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An efficient virus concentration method and RT-nested PCR for detection of rotaviruses in environmental water samples

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Abstract

Water samples were concentrated by the modified adsorption–elution technique followed by speedVac reconcentration of the filter eluates. Reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) was used to detect rotavirus RNA in concentrates of the water. The detection limit of the rotavirus determined by RT-nested PCR alone was about 1.67 plaque forming units (PFU) per RT-PCR assay and that by RT-nested PCR combined with concentration from 1 l seeded tap water sample was 1.46 plaque forming units per assay. Water samples were collected from various sources, concentrated, and determined rotavirus RNA. Of 120 water samples, rotavirus RNA was detected in 20 samples (16.7%); 2/10 (20%) of the river samples, 8/30 (26.7%) of the canal samples, and 10/40 (25%) of the sewage samples but was not found in any tap water samples (0/40). Only three water samples were positive for rotavirus antigen determined using an enzyme-linked immunosorbent assay (ELISA). Alignment analysis of the sequenced PCR product (346-bp fragment) was performed in eight rotavirus-positive samples using the rotavirus sequence deposited in the GenBank. All samples gave the correct VP7 sequence. Results of analysis showed two samples similar to human rotavirus (97–98%), five similar to rotavirus G9 sequence (94–99%), and one sample similar to animal rotavirus (97%). PCR inhibitors were not observed in any concentrated water samples. In all 20 (of 120) samples where rotaviruses were found, fecal coliforms including *Escherichia coli* were also found, but of the samples testing negative for rotaviruses, 76 were fecal coliforms positive and 69 were *E. coli* positive. The combination of the virus concentration method and RT-nested PCR described below made it possible to effectively detect rotaviruses in environmental water samples.

Keywords: Virus concentration method; RT-nested PCR; Rotaviruses; Water samples

1. Introduction

Rotaviruses have been recognized as the major cause of acute gastroenteritis in young children (Kapikian and Chanock, 1996). In Thailand, the prevalence of rotavirus infection was found to be between 27 and 34% (Maneekarn and Ushijima, 2000). Although the fecal–oral route is the predominant mode of transmission for rotaviruses, fecally polluted water has been implicated as a possible source of gastroenteritis (Hopkins et al., 1984). The viruses are excreted in large number in the feces of infected individuals and may be dispersed in environmental waters (Santos et al., 1994). Outbreaks of gastroenteritis caused by rotaviruses waterborne have been reported (Hopkins et al., 1984; Kukkula et al., 1997). The presence of rotaviruses have been found in various sources of water such as sewage (Baggi and Peduzzi, 2000; Dubois et al., 1997; Gajardo et al., 1995; Kittigul et al., 2000), river water (Baggi and Peduzzi, 2000; Gilgen et al., 1997), ground water (Abbaszadegan et al., 1999), and even drinking water (Gratacap-Cavallier et al., 2000). The stability of rotaviruses in environmental

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water and their resistance to water treatment may facilitate transmission to humans (Ansari et al., 1991; Raphael et al., 1985; Sattar et al., 1984). However, there is little data on the presence of rotaviruses in environmental water used for domestic and recreational purposes in Thailand.

Since only a few virus particles are present in water samples, detection of viruses requires the concentration of a large volume of water. The adsorption–elution technique has successfully been used to concentrate the virus (Abbaszadegan et al., 1999; Gilgen et al., 1997) although in one study rotavirus double-stranded RNA was detected directly from water samples (Dubois et al., 1997). In our laboratory, we have studied the factors affecting the concentration procedure and modified the method of concentrating the rotavirus from water samples (Kittigul et al., 2001). A highly sensitive technique for virus detection is prefered to ascertain the presence of the virus in environmental samples. Although cell culture-based methods are capable of isolating infectious viruses, they are laborious and time-consuming, and the sensitivity is low (Hot et al., 2003). Enzyme immunoassay has been used, but to a lesser extent, to determine the presence of the rotavirus antigen in water samples and sewage samples (Dahling et al., 1993; Kittigul et al., 2000). During the past decade, reverse transcriptase polymerase chain reaction (RT-PCR) emerged as a means to detect enteric viruses including rotaviruses in water samples (Abbaszadegan et al., 1999; Baggi and Peduzzi, 2000; Dubois et al., 1997; Fout et al., 2003; Gajardo et al., 1995; Gilgen et al., 1997; Gratacap-Cavallier et al., 2000).

An improved concentration method and an RT-nested PCR method were compared with the enzyme-linked immunosorbent assay (ELISA) for determining the presence of rotaviruses. The relation between presence of rotaviruses and the presence of fecal bacterial indicators in environmental water samples collected from several sources in Bangkok, Thailand were also examined.

2. Materials and methods

2.1. Water samples

From August to December 2001, 1 l water samples were collected from different sites of the Chaophraya River and from the canal beside a congested community in Bangkok, Thailand. Raw domestic sewage samples and samples from a swamp containing sewages were also collected (1 l each). Five litres of tap water samples were collected from domestic use storage containers in this community. The total of 120 water samples included 40 samples from sewage sources, 30 from the canal, 10 from the river, and 40 from storage containers. All samples were stored in a cooler and transported to the lab for processing within 2 h.

2.2. Concentration of rotaviruses from water samples

Viruses were concentrated from water samples using an adsorption–elution technique with negatively charged membrane filtration and reconcentrated using a speedVac concentrator. The method was established in our laboratory and published previously (Kittigul et al., 2001). Briefly, tap water was dechlorinated by sodium thiosulfate with a final concentration of 50 mg/l. All water samples, including tap water, domestic sewage, canal, and river water, were acidified to pH 3.5 with 1N HCl and aluminum chloride was added to a final concentration of 0.0015N. The mixture was stirred at room temperature for at least 30 min. Then, the water was passed through a GN-6 Metricel® filter, 47 nm in diameter and 0.45 μm porosity (Gelman, Ann Arbor, MI). The membrane filter was washed with 0.14N NaCl (pH 3.5). The adsorbed virus was eluted by using 2.9% tryptose phosphate broth containing 6% glycine, pH 9.0. The eluate was neutralized to pH 7.0–7.4 by the addition of 4N HCl. The volume of the eluate was further reduced by using a SpeedVac concentrator for 4–5 h. After reconcentration, the eluate was adjusted again to pH 7.0–7.4 with 4N HCl. The concentrated samples were stored at −80 °C until use.

2.3. Viruses

Bovine rotavirus or human rotavirus was used as positive control for the RT-nested PCR of rotavirus. The human rotavirus (Ito strain) serotype 3 was kindly provided by Dr. A. Bosch (University of Barcelona, Spain). Sequence data accessed from the National Center for Biotechnology Information/GenBank has the accession number K02033 (human WA rotavirus gene 9).

2.4. Extraction of RNA

RNA was extracted from 140 μl of concentrated water samples using the QIAamp® Viral RNA kit (QIAGEN AG, Basel, Switzerland) according to the manufacturer's instructions. The 60-μl RNA eluates were stored at −20 °C until further amplification of nucleic acids.

2.5. Primers

Primers RV1, RV2, RV3, and RV4 first published by Gilgen et al. (1997) were used for the amplification of sequences from the VP7 gene of group A rotaviruses.

2.6. RT-nested PCR

RNA used for reverse transcription was denatured for 3 min at 94 °C and chilled on ice for 10 min. Reverse transcription was undertaken with a 20-μl mixture containing 2 μl of nucleic acid extract and 18 μl of reaction mixture. With some modifications, we followed the instructions of the RT-PCR manufacturer (Promega, Madison, MI) and the method described by Gilgen et al. (1997). All the reagents required were included in a RT-PCR kit. The samples were added to a mixture consisting of 5 mM of $MgCl_2$, 1× RT buffer (10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton®

X-100), 1 mM of each dNTP, 1 U/μl recombinant Rnasin® ribonuclease inhibitor, 15 U/μg AMV reverse transcriptase, 1.25 μM of primer RV1 (downstream) and pyrocarbonic acid diethyl ester (DEPC)-treated water to a final volume of 20 μl. The RT mixture was incubated for 1 h at 41 °C, heated to 99 °C for 5 min, and then placed on ice. First-strand cDNA diluted 1:5 (10 μl) was added to 40 μl of PCR mixture. The final concentrations were: 200 μM of each dNTP, 2 mM of $MgCl_2$, 1× PCR buffer (20 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Nonidet® P40 and 0.5% Tween® 20), 0.25 μM of primer RV1, 0.25 μM of primer RV2, 2.5 U of *Taq* DNA polymerase. All were mixed with DEPC-treated water. Amplifications were performed with a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, Conn.). The cycling conditions were as follows: 60 s at 94 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, and the final extension step at 72 °C for 3 min. Then, the temperature was decreased to 4 °C. For nested PCR, 1 μl of the first amplification reaction was further amplified under the same conditions of amplification as for the first PCR except for changing the primer pair to RV3 and RV4 and their concentrations to 0.5 μM and the concentration of $MgCl_2$ to 3.5 mM. We expected to see a fragment of 346 bp. A positive control (rotavirus RNA) and negative controls (extraction, RT-nested PCR reagents, phosphate-buffered saline, rotavirus-negative tap water concentrate) were included for each PCR assay.

2.7. Gel electrophoresis and DNA sequencing

PCR products were electrophoresed in 1.5% agarose gels in Tris-borate–EDTA buffer along with a 100-bp DNA ladder (BioLabs, Beverly, MA) as a standard marker. The gels were stained with ethidium bromide and illuminated by an UV transilluminator.

Amplified products (346 bp) were purified and sequenced at the Bioservice Unit of the National Science and Technology Development Agency, Bangkok using the same forward (RV3) primer. The sequences were aligned and searched for nearly identical sequences using the Basic Local Alignment Search Tool (BLAST) program available on the NCBI network server.

2.8. ELISA

The presence of rotavirus antigen in the concentrated water samples was determined by a commercial rotavirus test kit (IDEIA™ Dako, Cambridshire, UK) following the procedure recommended by the manufacturer.

2.9. Bacteriological analysis

All water samples were tested for bacterial indicators of faecal pollution according to a standard method (Clesceri et al., 1998). Values for fecal coliform and *Escherichia coli* were calculated from most probable number (MPN) tables. The standard or acceptable levels of fecal coliforms in canal water for recreational use are 4×10^3 MPN/100 ml (Pairoj-Boriboon, 1989).

3. Results

3.1. Sensitivity and specificity of RT-nested PCR

The detection limit of rotavirus concentration was determined by serial two-fold dilutions of bovine rotavirus in phosphate-buffered saline. The RT-nested PCR was capable of detecting bovine rotavirus at the concentration of 3.57×10^2 plaque forming units (PFU)/ml or 1.67 PFU per RT-PCR reaction (Fig. 1). No cross-reactivity was found between the primers of rotavirus and poliovirus at concentration of 7.14×10^5 50% tissue culture infective doses/ml or hepatitis A virus at concentration of 7.71×10^2 radioimmunofocus assay units/ml when they were used as target RNA. After the addition of known concentrations of rotavirus in 1 l tap water, the water samples were concentrated, and the level of rotavirus was determined. No rotavirus DNA band was observed in any concentrations of rotavirus with RT-PCR products, whereas the nested PCR products of the seeded samples displayed the 346-bp fragment at the lowest concentration of 5×10^2 PFU/1 l tap water. The detection limit of rotavirus determined by the RT-nested PCR in combination with concentration from 1 l of seeded tap water was 1.46 PFU per assay.

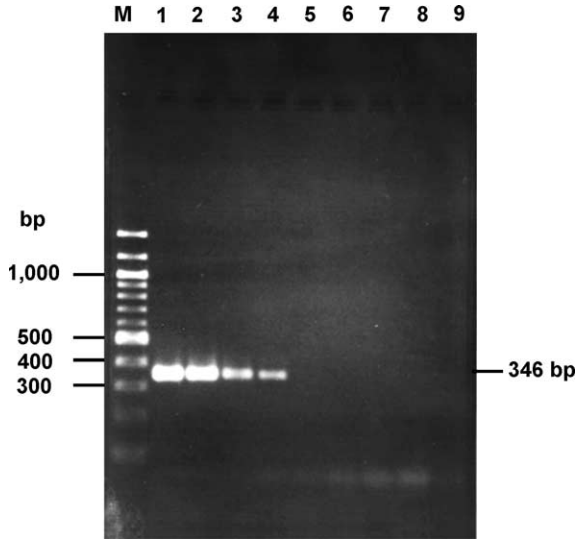

Fig. 1. The detection limit of rotavirus concentration in phosphate-buffered saline, as analyzed by agarose gel electrophoresis of amplification products. Lanes: (1) 6.67; (2) 4.99; (3) 3.33; (4) 1.67; (5) 0.83 PFU/RT-PCR assay; (6) phosphate-buffered saline; (7–9), RT, PCR, and nested PCR negative controls. M, 100-bp DNA ladder for molecular size standard.

3.2. RT-nested PCR in environmental water samples

A total of 120 water samples collected from the river (10 samples), a canal (30 samples), sewage sources (40 samples), and tap water (40 samples) were concentrated in a range of 125–2500-fold and the presence of rotavirus RNA was determined using RT-nested PCR. After secondary amplification, 20 samples (16.7%) displayed the 346-bp fragment. Rotaviruses were detected in 20% (2/10) of the river samples, 26.7% (8/30) of the canal samples, and 25% (10/40) of the sewage samples, but were not found in any tap water samples, as shown in Fig. 2. Of 20 rotavirus-positive samples, only three samples were also positive for rotavirus antigen determined using ELISA.

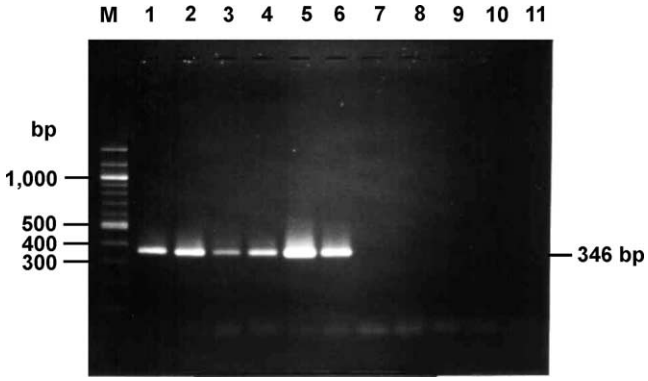

Fig. 2. The detection of rotaviruses in water samples. Five litres of water samples were collected from the storage containers for domestic uses in a congested community, Bangkok. One litre of water samples were collected from the river, canal and sewage sources surrounding the community. The water samples were concentrated and detected rotavirus RNA using the RT-nested PCR. Nested PCR products were visualized by agarose gel analysis and ethidium bromide staining. Lanes: (1) rotavirus-positive control of RT-nested PCR (346-bp); (2 and 4) sewage; (3) river; (5 and 6) canal; (7 and 8) tap water samples; (9–11) RT, PCR, and nested PCR negative controls. M, 100-bp DNA ladder for molecular size standard.

3.3. Sequence analysis

Among 20 rotavirus-positive concentrated samples, the nested PCR products of eight samples were sequenced with RV3 primer. Alignment analysis of the 346-bp fragment was performed using the BLAST program. Results of analysis indicated human rotavirus VP7 sequence in two samples (97–98%), G9 sequence in five samples (94–99%), and animal rotavirus in one sample (97%).

3.4. PCR inhibitors in environmental water samples

To eight concentrated sewage samples that did not show amplification, rotavirus was added and then the samples were amplified using RT-nested PCR. After amplification, positive results were obtained when the rotavirus was present in the water samples of 6.67, 3.33, and 1.67 PFU per RT-PCR assay. The detection limit was still the same as for the concentration of rotavirus in phosphate-buffered saline.

Table 1
Characteristics and bacteriological results of 20 water samples which rotaviruses were detected

No.	Sample code	Sample date	pH	Temperature	Fecal coliforms[a] (MPN/100 ml)	*E. coli* (MPN/100 ml)
Sewage						
1	SW05[b]	22 August	7.1	28	4.9×10^5	3.3×10^4
2	SW19	17 September	8.3	31	1.7×10^6	1.1×10^6
3	SW23	19 September	8.7	29	3.3×10^5	3.3×10^5
4	SW24	19 September	8.3	30	1.4×10^6	1.4×10^6
5	SW30	26 September	7.4	28	1.7×10^6	1.7×10^6
6	SW35	1 October	6.9	30	4.3×10^4	3.5×10^4
7	SW36	10 October	7.9	28	4.9×10^5	1.3×10^5
8	SW37	10 October	7.8	27.5	3.5×10^6	1.1×10^6
9	SW38	10 October	7.7	28	1.3×10^6	4.5×10^4
10	SW39	10 October	7.7	28	2.2×10^6	2.3×10^5
Canal						
11	CW06	15 October	7.3	30	2.2×10^6	1.7×10^6
12	CW13	22 October	7.7	29	7.9×10^5	1.7×10^5
13	CW17[b]	22 October	7.3	30	3.3×10^6	3.3×10^6
14	CW18[b]	22 October	7.3	30	2.3×10^6	3.4×10^5
15	CW25	31 October	7.4	29	2.3×10^6	1.3×10^6
16	CW26	31 October	7.4	29	1.3×10^6	2.7×10^5
17	CW27	31 October	7.4	29	2.2×10^6	1.1×10^5
18	CW30	31 October	7.7	29	4.9×10^5	3.3×10^5
River						
19	RW08	5 September	7.5	31	4.9×10^3	4.9×10^3
20	RW10	5 September	7.5	33	1.3×10^4	3.4×10^3

[a] Value of acceptable level for fecal coliforms: 4×10^3 MPN/100 ml for canal samples.
[b] The water samples positive for rotavirus by both RT-nested PCR and ELISA.

3.5. Characteristics of rotavirus-positive samples

Twenty water samples that gave positive results for rotavirus RNA had been concentrated approximately 150–1000-fold. A number of samples were collected from different sites on the same day. Most rotavirus-positive samples were collected in October 2001. The pH and temperature of sewage samples varied when compared with canal and river water samples. Bacteriological data shows that all water samples had fecal coliforms and the canal samples contained such bacteria above the acceptable level (4×10^3 MPN/100 ml). *E. coli* was present in all samples (Table 1).

The results of all 120 samples were examined for presence of rotaviruses and/or bacterial indicators. In all the 20 samples, where rotaviruses were detected fecal coliforms (including *E. coli*) were also detected. However, of the samples testing positive for fecal coliforms there were 76 samples in which rotaviruses could not be detected, and of the samples testing positive for *E. coli* there were 69 samples in which rotaviruses were not detected.

4. Discussion

In this study, the virus concentration method using the adsorption–elution technique along with speedVac reconcentration was evaluated in its application together with RT-nested PCR for the detection of rotavirus RNA in water samples collected from several sources in Bangkok, Thailand. In the test of the concentration method by the seeding experiment, rotavirus was detectable at the concentration of 5×10^2 PFU/1 l tap water or 1.46 PFU per assay, whereas the experimentally determined RT-nested PCR detection limit was 3.57×10^2 PFU/ml of phosphate-buffered saline or 1.67 PFU per assay. It seems that no rotavirus lost during the concentration process as measured by the RT-nested PCR. The concentration method is more efficient than previously reported (Kittigul et al., 2001) due to the difference in the volume of water and the method used for detecting rotaviruses. Previously, we studied the virus concentration method in 100 ml of tap water and the detection by the less sensitive ELISA.

The RT-nested PCR system was carried out specifically to detect group A rotaviruses. The primers used in this study were synthesized following the oligonucleotide sequence published by Gilgen et al. (1997). They selected those primers because they can hybridize to highly conserved regions of the viral genome. In the present study, the specificity of the RT-nested PCR for rotavirus was carried out by use of different enteric viruses (hepatitis A virus and poliovirus) and proved to be highly specific.

The sensitivity of RT-nested PCR makes it suitable for application to environmental water samples. After testing 120 water samples for the presence of rotaviruses, we were able to detect them in 20 samples: 20% of the river samples, 26.7% of the canal samples, and 25% of the sewage samples. A greater percentage of the canal samples contained rotaviruses than did the sewage samples. This might be due to sewage draining into the canal from which the samples were taken. No rotaviruses were detectable in any of the tap water samples from the storage containers used for domestic purposes. Although a serological test kit was applied for detection of rotavirus in environmental samples (Dahling et al., 1993), we found that the RT-nested PCR giving 20 rotavirus-positive samples had approximately seven times higher sensitivity than ELISA (three rotavirus-positive samples). Computer aided DNA sequence analysis comparison of rotavirus-positive water samples with data banks enabled us to classify eight rotavirus samples: human rotavirus (two samples), rotavirus G9 (five samples), and animal rotavirus (one sample). The DNA sequence of rotavirus G9 from all five samples was found to be 90% identical to the human rotavirus sequence. So, it seems that those samples were of human origin. The emergence of serotype G9 human rotavirus strains has been reported worldwide and it has been suggested that they originated through genetic reassortment (Ramachandran et al., 2000.), possibly involving a genetic recombination between human and animal rotaviruses (Gratacap-Cavallier et al., 2000).

Inhibitors of PCR reaction might be already present in highly polluted water or might be introduced during the concentration procedure (Abbaszadegan et al., 1993; Kopecka et al., 1993; Shieh et al., 1995). In the present study, the detection limit of RT-nested PCR for detection of rotaviruses added to rotaviruses negative sewage samples was the same as detection of rotaviruses added to phosphate-buffered saline. So, no PCR inhibitors were present. Previously, the method of concentrating virus by the adsorption–elution technique through an electropositive membrane filter has been found to eliminate PCR-interfering substances (Queiroz et al., 2001). In the present study, using a different concentration procedure involving a negatively charged membrane filter, no inhibitors for PCR were detected in concentrated sewage samples.

Although PCR has a limitation in its inability to discriminate between viable and noninfectious viruses, the process described here (virus concentration, RNA purification, and RT-nested PCR) can be applied successfully for the detection of rotaviruses in environmental samples and considered as a method for revealing the enteric virus contamination in water samples. The detection of rotaviruses in the environment or in drinking water is needed to help in the risk assessment for viral gastroenteritis. This powerful tool could prove to be valuable in the testing of water quality and in the epidemiological investigation of the source of fecal waste contamination.

Although standard bacterial indicators are used currently as safety criteria for water and the presence of coliphages is used as a parameter for the virological quality of water, no association between the presence of certain viruses and bacterial indicators had been previously found (Grabow et al., 2001; Hot et al., 2003). Nevertheless, in the present study, in every case where rotaviruses were found, fecal coliforms including *E. coli* were also found (although in the majority

of fecal coliform positive samples rotaviruses were not detected). The high degree of bacterial contamination-possibly due to the low standard of hygiene among people in the community where the samples were taken-may account for the absence of rotavirus-positive samples without fecal bacteria present.

In conclusion, the present study demonstrates the effectiveness of the described method for concentrating rotavirus and the sensitivity of RT-nested PCR for detecting the VP7 gene of rotaviruses in environmental water samples. The presence of rotaviruses in the river and canal could constitute a potential health risk for people.

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References

Abbaszadegan, M., Huber, M.S., Gerba, C.P., Pepper, I.L., 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. Appl. Environ. Microbiol. 59, 1318–1324.

Abbaszadegan, M., Stewart, P., LeChevallier, M., 1999. A strategy for detection of viruses in groundwater by PCR. Appl. Environ. Microbiol. 65, 444–449.

Ansari, S.A., Springthorpe, V.S., Sattar, S.A., 1991. Survival and vehicular spread of human rotaviruses: possible relation to seasonality of outbreaks. Rev. Infect. Dis. 13, 448–461.

Baggi, F., Peduzzi, R., 2000. Genotyping of rotaviruses in environmental water and stool samples in Southern Switzerland by nucleotide sequence analysis of 189 base pairs at the 5′ end of the VP7 gene. J. Clin. Microbiol. 38, 3681–3685.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998. Standard methods for examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.

Dahling, D.R., Wright, B.A., Williams, F.P., 1993. Detection of viruses in environmental samples: suitability of commercial rotavirus and adenovirus test kit. J. Virol. Methods 45, 137–147.

Dubois, E., Le Guyader, F.S., Haugarreau, L., Kopecka, H., Cormier, M., Pommepuy, M., 1997. Molecular epidemiological survey of rotaviruses in sewage by reverse transcriptase seminested PCR and restriction fragment length polymorphism assay. Appl. Environ. Microbiol. 63, 1794–1800.

Fout, G.S., Martinson, B.C., Moyer, M.W., Dahling, D.R., 2003. A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater. Appl. Environ. Microbiol. 69, 3158–3164.

Gajardo, R., Bouchriti, N., Pinto, R.M., Bosch, A., 1995. Genotyping of rotaviruses isolated from sewage. Appl. Environ. Microbiol. 61, 3460–3462.

Gilgen, M., Germann, D., Luthy, J., Hubner, Ph., 1997. 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. 37, 189–199.

Grabow, W.O., Taylor, M.B., de Villiers, J.C., 2001. New methods for the detection of viruses: call for review of drinking water quality guidelines. Water Sci. Technol. 43, 1–8.

Gratacap-Cavallier, B., Genoulaz, O., Brengel-Pesce, K., Soule, H., Innocenti-Francillard, P., Bost, M., Gofti, L., Zmirou, D., Seigneurin, J.M., 2000. Detection of human and animal rotavirus sequences in drinking water. Appl. Environ. Microbiol. 66, 2690–2692.

Hopkins, R.S., Gaspard Jr., G.B., Williams, F.P., Karlin, R.J., Cukor, G., Blacklow, N.R., 1984. A community waterborne gastroenteritis outbreak: evidence for rotavirus as the agent. Am. J. Public Health. 74, 263–265.

Hot, D., Legeay, O., Jacques, J., Gantzer, C., Caudrelier, Y., Guyard, K., Lange, M., Andreoletti, L., 2003. Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water. Water Res. 37, 4703–4710.

Kapikian, A.Z., Chanock, R.M., 1996. Rotaviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), Fields Virology, third ed. Lippincott–Raven, Philadelphia, pp. 1657–1708.

Kittigul, L., Raengsakulrach, B., Siritantikorn, S., Kanyok, R., Utrarachkij, F., Diraphat, P., Thirawuth, V., Siripanichgon, K., Pungchitton, S., Chitpirom, K., Chaichantanakit, N., Vathanophas, K., 2000. Detection of poliovirus, hepatitis A virus and rotavirus from sewage and water samples. Southeast Asian J. Trop. Med. Public Health 31, 41–46.

Kittigul, L., Khamoun, P., Sujirarat, D., Utrarachkij, F., Chitpirom, K., Chaichantanakit, N., Vathanophas, K., 2001. An improved method for concentrating rotavirus from water samples. Mem. Inst. Oswaldo. Cruz. 96, 815–821.

Kopecka, H., Dubrou, S., Prevot, J., Marechal, J., 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.

Kukkula, M., Arstila, P., Klossner, M.L., Maunula, L., Bonsdorff, C.H., Jaatinen, P., 1997. Waterborne outbreak of viral gastroenteritis. Scand. J. Infect. Dis. 29, 415–418.

Maneekarn, N., Ushijima, H., 2000. Epidemiology of rotavirus infection in Thailand. Pediatr. Int. 42, 415–421.

Pairoj-Boriboon, S., 1989. Laws and standards on pollution control in Thailand, second ed. Environmental Quality Standards Division Office of the National Environment Board, Bangkok.

Queiroz, A.P.S., Santos, F.M., Sassaroli, A., Harsi, C.M., Monezi, T.A., Mehnert, D.U., 2001. Electropositive filter membrane as an alternative for the elimination of PCR inhibitors from sewage and water samples. Appl. Environ. Microbiol. 67, 4614–4618.

Ramachandran, M., Kirkwood, C.D., Unicomb, L., Cunliffe, N.A., Ward, R.L., Bhan, M.K., Clark, H.F., Glass, R.I., Gentsch, J.R., 2000. Molecular characterization of serotype G9 rotavirus strains from a global collection. Virology 278, 436–444.

Raphael, R.A., Sattar, S.A., Springthorpe, V.S., 1985. Long-term survival of human rotavirus in raw and treated river water. Can. J. Microbiol. 31, 124–128.

Santos, N., Riepenhoff-Talty, M., Clark, H.F., Offit, P., Gouvea, V., 1994. VP4 genotyping of human rotavirus in the United States. J. Clin. Microbiol. 32, 205–208.

Sattar, S.A., Raphael, R.A., Springthorpe, V.S., 1984. Rotavirus survival in conventionally treated drinking water. Can. J. Microbiol. 30, 653–656.

Shieh, Y.S.C., Wait, D., Tai, L., Sobsey, M.D., 1995. Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by the polymerase chain reaction. J. Virol. Methods 54, 51–66.