Detection of infectious pathogenic viruses in water and wastewater samples from urbanised areas

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Abstract Regardless of the remarkable improvement in sanitary conditions in urbanised areas, infectious diseases caused by pathogenic viruses in water have been reported year after year. The actual situation for the behaviour of pathogenic viruses in urbanised society should be understood. In this study, infectious enteroviruses and adenoviruses in water and wastewater samples from urbanised areas were investigated. Infectious enteroviruses were continuously detected from sewage and sewage sludge during the summer season. The detection of infectious viruses in sewage and sewage sludge was facilitated by the enzymatic virus elution (EVE) method. The concentration of infectious enteroviruses in positive samples of sewage and sewage sludge was >400 virions/L. Infectious viruses were also detected from water samples at levels >4 virions/L.

Keywords Adenovirus; echovirus; enzymatic virus elution method; infectious pathogenic viruses; poliovirus

Water Science and Technology **Vol 50 No 1 pp 247–251** © IWA Publishing 2004

Introduction

Regardless of the remarkable improvement of sanitary conditions in urbanised areas, infectious diseases caused by pathogenic viruses in water have been frequently reported year after year (NIID, 2002). In addition, pathogenic viruses have been suspected of being agents of a type of gastroenteritis of unknown cause which dominates in a number of reports about waterborne infectious diseases. Although investigations into the presence of pathogenic viruses in environmental waters have been conducted by many researchers (e.g. Taylor *et al*., 2001), information on the amount of pathogenic viruses in environmental waters is still insufficient, because monitoring has not been a legal duty. Accumulation of information on the concentration and fate of such viruses in environmental waters is of primary importance for addressing the problem of pathogenic viruses in our society.

However, there has been difficulty in detecting infectious viruses from environment because it is considered that most of the viruses in water samples are adsorbed to solids. In particular, sewage and sewage sludge contain large amounts of solids consisting of numerous substances, including bacteria, organic polymers, silica, etc. In order to overcome this obstacle, the enzymatic virus elution (EVE) method has been proposed to facilitate virus detection from solid-rich samples (Sano *et al*., 2001, 2003). In the EVE method, hydrolytic enzymes and cation-exchange resin (CER) are used for the enhancement of virus elution from solids. Since samples prepared by the EVE method have no inhibitory effects on cell culture (Sano *et al*., 2001) and RT–PCR (Sano *et al*., 2003), EVE is a powerful tool for the detection of viruses adsorbing to solids. The objectives of this research were to investigate virus occurrences in water and wastewater samples and to evaluate the EVE method for recovering indigenous pathogenic viruses from environmental samples.

Materials and methods

Sample sites

Sewage, sewage sludge and treated wastewater were sampled from a municipal wastewater treatment plant and a pilot plant for wastewater treatment plant. The municipal wastewater

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treatment plant processed 10,000 m^3 domestic wastewater/d, providing a service for a population of 36,000. The pilot plant was constructed to evaluate nutrient removal by a membrane separation activated-sludge process. Raw water for water treatment was sampled from a water treatment plant. The water supply capacity of this plant is about $1.8 \times 10^6\ m^3$/d.

Recovery of viruses from sewage, sewage sludge and treated wastewater at wastewater treatment plants

Sewage and sewage sludge (400 mL) were centrifuged at 9,000×*g* for 15 min at 4°C. The supernatant was transferred into a sterilised 500 mL glass bottle. Viruses in the pellet were eluted by the EVE method as previously described (Sano *et al.*, 2001). Briefly, pellets were suspended in 400 mL of EVE buffer, containing 1% lysozyme and 0.1 meq cation exchange resin, and stirred lightly for 30 min at room temperature before recentrifugation (9,000×*g*, 30 min, 4°C) and recovery of the supernatant. Viruses were further concentrated by polyethylene glycol 6000 precipitation (Lewis and Metcalf, 1989) with slight modifications. At first, 8% (w/v) polyethylene glycol (PEG) 6000 (solid) was added to each sample. After dissolving by mixing with a magnetic stirrer at 4°C, the mixture was kept at 4°C overnight before being centrifuged (9,000×*g*, 90 min, 4°C). The pellet was suspended in 16 mL 0.1 M phosphate buffer (pH 9.0) and filtered (0.22 μm membrane). Finally, the pH of each sample was adjusted to 7.0, with 1 M HCl and 160 μL of antibiotic–antimycotic solution added for decontamination. Samples were stored at –20°C until further analysis.

Viruses in 40 L treated wastewater were concentrated by the DEAE-cellulose method (Yano *et al.*, 1993). The cellulose, in a chilled box, was transferred to the laboratory and cooled at 4°C. Viruses were eluted from the cellulose by 10 mL 5% beef extract solution (pH 9.0) containing 15% sodium nitrate and 1% sodium chloride. Viruses were concentrated and decontaminated as described above, and concentrates were stored at –20°C until further analysis.

Recovery of viruses from raw water at the water treatment plant

Viruses in 80 L of raw water were concentrated with the DEAE-cellulose method as above.

Virus detection with cell culture

Infectious viruses were detected by inoculating decontaminated samples into cell cultures of BGM (kindly supplied by Dr Yano, Tokyo Metropolitan Research Laboratory of Public Health, Japan), Hela, HEp-2 and RD-18S (kindly supplied by Dr Yoshida, Tokyo Metropolitan Research Laboratory of Public Health, Japan). These cells were grown in 24-well dishes in Eagle's minimum essential medium (MEM, NISSUI Pharmaceutical Co., Ltd, Tokyo, Japan) containing 5% foetal bovine serum (JRH Bioscience, Lenexa, USA), 2 mM L-glutamine (GIBCO BRL, New York, USA), 0.1% (w/v) $NaHCO_3$ (GIBCO BRL) and 1% antibiotic-antimycotic solution in an atmosphere of 5% CO_2 at 37°C. Three wells were employed for each sample. Each well was filled with 100 μL of inoculum and 1 mL of Eagle's MEM containing 1% foetal bovine serum, 2 mM L-glutamine, 0.1% (w/v) $NaHCO_3$ and 1% antibiotic–antimycotic solution.

Adenovirus detection

All samples showing apparent CPE twice were processed to Adenoclone (Meridian Bioscience, Inc. Cincinnati, OH, USA) for the detection of adenovirus antigen.

Enterovirus detection

Samples that gave negative results in the adenovirus detection were processed by RT–PCR

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for the detection of enterovirus genome (Kuan, 1997) with some modifications. Products were visualised using gel electrophoresis (1.5% agarose stained with ethidium bromide) and UV light. PCR products were purified with GENECLEAN II Kit (GL-1131-05, Q-BIO gene) and ligated into pGEM-T Easy (A3600, Promega, WI, USA). The ligation product was used for the transformation of *E. coli* DH5α (9052, TaKaRa, Shiga, Japan). The sequences of PCR products were analysed by direct sequencing of both strands of PCR products with the ABI PRISM310 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) using the PCR cycle sequencing big-dye terminator protocol. Homology searches were conducted using the BLAST server at the National Center for Biotechnology Information (NCBI).

Results and discussion

Detection of viruses in samples at the wastewater treatment plant

In Japan, poliovirus vaccination is routinely carried out twice for children between the ages of 3 months and 90 months. The live oral polio vaccine (OPV) grows in the intestine and enhances the production of anti-poliovirus antibodies. Accordingly, a large number of virions are excreted in faeces, and flow into the sewage stream. As shown in Figure 1, vaccine-derived polioviruses were isolated from treated wastewater in April, raw sewage in June, September and October. The investigation by Matsuura *et al*. (2000) showed that the occurrence of vaccine-derived polioviruses in river water and raw sewage had a seasonal variation with two peaks (around June and October). Due to this seasonal variation, the isolation of polioviruses was relatively difficult in March, April and September, as these periods were just before vaccination (Matsuura *et al*., 2000). However, poliovirus 1 (PV1) and poliovirus 3 (PV3) were isolated in April (treated wastewater) and September (raw sewage) in this investigation. These results indicated that vaccine-derived polioviruses were robust in environmental water and prevalent at these sampling sites.

Not only vaccine-derived polioviruses but also echoviruses were isolated from raw sewage in September (Figure 1). The genome of echovirus type 11 (E11, strain: Kar/87, accession number: AF447473) and type 19 (E19, strain: Djum/91, accession number: AF447480) gave the highest homology (95.6%). The concentration of echovirus in the positive sample was also calculated to be >400 infectious virions/L.

Detection of viruses in samples at the pilot plant for wastewater treatment

Table 1 shows the results of virus detection from sewage, sewage sludge and treated wastewater from the pilot plant for wastewater treatment. Samples that showed CPE were obtained from raw sewage and sewage sludge. However, both adenoviruses and

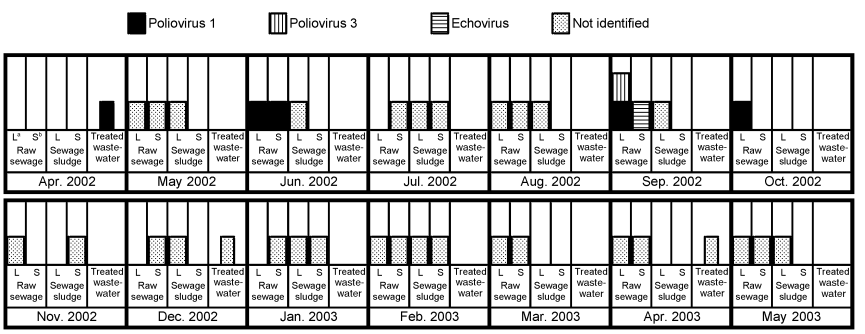

Figure 1 Detection of the infectious viruses in samples from the wastewater treatment plant in Sendai, Japan (April 2002 to May 2003). (L, Liquid phase; S, solid phase)

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Table 1 Detection of viruses in raw sewage, sewage sludge and treated wastewater from the wastewater treatment plant in Chiba, Japan

Sample date	Sample	Fraction	Virus detection in cell cultures[1]				Adenovirus	Enterovirus genome
			BGM	HeLa	Hep-2	RD-18S		
23 September 2002	Raw sewage	Supernatant[2]	–	–	–	+	–	–
		Pellet[3]	–	–	–	–	nt	nt
	Sewage sludge	Supernatant	–	–	+	–	–	–
		Pellet	–	–	–	+	–	–
	Treated water	1st concn[4]	–	–	–	–	nt	nt
		2nd concn[5]	–	–	–	–	nt	nt

[1] Inoculum of 200 μL equivalent to 5 mL raw sewage and sewage sludge and 500 mL treated water; [2] After centrifugation (9,000×*g*, 15 min, 4°C; [3] pellet after centrifugation; [4] concentrated by DEAE-cellulose; [5] concentrated by PEG; nt = not tested

Table 2 Detection of viruses in raw water from the water treatment plant in Osaka, Japan

Sample date (weather)	Virus detection in cell cultures			Adenovirus	Enterovirus genome
	BGM	HeLa	Hep-2		
24 January 2001 (Fair)	–	nt	nt	nt	nt
5 June 2001 (Fair)	–	nt	nt	nt	nt
5 September 2001 (Fair)	–	–	–	nt	nt
13 December 2001 (Rain)	–	–	+	–	–
20 March 2002 (Fair)	–	+	+	–	+ (PV1)
20 June 2002 (Fair, occasional showers)	–	+	+	–	–

enteroviruses were not detected from these sludge samples. Additionally, no samples showing CPE were obtained from treated wastewater.

Detection of viruses in samples at the water treatment plant

Table 2 shows results of virus detection from the raw water from the water treatment plant. One genome of enterovirus was obtained from the sample taken on March 20th 2002. The estimated concentration of PV1 in this positive sample was four infectious particles/L. The DNA sequencing analysis revealed that this genome was derived from PV1. Neither adenoviruses nor the genome of the enterovirus were detected from two samples that showed CPE in cell culture.

No adverse effects on cell cultures by samples from sewage and sewage sludge were observed during this study. As described previously, the EVE method made it possible to recover solid-embedded viruses from environmental samples without any inhibition. This is the first time the EVE method has been applied to a field survey for infectious pathogenic viruses. The results of this investigation indicated that the EVE method was able to recover indigenous pathogenic viruses that were bound to solids in environmental samples.

Conclusions

The following conclusions were derived from this study:

1. Vaccine-derived polioviruses were isolated from sewage and sewage sludge, especially in the summer season. The concentration of infectious polioviruses in sewage and sewage sludge was estimated to be >400 infectious particles/L. PV1 was detected from raw water for water treatment at the concentration of >4 infectious particles/L.
2. Not only vaccine-derived polioviruses but also infectious echovirus were isolated from raw sewage from the wastewater treatment plant. This echovirus was estimated to be type 11 or 19 by DNA sequencing analysis.

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3. The EVE method was effective in recovering indigenous viruses from solid-rich samples, including samples of sewage and sewage sludge.

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