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Development of a Virus Concentration Method and its Application for the Detection of Noroviruses in Drinking Water in China

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A new procedure for the concentration of nonoviruses from water samples has been developed. This procedure (calcium flocculation-citrate dissolution method) uses the following steps: virus flocculation formed by treatment with 1 M $CaCl_2$ and 1 M Na_2HPO_4, virus release by sodium citrate dissolution (0.3 M Na citrate, pH 3.5), and virus re-concentration by ultrafiltration. When reverse transcription (RT)-PCR was performed after the procedure, the overall detection sensitivity for seeded noroviruses in a one liter drinking water sample was as low as 1 RT-PCR unit, which is equal to a 10^{-6} dilution of the virus sample. This approach showed at least a 5-fold-higher sensitivity than the current method with its three steps of adsorption-elution-concentration. The newly developed procedure was used to test different brands of bottled drinking water from China for putative contamination with noroviruses. A total of 144 samples were analyzed; all of the samples were negative for norovirus specific nucleic acids.

Keywords: Noroviruses, concentration, drinking water

Noroviruses (NVs), formerly called Norwalk-like viruses or small round structured viruses, are single-stranded RNA viruses which form a genus *norovirus* within the *Caliciviridae* family. Based on comparisons with the genetic sequences of viral RNA-dependent RNA polymerases and capsid proteins, noroviruses have been subdivided into five genogroups (GI-GV). The two largest genogroups, GI and GII, include most of the diverse and common noroviruses (Ando *et al.*, 2000; Pang *et al.*, 2005; Zheng *et al.*, 2006). NVs are a major cause of viral acute gastroenteritis worldwide. Transmission through water is one of the major exposure routes (Koopmans *et al.*, 2002) as is the fecal-oral route and transmission via foodstuffs. Large outbreaks of waterborne acute gastroenteritis caused by NVs have been well documented (Beuret *et al.*, 2000; Haefliger *et al.*, 2000; Beuret *et al.*, 2002; Borchardt *et al.*, 2003; Parshionikar *et al.*, 2003). To limit and/or prevent such outbreaks, rapid and reliable methods to detect NVs are required. To date, noroviruses have not been cultivated *in vitro*. Laboratory detection methods depend primarily on electron microscopy (EM) and the use of the reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR has been demonstrated to be one of the most-sensitive methods available for NV monitoring (Green *et al.*, 1998; Vinjé *et al.*, 2000; Vinjé *et al.*, 2003).

Since only a few viral particles are typically present in water samples, efficient methods to concentrate the viruses from water samples are urgently needed. For drinking water surveys, the noroviruses infectious dose can be as low as 10-100 virus particles (Koopmans *et al.*, 2002). The concentration method currently used, the three-step isolation method, or, the adsorption-elution-concentration method, concentrates the viruses in water by adsorption to and subsequent elution from a positively charged membrane and then reconcentrates the viruses by ultrafiltration (Gilgen *et al.*, 1997; Beuret *et al.*, 2002). This method has been widely used in the analysis of bottled and natural mineral waters for the presence of noroviruses in recent years (Gassilloud *et al.*, 2003; Lamothe *et al.*, 2003), and we define it as the "current method" in this study. However, this method has limitations. First, beef extract (1 to 3%, pH 9 to 11) is used to elute viruses in this procedure, and the contents of beef extract are suspected to have an inhibitory effect on cDNA synthesis and PCR amplification (Abbaszadegan *et al.*, 1993), Second, it may cause significant loss of viruses as the filter cannot retain viral particles efficiently and the viral elution from the filter is not complete (Lamothe *et al.*, 2003). In order to reduce the inhibitory effects of the eluate, instead of using an organic eluate, a method has been developed where the use of beef extract was substituted with a step of rinsing the membrane with H_2SO_4, followed by NaOH (pH 10.8) elution (Katayama *et al.*, 2002; Haramoto *et al.*, 2004). In addition, a one-step method whereby viruses are directly lysed after filtration on a negatively charged membrane has also been reported (Beuret, 2003). Little effort has been spent to reduce the loss of viruses in the filtration procedure except for using charged filter membranes.

In this study, we established a new method to concentrate viruses in water samples by using a flocculation-dissolution-concentration procedure which is beef extract-free and by

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using a non-charged filter. This new method was applied to the detection of NVs in drinking water in China.

Materials and Methods

Virus samples

Stool samples that tested positive for noroviruses (NV GII) by EM and RT-PCR were obtained from the Center for Viral Disease Control and Prevention, China CDC (Fang *et al.*, 1995). Each stool sample was prepared by making a 1:10 (wt/vol) dilution in phosphate-buffered saline (0.1 M PBS, pH 7.2) followed by thorough mixing and centrifugation at 3,000×g for 5 min. Supernatants were used as virus samples and were stored at -80°C until use. The titer of NVs in the virus samples was determined to be 10^7 RT-PCR units (RT-PCRU) per milliliter by measuring the serial diluted virus samples using the selected RT-PCR method (Sair *et al.*, 2002).

Concentration of viral particles

Each virus sample (100 μl) was ten-fold diluted to 10^{-5} and 10^{-6} with 0.1 M PSB, pH 7.2, then 100 μl of the 10^{-5} dilution, 50 μl of the 10^{-5} dilution and 100 μl of the 10^{-6} dilution would contain 10, 5 and 1 RT-PCRU NVs, respectively, and could be used for the seeding experiments. A one liter drinking water sample was seeded with the above diluted virus samples that containing 10, 5, and 1 RT-PCRU NVs, respectively. To the water sample was added 1 ml 1 M $CaCl_2$ followed by the addition of 1 ml 1 M Na_2HPO_4. The water sample was stirred for 5 min to allow for the formation of flocculation, and subsequently filtrated through a non-charged hybrid cellulose ester membrane with a 0.45 μm pore size and a 50 mm diameter (Xinya Co., Ltd, China). After filtration, the membrane was transferred to a 9 cm-diameter Petri dish, and was rinsed with 4 ml 0.3 M citrate buffer (pH 3.5) for 3 min to dissolve the virus-contained flocculate. The 4 ml virus-contained buffer was further concentrated to less than 100 μl by using a micro-concentrator (Ultrafilter, Millipore, Ireland) at 7,500×g at room temperature. The retained concentrate was adjusted to 100 μl with sterilized double distilled water.

The above concentration procedure is termed the "calcium flocculation-citrate dissolution method."

RNA extraction

RNA was isolated from the retained concentrate using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The extracted RNA was air-dried and redissolved in 10 μl of diethyl-pyrocarbonate (DEPC) treated water prior to the reverse transcription reaction.

Oligonucleotide primers

The primers used for NVs detection in this study are JV12 and JV13 as described previously (Vinjé *et al.*, 2000). The primers are suitable for both detection of NVs GI and GII. Primers were synthesized by the Shanghai Shenggong Biological Engineering Technology and Service CO. Ltd. The primers are shown in Table 1.

Reverse transcription

For the reverse transcription reaction, RNA (10 μl) was incubated for 60 min at 40°C and for 5 min at 95°C, then chilled on ice for 10 min. In a final volume of 20 μl, the reaction contained 1× RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 0.5 mM dNTPs, 1 μM JV13, 10 U Rnasin (Promega, USA) and 100 U M-MLV reverse transcriptase (Promega, USA).

PCR

The reverse transcription product (15 μl) was used for PCR in a total volume of 50 μl containing 1× PCR buffer (50 mM KCl, 100 mM Tris-HCl pH 9.0, 1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of *Taq* polymerase (Promega), and 0.3 μM primers (JV12, JV13). After an initial denaturation step at 94°C for 3 min, 40 amplification cycles (94°C, 1 min; 37°C, 1.5 min; and 74°C, 1 min) were performed followed by a final extension step at 74°C for 7 min.

Analysis of the PCR products

Each amplicon (10 μl) was mixed with 10 μl loading buffer and was analyzed on a 2.6% agarose gel. Products were visualized by ethidium bromide staining and UV transillumination. Product sizes were determined by comparison with a 1 kb DNA ladder (Promega). RT-PCR performed in this study generated a 327 bp amplicon from a NV positive sample.

Evaluation of the new concentration method

Each 100 μl virus sample was further diluted to 10^{-1} to 10^{-7} with PBS (0.1 M, pH 7.2) to determine the experimental detection limit for RT-PCR.

In order to confirm the recovery efficiency in our new method, a comparison study was conducted by using the current three-step isolation method (Gilgen *et al.*, 1997; Beuret *et al.*, 2002). The three-step isolation procedure combines filtration with a positively charged nylon membrane, ultrafiltration and clean-up of the viral RNA with a silica based membrane. Briefly, water samples were first filtrated through a positively charged 0.45 μm pore size, 47 mm diameter membrane (Zetapor filter membrane; CUNO Inc., USA), then 4 ml 50 mM glycine-NaOH (pH 9.5) containing 1% beef extract was used for virus elution. The elution buffer was adjusted to pH 8.0 with 20 μl of 1.0 M HCl, and then the 4 ml virus-containing buffer was concentrated to 100 μl by use of a microconcentrator. RNA extraction and RT-PCR was then carried out as described above.

Table 1. Oligonucleotides primers of the Noroviruses

Primer	Orientation	DNA sequence (5'-3')	nt Position (range)	Product size (bp)
JV12	+	ATACCACTATGATGCAGATTA	4552-4572	327
JV13	-	TCATCATCACCATAGAAAGAG	4858-4878	

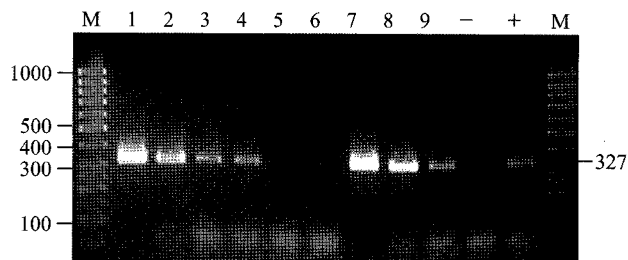

Fig. 1. RT-PCR analysis with norovirus sequences (amplication of 327 bp) in one liter seeded water samples. The 2.6% agarose gel was stained with ethidium bromide. Lanes 1, 4, 7 show virus samples containing 10 RT-PCRU NVs; lanes 2, 5, 8 show virus samples containing 5 RT-PCRU NVs; lanes 3, 6, 9 virus samples containing 1 RT-PCRU NVs. Our described method was used for one liter seeded samples in lanes 1-3, the current three-step isolation method was used for samples in lanes 4-6, and RNA extraction was performed without seeding and concentration steps for samples in lanes 7-9. +, positive control; -, negative controls; M, 1 kb size markers

The new method compared with the current method in seeding experiments for triplicate. For the seeding experiment, 100 μl of a 10^{-5} dilution, 50 μl of a 10^{-5} dilution and 100 μl of a 10^{-6} dilution of the virus samples were seeded to 1 liter purified (virus-free) drinking water samples to ensure the presence of 10, 5, and 1 RT-PCRU NVs in the test samples, respectively.

Drinking water samples

Water samples were obtained from 18 different brands of bottled waters. These drinking waters were packaged in PVC bottles with a volume ranging from 350 ml to 2 liter or were filled into 20 liter PVC barrels. These samples include 2 brands of imported bottled mineral water, 2 brands of domestic bottled mineral water, 7 brands of bottled purified water, 1 brand of bottled distilled water, and 6 brands of barreled purified water. All water samples were bought from a local grocery in January, March, May, and July of 2006, respectively, and surveys were taken at the time of purchase. A one liter volume of each water samples was used in each test; a one liter sample of sterilized purified water was used as a negative control, and a one liter sterilized purified water sample inoculated with 100 μl of a 10^{-4} dilution of the virus sample was used as positive control. A total of 144 samples were tested. The tested water samples are described in Table 2.

Controls

All precautions were taken to prevent false-positive or false-negative results. Amplifications were performed in different rooms, and filter-equipped pipette tips were used throughout the assay. All experiments were repeated at least twice, and a negative control sample (*i.e.*, containing no nucleic acid) was run with each test.

Results

Concentration procedure

Our method includes two subsequent concentration steps. The first concentration step is to absorb virus particles to an inorganic flocculate formed by the reaction of Ca^{2+} and HPO_4^{2-}. By using a low pH buffer (pH 3.5) of sodium citrate, the subsequent dissolution step rapidly and thoroughly

Table 2. Water samples investigated during the survey

Brand code	Water type	Origin	Production date	Tested samples
A	Bottled mineral water	France	2005.11, 2006.1, 2006.2, 2006.3	8
B	Bottled mineral water	Japan	2005.12, 2006.1, 2006.3, 2006.5	8
C	Bottled mineral water	Jilin, China	2005.12, 2006.2, 2006.4, 2006.6	8
D	Bottled mineral water	Guangdong, China,	2006.1, 2006.3, 2006.5, 2006.7	8
E	Bottled purified water	Hubei, China	2005.11, 2006.1, 2006.2, 2006.5	8
F	Bottled purified water	Guangdong, China	2005.10, 2006.1, 2006.2, 2006.6	8
G	Bottled purified water	Guangdong, China	2005.12, 2006.1, 2006,3, 2006.7	8
H	Bottled purified water	Guangdong, China	2005.11, 2006.2, 2006.2, 2006.5	8
I	Bottled purified water	Guangdong, China	2005.12, 2006.1, 2006.2, 2006.6	8
J	Bottled purified water	Guangxi, China	2006.1, 2006.3, 2006.5, 2006.7	8
K	Bottled purified water	Zhehiang, China	2006.1, 2006.3, 2006.5, 2006.7	8
L	Bottled distilled water	Guangdong, China	2005.10, 2005.12, 2006.2, 2006.5	8
M	Barreled purified water	Guangdong, China	2006.1, 2006.3, 2006.5, 2006.7	8
N	Barreled purified water	Guangdong, China	2006.1, 2006.3, 2006.5, 2006.7	8
O	Barreled purified water	Guangdong, China	2006.1, 2006.3, 2006.5, 2006.7	8
P	Barreled purified water	Guangdong, China	2006.1, 2006.3, 2006.5, 2006.7	8
Q	Barreled purified water	Guangdong, China	2006.1, 2006.3, 2006.5, 2006.7	8
R	Barreled purified water	Guangdong, China	2006.1, 2006.3, 2006.5, 2006.7	8
Total			From 2005.10 to 2006.7	144

dissolved the flocculate. This procedure can concentrate the water samples from one liter to 4 ml, and is beef extract-free and thus avoids its inhibitory effects on RT-PCR. The next concentration step is to concentrate the above solution from 4 ml to 100 μl by ultrafiltration with a commercially available microconcentrator. These two concentration steps together can easily concentrate the NVs 10,000 times for one liter water samples.

Sensitivity of the newly developed concentration method

The experimental detection limit of RT-PCR was determined by incorporating serial ten-fold dilutions of the virus sample. The virus sample was still detectable at the last dilution of 10^{-6}.

A result of the comparison study between use of the novel concentration method and the current three-step isolation method is shown in Fig. 1. There was a similar recovery rate of our new method (lanes 1-3) to the RT-PCR (lanes 7-9), and there was an evident recovery loss using the current method (lanes 4-6). All of the seeding samples were detectable using our new concentration method; the overall detection sensitivity for seeded NVs in a one liter drinking water sample was as low as 1 RT-PCR unit which is equal to the 10^{-6} dilution of the positive virus sample, indicating that the detection limit in the seeding experiment is the same as the experimental detection limit. The comparison experiments were performed three times with consistent results. The new method detected as little as one RT-PCRU in one liter water samples while the current method could only detect 10 RT-PCRU but failed to detect 5 RT-PCRU in one liter samples. These results demonstrate that the newly developed method has at least a 5 fold-higher sensitivity than the current method.

Detection of NVs in drinking water

The new concentration method was applied to detect NVs in different drinking water samples. The tested samples included mineral water, distilled water and purified drinking water, and originated from the North (2 brands) and the South (14 brands) of China as well as from overseas sources (2 brands). The samples were produced on different dates from November 2005 to July 2006. The tests were performed in January, March, May, and July, respectively (36 samples for each test). A total of 144 samples were assayed, and all of the tested samples were negative for NV specific nucleic acids.

Discussion

Noroviruses are currently unable to be cultured *in vitro* and hence are impossible to be detected using classical cell culture methods. PCR has been demonstrated to be the most powerful tool to detect NVs, and many PCR methods, including reverse transcription (RT)-PCR, nested RT-PCR and seminested RT-PCR, have been developed in recent years (Afliger *et al.*, 1997; Green *et al.*, 1998; Schwab *et al.*, 1998; Vinjé *et al.*, 2000; Sair *et al.*, 2002). In an initial study, two PCR methods were selected and their detection limits were compared (data not shown). One is direct RT-PCR using the primers JV12 and JV13 (Vinjé *et al.*, 2000), which can detect a 10^{-6} dilution of the tested virus sample. The other method, nested RT-PCR, which now is used as the CRL standard for NVs detection by CEFAS (Green *et al.*, 1998), was demonstrated to be less sensitive and only detected a 10^{-4} dilution of the same virus sample. In this study, we focused on using direct RT-PCR for detection.

As there only a few virus particles present in water, it is essential to establish a method to concentrate viruses efficiently from the water samples and to exclude any existing RT-PCR inhibitors. Previously studies have demonstrated that a positively charged filter membrane possesses a high ability to recover polioviruses from water samples (>60%) (Sobsey and Jones, 1979; Sobsey and Glass, 1980). Based on these findings, the adsorption-elution-concentration method was developed for NV concentration and was widely used. However, it was latter revealed that this adsorption-elution-concentration method recovered NVs inefficiently, and the detection limit in seeding experiment was two orders of magnitude less sensitive than the RT-PCR detection limit for virus samples (Gilgen *et al.*, 1997). As we found in our laboratory, by using the positively charged membrane concentrating method, the detection limit were 10 RT-PCRU NVs in one liter seeded water sample, one order of magnitude less sensitive than the experimental RT-PCR detection limit. Beef extract, which is commonly used to elute NVs in the current concentration method, has been suggested to have inhibitory effect on cDNA synthesis and PCR amplification and may account for the lower sensitivity of detection. Accordingly, many methods were used to remove inhibitors from the beef extract, such as resin treatments (Abbaszadegan *et al.*, 1993), glass purification (Ijzerman *et al.*, 1997), and directly lysed viruses form the membrane with special equipment and special kit (Beuret, 2003). But all these procedures are expensive and labor-intensive.

Our new concentrating method has a high ability to recover NVs from water samples. The detection limit of this new protocol in seeding experiment is 1 RT-PCRU NVs per one liter seeded water, which is no less than the experimental detection limit of the direct RT-PCR on virus samples without any seeding and concentration steps. There are several advantages of our newly established method. First, the viruses are not adsorbed to a positively charged filter membrane as in the current method but rather are adsorbed and enveloped by an inorganic flocculate formed by Ca^{2+} and HPO_4^{2-}. This flocculation step is far more efficient than the normal filter adsorption step that is unable to retain viral particles efficiently. Second, the viruses are not released from the membrane by elution with beef extract, which may be not complete, but rather by dissolution with a citrate buffer in which the viruses can be sufficiently and rapidly released. Furthermore, the inhibitory problems caused by beef extract are avoided. No special materials or equipment, such as charged membrane filters and multiple vacuum filtration apparatus are required. Our study demonstrates that the approach is a rapid, simple and efficient way to concentrate NVs, and that the procedure is more sensitive but consumes less time and is less expensive.

NVs sequences have been detected in bottled water in a Swiss study (Beuret *et al.*, 2002) in which NVs sequence were detected in 53 (33%) of 159 samples and the sequences belonged entirely to genogroup II. In our study of 144

bottled drinking water samples, no NV RNA was detected. There are no obvious technical reasons to explain the large discrepancy between the results of the Swiss investigations and those presented here. In fact, the RT-PCR method with primers JV12 and JV13 has been demonstrated to be reliable in an international collaborative study (Vinjé *et al.*, 2003), and exhibited a high sensitivity of detecting virus sample in our study.

Mineral water that originates from deep groundwater reservoirs is well protected from fecal contamination. Purified water is processed by ion-exchange, micro-filtrate, and reverse osmosis technology, and no microorganisms will exist. Distilled water is obviously free from viruses. Considering these reasons, the result that NV RNA was not present in the investigated drinking water is quite reasonable. In fact, our results of this study are in accord with a multicenter study (Lamothe *et al.*, 2003).

In conclusion, the results of this study demonstrate that the calcium flocculation-citrate dissolution method is a rapid, effective and inexpensive method to concentrate NVs from drinking water. The water survey results also imply that NVs may not be a concern for drinking water safety in China.

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