An Improved Method for Concentrating Rotavirus from Water Samples

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A modified adsorption-elution method for the concentration of seeded rotavirus from water samples was used to determine various factors which affected the virus recovery. An enzyme-linked immunosorbent assay was used to detect the rotavirus antigen after concentration. Of the various eluents compared, 0.05M glycine, pH 11.5 gave the highest rotavirus antigen recovery using negatively charged membrane filtration whereas 2.9% tryptose phosphate broth containing 6% glycine; pH 9.0 was found to give the greatest elution efficiency when a positively charged membrane was used. Reconcentration of water samples by a speedVac concentrator showed significantly higher rotavirus recovery than polyethylene glycol precipitation through both negatively and positively charged filters (p-value <0.001). In addition, speedVac concentration using negatively charged filtration resulted in greater rotavirus recovery than that using positively charged filtration (p-value = 0.004). Thirty eight environmental water samples were collected from river, domestic sewage, canals receiving raw sewage drains, and tap water collected in containers for domestic use, all from congested areas of Bangkok. In addition, several samples of commercial drinking water were analyzed. All samples were concentrated and examined for rotavirus antigen. Coliforms and fecal coliforms (0->1,800 MPN/100 ml) were observed but rotavirus was not detected in any sample. This study suggests that the speedVac reconcentration method gives the most efficient rotavirus recovery from water samples.

Key words: rotavirus - speedVac reconcentration - water samples

Rotaviruses cause acute gastroenteritis mainly in infants and young children admitted to hospital (Kapikian & Chanock 1996). The viruses are excreted in large numbers in the feces of infected individuals. Waterborne outbreaks of rotavirus have been reported (Hopkins et al. 1984, Kukkula et al. 1997). Rotavirus was detected in sewage (Mehnert & Stewien 1993, Gajardo et al. 1995, Dubois et al. 1997), river water (Gilgen et al. 1997), groundwater (Abbaszadegan et al. 1999), and drinking water (Jothikumar et al. 1995, Gratacap-Cavallier et al. 2000). The presence of rotavirus in water and sewage indicates contamination of the virus in the environment. However, the density of virus in water is so low that virus concentration is necessary for detection. Several methods for concentrating waterborne viruses have been suggested but adsorption-elution from microporous filters seems to be the most promising technique (Guttman-Bass & Armon 1983, Lewis & Metcalf 1988, Li et al. 1998, Abbaszadegan et al. 1999). Although the reverse transcriptase-polymerase chain reaction (RT-PCR) technique was developed for direct detection of rotavirus double-stranded RNA (ds RNA) from water samples (Dubois et al. 1997), the virus concentration technique by adsorption-elution continues to be used for detection of rotavirus ds RNA (Gilgen et al. 1997, Abbas zadegan et al. 1999). Reconcentration methods carried out by aluminum hydroxide precipitation, polyethylene glycol precipitation, or organic flocculation, require the addition of polycationic salts and acidification of the primary eluate, thus leading to the possibility of virus loss (Lewis & Metcalf 1988, Toranzos & Gerba 1989). The use of evaporation together with centrifugation by a speedVac concentrator as secondary concentration presented here could decrease the volume of the eluate efficiently and overcome the drawbacks of other reconcentration techniques.

The purpose of this study was to determine the factors that influence the concentration of rotavirus

The study was supported by a grant from the China Medical Board, Faculty of Public Health, Mahidol University.

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Received 14 November 2000
Accepted 9 May 2001

from water samples by using a modified adsorption-elution technique. The recoveries of rotavirus antigen in concentrated samples were examined by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Virus concentration method - The technique for concentration of virus was carried out using a standard method (American Public Health Association 1992), with some modification. Inactivated bovine rotavirus was seeded in a tap water sample of 100 ml. Prior to inactivation, the material contained approximately 10^5 infectious forming units (IFU) per ml, as determined by cell culture. Therefore, the amount of inactivated rotavirus in 100 ml was ~$3.5X10^4$ IFU. The tap water was dechlorinated by sodium thiosulfate with a final concentration of 50 mg/l. For the negatively charged filter, the seeded tap water was preconditioned to acid pH at 3.5 with 1N HCl and its ionic condition was adjusted with $AlCl_3$ to a final concentration of 0.0015 N. The mixture was stirred for at least 30 min. The tap water was placed in the adsorbent filter holder and passed through a GN-6 Metricel® filter, 47 mm in diameter and 0.45 µm porosity (Gelman, Ann Arbor, MI). The membrane filter was washed with 0.15 N NaCl, pH 3.5, to remove excess Al^{3+}. The adsorbed virus was eluted by passing an eluent solution (7.5 ml) through the filter. The primary eluate was neutralized to pH 7 and the volume of the eluate was further reduced by reconcentration methods.

For the positively charged filter, seeded tap water at pH 8.0 was passed through a Zetapor® 1 MDS adsorbent filter, 47 mm in diameter and 0.2 µm porosity (AMT Cuno, Meriden, CT) without preconditioning the sample. The adsorbed virus was eluted with an eluent solution and the primary eluate was reconcentrated.

Eluents - Three groups of eluents containing tryptose phosphate broth (TPB), beef extract (BE), or glycine, were included in the virus concentration technique. The TPB eluates were 1% TPB + 6% glycine + 6% arginine pH 9.0, and 2.9% TPB + 6% glycine pH 9.0. The BE eluates were 3% BE pH 9.0 and 3% BE + 0.05M glycine pH 9.5. In the glycine group, 0.05M glycine pH 10.5, and 0.05M glycine pH 11.5, were used in the experiments.

Reconcentration methods

Organic flocculation - The method of reconcentration by organic flocculation was performed as described by Guttman-Bass and Armon (1983). Briefly, the primary eluate was seeded with rotavirus ($3.5X10^4$ IFU) and the pH was lowered to 3.5, while shaking at room temperature for 30 min. The eluate was centrifuged at 3,000 x g for 10 min. Then, the pellet was dissolved in a 1/20 vol. of 0.15M NaH_2PO_4/ Na_2HPO_4, pH 7.5 and stored at -80°C until viral assay.

Polyethylene glycol precipitation - Reconcentration by polyethylene glycol (PEG) precipitation was carried out essentially as described by Lewis and Metcalf (1988), with some modification. The primary eluate was brought to 3% beef extract and adjusted to pH 7.5. PEG (MW 6,000) was added to a final concentration of 8%. The eluate was stirred at 4°C for 2 h and centrifuged at 10,000 x g for 20 min. The pellet was suspended in 0.15M NaH_2PO_4/Na_2HPO_4, pH 9.0 and sonicated at high frequency (20 kHz ± 50 Hz) with a sonicator (Sonics & Materials, Danbury, CT) for 30 sec. Next, the suspension was shaken at room temperature for 20 min, and centrifuged again at 10,000 x g for 30 min. Finally, the supernatant was collected and stored at -80°C until use.

SpeedVac concentration - A speedVac instrument (Savant, Famingdale, NY) was used for reconcentrating the primary eluate. The technique combined centrifugal force, vacuum, and heating at medium temperature (43°C) for solvent removal and sample concentration. It took 4 h for concentration of the eluate (7.5 ml) to a final volume of 0.5-1 ml. The concentrated eluate was stored at -80°C until assay.

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

Water samples - Water samples were collected in sterile 1-liter bottles from a river (4 samples), domestic sewage (8 samples) and canals receiving raw sewage drains (12 samples), all located in congested areas of Bangkok. Tap water collected in containers for domestic use (10 samples) and commercial drinking water (4 samples from different producers) were also obtained in sterile 5-liter bottles. Temperature and pH of all 38 samples were measured. The samples were transported to the laboratory in an ice box. Coliforms and fecal coliforms were identified by bacteriological methods (Hitchins et al. 1992). The water samples were concentrated in optimal conditions and determined for rotavirus antigen by using the IDEIA™ kit.

The experiments comparing virus concentration methods are described in Figs 1, 2, 3. The environmental water samples were processed according to the scheme shown in Fig. 4.

Data analysis - Recovery rates of rotavirus antigen were calculated as the percentage of OD value of the spiked virus after concentration compared with that before the concentration process. Analysis of variance was used to determine the

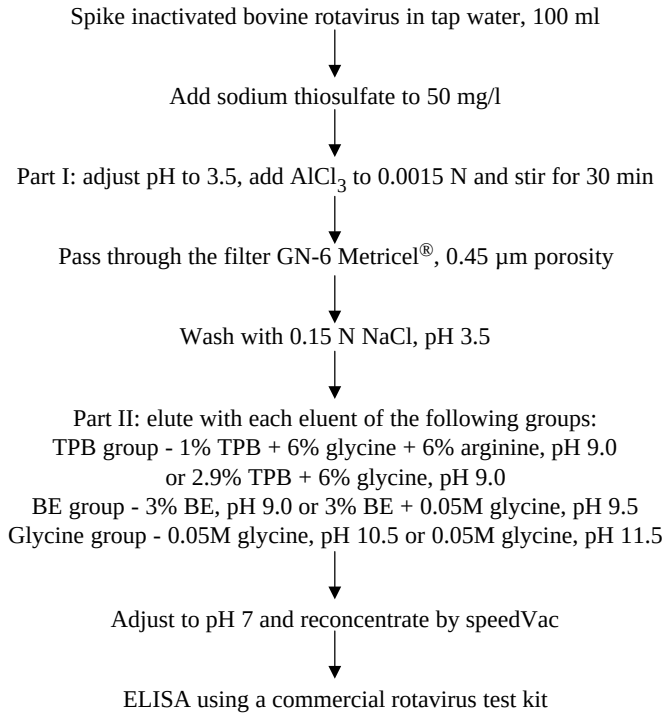

Fig. 1: rotavirus concentration method using negatively charged membrane filtration: various eluents

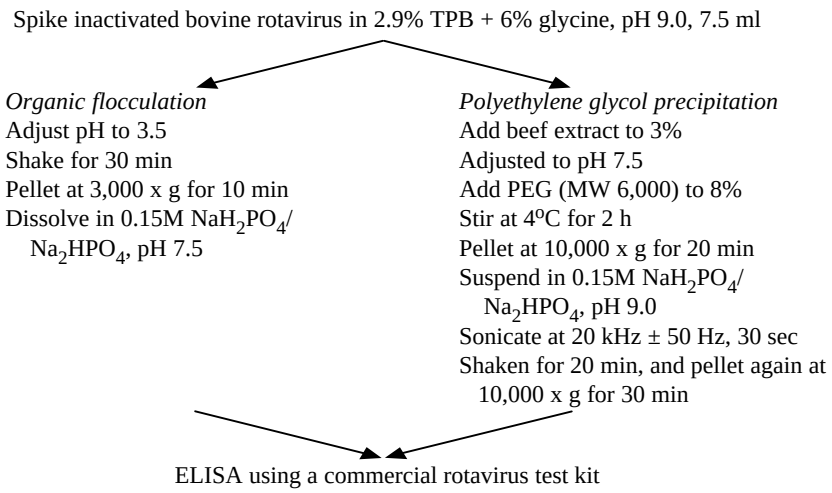

Fig. 2: rotavirus reconcentration methods

effects of various factors. Such effects were confirmed by analysis of one way ANOVA for three variables or t-test for two variables in each factor.

RESULTS

Effect of eluents, and reconcentration methods - Various eluents were compared using a negatively charged membrane filter (Fig. 1). After triplicate experiments, 0.05 M glycine, pH 11.5, gave the highest mean recovery (53%) followed by 2.9% TPB + 6% glycine, pH 9.0 (47.9%) and 3% BE + 0.05 M glycine, pH 9.5 (37.7%). The use of 6% arginine in 1% TPB and 6% glycine gave a rotavirus recovery of 43.1%. After passing the same eluent five times, the rotavirus recovery was lower than passing just once in both 2.9% TPB + 6% glycine, pH 9.0 and

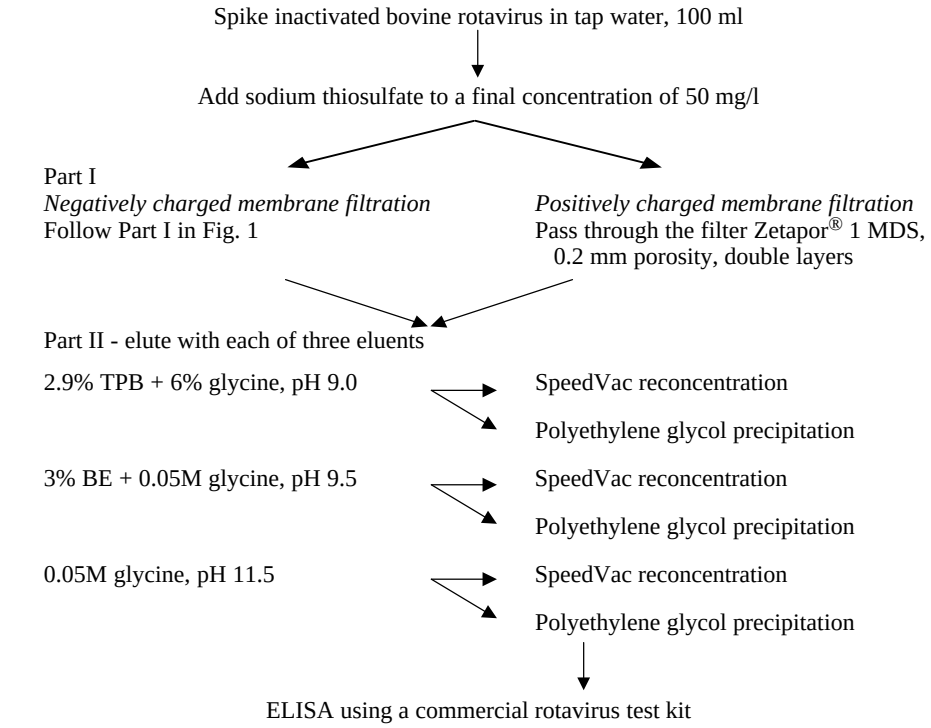

Fig. 3: rotavirus concentration using different membrane filters, eluents and reconcentration methods.

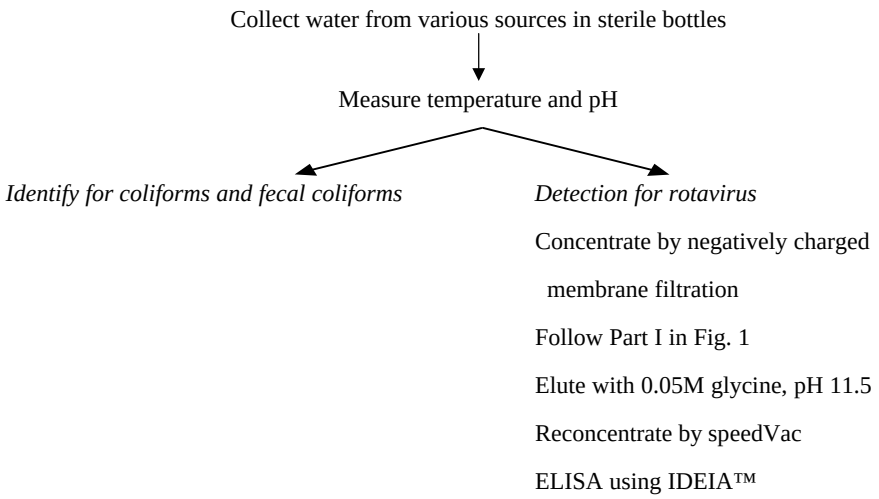

Fig. 4: processing of environmental water samples (1l or 5l)

0.05 M glycine, pH 10.5, as shown in Table I. The three different kinds of eluents which gave the highest recoveries of rotavirus were further assayed. These eluents included 0.05M glycine pH 11.5, 2.9% TPB + 6% glycine pH 9.0, and 3% BE + 0.05M glycine pH 9.5. In the comparison of the two reconcentration methods (Fig. 2), less rotavirus was recovered using organic flocculation (23.3%) than with PEG precipitaion (45.7%). The three eluents and the two reconcentration methods (PEG precipitation and speedVac concentration) were compared during the use of positively and negatively charged membrane filtration (Fig. 3).

Rotavirus recovery on different conditions - When negatively charged filtration and speedVac reconcentration were used, 0.05M glycine, pH 11.5

gave the highest rotavirus recovery of 56.6 ± 2.7% (mean ± SD) followed by 2.9% TPB + 6% glycine, pH 9.0 (41.2 ± 13.6%) and 3% BE + 0.05M glycine, pH 9.5 (37.6 ± 5.3%). Whatever eluents were used, reconcentration by PEG precipitation recovered less than half of the virus, as shown in Table II.

Lower recoveries were observed using positively charged filtration. With speedVac reconcentration, 2.9% TPB + 6% glycine, pH 9.0 gave the highest rotavirus recovery of 34.9 ± 8.4% followed by 3% BE + 0.05M glycine, pH 9.5 (28.2 ± 5.7%) and 0.05M glycine, pH 11.5 (25.3 ± 0.1%) whereas PEG precipitation gave a much lower rotavirus recovery (Table III).

Factors influencing virus concentration method - The three different kinds of eluent did not affect the efficiency of rotavirus concentration. The use of speedVac concentration increased the efficiency of the virus concentration method significantly over PEG precipitation when using both negatively and positively charged membranes, *p*-value <0.001. Moreover, reconcentration by a speedVac concentrator gave a significantly higher virus recovery in negatively charged filtration than that in positively charged filtration with a *p*-value of 0.004, as shown in Table IV.

Rotavirus antigen in environmental water samples - The modified adsorption-elution method was used to determine rotavirus antigen in water samples collected from various sources with a use of a negatively charged filter, 0.05M glycine, pH 11.5, and speedVac concentration (Fig. 4). The environmental water samples had pH and temperature in the range of 7.23-8.75 and 28-32, respectively. After reconcentration, the water samples were concentrated 2,000-10,000 fold. Although coliforms and fecal coliforms (0->1,800 MPN/ml) were found in water samples collected from sewage, canals, river and tap water in containers for drinking or domestic uses, rotavirus antigen was not detected by a commercial ELISA kit.

TABLE I

Recovery of rotavirus antigen using negatively charged filters with different eluents

Eluent	Rotavirus antigen recovered (%)[a]			
	No. 1	No. 2	No. 3	Mean ($\bar{x}$)
1% TPB + 6% glycine + 6% arginine, pH 9.0	43.13	ND	ND	43.13
2.9% TPB + 6% glycine, pH 9.0	52.42	50.58	40.85	47.95
2.9% TPB + 6% glycine, pH 9.0, 5 times[b]	30.22	ND	ND	30.22
3% BE, pH 9.0	41.64	35.73	34.70	37.36
3% BE + 0.05 M glycine, pH 9.5	42.68	33.46	37.09	37.74
0.05 M glycine, pH 10.5	59.56	ND	ND	59.56
0.05 M glycine, pH 10.5, 5 times[b]	43.69	ND	ND	43.69
0.05 M glycine, pH 11.5	62.03	50.99	46.03	53.02

a: tap water, 100 ml was spiked with 350 µl of rotavirus (10^5 infectious forming units/ml); *b*: the filter was eluted with the same eluent for 5 times; ND: not determined.

TABLE II

Recovery of rotavirus concentrated by negatively charged filtration with three different eluents on speedVac concentration or polyethylene glycol (PEG) 6000 precipitation

Eluents	Rotavirus recovered (%)					
	2.9% TPB + 6% glycine pH 9.0		3% BE + 0.05 M glycine pH 9.5		0.05 M glycine pH 11.5	
Experiment[a]	SpeedVac concentration	PEG precipitation	SpeedVac concentration	PEG precipitation	SpeedVac concentration	PEG precipitation
No. 1	30.41	18.18	34.57	10.13	57.77	17.80
No. 2	36.86	14.82	34.48	16.14	58.46	22.76
No .3	56.32	23.29	43.66	19.85	53.55	23.08
Mean ($\bar{x}$) ± SD	41.22 ± 13.58	18.76 ± 4.27	37.57 ± 5.27	15.37 ± 4.91	56.59 ± 2.66	21.21 ± 2.96

a: tap water, 100 ml was spiked with 350 µl of rotavirus (10^5 infectious forming units/ml), concentrated by adsorption-elution technique using a negatively charged membrane (GN-6, Metricel with 0.45 µm porosity) and reconcentrated by speedVac concentration or polyethylene glycol (PEG) 6000 precipitation.

TABLE III

Recovery of rotavirus concentrated by positively charged filtration with three different eluents and speedVac concentration or polyethylene glycol (PEG) 6000 precipitation

Eluents	Rotavirus recovered (%)					
	2.9% TPB + 6% glycine pH 9.0		3% BE + 0.05 M glycine pH 9.5		0.05 M glycine pH 11.5	
Experiment[a]	SpeedVac concentration	PEG precipitation	SpeedVac concentration	PEG precipitation	SpeedVac concentration	PEG precipitation
No. 1	44.57	17.03	34.74	23.51	25.14	16.30
No. 2	30.13	13.73	24.59	20.73	25.32	13.46
No. 3	29.87	12.93	25.32	20.20	25.32	13.73
Mean ($\bar{x}$) ± SD	34.86 ± 8.41	14.56 ± 2.17	28.22 ± 5.66	21.48 ± 1.78	25.26 ± 0.10	14.50 ± 1.57

a: tap water, 100 ml was spiked with 350 µl of rotavirus (10^5 infectious forming units/ml), passed through positively charged membranes (double layers of Zetapor, 0.20 µm). After elution, the sample was reconcentrated by speedVac concentration or polyethylene glycol (PEG) 6000 precipitation.

TABLE IV

Recovery of rotavirus concentrated by negatively or positively charged membrane filtration with different eluents and reconcentration methods

Characteristics	Negative charged filter Mean ± SD	Positive charged filter Mean ± SD	*p*-value
Eluents			
2.9% TPB + 6% glycine, pH 9.0	29.99 ± 15.22	24.71 ± 12.40	NA
3% BE + 0.05 M glycine, pH 9.5	26.47 ± 12.98	24.85 ± 5.26	NA
0.05 M glycine, pH 11.5	38.90 ± 19.54	19.88 ± 5.98	NA
p-value	0.411[a]	0.529[a]	
Reconcentration methods			
SpeedVac concentration	45.13 ± 11.44	29.44 ± 6.62	0.004[b]
PEG precipitation	18.45 ± 4.38	16.85 ± 3.83	0.421[b]
p-value	< 0.001[b]	< 0.001[b]	

NA: not analyzed; *a*: one way ANOVA; *b*: t-test

DISCUSSION

Of the eluents used, 0.05 M glycine, pH 11.5 gave the highest mean recovery after triplicate experiments. The primary eluate was simply adjusted to pH 7.0 to avoid the possibility of appreciable virus inactivation because it contained phenol red, as a pH indicator. To reduce the possibility of viral inactivation at pH 11.5, elution with glycine, pH 10.5, was attempted but it was observed that elution with this solution gave lower and more erratic recoveries than did elution at pH 11.0 (Farrah et al. 1976). Three percent BE gave a rotavirus recovery lower than elution with glycine buffer although BE was observed to be a more efficient eluent than glycine buffer in other studies (Sobsey & Glass 1980, Guttman-Bass & Armon 1983). In positively charged filtration, the efficiency of virus concentration method was greatly reduced by elution with 0.05 M glycine, pH 11.5 but was not when 2.9% TPB + 6% glycine, pH 9.0 was used. This indicates that the latter might be an appropriate eluent for both negatively and positively charged membranes.

SpeedVac reconcentration yielded the highest rotavirus recovery. The evaporation combined with centrifugation simultaneously reduced the volume of the eluate and concentrated the samples 2,000-10,000 fold. The use of a speedVac concentrator in the second step of rotavirus concentration allowed for the reduction of eluate volume without the need for the addition of cationic salts and acidification. This method has several advantages over the existing PEG precipitation and organic flocculation procedures. Furthermore, we found that at least one virus (poliovirus) is not inactivated during the reconcentration process, as determined by cell culture isolation (unpublished observation).

The positively charged membrane could absorb virus without preconditioning of water at acid pH or addition of $AlCl_3$ but it gave a lower recovery and significantly different results compared with the negatively charged membrane. Therefore the

optimum condition for rotavirus concentration method was negatively charged filtration, elution with 0.05 M glycine, pH 11.5, 2.95 TPB + 6% glycine, pH 9.0 or 3% BE + 0.05 M glycine, pH 9.5, and reconcentration by speedVac. Using this technique, rotavirus antigen has been detected in domestic sewage (Kittigul et al. 2000). In this study, rotavirus antigen was not detected in any sample. This may be due to the different sites of sample collection. Eluents containing BE should be avoided because it can cause non-specific reaction in ELISA (Jansons & Bucens 1986) and inhibition of virus detection by the RT-PCR technique (Kopecka et al. 1993, Schwab et al. 1993). The application of a commercial ELISA was reported for determination of rotavirus antigen in environmental water samples with high sensitivity and specificity (Dahling et al. 1993). Although rotavirus was not detected by a commercial ELISA in the present study, it is emphasized that the use of modified adsorption-elution technique in optimum condition and speedVac reconcentration is an effective method for rotavirus concentration from water samples.

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