutes a potential public health hazard.

Introduction

Enteric viruses are excreted in large numbers in feces and have been found in surface water, ground water and even in the treated drinking water (6, 10). Their presence in these waters is a public health concern because even at low concentrations they can cause illness when ingested (35). Generally, microbiological water quality is assessed using bacterial indicators such as heterotrophic bacteria and coliforms. These parameters are also accepted for evaluating water quality in Korea. However, they do not seem to properly reflect the risk from viruses. Previous studies have repeatedly revealed the presence of enteric viruses in finished water meeting standards for coliform bacteria and that several outbreaks were caused by tap water contamination associated with viruses in spite of good compliance with water treatment procedures (6, 10).

In addition, the quality of surface water and tap water in Korea is very low. The BOD value of surface water ranges from 0.8 to 7.2 mg/L and thus in some areas, since the pollution level of surface water is very high, the water is not adequate for using source water. The high BOD value is due to the low rate of wastewater treatment, particularly in live-stock wastewater treatment (19). In the case of tap water, there are some reports that fecal coliforms and injured coliforms were detected in many household tap water samples with high concentrations of residual chlorine (0.8 mg/L) and even in the finished water immediately after chlorine treatment at the water plant in the Seoul metropolitan area of Korea (18, 24). In Korea, almost every year, enteric viral disease such as gastroenteritis and aseptic meningitis have also been prevalent (12, 13, 22).

The situation in Korea described above indicates that there is a possibility of viral presence in and viral infection through the drinking water. However, there are few reports on the virus level in the water environment in Korea. The limited data on viral occurrence in water makes it

difficult to determine the risks of viruses and this state of affairs precludes the preparation of preventative plans against viral transmission through the water environment. Therefore, to prevent diseases induced by enteric viruses, a survey of viral contamination in the water environment including tap water, which is used as drinking water, is needed.

Among enteric viruses, the enteroviruses and adenoviruses have been known to be prevalent in sewage and polluted waters (5, 8, 9, 25, 33) and thus these viruses are accepted as viral indicator. Enteroviruses comprise more than 70 distinct serotypes of human pathogens, and are known to be the main causative agent (> 85%) of aseptic meningitis (3, 21). Nevertheless, vaccinations are not available for many serotypes, with the exception of the poliovirus, so the prevention of diseases caused by these viruses is very difficult (21). Adenoviruses also comprise many serotypes. In particular, adenovirus subgenus F (Ad 40/41) has been recognized as an agent of gastroenteritis and there is a suggestion that these viruses are a major cause of undocumented waterborne disease (8). In this respect, enteroviruses and adenoviruses are not only indicators but also pathogenic viruses themselves. In addition, the seasonal distributions of cultivable enteroviruses and adenoviruses in sewage and polluted river water have been shown to be different from each other (31). Therefore, the simultaneous detection of adenoviruses and enteroviruses could indicate the presence of a broader range of pathogenic viruses. To detect two viruses simultaneously, we develop and apply the multiplex PCR including a reverse transcription step followed by nested multiplex in the previous study (6).

In this study, the simultaneous detection of adenoviruses and enteroviruses was performed with cell culture followed by PCR amplification to determine the viral contamination in tap water in urban areas in Korea. To identify the recovered viruses from tap water, sequence analysis of PCR products was performed

Materials and Methods

River water and tap water sample collection

The river water samples were collected about monthly over 26-months period (Nov.1997 - Dec.1999) from two different sites (PD, JS) located in mainstream on the Han river. The yearly average values of BOD, SS, and total coliforms of the year 1997 were 1.5, 8.2 mg/L, and 6.0×10^3 MPN/100mL for PD, 2.6, 8.8 mg/L, and 1.4×10^3 MPN/100mL for JS, respectively (20). A total of 22 tap water samples were collected in ten sites in two urban areas in Korea from September, 1997 to July, 1998. All areas are in cities that harbor populations above one million and in particular, area one is the Seoul metropolitan city whose population is above 10 million. The tested tap water was processed through flocculation/sedimentation, filtration, and chlorination. All sampling sites were faucets in private household except site 6 in area one, faucet in our laboratory. All sampling sites were above 10 km distant from the water plant and in particular, most sites were the end points in the distribution system. Tap water samples were tested for the presence of coliform bacteria with membrane filtration method and residual chlorine was measured according to the N, N diethyl-p-phenylenediamine (DPD) colorimetric method (2).

Sample concentration.

Seventy to three hundred liters of water were concentrated by filtration through a 1MDS filter (CUNO Inc., Meriden, Conn.). Pre-filters (5 and 1 micrometer cartridge filters) were used to prevent clogging of the virus adsorption filter with the particles present in the river water samples. The collection of virus in water samples was performed according to the ICR (Information Collection Rule) method (32) with minor modification in secondary concentration step. Secondary concentration was performed as described by Lewis and Metcalf (15). The viruses adsorbed to the filter were eluted with 0.05 M glycine buffer, pH 9.5, containing 1.5% beef extract. The eluates were immediately adjusted to neutral pH with 1 N HCl. The 1-l

volumes of eluate were incubated overnight with gentle stirring at 4℃ after adding 13% (w/v) polyethylene glycol (PEG) 8000 (plus 0.2 M NaCl) at pH 7.2. After incubation, the eluates were centrifuged at 7,000 ×g for 30 min. The resulting pellets were resuspended in 20-30 ml of 10 mM phosphate buffered saline (PBS). The resulting sample was filtered through a 0.2 μm pore-size filter and stored at -70℃ until it was used for cell culture analysis.

Cell culture assay of water concentrates

For each sample, a 0.8 ml portion of the final concentrate was inoculated into each of five individual tissue culture dishes of 4-day-old BGM cells, each with a growth area of 17.5 cm^2. Tissue culture dishes were incubated at 37℃ for 2 weeks. Poliovirus type 1 strain (CHAT strain) stock was used as positive control and phosphate buffered saline (PBS) solution was used as negative control. Following primary passage and assay, all cell culture samples were frozen and thawed three times and secondary passages on fresh monolayers of BGM cells were performed. Positive cell culture samples were confirmed using RT multiplex PCR for adenoviruses and enteroviruses on the cell culture lysates. The virus concentrations were calculated by using the MPN software program supplied by the U.S. EPA.

Reverse transcription (RT)

Prior to PCR amplification, virus was purified from cell culture lysates as described by Reynolds *et al.* (27), except that Sepadex G-50 columns was used. The resulting supernatant was immediately subjected to RT. The oligonucleotide primer sequences used for the detection of adenoviruses and enteroviruses were identical to those described by Allard *et al.*, and Leparc *et al.*, respectively (1, 14). The target viral genome was heat released, and reverse-transcribed. Five microliters of cell culture lysates were added to 5 μl of RT mixture consisting of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 1.25 μM antisense primer, 50 U M-MLV reverse transcriptase (Promega), and 10 U RNasin. RT was carried out at 42℃ for 45 min and then the tubes were heated to 95℃ for 5 min to inactivate the enzyme.

Multiplex nested PCR

The completed RT reaction (10 μl) was mixed with 40 μl of PCR mixture (final concentration: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton-X-100, 200 μM each dNTP (Promega), 1.25 U of *Taq* Polymerase (Promega), 0.25 μM of the enterovirus primer (EV1), 0.1 μM of the adenovirus primer pair, and 1.5 mM $MgCl_2$). The PCR was carried out by the following protocol: initial denaturation step at 94℃ for 4 min; 35 cycles, with 1 cycle consisting of 30 sec at 95℃, 30 sec at 55℃, and 1 min at 72℃; final extension step at 72℃ for 7 min. For the nested PCR amplification, one microliter was taken from a first round amplification and added to a new batch of 30 μl PCR reaction mixture containing 0.25 μM of the enterovirus semi-nested primer pair and 0.1 μM of the adenovirus nested primer pair. Amplifications were carried out in the same conditions as used for the first amplification.

Quality control of the amplification method

To avoid false positive results due to contamination with amplified DNA by the previous PCR, separate areas and apparatus were used for sample preparation, reagent preparation, and amplified samples. Virus-seeded positive controls (0.1 $TCID_{50}$ adenovirus and 0.1 $TCID_{50}$ enterovirus) as well as negative controls were incorporated in all PCR assays to ensure the propriety of the PCR assay. Negative controls were used to ensure the absence of carryover contamination. Positive controls were incorporated because false-negative results can arise from various causes such as the loss of templates in nucleic acid purification, contamination of RNases, or insufficient removal of PCR inhibitors.

Sequencing of PCR product

In order to characterize the virus type, the nested PCR products of adenoviruses and enteroviruses from water samples were sequenced. The purified nested PCR products were ligated into the pGEM-T vector (Promega Co., Madison, WI) and transformed into *Escherichia coli* DH5α competent cells. Sequentially, blue/white screening was performed according to the manufacturer's instructions. Plasmid preparations for DNA sequencing were made with Wizard mini-Preps (Promega Co., Madison, WI). All the clones (clones of adenovirus and clones of enterovirus) were sequenced by the chain termination method on an ALFexpress DNA autosequencer (Pharmacia Biotech, Uppsala, Sweden) using the T7 primer and the Cy5 AutoRead sequencing kit. The sequences were compared with those available in the EMBL/GenBank database.

Results and Discussion

Sensitivity of the RT multiplex PCR assay

In the previous study (6), we showed that the sensitivity of the RT multiplex PCR assay on adenovirus and enterovirus was the same (0.01 $TCID_{50}$) as that determined by each monoplex PCR assay and the detection limit of each viral species was not affected by the amount of nucleic acids of the other viral species, and when the method was applied to environmental water samples, the results of multiplex assay were generally similar to those of monoplex assay.

Viral analyses on river water and tap water samples

The presence of infectious viruses was determined through cell culture followed by PCR. The levels of viruses in river water samples were from 0.93 to 17.3 MPNIU/100L in PD or JS (Fig. 1). These results were comparable to other results showing (9). There was no seasonal distribution of viral presence in river water. Viruses were detected in surface water all year round. These results showed that viral contamination of surface water always occurred. The level of virus was not different from each sites significantly. However, the frequency of viral occurrence is higher in JS than that in PD.

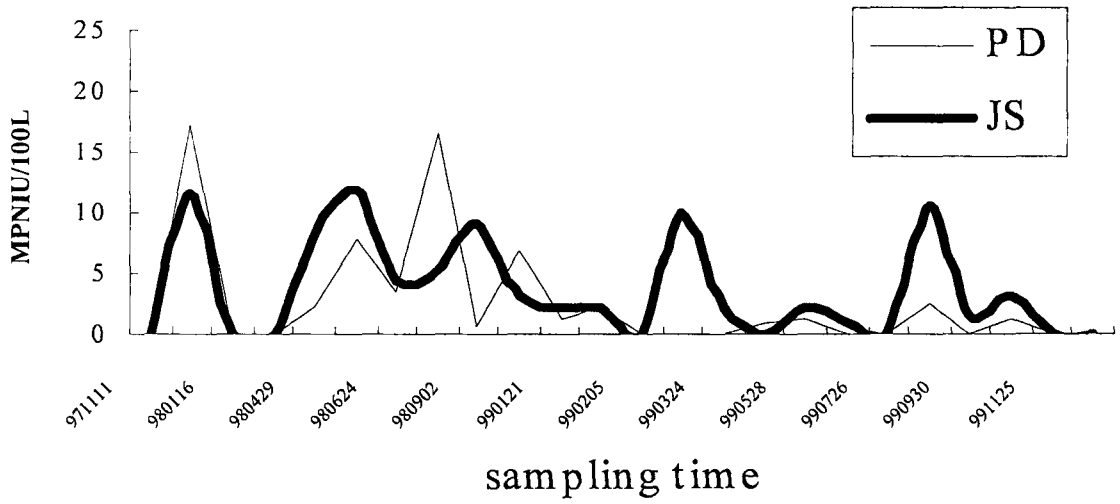

Fig. 1. The seasonal distribution of enteric viruses in surface water

We found the presence of infectious viruses in 14 (63.6%) of 22 tap water samples. Enteroviruses and adenoviruses were detected in 11 (50.0%) and 8 (36.7%) of the tested water samples, respectively. Both enteroviruses and adenoviruses were detected in five samples (22.7%). The virus level was mostly below 10 MPN infectious units per 1000 liters in all areas (Table 1). No tap water samples did not contain coliform bacteria and residual chlorine was not found in all samples.

The frequency of viral contamination was very high compared with other studies, which showed a rate of occurrence below ten percent (10). Viruses were detected almost every month at one site in the Seoul area. This showed that the tap water at this site was chronically exposed to viral contamination. Two samples from the Seoul area showed a high level of viral contamination above twenty MPNIU per 1000 liters (Table 1). It is interesting to note that these two samples were collected in the summer season (June and July, 1998). In fact, it is not confirmed that this level of viral load in tap water can cause infection in humans, since the minimum infectious dose of virus for humans has not been determined. However, risk assessment study of rotavirus has shown that one infectious unit of virus could cause infection (34) and the study of adenoviruses showed that annual risks of infection inrecommended that drinking water should not present a risk of infection >1:10000 /year (17), suggesting that the viral load of the tap water in this study is significantly harmful to human health.

Although this study examined samples from a limited geographical area and the number of tested samples was small, the percentage of viral occurrence in tap water was high compared to other studies and the enteric adenovirus was detected in tap water.

The reason for the high quantity of viruses in tap water is not clear. Although no coliforms were not detected in tap water samples of this study, however, the previous studies revealing the bacterial contamination of tap water produced at the same water plant in this study (18) indicates the insufficient water treatment process, particularly in disinfection process. The injured bacteria are detected even at the start point of distribution system (18) and also in this study, free chlorine residue was not found in all samples. In addition, contamination with

Table 1. Detection of enteroviruses and adenoviruses in tap water samples by the integrated cell culture-PCR.

Sampling site	Sample No.	Sampling time (day/mo/yr)	Virus conc. (MPNIU/1000L)	Virus type[b]
Area 1 (Seoul)				
Site 1	1101	7/10/97	9	PV1[c]
	1102	8/10/97	ND[a]	
	1103	9/10/97	ND	
Site 2	1201	10/10/97	9	PV1
	1202	11/10/97	ND	
Site 3	1301	14/10/97	2	PV1
Site 4	1401	8/4/98	ND	
Site 5	1501	24/6/98	ND	
Site 6	1601	22/9/97	ND	
	1602	13/10/97	3	PV1
	1603	14/10/97	5	CVB[d]
	1604	26/11/97	9	PV1
	1605	19/12/97	6	PV1, NEAd[e]
	1606	20/1/98	6	NEAd, EAd[f]
	1607	15/3/98	ND	
	1608	8/4/98	3	PV1, NEAd, EAd
	1609	18/6/98	20	NEAd, EAd
	1610	31/7/98	29	NEAd, EAd
Area 2 (Pusan)				
Site 1	2101	1/5/98	11	PV1, EAd
Site 2	2201	1/5/98	6	CVB or EC6[g], NEAd, EAd
Site 3	2301	1/5/98	5	PV1, EAd
Site 4	2401	1/5/98	ND	

[a] ND, Not detected.
[b] virus type was determined by sequence analysis with clones of cell culture-PCR products.
[c] poliovirus type 1, [d] coxsackievirus type B, [e] non-enteric adenovirus (Ad 1 or Ad 5 or Ad 6).
[f] enteric adenovirus (Ad 40 or 41), [g] echovirus type 6.

viruses and bacteria were not correlated, as showed by recent study in tap water (30) This situation and the present data suggest that either the water treatment processes are considered to be insufficient for the removal and inactivation of enteric viruses or the distribution system of tap water is exposed to unusual contamination originated from the leakage of distribution system (19).

The distribution of adenoviruses and enteroviruses is different, namely, the co-occurrence of the two virus groups was not easily detected. This is due to the growth pattern and the survival of each group. Viral proliferation may be affected by competition between each virus (29). It seems likely that cell culture with BGM selects for enteroviruses that replicate more rapidly than adenoviruses, therefore adenoviruses were infrequently detected in the presence of enteroviruses. The survival rate of adenoviruses, however, is higher than that of enteroviruses; adenoviruses were frequently detected in samples without enteroviruses (31).

Analysis of the sequences of the adenoviruses and enteroviruses detected

Sequence analysis of the PCR product demonstrated that for enteroviruses, all the clones except for two were similar to type 1 poliovirus and most likely vaccine related. The remainder two clones were similar to coxsackievirus B type or echovirus type 6 (Table 1). In the case of adenoviruses, seven clones were similar to enteric adenovirus (Ad 40/41) and eight clones were found to be non-enteric adenoviruses (Table 1).

The types of virus found in this study were similar to the causative agent of enteroviral disease in Korea (12). In particular, echovirus type 6 was reported to be the main cause (47.4%) of aseptic meningitis in 1998 in Korea (12). Previous study showed that adenovirus type 5 was frequently detected in the water environment (31). This may be due to the high load of adenovirus type 5 excreted from patients. It was documented that adenovirus type 5 accounted for 11 percent of all adenovirus strains reported to the World Health Organization (28).

The reason for the presence of enteric adenovirus in tap water is supposed that enteric adenoviruses can survive and persist longer than other enteric viruses in tap water (8). It is a very interesting result that Ad 40 and 41 were recovered through cell culture assay with BGM cell because Ad 40 and 41 have been recognized as fastidious viruses and could be cultivated with only few cell lines (CaCo-2 or PLC/PRF/5) (26). One explanation of this phenomenon is that Ad 40 and 41 could infect the cell line but not propagate sufficiently to produce progeny viruses and showed no cytopathic effect. Brown *et al.* (4) indicated that a high particle-to-infectious unit ratio contributed to poor growth of enteric adenovirus types 40 and 41 in cell culture.

The reason for the frequent isolation of polioviruses is supposed that strains of vaccine viruses are always excreted from vaccinees. The oral poliovirus vaccine is a source of dissemination of polioviruses, which discharge into the water environment. The nucleotide sequences of the PCR product from each sample differed slightly from each other.

There is a possibility that the level of viral contamination of water samples tested in the present study is higher than the estimated value. The viral detection method in water samples has been continuously improved and enables many researchers to monitor viral contamination in water samples. Nevertheless, the present detection technique has several limitations (2). The efficiency of virus recovery after filtration of the water samples through electropositive filters differs according to the type of sample and target virus (16). In addition, as our results also showed, mixed infections involving several enteric viruses are not uncommon (23). Finally, there are many types of enteric viruses besides enterovirus and adenovirus. Among them, there are uncultivated viruses such as caliciviruses, HEV (11, 36).

Overall, it is concluded that surface water and tap water in Korea may be exposed to the risk of viral contamination, especially from recently recognized viruses and this constitutes a potential public health hazard. To prevent waterborne diseases, further studies should be carried out with a broad range of samples and a wide spectrum of target viruses.

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