Detection of adenoviruses and enteroviruses in tap water and river water by reverse transcription multiplex PCR

Hong Baek Cho, Seung-Hoon Lee, Jang-Cheon Cho, and Sang-Jong Kim

Abstract: A reverse transcription (RT) multiplex polymerase chain reaction (PCR) assay was developed to simultaneously detect adenoviruses and enteroviruses, both of which have attracted much attention as molecular indices of viral pollution in environmental samples. The method involves a reverse transcription step, followed by a multiplex nested PCR in which the combination of primers amplifies cDNA from enteroviruses and adenoviruses. The sensitivity of this assay was found to be similar to that of each monoplex PCR or RT-PCR assay, and to be consistent regardless of relative concentrations of adenoviruses and enteroviruses. To assess suitability and environmental application of the RT multiplex PCR assay, a total of 12 river water samples and 4 tap water samples were analyzed by RT multiplex PCR, each monoplex PCR or RT-PCR, and cell culture assay on the Buffalo Green Monkey kidney cell line. The sensitivity of the RT multiplex PCR was also found to be similar to that of each monoplex PCR in environmental samples. This suggests the RT multiplex PCR assay could be applied to the routine monitoring of viral pollution in environmental waters.

Key words: adenoviruses, enteroviruses, multiplex PCR, tap water.

Résumé : Un test utilisant la réaction en chaîne de la polymérase (PCR) multiplex et la transcription inversée (RT) a été mis au point pour détecter simultanément les adénovirus et les entérovirus qui reçoivent beaucoup d'attention comme indicateurs de pollution virale dans les échantillons environnementaux. Ce test comprend une étape de transcription inversée suivie d'une PCR multiplex nichée où une combinaison d'amorces permet d'amplifier l'ADNc à partir de l'ADN des entérovirus et des adénovirus. La sensibilité de ce test était la même que celle de chaque PCR monoplex ou RT-PCR et il demeurait reproductible peu importe les concentrations relatives d'adénovirus ou d'entérovirus. Pour vérifier l'applicabilité de ce test PCR multiplex RT au domaine environnemental, un total de 12 échantillons d'eau de rivière et de 4 échantillons d'eau du robinet ont été analysés par PCR multiplex RT, chacune des PCR monoplex ou RT-PCR et par culture sur des cellules de rein du singe vert de la lignée Buffalo. Dans ces échantillons, la sensibilité de la PCR multiplex RT était la même que celle de chaque PCR monoplex. Les résultats obtenus suggèrent que la PCR multiplex RT pourrait être utilisée de routine pour mesurer la pollution virale dans les eaux environnementales.

Mots clés : adénovirus, entérovirus, PCR multiplex, eau potable.

[Traduit par la Rédaction]

Introduction

Enteric viruses are excreted in large numbers in feces and have been found in surface water, ground water, and even in treated drinking water (Gerba and Rose 1990; Keswick et al. 1984). Their presence in these waters is a public health concern, because even at low concentrations, they can cause illness when ingested (Ward and Akin 1984). However, current microbiological parameters of water, which consist of bacterial indicators, do not seem to reflect the risk from viruses properly because viruses are generally more resistant to treatment processes (Sobsey 1989), survive longer than bacteria in natural environments (Berg and Metcalf 1978), and seasonal distribution of many types of virus are significantly different from bacterial indicators (APHA 1995). Therefore, there is a clear need for monitoring virological quality of water.

The traditional detection of enteric viruses in environmental samples involves cell culture (APHA 1995), which is expensive, labor-intensive, and time-consuming. Moreover, many important enteric viruses such as caliciviruses or hepatitis A virus cannot be detected, or can be detected only with great difficulty. Due to these limitations, much research has been directed to the detection of viral nucleic acid, and the detection of viruses by PCR. Although it cannot be used to determine the infectious state of the detected viruses, it is

Received August 9, 1999. Revision received November 15, 1999. Accepted January 28, 2000.

H.B. Cho, S.-H. Lee, J.-C. Cho, and S.-J. Kim.[1]
Department of Microbiology, College of Natural Sciences, Seoul National University, Kwanak-Gu San 56–1, Seoul 151–742, South Korea.

[1]Author to whom all correspondence should be addressed (e-mail: sjkimm@plaza.snu.ac.kr).

employed extensively nowadays because of its sensitivity, low cost, and short assay time.

Analysis of water samples for viral pollution has been directed toward the detection of enteroviruses, since most of them can be detected by traditional cell culture methodology. Enteroviruses have also been used as targets of PCR assay for the assessment of viral pollution, since they are well characterized for the nucleic acid-based detection methods, and have been shown to be prevalent in sewage and polluted waters (Castignolles et al. 1998; Gantzer et al. 1998; Kopecka et al. 1993; Pina et al. 1998; Puig et al. 1994). Although enteroviruses have been widely used to assess the virological quality of environmental waters, there have been some reports showing that the presence of enteroviruses did not correlate well with the presence of important pathogens such as hepatitis A virus in some environmental samples (Dubrou et al. 1991; Pina et al. 1998). Recently, the detection of adenoviruses by PCR methodology has attracted much attention in the evaluation of viral water quality, because they are also well characterized for the nucleic-acid-based detection methods, more stable in various environments (including wastewater, seawater, and tap water) than enteroviruses, and have been detected more prevalently in sewage and polluted waters than enteroviruses by PCR methodology (Castignolles et al. 1998; Enriquez et al. 1995; Puig et al. 1994; Vantarakis and Papapetropoulou 1998). 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 (Irving and Smith 1981; Krikelis et al. 1984; Tani et al. 1995). Therefore, the simultaneous detection of adenoviruses and enteroviruses could indicate the presence of a broader range of pathogenic viruses.

Conventional PCR uses one set of primers and can detect only one target sequence in a sample. Multiplex PCR uses several primer pairs, and allows the simultaneous detection of different virus groups. This technique has mainly been used on clinical samples (Casas et al. 1997; Ellis et al. 1997; Pozo and Tenorio 1999; Stockton et al. 1998). A few studies (Tsai et al. 1994) have also been dedicated to multiplex PCR for the simultaneous detection of different enteric viruses in environmental samples. In this report, we describe the development and application of multiplex PCR, including a reverse transcription step followed by nested multiplex PCR for the simultaneous detection of adenoviruses and enteroviruses. This RT multiplex PCR assay provides a rapid and cost-effective way to simultaneously detect adenoviruses and enteroviruses, both of which are attractive as molecular indices of viral contamination.

Materials and methods

Virus strains

Poliovirus type 1(Chat strain) and adenovirus type 5 were propagated in Buffalo Green Monkey kidney (BGM) cells, and PLC/PRF/5 cells growing in minimum essential medium (Gibco BRL, Life Technologies) containing 10% fetal bovine serum, respectively. Virus titration was carried out by the tissue culture infectious dose fifty ($TCID_{50}$) method in 96-well plates (Payment and Trudel 1993).

River water and tap water sample collection

The river water samples were collected quarterly over a one-year period (April 1998 – February 1999) from three different sites on the Han river. Two sampling sites (PD, JS) were located in mainstream, and the other one (WS) was located in the tributary flowing into the Han river between PD and JS (Fig. 1). These sites were selected because all water intakes for the tap water supply of the Seoul metropolitan area are located between PD and JS. 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; 3.2, 10.9 mg/L, and 2.6×10^5 MPN/100mL for WS; respectively (Ministry of Environment, Republic of Korea 1998). 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. Tap water samples were collected in our laboratory four times from December 1997 – July 1998. The tap water was originally taken from the PD site, and processed through flocculation/sedimentation, filtration, and chlorination, but no residual chlorine was detected in the tap water. One thousand to three thousand liters of water were concentrated without using pre-filters.

Filter elution and concentration

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 M HCl. The 1-L volumes of eluate were incubated overnight with gentle stirring at 4°C, 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 7000 × *g* for 30 min. The resulting pellets were resuspended in 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°C until it was used for cell culture analysis or nucleic acid extraction.

Cell culture assay

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°C for 2 weeks. 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. Environmental Protection Agency.

Nucleic acid extraction

Nucleic acid extraction was performed according to the method of Boom et al. (1990) with minor modifications. Twenty-five microliters of viral suspension was added to 20 μL of the silica matrix solution (Bioneer Co., Chungbuk, Korea) and 450 μL of lysis buffer (120 g of GuSCN in 100 mL of 0.1 M Tris-HCl, pH 6.4, with 22 mL of 0.2 M EDTA adjusted with NaOH to pH 8.0 and 2.6 g of Triton X-100 added), left for 10 min at room temperature, and washed twice in 500 μL of washing buffer (120 g of GuSCN in 100 mL of 0.1 M Tris-HCl, pH 6.4), twice more with 70% ethanol, and once with acetone. The pellet obtained after the complete evaporation of acetone was resuspended with 25 μL of diethyl pyrocarbonate-treated deionized water containing 10 mM dithiothreitol (Promega, Madison, Wis.) and 1 U/μL RNasin (Promega). The resulting supernatant was immediately subjected to RT or PCR.

Fig. 1. Map of sampling sites in Han river, Korea. The small rectangular area in the map of Korea placed in the upper right side corresponds to the larger map. The water intakes for tap water supply (▲) and sampling sites (●) are shown.

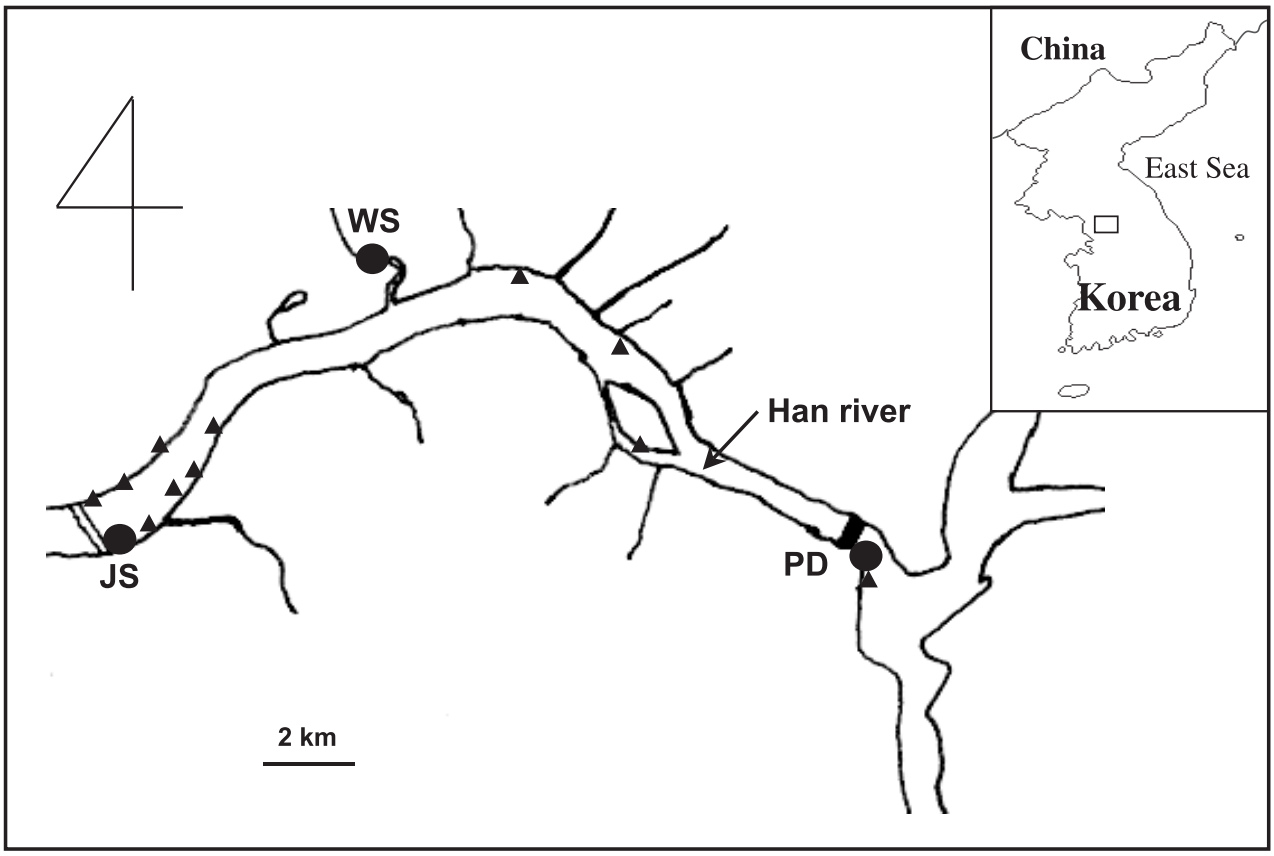


Table 1. Oligonucleotide primers used in this study for PCR amplification of adenoviruses and enteroviruses.

Virus and oligonucleotide	Region	Sequence 5′ → 3′	Localization*
Adenovirus	Hexon		
AV1		GCC GCA GTG GTC TTA CAT GCA CAT C	18858–18883
AV2		CAG CAC GCC GCG GAT GTC AAA GT	19136–19158
AV3		GCC ACC GAG ACG TAC TTC AGC CTG	18937–18960
AV4		TTG TAC GAG TAC GCG GTA TCC TCG CGG TC	19051–19079
Enterovirus	5′NTR		
EV1		CAA GCA CTT CTG TTT CCC CGG	164–184
EV2		ATT GTC ACC ATA AGC AGC CA	599–578
EV3		CTT GCG CGT TAC GAC	526–511

*Sequence positions for adenovirus and enterovirus are in reference to the genomes of coxsackie virus B3 and adenovirus type 2.

Specific primers

The oligonucleotide primer sequences (Table 1) used for the detection of adenoviruses and enteroviruses were identical to those described previously (Allard et al. 1992; Leparc et al. 1994). Primer sequences for adenoviruses were from the hexon gene region, and their specificity was evaluated against the 47 human adenovirus serotypes (Puig et al. 1994). The external primers (AV1, AV2) generate a 300 bp PCR product, and the internal primers (AV3, AV4) generate a 142 bp PCR product. Primer sequences for enteroviruses were from the highly conserved 5′ nontranslated region, which is highly conserved among the enterovirus serotypes. The external primers (EV1, EV2) generate a 435 bp fragment, whereas the use of the primer EV1 and internal primer EV3 generate a 362 bp PCR product.

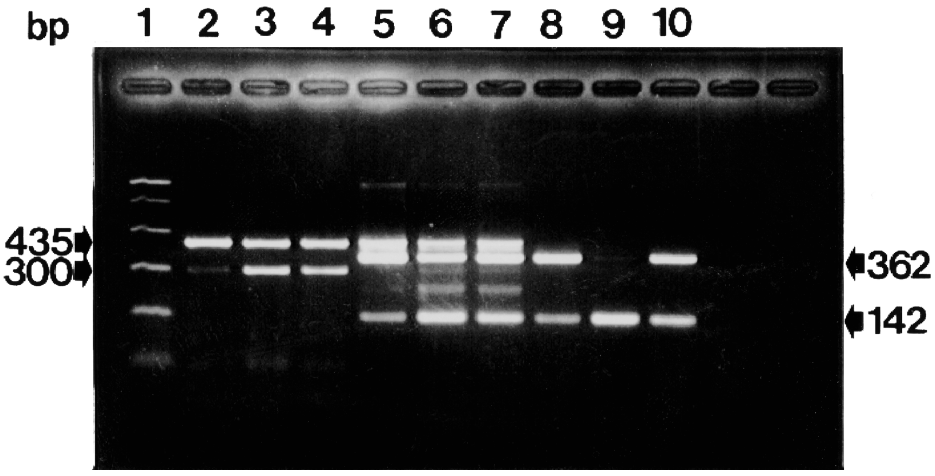
Reverse transcription

Five microliters of nucleic acid extract 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 (EV2), 50 U M-MLV reverse transcriptase (Promega), and 10 U RNasin. RT was carried out at 42°C for 45 min, and then the tubes were heated to 95°C 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

Fig. 2. Effect of primer concentrations on the RT multiplex PCR and subsequent multiplex nested PCR for the detection of enteroviral RNA and adenoviral DNA. Primers for enteroviruses were maintained at 250 nM in the first round PCR and nested PCR. Primers for adenoviruses were applied in concentrations of 50 nM (lanes 2, 5, 8), 150 nM (lanes 3, 6, 9), 100 nM (lanes 4, 7, 10) in the first round of nested PCR. Lanes 2–4, first round multiplex PCR products of 100 $TCID_{50}$ each virus; lanes 5–7, multiplex nested PCR products using the products of lanes 2–4 as templates in order; lanes 8–10, multiplex nested PCR products using 0.1 $TCID_{50}$ each virus as templates; lane 1, size marker.



primer (EV1), 0.1 μM of the adenovirus primer pair (AV1, AV2), and 1.5 mM $MgCl_2$). The PCR was carried out by the following protocol: initial denaturation step at 94°C for 4 min; 35 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; final extension step at 72°C 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 (EV1, EV3) and 0.1 μM of the adenovirus nested primer pair (AV3, AV4). Amplifications were carried out in the same conditions as used for the first amplification. RT monoplex PCR for the detection of enteroviruses was done in the same conditions as the RT multiplex PCR, and monoplex PCR for adenoviruses was done according to the conditions presented in Allard et al. (1992).

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 as well as negative controls were incorporated in all PCR assays to ensure the propriety of the PCR assay. A negative control was added every three samples 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 (positive controls are the final concentrates of each environmental sample, which is seeded with both adenoviruses and enteroviruses in concentrations of 20 $TCID_{50}$/mL, so that the positive controls subjected to PCR assay (5 μL) contain 0.1 $TCID_{50}$ adenovirus and 0.1 $TCID_{50}$ enterovirus).

Sequencing of the nested PCR products

To evaluate the variability of the detected adenovirus and enterovirus strains, and also as an extra control for testing the specificity of the applied technique, and for testing for the absence of carryover contamination, nested PCR products of adenoviruses and enteroviruses from 4 tap water samples were sequenced. The amplified products were cut out of the gel, purified, and concentrated. The purified nested PCR products were ligated into the pGEM-T vector (Promega Co., Madison, Wis.) and transformed into *Escherichia coli* DH5α competent cells. Subsequently, 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, Wis.). A total of 8 clones (4 clones of adenovirus and 4 clones of enterovirus) were sequenced by the chain termination method on a ALFexpress DNA autosequencer (Pharmacia Biotech, Uppsala, Sweden) using the T7 primer and the Cy5 AutoRead sequencing kit. The sequences were compared with the sequences available in the EMBL/GenBank database.

Statistical analysis

Statistical analyses were done with the SAS system (release v. 6.08). The PCR and cell culture results were analyzed by Student's *t*-test at the 5% level of significance.

Results

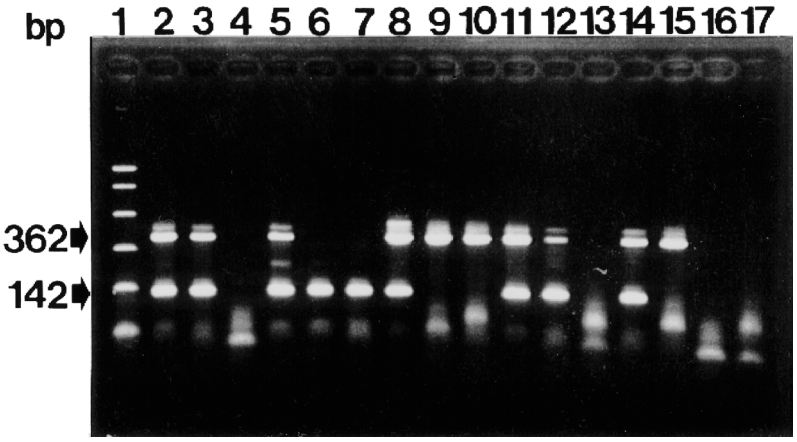
RT multiplex PCR with various primer concentrations

To determine the optimal primer concentrations for RT multiplex PCR, we tested several combinations of adenovirus and enterovirus primer concentrations. All experiments were performed at least two times. Figure 2 shows the effects of various adenovirus primer concentrations on the RT multiplex PCR assay. The amplification of the enterovirus target sequence was inhibited by the adenoviral DNA amplification in the presence of high concentrations of adenovirus primers when the enterovirus templates were present at lower concentrations. The best amplification results were obtained at the 100 nM concentration of adenovirus primers when the concentration of enterovirus primers was maintained at 250 nM.

Sensitivity of the RT multiplex PCR assay

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 (data not shown). To ascertain if the sensitivity of RT multiplex PCR assay is maintained with the varying concentrations of each

Fig. 3. RT multiplex PCR assay on the varying concentrations of each virus template. Lanes 2–4, 0.1 $TCID_{50}$ adenovirus and 0.1 $TCID_{50}$ enterovirus and tenfold serial dilutions; lanes 5–7, 1 $TCID_{50}$ adenovirus and 0.01 $TCID_{50}$ enterovirus and tenfold serial dilutions; lanes 8–10, 0.01 $TCID_{50}$ adenovirus and 1 $TCID_{50}$ enterovirus and tenfold serial dilutions; lanes 11–13, 0.1 $TCID_{50}$ adenovirus and 0.01 $TCID_{50}$ enterovirus and tenfold serial dilutions; lanes 14–16, 0.01 $TCID_{50}$ adenovirus and 0.1 $TCID_{50}$ enterovirus and tenfold serial dilutions; lane 17, negative control; lane 1, size marker.



viral species in the reaction conditions employed in this study, 10-fold serial dilutions of each virus in distilled water were subjected to nucleic acid extraction. We mixed varying concentrations of each viral species and performed RT multiplex PCR followed by multiplex nested PCR on each sample (Fig. 3). The detection limit of each viral species was not affected by the amount of nucleic acids of the other viral species.

PCR and cell culture analyses on river water and tap water samples

Twelve river water concentrates and four tap water concentrates were analyzed by PCR assay (Table 2). To determine the approximate concentrations of these viruses, tenfold and hundred-fold dilutions of nucleic acid extracts were also subjected to a PCR assay. PCR for adenoviruses and RT-PCR for enteroviruses were done in addition to the RT multiplex PCR, to compare the results of multiplex PCR and monoplex PCR. Typical results obtained from each monoplex PCR or RT-PCR and RT multiplex PCR are shown in Fig. 4. The results of the RT multiplex PCR assay coincided with those of each monoplex PCR or RT-PCR assay in 10 samples out of 16 samples for both adenoviruses and enteroviruses. In addition, the level of viruses determined by multiplex PCR as a whole was the same as that by each monoplex PCR.

The viruses were found in river water samples year-round, with the highest levels in the February samples. The amounts of viruses estimated by PCR assay were higher in the tributary (WS) than in the mainstream (PD, JS) ($P < 0.02$, *t*-test). Both adenoviruses and enteroviruses were detected in all analyzed tap water samples.

The same samples were analyzed by cell culture assay. The samples collected from the tributary were also shown to contain higher levels of viruses (from 6.4×10^{-5} MPN/mL to 8.4×10^{-4} MPN/mL in WS) than the samples from the mainstream (from 2.3×10^{-5} MPN/mL to 1.1×10^{-4} MPN/mL in PD or JS) ($P < 0.06$, *t*-test). However, the amount of viruses estimated by cell culture assay were the lowest in February samples, and thus there was some discrepancy between the amounts of viruses estimated by PCR assay and cell culture assay. The amounts of viruses in tap water samples were in the range 3.3×10^{-6} to 6.9×10^{-6} MPN/mL.

Analysis of the sequences of the adenoviruses and enteroviruses detected

The nested amplicons of 4 tap water samples for adenovirus and enterovirus were sequenced. Of four adenovirus isolates, two were closely related to adenovirus type 41, one to type 40, and one to type 5. All four isolates of enterovirus were related to poliovirus type 1 (Mahoney strain v. 01149) but showed 5, 16, 5, and 3 nucleotide differences from it.

Discussion

The RT multiplex PCR assay established in this study allows the simultaneous detection of adenoviruses and enteroviruses, both of which have attracted much attention as molecular indices of viral pollution of environmental waters. The RT multiplex PCR assay of this study was followed by another multiplex PCR, which comprised of the nested PCR for adenoviruses and the seminested PCR for enteroviruses, to increase the sensitivity and to confirm the results of the first multiplex PCR. The detection of pathogenic microorganisms by PCR in environmental samples requires a subsequent assay, which increases the sensitivity of the PCR, and confirms the amplified DNA because the pathogenic microorganisms are usually present in levels too low to be detected by one-step PCR (Puig et al. 1994), and spurious PCR products may arise from the unidentified nucleic acids present in the environmental samples. DNA hybridization or nested PCR (or seminested PCR) has been used for this purpose. Although nested (or seminested) PCR is more susceptible to contamination with amplified DNA, causing the false positives, it is more rapid to perform than DNA hybridization. In addition, nested (or seminested) PCR can be done in a multiplex fashion, and thus the simultaneous confirmation of the first-round PCR products is possible, which makes the assay more effective in terms of time and cost.

Table 2. Detection of viruses by PCR and cell culture assay in river water and tap water samples.

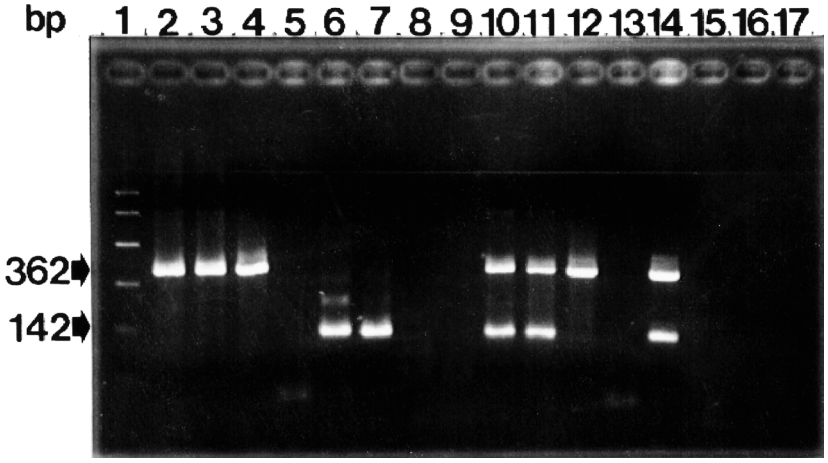
Sample description	PCR result: Equivalent volume examined (mL)*	AV: Monoplex	AV: Multiplex	EV: Monoplex	EV: Multiplex	Cell culture result: Average MPN/mL (range) ‡
Site PD						
April, 1998	60	+ – –†	+ – –	+ – –	– – –	2.3×10^{-5} (3.6×10^{-6} – 7.7×10^{-5})
July, 1998	50	+ – –	+ – –	– – –	+ – –	1.1×10^{-4} (1.5×10^{-5} – 2.7×10^{-4})
October, 1998	36	– – –	– – –	– – –	+ – –	$<8.0 \times 10^{-5}$ ($<1.2 \times 10^{-5}$ – $<2.7 \times 10^{-4}$)
February, 1999	50	+ – –	+ – –	+ + –	+ + –	$<2.8 \times 10^{-5}$ ($<4.2 \times 10^{-6}$ – $<9.4 \times 10^{-5}$)
Site JS						
April, 1998	40	+ – –	+ + –	– – –	– – –	8.0×10^{-5} (5.3×10^{-6} – 2.1×10^{-4})
July, 1998	50	+ – –	– – –	– – –	– – –	6.4×10^{-5} (7.1×10^{-5} – 7.7×10^{-4})
October, 1998	40	– – –	– – –	– – –	+ – –	$<3.5 \times 10^{-5}$ ($<5.3 \times 10^{-6}$ – $<1.2 \times 10^{-4}$)
February, 1999	50	+ + –	+ + –	+ – –	+ – –	$<3.5 \times 10^{-5}$ ($<5.3 \times 10^{-6}$ – $<1.2 \times 10^{-4}$)
Site WS						
April, 1998	36	+ – –	+ – –	+ – –	+ – –	8.4×10^{-4} (2.0×10^{-4} – 2.1×10^{-3})
July, 1998	33	+ – –	+ – –	+ – –	+ – –	3.0×10^{-4} (7.1×10^{-5} – 7.7×10^{-4})
October, 1998	28	+ – –	+ – –	+ – –	+ – –	3.6×10^{-4} (8.5×10^{-5} – 9.2×10^{-4})
February, 1999	50	+ + –	+ + –	+ + +	+ + +	6.4×10^{-5} (4.3×10^{-6} – 1.7×10^{-4})
Tap water						
December, 1997	500	+ + +	+ + –	+ – –	+ + –	6.4×10^{-6} (4.3×10^{-7} – 1.7×10^{-5})
January, 1998	500	+ + –	+ – –	+ + –	+ – –	6.4×10^{-6} (4.3×10^{-7} – 1.7×10^{-5})
April, 1998	1700	+ – –	+ + –	+ – –	+ – –	3.4×10^{-6} (4.5×10^{-7} – 8.1×10^{-6})
July, 1998	200	+ + –	+ + +	+ – –	+ – –	7.0×10^{-6} (1.1×10^{-6} – 2.4×10^{-5})

*Equivalent volume of original environmental water examined per 5 μL of a final concentrate subjected to PCR assay.

‡For samples that were never positive, the average value is calculated using detection limits. The range in parentheses represent the lower and upper 95% confidence limits. Most of the detected viruses in cell culture assay were enteroviruses. Adenoviruses were detected in one tap water sample (January) and two river water samples (July PD and WS).

†The three + or – signs represent the PCR amplification results of a final concentrate, a ten-fold diluted sample, and a hundred-fold diluted sample, sequentially.

Fig. 4. Detection of enteroviruses and adenoviruses in the river water sample (WS, February). Lanes 2–4, enterovirus detection in the undiluted sample and serial 10-fold diluted samples; lane 5, negative control for the enterovirus PCR; lanes 6–8 and 9, adenovirus detection for the same samples; lanes 10–12 and 13, multiplex detection of enteroviruses and adenoviruses for the same samples; lane 14, positive control seeded with 0.1 $TCID_{50}$ of each virus; lane 1, size marker.



In the optimization of the RT multiplex PCR assay, it was observed in the preliminary experiment that the amplification of enterovirus target sequence was inhibited in the multiplex nested PCR when equimolar primer concentrations were applied. The adjustment of primer concentrations has been shown to be important in the success of multiplex PCR in other studies (Cacas et al. 1997; Tsai et al. 1994). Therefore, we determined the optimum primer concentrations for the multiplex PCR and subsequent multiplex nested PCR by testing varying concentrations of adenovirus primer sets (Fig. 2). By adjusting primer concentrations to optimal levels, enteroviruses and adenoviruses could be detected simul-

taneously without losing sensitivity, which is critical in the detection of low levels of viruses in the environmental samples.

Under optimized primer concentrations, the detection sensitivity of the multiplex assay was comparable to that of each monoplex assay when similar amounts of each virus were subjected to PCR amplification. However, the amount of one viral species can be much higher than the other viral species in the environmental samples and the PCR amplification of a more abundant virus group might interfere with that of a minor group. We think the reliability of multiplex PCR assay needs to be examined for this kind of interference in the development of the assay. Several combinations of virus mixtures, which contained different concentrations of each virus group, were subjected to multiplex assay and found to yield consistent results regardless of varying concentrations of each virus (Fig. 3). In addition, we subjected river water and tap water samples to each monoplex and multiplex assay, to compare their results and ascertain the reliability of the RT multiplex PCR assay. Although there were small differences between the results of the two assays, the results of multiplex assay were generally similar to those of monoplex assay (Table 2). A small discrepancy between the results of the two assays was presumed to occur because samples cannot be thoroughly uniform in their composition and thus the detection of low levels of viruses in the environmental samples depends somewhat on the probability.

We performed cell culture assay in addition to PCR assay on river water and tap water samples to get more comprehensive information on the virological status of these waters. Viruses were detected in most samples, especially in all tap water samples, by either cell culture assay or PCR assay. The reason for the high quantity of virus in tap water is not clear. However, some investigations on tap water in the Seoul metropolitan area reported that injured coliforms were detected even in water treatment plants, and fecal coliforms were detected in many samples with high concentrations of residual chlorine (Ministry of Environment, Republic of Korea 1997; Park et al. 1993). These reports suggest that the water treatment processes are not enough, the tap water of this area is chronically exposed to microorganisms, and viruses can also be present in high concentrations in tap water.

The detection results of cell culture and PCR assay did not correspond in some samples, especially in the river water samples collected in February 1999, where the results of PCR assay indicated higher level of viral contamination but less amount of viruses were detected by cell culture assay than the other samples. The results of PCR assay and cell culture assay did not correlate well in the experiments of other researchers (Abbaszadegan et al. 1999; Reynolds et al. 1998), that is, some samples which were positive in PCR assay were negative in cell culture assay and vice versa. This inconsistency between PCR- and cell-culture-assays might be explained if we consider the following facts. Firstly, although PCR assay is much more sensitive than cell culture assay, the results of PCR assay can be negative in some samples that were positive in cell culture assay, because the assay volumes used by PCR are much (about several hundred times) smaller than those of the cell culture assay. Secondly, it has been widely accepted that some infectious viruses cannot be detected by cell culture assay, since cell culture technique can detect only cultivable viruses in employed cell lines. In addition, PCR assay detects the non-infectious virus particles or viral nucleic acids which cannot be detected by cell culture assay. Therefore, some samples that were positive by PCR assay can be negative by cell culture assay. Despite this, the detection of enteroviruses and adenoviruses by either PCR assay or cell culture assay from tap water, as well as from river water, which is used as source water, illustrates the potential public health hazard.

Although the determination of the quantity of infectious viruses is important in assessing the risk from viruses present in the environmental samples, cell culture technique, which is employed to determine the quantity of infectious viruses, is restricted to routine use as an environmental monitoring tool of viral water quality, because it requires long analysis time and high cost. Moreover, the quantity of infectious viruses present in the environment cannot be accurately determined by cell culture assay, since only cultivable viruses can be detected in cell culture assay, and the range of cultivable viruses is variable according to the cell line used in the assay (Tani et al. 1992). On the other hand, PCR assay is rapid and cost-effective to perform. Although a positive result in PCR assay does not indicate the presence of infectious viruses, it does indicate viral contamination and thus a potential health risk. Therefore, at present, PCR assay seems desirable as a routine monitoring tool for viral contamination.

The monitoring of adenoviruses and enteroviruses in the environmental water samples is useful in the assessment of the public health risk associated with exposure to these pathogens. Besides, the detection results of these viruses by PCR assay can be useful as molecular indices of viral contamination, because of their prevalence in environmental waters where fecal pollution is suspected (Castignolles et al. 1998; Gantzer et al. 1998; Kopecka et al. 1993; Pina et al. 1998; Puig et al. 1994). The RT multiplex PCR assay established in this study provides a more rapid and efficient way to detect these viruses, and thus could be applied broadly as a monitoring tool of viral pollution in environmental waters.

Acknowledgements

This work was supported by Daesang Foundation.

References

Allard, A., Albinsson, B., and Wadell, G. 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.

American Public Health Association. 1995. Standard methods for the examination of water and wastewater. 19th ed. American Public Health Association, Washington, D.C.

Berg, G., and Metcalf, T.G. 1978. Indicators of viruses in water. *In* Indicators of viruses in water and food. *Edited by* G. Berg. Ann Arbor Science. Ann Arbor, Mich.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., and van der Noordaa, J. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**: 495–503.

Casas, I., Tenorio, A., Echevarria, J.M., Klapper, P.E., and Cleator, G.M. 1997. Detection of enteroviral RNA and specific DNA of

herpesviruses by multiplex genome amplification. J. Virol. Methods, **66**: 39–50.

Castignolles, N., Petit, F., Mendel, I., Simon, L., Cattolico, L., and Buffet-Janvresse, C. 1998. Detection of adenovirus in the waters of the Seine River estuary by nested-PCR. Mol. Cell. Probes, **12**: 175–180.

Dubrou, S., Kopecka, H., Lopez-Pila, J.M., Marechal, J., and Prevot, J. 1991. Detection of hepatitis A virus and other enteroviruses in wastewater and surface water samples by gene probe assay. Water Sci. Technol. **24**: 267–72.

Ellis, J.S., Fleming, D.M., and Zambon, M.C. 1997. Multiplex reverse transcription-PCR for surveillance of Influenza A and B viruses in England and Wales in 1995 and 1996. J. Clin. Microbiol. **35**: 2076–2082.

Enriquez, C.E., Hurst, C.J., and Gerba, C.P. 1995. Survival of the enteric adenoviruses 40 and 41 in tap, sea, and wastewater. Water Res. **29**: 2548–2553.

Gantzer, C., Maul, A., Audic, J.M., and Schwartzbrod, L. 1998. Detection of infectious enterovirus genomes, somatic coliphages, and *Bacteroides fragils* phages in treated wastewater. Appl. Environ. Microbiol. **64**: 4307–4312.

Gerba, C.P., and Rose, J.B. 1990. Viruses in Source and Drinking Water. *In* Drinking water microbiology. *Edited by* G.A. McFeters. Spriger-Verlag, New York.

Irving, L.G., and Smith, F.A. 1981. One-year survey of enteroviruses, adenoviruses, and reoviruses isolated from effluent at an activated-sludge purification plant. Appl. Environ. Microbiol. **41**: 51–59.

Keswick, B.H., Gerba, C., DuPont, H.L., and Rose, J. 1984. Detection of enteric viruses in treated drinking water. Appl. Environ. Microbiol. **47**: 1290–1294.

Kopecka, H., Dubrou, S., Prevot, J., Marechal, J., and Lopez-pila, J.M. 1993. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl. Environ. Microbiol. **59**: 1213–1219.

Krikelis, V., Spyrou, N., Markoulatos, P., and Serie, C. 1984. Seasonal distribution of enteroviruses and adenoviruses in domestic sewage. Can. J. Microbiol. **31**: 24–25.

Leparc, I., Aymard, M., and Fuchs, F. 1994. Acute, chronic and persistent enterovirus and poliovirus infection: detection of viral genome by semi-nested PCR amplification in culture-negative samples. Mol. Cell. Probe, **8**: 487–495.

Ministry of Environment, Republic of Korea. 1997. Control technology of drinking water quality in the pipeline network. Ministry of Environment, Republic of Korea.

Ministry of Environment, Republic of Korea. 1998. Environmental Statistics Yearbook Vol. 11. Ministry of Environment, Republic of Korea.

Park, S.J., Cho, J.C., and Kim, S.J. 1993. Bacterial distribution and variation in water supply systems. Korea J. Microbiol. **31**: 245–254.

Payment, P., and Trudel, M. 1993. Isolation and identification of viruses. *In* Methods and techniques in virology. *Edited by* P. Payment and M. Trudel. M. Marcel Dekker, Inc., New York.

Pina, S., Puig, M., Lucena, F., Jofre, J., and Girones, R. 1998. Viral pollution in the environment an in shellfish: Human adenovirus detection by PCR as an index of human viruses. Appl. Environ. Microbiol. **64**: 3376–3382.

Pozo, F., and Tenorio, A. 1999. Detection and typing of lymphotropic herpesviruses by multiplex polymerase chain reation. J. Virol. Methods, **79**: 9–19.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G., and Girones, R. 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. Appl. Environ. Microbiol. **60**: 2963–2970.

Sobsey, M.D. 1989. Inactivation of health-related microorganisms in water by disinfection process. Water Sci. Technol. **21**: 179–195.

Stockton, J., Ellis, J.S., Saville, M., Clewley, J.P., and Zambon, M.C. 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J. Clin. Microbiol. **36**: 2990–2995.

Tani, N., Shimamoto, K., Ichimura, K., Nishii, Y., Tomita, S., and Oda, Y. 1992. Enteric virus level in river water. Water Res. **26**: 45–48.

Tani, N., Dohi, Y., Kurumatani, N., and Yonemasu, K. 1995. Seasonal distribution of adenoviruses, enteroviruses and reoviruses in urban river water. Microbiol. Immunol. **39**: 577–580.

Tsai, Y.-L., Tran, B., Sangermano, L., and Palmer, C. 1994. Detection of poliovirus, hepatitis A virus, and rotavirus from sewage and ocean water by triplex reverse transcriptase PCR. Appl. Environ. Microbiol. **60**: 2400–2407.

Vantarakis, A.C., and Papapetropoulou, M. 1998. Detection of enteroviruses and adenoviruses in coastal waters of SW Greece by nested polymerase chain reaction. Water Res. **32**: 2365–2372.

Ward, R.L., and Akin, E.W. 1984. Minimum infective dose of animal viruses. CRC Crit. Rev. Environ. Contam. **14**: 297–310.