DETECTION OF POLIOVIRUS, HEPATITIS A VIRUS AND ROTAVIRUS FROM SEWAGE AND WATER SAMPLES

Leera Kittigul[1], Boonyos Raengsakulrach[2], Sontana Siritantikorn[3], Raweewan Kanyok[3], Fuangfa Utrarachkij[1], Pornpan Diraphat[1], Vipa Thirawuth[2], Kanokrat Siripanichgon[1], Supornvit Pungchitton[1], Kitja Chitpirom[1], Nattasai Chaichantanakit[1] and Kanda Vathanophas[1]

[1]Department of Microbiology, Faculty of Public Health, Mahidol University; [2]Department of Virology, Armed Forces Research Institute of Medical Sciences; [3]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract. A modified adsorption-elution technique for concentration of enteric viruses from sewage and water samples was developed. The viruses in water were concentrated by negatively charged membrane filtration, eluted with 2.9% tryptose phosphate broth containing 6% glycine pH 9.0, and reconcentrated using centrifugation by a speedVac concentrator. The presence of poliovirus, hepatitis A virus (HAV) RNA, and rotavirus antigen was determined by cell culture isolation, nested polymerase chain reaction (nested PCR), and enzyme-linked immunosorbent assay (ELISA), respectively. A total of 100 sewage and water samples were collected from various sources in congested communities in Bangkok, concentrated and examined for those enteric viruses. Of 20 surface water samples from canals which located near sewage drains, 15% were positive for HAV RNA by nested PCR. Of 48 domestic sewage samples from man-holes of underground sewers, 8% were positive for rotavirus antigen by ELISA. Even though the samples were concentrated 256-2,000 fold, poliovirus was not found by isolation in cell culture.

INTRODUCTION

Enteric viruses including polioviruses, hepatitis A virus (HAV) and rotaviruses are found in fecally-contaminated water and food and are an important public health concern (Metcalf *et al*, 1995). Water borne outbreaks of viral hepatitis and gastroenteritis caused by rotaviruses have been reported (Hopkins *et al*, 1984; Wen *et al*, 1992; De Serres *et al*, 1999). The outbreaks were attributed to the consumption of drinking water and food that was considered safe by bacteriological standard methods (Hejkal *et al*, 1982). The resistance of enteroviruses to chlorine is greater than that of the coliform bacteria group used to indicate the sanitary quality of water (Trask *et al*, 1945). Furthermore, rotaviruses can survive well enough in treated drinking water (Sattar *et al*, 1984), raw and treated river water (Raphael *et al*, 1985). The methods for determination of enteric viruses contaminated in water samples are required for monitoring of food and water quality in the presence of viral pollution in order to enable effective management of public water supplies, implementation of appropriate preventive control and curative measures.

The determination of viruses in environmental water samples is, however, rather difficult because their densities are so low that the efficient virus concentration is necessary. Several methods for concentration of enteric viruses from water have been proposed and the most promising method is the virus adsorption-elution technique (Toranzos and Gerba, 1989; Li *et al*, 1998; Abbaszadegan *et al*, 1999). Detection of enteric viruses in environmental samples includes viral isolation in cell culture (Abbaszadegan *et al*, 1999), solid-phase immunoassay (Guttman-Bass *et al*, 1987) and nucleic acid amplification by the reverse transcriptase-polymerase chain reaction (RT-PCR) (Tsai *et al*, 1994; Gilgen *et al*, 1997; Sellwood *et al*, 1998).

In this study a modified virus adsorption-elution method was applied for the concentration of poliovirus, HAV and rotavirus from sewage and water samples collected from congested areas. The concentrated samples were assayed for poliovirus by isolation in cell culture, HAV RNA by nested PCR and rotavirus antigen by enzyme-linked immunosorbent assay (ELISA).

Correspondence: Dr Leera Kittigul, Department of Microbiology, Faculty of Public Health, Mahidol University, 420/1 Rajvithi Road, Bangkok 10400, Thailand. Tel: 662-2455525; Fax: 662-2458351; E-mail: phlkt@mahidol.ac.th

MATERIALS AND METHODS

Water samples

During February-June, 1998, 68 water samples were collected in sterile 1-liter bottles from domestic sewage (48 samples) and canals receiving raw sewage drains (20 samples), located in the congested areas of Bangkok. Moreover, tap water samples (2 samples), drinking water (4 samples) and water samples collected in the containers for domestic uses (26 samples) were also obtained in sterile 5-liter bottles from the houses in these congested communities to determine the presence of enteric viruses. Temperature and pH of all 100 water samples were measured. The samples were transported to the laboratory in an ice box and concentrated within 2 hours after collection.

Virus concentration

Virus particles were concentrated by adsorption to and elution from microporous filters according to the standard method for the examination of water and wastewater (American Public Health Association, 1992) with some modification. For sewage and water samples from canals (1 liter), large suspended particles were first removed by filtration through adsorbent pad (Whatman, Maidstone, United Kingdom). Residual free chlorine in tap water samples (5 liters) was neutralized at the time of collection by the addition of sodium thiosulfate solution to a final concentration of 50 mg/l before processing with electronegative filters. Briefly, the water sample was adjusted to pH 3.5 with 1N HCl and 0.0015 N $AlCl_3$ and gently mixed on a magnetic stirrer at room temperature for 30 minutes. Next, the sample was filtered through 0.45 μm porosity of cellulose nitrate filter with electronegative charge (GN-6, Gelman Sciences, Ann Arbor, M,USA) on 47-mm-diameter of filter holder at 15 bars pressure. The virus adsorbent filter was washed by passing 0.14 N NaCl, pH 3.5 through the filter to remove excess Al^{3+}. Then the virus was eluted by 10-30 ml of 2.9% tryptose phosphate broth (TPB) containing 6% glycine, pH 9.0. Sample was then neutralized to pH 7.0-7.5 with 4N HCl and stored at 4ºC overnight. The eluate was further concentrated using centrifugation by a speedVac concentrator (Savant Instruments, Farmingdale, NY) for approximately 5 hours to obtain a final volume of 3-4 ml each. The sample was added 1/10 of the measured volume each of 20x penicillin-streptomycin, 10x Hanks balanced salt solution and 10x nutrient broth. Finally, the sample was adjusted to pH 7.4 with 4N HCl while mixing vigorously and stored at -80ºC until virus assays.

Isolation of poliovirus in cell cultures

The continuous cell lines: human, epidermoid carcinoma of larynx (HEp-2) and Rhesus monkey kidney cell (LLC-MK2) were grown in minimal essential media (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics and maintained in minimal essential media supplemented with 2% fetal bovine serum and antibiotics (MM). Concentrated water samples (200 μl) for poliovirus detection were inoculated onto monolayers of cell cultures, incubated at 35ºC for one week and observed for cytopathic effect (CPE) every day. Samples which did not produce CPE were passed an additional two times on monolayers of cell cultures and observed for CPE. When CPE were not shown in cell cultures after three time subpassages, the sample was considered negative for poliovirus. Cell and virus controls were performed in parallel with samples by adding 200 μl of MM and inoculating 200 μl of poliovirus type 1 onto monolayer cell cultures, respectively. For samples showing CPE in cell cultures after inoculation, the virus was further identified and typed with poliovirus type 1, 2 and 3 antisera by neutralization tests (Schmidt, 1989). To determine the efficiency of the concentration method, poliovirus type 1 with 10^{13} $TCID_{50}$ (50% of tissue culture infective dose) per 100 μl were seeded to 5 liters tap water, concentrated and determined by isolation in cell cultures.

RNA extraction and HAV RT-PCR

Nucleic acids (RNA and DNA) were extracted from 100 μl of each concentrated water sample using the guanidinium thiocyanate acid method (GuSCN-DNA/RNA) described by Casas *et al* (1995). The extract equivalent to 20 μl of the original sample was subjected to the HAV RT-PCR by a method modified from that of Robertson *et al* (1991). Briefly, 2.5 μl of the extract was mixed with 5 pmol of primer HAV-2389 (5´-GGA AAT GTC TCA GGT ACT TTC TTT G-3´) in a 5-μl volume. The mixture was heated at 95ºC for 5 minutes and then cooled on ice. The RT-PCR was performed in a 50-μl volume containing the denatured RNA, 10 mM Tris, 50 mM KCl, 3.5 mM $MgCl_2$, 200 μM of each dNTPs, 12.5 pmol of primer HAV-2389, 12.5 pmol of primer HAV-2167 (5´-GTT TTG CTC CTC TTT ATC ATG CTA TG-3´), 1 unit of avian my-

eloblastosis virus reverse transcriptase (Promega, Madison, WI), 20 units of RNAsin (Promega), and 1.25 units of AmpliTag (Perkin Elmer, Norwalk, CT). The RT and PCR were carried out with following steps: RT at 42ºC for 45 minutes; cycle 1, 94ºC for 3 minutes, 55ºC for 1 minute, 72ºC for 75 seconds; cycles 2-29, 94ºC for 35 seconds, 55ºC for 1 minute, 72ºC for 75 seconds; cycle 30, 94ºC for 1 minute, 55ºC for 1 minute, 72ºC for 5 minutes. The nested PCR was then performed in a 50-µl reaction containing 5 µl of the first PCR product (diluted 1:100), 10 mM Tris, 50 mM KCl, 3.5 mM $MgCl_2$, 200 µM of each dNTPs, 12.5 pmol of primer HAV-2389, 12.5 pmol of primer HAV-2232 (5´-TCA ACA ACA GTT TCT ACA GA-3´), and 1.25 units of AmpliTag. The nested PCR cycles were as follows: cycles 1-24, 94ºC for 35 seconds, 49ºC for 1 minute, 72ºC for 1 minute; and cycle 25, 94ºC for 1 minute, 49ºC for 1 minute, 72ºC for 5 minutes. The nested PCR product (18 µl) was then analyzed by agarose gel electrophoresis. The product of 183 base pairs in size indicated the presence of HAV sequence in the samples. Positive controls included in each run were dilutions of HAV strain HM-175 [0.01, 0.1, 1, and 100 radioimmunofocus assay or RIFA units/ml (Lemon *et al*, 1983)] which were extracted and subjected to the RT-PCR and nested PCR along with samples.

To assess the sensitivity of the concentration technique, different amount of HAV : $2x10^5$, $2x10^4$, $2x10^3$, $2x10^2$ and 2x10 RIFA units were spiked to 1 liter tap water samples. The spiked samples were then concentrated and assayed for HAV RNA by the RT-PCR described above.

Rotavirus ELISA

The presence of rotaviral antigens in concentrated water samples was determined by the commercial IDEIA™ Rotavirus test (DAKO, Cambridgeshire, United Kingdom). The microtiter 96-well plate was coated with rotavirus specific rabbit polyclonal antibody. The concentrated samples were added to the microtiter plates wells and incubated for 1 hour with horseradish peroxidase conjugated to rotavirus specific rabbit polyclonal antibody. The wells were then washed 5 times with Tris-buffered solution. This was followed by the addition of substrate buffer containing tetramethylbenzidine (TMB) and hydrogen peroxide. The wells were mixed gently and incubated at room temperature for 10 minutes. The reaction was stopped by adding sulfuric acid to each well. The absorbance of the yellow product was measured at 450 nm on an ELISA plate reader (Biotek, Winooski, VT). Bovine rotavirus with $5.7x10^4$ infectious forming units were seeded to 5 liters tap water, concentrated and determined by ELISA.

RESULTS

The effectiveness of nested PCR for detecting HAV corresponded to recovered value of 20 RIFA units per 1 liter of original sample before concentration. Rotavirus with $5.7x10^4$ infectious forming units per 5 l of unconcentrated water sample was detected in the eluate by ELISA whereas poliovirus type 1 starting with 10^{13} $TCID_{50}$ per 100 µl decreased to 10^5 $TCID_{50}$ per 100 µl after concentration.

One hundred water samples collected from various sources in the congested communities in Bangkok were analysed for poliovirus, HAV and rotavirus. The sewage and water samples from canal were concentrated 256-526 fold with reduced volume of water. The tap water was concentrated 256-2,000 fold. Out of 20 surface water samples from canals, 3 (15%) were positive for HAV RNA. Out of 48 sewage samples, 4 (8%) were positive for rotavirus antigen. Poliovirus, however, was not found in any sample by virus isolation (Table 1).

Three samples with positive HAV RNA and 4 samples with positive rotavirus antigen were characterized as shown in Table 2. The temperature was 30-31ºC. The pH of the surface water samples was 7.8-7.9 but of the sewage was rather acid in 1 sample (pH 6.8) and more alkali in 3 samples (pH 8.3-8.4). The samples were concentrated in the range of 256-526 fold. Each of the two samples: no.19, 20 and 73, 74 collected at the same site were positive for HAV and rotavirus, respectively. Surprisingly, all HAV RNA positive samples were surface water collected from the same canal receiving raw sewage drain though sample no. 18 was collected at different site from no. 19 and 20. All samples containing rotavirus antigen were domestic sewage collected from man-holes of underground sewers around the communities.

DISCUSSION

Since the densities of viruses in water are usually low, a virus concentration method is required. In another experiment, we evaluated the recovery efficiency of rotavirus in the adsorption-

Table 1
Virological analysis of sewage and water samples concentrated by the adsorption-elution technique.

Water source	No. of samples[a]	Type of water	No. of positive samples (%)		
			Poliovirus	HAV	Rotavirus
Tap	2	Tap water	0	0	0
Containers for					
drinking	4	Tap water	0	0	0
using	26	Tap water	0	0	0
Man-hole	48	Sewage	0	0	4 (8%)
Canals	20	Surface water	0	3 (15%)	0
Total	100		0	3 (3%)	4 (4%)

[a]Five liters of tap water and 1 liter of sewage and surface water were collected in duplicate and concentrated to 3-4 ml. Each 100 µl of the concentrated eluate was assayed for enteric virus.

Table 2
Characterization of samples contaminated by HAV and rotavirus.

Sample No.	Type of water/ source	Temp (ºC)	pH	Original/ concentrate volume	Virus detection[a]	
					HAV RNA	Rotavirus antigen
18	Surface water/canal	31	7.9	1,000 ml/3.6 ml	+	-
19[b]	Surface water/canal	30	7.8	1,000 ml/1.9 ml	+	-
20[b]	Surface water/canal	30	7.8	1,000 ml/3.6 ml	+	-
25	Sewage/man-hole	30	6.8	1,000 ml/3.5 ml	-	+
73[c]	Sewage/man-hole	31	8.3	1,000 ml/3.5 ml	-	+
74[c]	Sewage/man-hole	31	8.3	1,000 ml/3.2 ml	-	+
91	Sewage/man-hole	31	8.4	1,000 ml/3.9 ml	-	+

[a] The sample volume used is 100 µl of concentrated eluate in either nested PCR for the detection of HAV RNA or an ELISA for the detection of rotavirus antigen.
[b,c] Two samples were collected at the same site.

elution system. The recovery of rotavirus antigen was found to be 41.22 ± 13.58% (mean ± SD) in water samples concentrated by a procedure used in this study (Kittigul *et al*, submitted).

The present data is of interest to examine various viruses using three different methods. The sensitivity of nested PCR for HAV was 20 RIFA units per 1 liter of unconcentrated water samples. Although a number of RT-PCR methods for HAV have been applied to environmental water samples (Tsai *et al*, 1994; Metcalf *et al*, 1995; Schwab *et al*, 1995), there is little information on their sensitivity and specificity. Three of twenty surface water samples (15%) collected from canal contained HAV. The presence of HAV in canal waters was reported by Griffin *et al* (1999). HAV was more persistent than poliovirus in environmental water at 25ºC (Sobsey *et al*, 1988) and seawater at 4ºC (Tsai *et al*, 1993). Because of the relatively short life of viral RNA in natural water, the detection of virus in environmental samples by the RT-PCR was mainly due to the presence of well-protected viral particles and not due to the presence of naked viral RNA (Tsai *et al*, 1995). The enrichment of impurities and organic compounds of natural origin, which were coconcentrated along with viruses in environmental waters, might cause interference in PCR assays (Kopecka *et al*, 1993). However, the interference did not occur with a use of modified adsorption-elution method.

Rotavirus antigen was detected in domestic sewage of 8%. The presence of rotavirus in sew-

age was also observed in other studies (Steinmann, 1981; Hejkal *et al*, 1984). The considerable numbers of the original rotavirus retained survival at day 19 in suspended solids and fluffy and compact sediments in estuarine water. The persistence of rotavirus was able to allow transmission of infectious virus from polluted to nonpolluted water areas (Rao *et al*, 1986).

For poliovirus with reduction of $TCID_{50}$ after virus concentration method (from 10^{13} to 10^{5}), it may be due to the loss of virus or its infectivity during processing. Poliovirus was not detected in any water samples. Although the cell culture isolation is the only method that determined infectivity of the virus and still recommended in the standard method, the sensitivity is quite low (Abbaszadegan *et al*, 1999). The fungal contamination in some water samples caused the virus isolation difficult but it did not pose any problem in RT-PCR or ELISA.

The detection of HAV in surface water from canal and rotavirus in domestic sewage samples reflected the fecal presence in the environment around the congested communities that the people in these areas have a risk for enteric viruses. This study has demonstrated the potential usefulness of the virus concentration method, the nested PCR for detecting HAV RNA, and the ELISA for determination of rotavirus antigen in environmental water samples.

ACKNOWLEDGEMENTS

We thank Dr T Endy, Department of Virology, Armed Forces Research Institute of Medical Sciences for collaboration on the HAV RT-PCR technique, Mr Suebpong Nirunpan, Department of Microbiology, Faculty of Public Health for assistance in collection of water samples and Mrs Saisamorn Prasarnsang for typing the manuscript. This study was supported by Thai Government budget Grant.

REFERENCES

Abbaszadegan M, Stewart P, LeChevallier M. A strategy for detection of viruses in groundwater by PCR. *Appl Environ Microbiol* 1999; 65: 444-9.

American Public Health Association. Standard methods for the examination of water and wastewater, 18th ed. Washington DC: American Public Health Association, 1992.

Casas I, Powell L, Klapper PE, Cleator GM. New method for the extraction of viral RNA and DNA from cerebrospinal fluid for use in the polymerase chain reaction assay. *J Virol Methods* 1995; 53: 25-36.

De Serres G, Cromeans TL, Levesque B, *et al*. Molecular confirmation of hepatitis A virus from well water: epidemiology and public health implications. *J Infect Dis* 1999; 179: 37-43.

Gilgen M, Germann D, Luthy J, Hubner P. Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. *Int J Food Microbiol* 1997; 37: 189-99.

Griffin DW, Gibson CJ, Lipp EK, Riley K, Paul JH, Rose JB. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the florida keys. *Appl Environ Microbiol* 1999; 65: 4118-25.

Guttman-Bass N, Tchorsh Y, Marva E. Comparison of methods for rotavirus detection in water and results of a survey of Jerusalem wastewater. *Appl Environ Microbiol* 1987; 53: 761-7.

Hejkal TH, LaBelle RL, Keswick BH, Gerba CP, Hafkin B, Sanchez Y. Viruses in a small community water supply associated with a gastroenteritis epidemic. *J Am Water Works Assoc* 1982; 74: 318-21.

Hejkal TW, Smith EM, Gerba CP. Seasonal occurrence of rotavirus in sewage. *Appl Environ Microbiol* 1984; 47: 588-90.

Hopkins RS, Gaspard GB, Williams FP, Karlin RJ, Cukor G, Blacklow NR. A community waterborne gastroenteritis outbreak: evidence for rotavirus as the agent. *Am J Public Health* 1984; 74: 263-5.

Kopecka H, Dubrou S, Prevot J, Marechal J, Loípez-Pila JM. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. *Appl Environ Microbiol* 1993; 59: 1213-9.

Lemon SM, Binn LN, Marchwicki RH. Radioimmunofocus assay for quantitation of hepatitis A virus in cell cultures. *J Clin Microbiol* 1983; 17: 834-9.

Li JW, Wang XW, Rui QY, *et al*. A new and simple method for concentration of enteric viruses from water. *J Virol Methods* 1998; 74: 99-108.

Metcalf TG, Melnick JL, Estes MK. Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology-a trip of over 50 years. *Ann Rev Microbiol* 1995; 49: 461-87.

Rao VC, Metcalf TG, Melnick JL. Development of a method for concentration of rotavirus and its application to recovery of rotaviruses from estuarine

waters. *Appl Environ Microbiol* 1986; 52: 484-8.

Raphael RA, Sattar SA, Springthorpe VS. Long-term survival of human rotavirus in raw and treated river water. *Can J Microbiol* 1985; 31: 124-8.

Robertson BH, Khanna B, Nainan OV, Margolis HS. Epidemiologic patterns of wild-type hepatitis A virus determined by genetic variation. *J Infect Dis* 1991; 163: 286-92.

Sattar SA, Raphael RA, Springthorpe VS. Rotavirus survival in conventionally treated drinking water. *Can J Microbiol* 1984; 30: 653-6.

Schmidt NJ. Cell culture procedures for diagnostic virology. In: Schmidt NJ, Emons RW, eds. Diagnostic procedures for viral, rickettsial and chlamydial infections. Washington, DC: American Public Health Association, 1989: 51-100.

Schwab KJ, Leon RD, Sobsey MD. Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A virus, and Norwalk virus by reverse transcription-PCR. *Appl Environ Microbiol* 1995; 61: 531-7.

Sellwood J, Shore J, Read S, Wyn-Jones P. The use of reverse transcriptase-polymerase chain reaction to investigate environmental samples for the presence of enteroviruses. *Commun Dis Public Health* 1998; 1: 58-60.

Sobsey MD, Shields PA, Hauchman FS, Davis AL, Rullman VA, Bosch A. Survival and persistence of hepatitis A virus in environmental sample In: Zuckerman AJ, ed. Viral hepatitis and liver disease. New York: Alan R Liss, 1988: 121-4.

Steinmann J. Detection of rotavirus in sewage. *Appl Environ Microbiol* 1981; 41: 1043-5.

Toranzos GA, Gerba CP. An improved method for the concentration of rotaviruses from large volumes of water. *J Virol Methods* 1989; 24: 131-40.

Trask JD, Melnick JL, Wenner HA. Chlorination of human, monkey-adapted, and mouse strains of poliomyelitis virus. *Am J Hyg* 1945; 41: 30-40.

Tsai Y-L, Sobsey MD, Sangermano LR, Palmer CJ. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. *Appl Environ Microbiol* 1993; 59: 3488-91.

Tsai Y-L, Tran B, Palmer CJ. Analysis of viral RNA persistence in seawater by reverse transcriptase-PCR. *Appl Environ Microbiol* 1995; 61: 363-6.

Tsai Y-L, Tran B, Sangermano LR, Palmer CJ. Detection of poliovirus, hepatitis A virus, and rotavirus from sewage and ocean water by triplex reverse transcriptase PCR. *Appl Environ Microbiol* 1994; 60: 2400-7.

Wen Y-M, Xu Z-Y, Melnick JL. Virus hepatitis in China: problems and control strategies. In: Wen Y-M, Xu Z-Y, Melnick JL, eds. Monographs in virology, Basel: Karger 1992; 19: 159.