The Journal of Microbiology (2012) Vol. 50, No. 5, pp. 726–734
DOI 10.1007/s12275-012-2279-y

Reliability of Non-Culturable Virus Monitoring by PCR-Based Detection Methods in Environmental Waters Containing Various Concentrations of Target RNA

Eung Seo Koo, Chang-Hoon Yoo, Youjin Na, Soo Young Park, Hey Rhyoung Lyoo, and Yong Seok Jeong*

Department of Biology, College of Sciences, Kyung Hee University, Seoul 130-701, Republic of Korea

(Received May 24, 2012 / Accepted June 7, 2012)

Owing to the lack of practical cell culture system for human noroviruses (HuNoV), various detection methods based on conventional reverse transcription-PCR (RT-PCR) and the quantitative real-time PCR have been major tools for monitoring environmental water safety. In this study, we showed that the proportion of water sample concentrates used for one-step RT-PCR significantly influences false-negative findings of the non-culturable viruses. In total, 59 archived samples of previously analyzed water concentrates were re-examined for HuNoV RNA by the one-step RT-PCR and semi-nested PCR. Using new aliquots for RNA extraction for every trial, up to 20 PCR trials were performed for each archive to determine whether the crosscheck results supported the previous determinations. We reconfirmed that 27.6% (8/29) of the samples were HuNoV-positive samples: 6.7% (1/15) from groundwater, 33.3% (3/9) from river water, and 80% (4/5) from treated sewage effluent (TSE). These results corresponded to the ratio of previously negative HuNoV samples now identified as positive (8/30): 6.7% (1/15) from groundwater, 20% (1/5) from river water, and 60% (6/10) from TSE. To elucidate the cause of these results, 16 different concentrations of murine norovirus (MNV) RNA (from 2×10^2 to 8×10^3 copies, divided into 10 tubes for each concentration) were subjected to one-step RT-PCR. The detection frequency and reproducibility decreased sharply when the number of MNV RNA copies fell below threshold levels. These observations suggest that the proportion of water concentrate used for PCR-based detection should be considered carefully when deciding viral presence in certain types of environmental water, particularly in regard with legal controls.

Keywords: non-culturable virus, human noroviruses, RT-PCR, false-negative

*For correspondence. E-mail: ysjeong@khu.ac.kr; Tel.: +82-2-961-0829; Fax: +82-2-961-0244

Introduction

Enteric viruses are shed in enormous quantities in the feces of infected patients (10^9 to 10^{10}/g) and can persist for several months in groundwater, particularly at low temperatures. As shown in previous studies, high concentrations of these viruses, spread through various routes and eventually reach different water settings, including groundwater, where the viruses persist at extremely low concentrations (Bosch *et al.*, 2008).

Numerous surveillance studies for environmental viruses have been performed worldwide to preserve various water resources. Viruses associated with human diseases transferred via water and food consumption include poliovirus, echovirus, enterovirus, coxsackievirus, hepatitis A, adenovirus, norovirus, hepatitis E, rotavirus, and astrovirus (Ventura *et al.*, 2000; Sanchez *et al.*, 2007; Schwab, 2007; Gerba, 2009; Meng, 2010; Rohayem *et al.*, 2010; Savolainen-Kopra and Blomqvist, 2010; Todd *et al.*, 2010; Mena and Rhoades *et al.*, 2011). In humans, these viruses cause mild to severe gastroenteritis, meningitis, respiratory disease, and hepatitis (Carter, 2005). In recent decades, human norovirus (HuNoV) infection has been the leading cause of non-bacterial gastroenteritis, and has become an increasing public concern worldwide (Kitajima *et al.*, 2009). HuNoV are non-enveloped, positive-sense, single-stranded RNA viruses belonging to the family *Caliciviridae*. They are further classified into 5 genogroups (GI to GV), of which GI, GII, and GIV infect humans of all ages (Chan *et al.*, 2006). While GII accounts for the majority of reported outbreaks of NoV-associated gastroenteritis, GI occurrences have been noted frequently in environmental water settings (Bull *et al.*, 2006; Lee and kim, 2008; La Rosa *et al.*, 2010). Increased urbanization and the ease and frequency of global travel in modern days therefore necessitate more proactive public health surveillance including monitoring for waterborne viruses (Wong *et al.*, 2007).

Cell culture and PCR techniques are common methods for the detection of pathogenic viruses in aquatic environments (Duizer *et al.*, 2004). Most cell culture-associated methods, including the Total Culturable Virus Assay (TCVA) used by the US Environmental Protection Agency (USEPA), detect infectious virions and have been considered effective (Fong and Lipp, 2005; Lambertini *et al.*, 2010). Meanwhile, PCR-based methods including nested PCR, multiplex PCR, and real-time quantitative PCR (RT-qPCR), provide much higher detection sensitivity (Fout *et al.*, 1996; Fong and Lipp, 2005) although these methods cannot determine the infectivity of

the detected viral nucleic acids. Integrated cell culture-PCR (ICC-PCR) is a combination of the cell culture- and PCR-based techniques and has been used for many years to detect infectious enteric viruses in environmental samples (Fong and Lipp, 2005). ICC-PCR provides several advantages for handling infectious viruses that do not show cytopathic effect (CPE) and increases sensitivity through replication and amplification of a limited number of infectious virions (Lee and Jeong, 2004). Although expensive and insufficiently sensitive for detection in environmental water, immunological methods may also be combined with cell culture systems to detect enteric viruses (Griffin *et al*., 2003; Lee *et al*., 2009).

Nevertheless, there has been no choice but to perform RT-PCR for the detection of non-culturable viruses such as HuNoV, typically followed by a slot blot or nucleotide sequencing, using RNA extracted from water (Borchardt *et al*., 2003; Parshionikar *et al*., 2003; Duizer *et al*., 2004; La Rosa *et al*., 2007; Cheong *et al*., 2009). Although electron microscopy and enzyme immunoassays have been adapted for HuNoV detection, the RT-PCR method is commonly employed for analyzing environmental water (Atmar and Estes, 2001). RT-qPCR following reverse transcription has also been used for HuNoV surveillance (Haramoto *et al*., 2005; Kitajima *et al*., 2009; Aw and Gin, 2010). Compared to conventional RT-PCR, the RT-qPCR technique often has higher sensitivity and ability to quantify target sequences (Bosch *et al*., 2008; Anbazhagi and Kamatchiammal, 2010).

Higher-sensitivity PCR methods are not completely free from the problems of "false-positives" (Kwok and Higuchi, 1989), which are mainly ascribed to the amplification of non-specific sequences or carry-over of target products (Burkardt, 2000). Many suggested modifications have been well applied to prevent false-positives in most PCR-based analyses. Meanwhile, PCR-based detection methods without prior amplification of target sequences are vulnerable to false-negative results, especially when researchers deal with environmental samples (Lee and Jeong, 2004). Thus far, efforts to solve the PCR false-negative problem have focused on removing PCR inhibitors such as RNase, humic acid, fulvic acids, heavy metals, phenolic compounds, and other experimental reagents (Scipioni *et al*., 2008). However, the very small volume of concentrates that can be assayed in an RT-PCR reaction could be one of the major obstacles to the use of RT-PCR for detecting viruses in environmental waters, which often contain very low virus titers (Fout *et al*., 2003).

Most waterborne viruses are present at concentrations too low to detect even after sample concentration (Wyn-Jones, 2007). There are several techniques for concentrating large volumes of water (up to hundreds of liters) to about 5–30 ml of final concentrate (Berg *et al*., 2001; Wyn-Jones, 2007). However, a limited amount of the final concentrate is often used for analysis. Therefore, a determination of whether a given water source is polluted with pathogenic viruses is made by looking at only a few liters of water, regardless of the source (Borchardt *et al*., 2003; Parshionikar *et al*., 2003; Powell *et al*., 2003; La Rosa *et al*., 2007; Cheong *et al*., 2009; Nakamura *et al*., 2009). In other words, the detection of non-culturable viruses in certain types of water such as tap water and groundwater could be subject to probability, misleading scientists and policymakers into making inappropriate decisions.

The aim of this study was to demonstrate that the portion of the concentrated environmental water applied to nucleic acid amplification is an important factor in determining whether the water is polluted by viruses, especially when the pollutants are below a certain threshold concentration. The problem regarding low target concentration in PCR may be well conceptualized in general, but this study, to our knowledge, is the first to attempt at elucidating the impact of the aforementioned problem and provide an opportunity to reconsider the reliability of PCR detection for monitoring non-culturable viruses in environmental samples.

Materials and Methods

Preparation of RNA extract from field water samples

Hundreds of environmental water samples were collected through NanoCeram filters (Argonide, USA) and concentrated according to USEPA method 1615 (Fout *et al*., 2010). Final concentrates were stored at -80°C prior to RNA extraction and analysis. We chose 59 samples from the final concentrate archives (30 groundwater, 13 river water, and 16 treated sewage effluent [TSE] samples for discharge) that had been analyzed for HuNoV contamination in 4 institutes (Pusan National University, Dankook University, Konkuk University, and Korea Water Resources Corporation) authorized by the National Institute of Environmental Research (NIER) for norovirus analysis in South Korea. Of 30 groundwater samples, 15 were identified as HuNoV-positive and the other 15 as HuNoV-negative. Of the 13 river water and 16 TSE samples, 9 and 5 were identified as HuNoV-positive, respectively. To extract the HuNoV genome, 140 μl of final concentrate was processed with a QIAamp viral RNA mini kit (QIAGEN, Netherlands). All extraction steps, including the previous analyses processed by the 4 above institutes, were performed according to the instruction manual of the kit.

Preparation and quantification of RNA extract from a murine norovirus isolate

A murine norovirus (MNV) was isolated from mouse feces and additional plaque cloning was performed twice by using RAW 264.7 cells supplemented with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). After second plaque cloning, reverse transcription-PCR (RT-PCR) and plaque assays were performed to confirm that the MNV isolate was infectious. The MNV isolate was proliferated in RAW 264.7 cells under the aforementioned conditions. The MNV genome was sequenced (Bionics, Korea) and registered to GenBank (accession number, JX048594).

To prepare MNV RNA, propagated MNV in RAW 264.7 cells was concentrated with Centriprep (Millipore, USA) and the viral RNA was extracted with a QIAamp viral RNA mini kit. All extraction steps were performed according to the instruction manual, and the concentration of the MNV genome extract was determined with a Biophotometer (Eppen-

dorf, Germany). For reverse transcription, 100 ng of the MNV genome extract was mixed with 100 pmole of primer MNVR (Table 1) and denatured at 95°C. Then, 6 µl of 5× MMLV RTase buffer, 3 µl of 2.5 mM dNTPs, 200 units of MMLV RTase (Promega, USA), and deionized sterile water were added (30 µl final volume) and incubated at 37°C for 16 h followed by heat inactivation at 95°C.

Ten percent of the MNV RT product was used for PCR in the presence of 1.5 µl of 2× GC buffer, 2.5 mM dNTPs, 25 pmole forward primer (MNVF), and reverse primer (MNVR) (Table 1), 1 unit of LA Taq (TaKaRa, Japan), and deionized sterile water (30 µl final volume). The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 35 amplification cycles (95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec), and a final extension step (72°C for 1 min). The obtained PCR product (503 bp) was purified with HiYield™ Gel/PCR DNA Fragment Extraction Kit (RBC, Taiwan) and used to generate standard curves for real-time qPCR.

To determine the copy number of the MNV RT product, iCycler iQ (BioRad, USA) was used. Triplicated reaction mixtures [2 µl of 10-fold diluted MNV RT product, 2× iQ™ SYBR Green Supermix (BioRad), 20 pmole each primer (MNVRTF and MNVRTR, Table 1), and sterile deionized water for a reaction volume of up to 30 µl] were incubated at 50°C for 2 min, and then at 95°C for 10 min. After the initial denaturation step, amplification was performed in 40 cycles (95°C for 15 sec, 60°C for 1 min), followed by a final round at 95°C for 1 min and 55°C for 1 min. MNV cDNA copy number calculations were performed as per the equations described in the "iCycler iQ Real-Time PCR Applications Guide" (BioRad).

One-step RT-PCR and semi-nested secondary PCR

Previously, 59 samples were analyzed for detecting the presence of HuNoV according to the Guide for "Detection of Norovirus in Groundwater" provided by NIER (Jung *et al.*, 2011). For this study, the one-step RT-PCR kit (QIAGEN) with minor modification was used to amplify a target sequence of the defined copy number MNV RNA or HuNoV RNA from the final concentrates of environmental water samples. Briefly, a defined copy number of MNV RNA in 10 µl of distilled water or 1/8 (10 µl) of RNA extract (80 µl) from each water concentrate archives were mixed with 5× RT buffer, 5× Q-solution, 2.5 mM dNTPs, 30 pmoles forward and reverse primer (MNVF and MNVR for MNV, GI-F1M and GI-R1M for HuNoV GI, GII-F1M and GII-R1M for HuNoV GII) (Table 1), and 2 µl of enzyme mix. Deionized sterile water was added for a reaction volume 50 µl. The PCR conditions were as follows: reverse transcription steps at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of amplification (94°C for 1 min, 50°C for 1 min, 72°C for 1 min). A final extension was performed at 72°C for 10 min.

One-step RT-PCR products from environmental water concentrates underwent a second PCR process. The first PCR products (2 µl or 4% v/v) were added to Maxime PCR premix (Intron, Korea) containing 20 pmole of each forward and reverse primer (GI-F2 and GI-R1M for HuNoV GI, GII-F3 and GII-R1M for HuNoV GII) (Table 1) and sterile deionized water (for 20 µl final reaction volume). The semi-nested PCR conditions were as follows: first denaturation at 95°C for 5 min, 25 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min 30 sec. A final extension was performed at 72°C for 7 min. PCR products corresponding to the size of the positive control were eluted and sequenced (Bionics, Korea).

Quality assurance for PCR

Several precautions were taken to minimize the occurrence of false-positive results. Separate rooms were used to perform NanoCeram filter elution-concentration, one-step RT-PCR preparation, semi-nested PCR preparation, and gel electrophoresis. In addition to using RNase- and DNase-free reagents and disposable wares, all instruments and locations for detection experiments were sterilized by UV radiation before every experiment.

In the HuNoV detection experiment, sterile deionized water, and non-specific RNA (bacteriophage MS2 genome) served as negative controls for one-step RT-PCR. For the semi-nested PCR, sterile deionized water and the one-step RT-PCR product served as negative controls. Cloned and run-off transcribed HuNoV GI or GII RNA sequences *in vitro*, and their one-step RT-PCR products were used as positive con-

Table 1. Primers used in this study

Virus	Primer ID	Sequences[a]	Target region[b]	Purpose	Primer citation
HuNoV (GI)	GI-F1M	5′-CTGCCCGAATTYGTAAATGATGAT-3′	5341–5364	One step RT-PCR	Park *et al.* (2010)
	GI-R1M	5′-CCAACCCARCCATTRTACATYTG-3′	5648–5670		
	GI-F2	5′-ATGATGATGGCGTCTAAGGACGC-3′	5357–5379	Semi nested PCR	
	GI-R1M	5′-CCAACCCARCCATTRTACATYTG-3′	5648–5670		
HuNoV (GII)	GII-F1M	5′-GGGAGGGCGATCGCAATCT-3′	5048–5063	One step RT-PCR	
	GII-R1M	5′-CCRCCIGCATRICCRTTRTACAT-3′	5366–5388		
	GII-F3	5′-TTGTGAATGAAGATGGCGTCGART-3′	5078–5101	Semi nested PCR	
	GII-R1M	5′-CCRCCIGCATRICCRTTRTACAT-3′	5366–5388		
MNV	MNVF	5′-GCCAACTCTTTCAAGCA-3′	6878–6894	One step RT-PCR	In this study
	MNVR	5′-AAAATGCATCTAAATACTAC-3′	7811–7830		
	MNVRTF	5′-CTTCGTGGAGGTTCCTG-3′	7196–7212	qPCR	
	MNVRTR	5′-TATGCCCTGCTACTCCC-3′	7287–7303		
	MNVR	5′-AAAATGCATCTAAATACTAC-3′	7811–7830	Reverse transcription	

[a] "Y" = C or T, "R" = A or G
[b] Corresponding nucleotide position of HuNoV GI, HuNoV GII, and MNV (accession nos. JX023285, JQ622197, and EU004683, respectively)

Table 2. Crosscheck for the presence of HuNoV in environmental water

Sample ID	Water type	Previous determination	Crosscheck[b]		
			Results	Trials	Total vol. of assayed concentrate (ml)
3	Ground	+	+	3	0.42
1	Ground	+	-	20	2.80
10	Ground	+	-	20	2.80
15	Ground	+	-	20	2.80
2	Ground	+	-	20	2.80
4	Ground	+	-	20	2.80
5	Ground	+	-	20	2.80
11	Ground	+	-	20	2.80
12	Ground	+	-	20	2.80
13	Ground	+	-	20	2.80
14	Ground	+	-	20	2.80
16	Ground	+	-	20	2.80
17	Ground	+	-	20	2.80
18	Ground	+	-	20	2.80
19	Ground	+	-	20	2.80
34	Ground	-	+	15	2.10
40	Ground	-	-	20	2.80
44	Ground	-	-	20	2.80
46	Ground	-	-	20	2.80
31	Ground	-	-	20	2.80
32	Ground	-	-	20	2.80
33	Ground	-	-	20	2.80
35	Ground	-	-	20	2.80
41	Ground	-	-	20	2.80
42	Ground	-	-	20	2.80
43	Ground	-	-	20	2.80
45	Ground	-	-	20	2.80
47	Ground	-	-	20	2.80
48	Ground	-	-	20	2.80
49	Ground	-	-	20	2.80
6	River	+	+	10	1.40
22	River	+	+	3	0.42
26	River	+	+	10	1.40
21	River	+	-	20	2.80
29	River	+	-	20	2.80
7	River	+	-	20	2.80
25	River	+	-	20	2.80
27	River	+	-	20	2.80
28	River	+	-	20	2.80
37	River	-	+	3	0.42
36	River	-	-	20	2.80
52	River	-	-	20	2.80
53	River	-	-	20	2.80
57	River	-	-	20	2.80
8	TSE[a]	+	+	10	1.40
9	TSE	+	+	3	0.42
23	TSE	+	+	3	0.42
24	TSE	+	+	4	0.56
30	TSE	+	-	20	2.80
38	TSE	-	+	4	0.56
39	TSE	-	+	4	0.56
50	TSE	-	+	10	1.40
51	TSE	-	+	1	0.14
55	TSE	-	+	2	0.28
56	TSE	-	+	1	0.14
54	TSE	-	-	20	2.80
58	TSE	-	-	20	2.80
59	TSE	-	-	20	2.80
60	TSE	-	-	20	2.80

[a] Treated sewage effluent
[b] A new detection (up to 20 trials) was processed using the new aliquot of the archives until a positive result was confirmed

trols for the one-step RT-PCR and semi-nested PCR, respectively. All PCR results were confirmed by agarose gel electrophoresis. All PCR products of sizes comparable to those of the positive controls were eluted and sequenced.

Results

HuNoV validation analysis in archival environmental water concentrates

In the preliminary experiment, 22 archives of concentrated groundwater that had been identified previously as HuNoV-positive (3 for GI and 19 for GII) by one-step RT-PCR, followed by semi-nested PCR and sequencing, were subjected to crosschecking by using the same methodologies. We then repeated the process with 3-fold more RNA extracts in the one-step RT-PCR and 3-fold more products in the semi-nested PCR than the amounts used in the previous attempts in order to verify the results of the first identification. Fourteen of the 22 samples were reconfirmed as HuNoV-positive in the first trial. For the "still-negative" 8 samples, we took another 140-μl fraction, prepared fresh RNA extract, and repeated the PCR but obtained positive results for only 2 samples. Twenty-one of the 22 archived samples were reconfirmed through multiple attempts with new 140-μl fractions but 1 archive could not be reconfirmed (data not shown).

These results prompted us to question the reliability of non-culturable virus detection in limited numbers of RT-PCR trials, especially when the target concentration is relatively low. Pusch *et al.* (2005) demonstrated that viral and bacterial contamination rates were higher in TSE than in downstream river water. Groundwater is better protected from contaminants than surface water (Katayama, 2008). Therefore, we sought to determine whether detectability is dependent upon the type of environmental water sample.

In total, 59 archives that had been tested previously for HuNoV by one-step RT-PCR, semi-nested PCR, and sequencing were obtained from the 2010 survey for water quality control in South Korea. Of 30 groundwater samples, 15 were HuNoV-positive for HuNoV contamination and the other 15 were negative. Of 14 river water and 15 TSE samples, 9 and 5 were positive, respectively (Table 2). Regardless of the previous HuNoV determination, our attempts to detect HuNoV RNA in each archive included up to 20 trials until HuNoV positivity was reconfirmed. The PCR detection procedure was the same as that used for the original determination (see 'Materials and Methods'). If positivity was not confirmed in the first detection experiment, a second trial was performed on a new aliquot of concentrate. Positivity was identified in 26.7% (8/30) of the samples originally identified as negative. These reversals (negative → positive) were observed in 6.7% (1/15) of groundwater samples, 20% (1/5) of river water samples, and 60% (6/10) of TSE samples (Table 2). In contrast, 72.4% (21/29) of the samples previously identified as positive were not validated as positive until 20 trials had been performed. These reversals (positive → negative) occurred in 93.3% (14/15) of groundwater samples, 66.7% (6/9) of river water samples, and 20% (1/5) of TSE samples. Finally, 27.6% (8/29) of originally positive samples were validated: 6.7% (1/15) of groundwater, 33.3%

(3/9) of river water, and 80% (4/5) of TSE samples were validated as positive. These results suggest that the likelihood of validating HuNoV presence in the archives is inversely correlated, in part, with the amount of viral RNA present. In addition, the possible protection of microbes by various particles and organic matter in certain water samples could not be excluded in this phenomenon (Sobsey and Meschke, 2003).

Detection of various copy numbers of MNV RNA by one-step RT-PCR

To determine the correlation between detection frequency and viral RNA concentration, a series of MNV RNA concentrations (from 2×10^2 to 8×10^3 copies) were subjected to the one-step RT-PCR. Total RNA copies (100 μl) were distributed into 10 tubes (10 μl per tube) so that each tube was assumed to contain from 20 to 800 target copies. Product intensity on gel electrophoresis was classified into 5 grades and plotted as small squares (Fig. 1A). Target detection was successful in all trials when the MNV RNA concentration was above 7×10^3. In other words, if there were 7×10^3 target copies in a final water concentrate (thought to contain 700 MNV RNA copies per tube) we would obtain positive PCR results regardless of which tube was chosen for analysis. Detection frequency remained >80% in samples with relatively higher concentrations of MNV RNA (from 6×10^3 to 1.8×10^3 target copies), whereas detection dropped to <50% at lower MNV RNA concentrations (below 1.6×10^3 target copies) in a concentration-dependent manner ($P<0.05$). There was less than an 8% chance of detecting 200 copies of MNV in a final water concentrate. These concentration-dependent detection experiments were repeated 3 times, and all results were highly concordant (Fig. 1A).

The concentration-dependent MNV RNA detection frequencies were subjected to regression analysis and plotted against the concentration ranges (Figs. 1B and 1C). Under these experimental conditions, $1.0–1.6\times10^3$ MNV RNA target copies appeared to be the threshold at which positive samples could be identified as positive or negative with comparable probability (Fig. 1C). While the detection rates at $8.0–1.8\times10^3$ MNV RNA target copies were >80% and gradually decreased

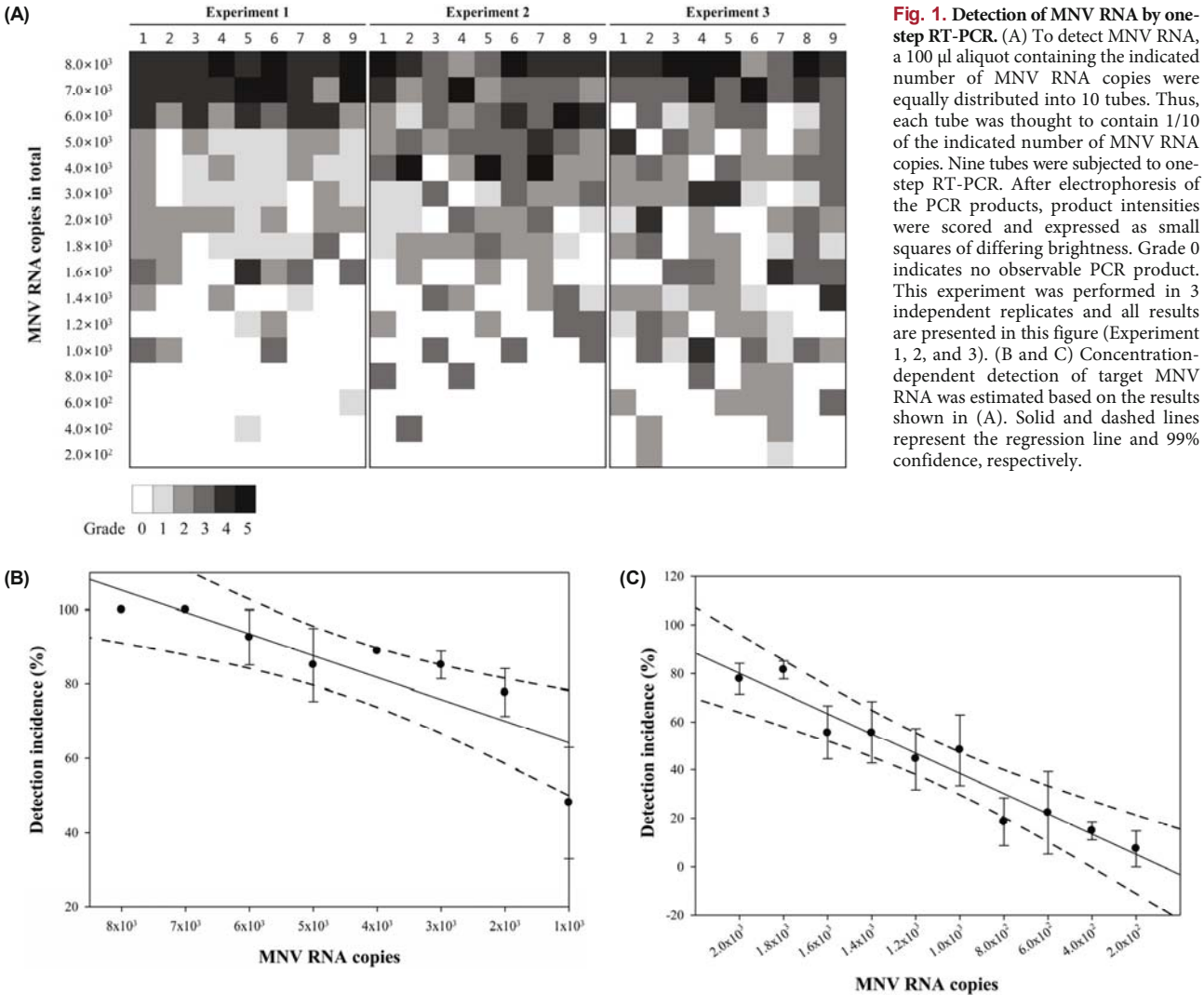

Fig. 1. Detection of MNV RNA by one-step RT-PCR. (A) To detect MNV RNA, a 100 μl aliquot containing the indicated number of MNV RNA copies were equally distributed into 10 tubes. Thus, each tube was thought to contain 1/10 of the indicated number of MNV RNA copies. Nine tubes were subjected to one-step RT-PCR. After electrophoresis of the PCR products, product intensities were scored and expressed as small squares of differing brightness. Grade 0 indicates no observable PCR product. This experiment was performed in 3 independent replicates and all results are presented in this figure (Experiment 1, 2, and 3). (B and C) Concentration-dependent detection of target MNV RNA was estimated based on the results shown in (A). Solid and dashed lines represent the regression line and 99% confidence, respectively.

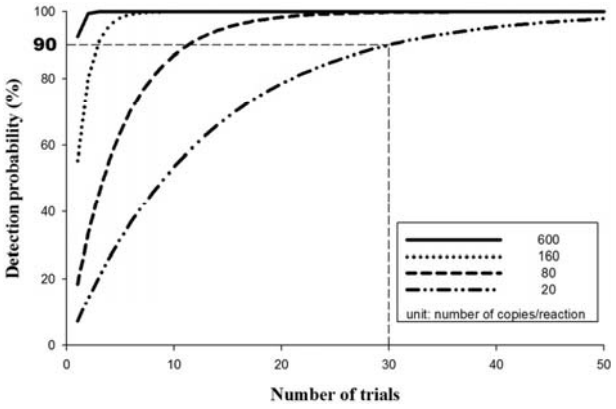

Fig. 2. Detection probabilities of selected target copy numbers by one-step RT-PCR. The probability values were calculated with the following formula: P(n) = P(n-1) + {1-P(n-1)} × P(1), where "n" is the number of detection trials. The unique detection probability "P(1)" at each copy number was obtained by averaging 3 detection frequencies in triplicate experiments as shown Fig. 1A. In the presence of 20 copies, more than 30 trials are required to reach 90% probability of MNV RNA detection.

with decreasing concentration, a sharp decline in the detection rate was observed at a concentration of $1.6–0.2\times10^3$ target copies (Fig. 1C).

Based on these results, we estimated the number of one-step RT-PCR trials (or portions of total concentrate) that must be attempted to optimize the reliability of detection (Fig. 2). As depicted in Fig. 1, we obtained PCR-positive results in 2 of 27 tubes (average of 0/9, 0/9, and 2/9; 7.4%) when each tube was assumed to contain 20 MNV RNA copies. Meanwhile, 25 of 27 tubes (average of 9/9, 9/9, and 7/9; 92.5%) were PCR-positive and each tube was assumed to have 600 target copies. As shown in Fig. 2, just one trial yielded a 90% probability of MNV RNA detection when the copy number was >600 per reaction. However, more than 30 trials would be necessary to obtain a positive signal when a copy number of <20 is used.

Discussion

Gene amplification is the primary tool for detection of pathogenic, non-culturable virus in water. Conventional PCR detection of waterborne pathogenic viruses uses a portion of whole concentrate, and the number of portions used varies (Powell *et al.*, 2003; Miagostovich *et al.*, 2008; Cheong *et al.*, 2009). As described above, virological analyses of water must recover low numbers of viruses from large volumes of water (Bosch *et al.*, 2008). Despite the concentration of water samples, the number of targets used in a PCR trial was not increased as long as we analyzed a limited portion from a whole concentrate. To reduce the effect of this problem on waterborne virus detection by PCR-based methods, a second concentration of the concentrated eluate was recently adopted by the USEPA (Fout *et al.*, 2010).

We studied the impact of PCR false-negatives due to low target concentration on the determination of whether a given water sample is contaminated with viral pathogens. Opel *et al.* (2010) reported that various PCR inhibitors affect PCR efficiency through a variety of mechanisms. We demonstrated, however, that the amount of final concentrated sample analyzed in a PCR trial should be considered in addition to the known PCR inhibitors in any PCR-based detection method targeting non-culturable viruses. RT-qPCR is one of the preferred methods for detecting viral nucleic acids in various environmental waters due to its improved sensitivity and quantification ability (Ngazoa *et al.*, 2008; Victoria *et al.*, 2010; Wolf *et al.*, 2010). RT-qPCR may alleviate the detection problem but cannot be an ultimate solution as long as only a portion of a concentrated sample is analyzed for non-culturable viruses.

Unlike fecal sample dilution or raw sewage, most cases of groundwater sampling for virus detection require collection of hundreds to thousands of liters to yield 20–30 ml of final concentrate. While there are no recommendations for the appropriate amount or portions of concentrate to be analyzed in PCR-based methods, we noticed that quite limited portions of the final concentrate are often used for PCR trials during experimental or monitoring analyses of environmental water. One of the popular commercial kits for target RNA preparation, for instance, uses a 140-μl aliquot of final concentrate for a single extraction. If 30 ml of final concentrate is obtained from a 500-L groundwater sample, the amount used for RNA extraction constitutes less than 0.47% of the original sample. Furthermore, most reverse transcription reactions use barely over 1/10 of the RNA extract thus, the final portion subjected to RT-PCR represents less than 1/2,000 of the original sample. As shown in Fig. 2, more than 30 one-step RT-PCR trials are required to reach 90% detection probability when 20 copies of MNV RNA are targeted in 1 trial. Thus, PCR negativity after analyzing a limited portion (e.g., a couple of 140-μl fractions) of the final concentrate may erroneously suggest that a given water supply is safe for consumption when the concentration of the pathogenic viruses is below a certain level. In addition, we obtained positive results intermittently after 27 (9 tubes for triplicate experiments) trials when one-step RT-PCR was performed using low copy numbers (10 aliquots of $1.6–0.2\times10^3$ copies) of MNV RNA (Fig. 1). These results suggest a possibility that the unequal distribution of low-concentration targets in aqueous solution often necessitates many more trials (or a much larger portion of the final concentrate) in PCR-based detection although little is known about the physical distribution of viral RNA molecules in aquatic conditions.

In the crosscheck study for HuNoV contamination, 59 archived water concentrates from 3 different environments were subjected to one-step RT-PCR: groundwater, river water, and TSE. Each type of water was expected to have relatively low, intermediate, and high degrees of HuNoV contamination, respectively, although this cannot be generalized. Limited availability made it impossible to have equal numbers of previously determined PCR-positive and PCR-negative archives for river water or TSE. Nevertheless, only 6.7% (1/15) of HuNoV-positive groundwater archives were reconfirmed by PCR and sequencing while 33.3% (3/9) and 80% (4/5) of the river water and TSE archives, respectively, were reconfirmed with fresh 140-μl fractions for

each trial. These results were somewhat paralleled by the percentages of archives for which the results changed from HuNoV-negative to positive meanwhile, the opposite seemed true for the percentage of determinations that changed from HuNoV-positive to negative (Table 2). Therefore the degree of HuNoV contamination in archives from different types of water corresponded well to general expectations (Katayama, 2008). They were also concordant with the results of the MNV RNA experiment (Fig. 1), which demonstrated that the reliability of detection in a couple of trials (or using quite limited portions of the final concentrates) is drastically diminished in water samples contaminated with a small number of non-culturable viruses that may yet be present in sufficient numbers to cause infection. These results suggest 2 important points that we need to consider in PCR-based detection of non-culturable viruses in environmental water samples. The first is that PCR-negative results for a given water sample with a low concentration of pathogenic viruses may not reliably indicate that the water is safe for consumption. The second is that the volume (or number of trials) should be adjusted carefully, depending on the type of water being examined.

Throughout this study, the one-step RT-PCR method was employed to detect target nucleic acids in various samples. Secondary semi-nested PCR and sequencing of amplified PCR products were used to obtain higher sensitivity and specificity for validation of concentrated archive determinations. There were several reasons for our choice of method. First, one-step RT-PCR is popular for detection of RNA virus pathogens in various environmental waters (Arraj *et al.*, 2008), so the results of this study can be compared to those of other studies. Second, this method has few process variations because it is well established in conjunction with a commercial viral RNA extraction kit (QIAGEN). Third, as the name "one-step" suggests, the entire volume of the RT product is used for the following amplification cycles; thus, the method allows analysis of the maximum amount of RNA extracted from each aliquot of the final concentrated water samples.

Most surveys for waterborne virus pathogens are utilized for academic purposes but some may serve as reference data for establishing policies and rules regarding water quality controls in communities (Borchardt *et al.*, 2003; Cheong *et al.*, 2009; Anbazhagi and Kamatchiammal, 2010). We noted that the portions of final concentrate used in reverse transcription ranged from 0.3–78% in 12 reports (Borchardt *et al.*, 2003; Fout *et al.*, 2003; Parshionikar *et al.*, 2003; Powell *et al.*, 2003; Bull *et al.*, 2006; La Rosa *et al.*, 2007, 2010; Miagostovich *et al.*, 2008; Rodríguez-Díaz *et al.*, 2008; Cheong *et al.*, 2009; Iwai *et al.*, 2009; Nakamura *et al.*, 2009). In addition, the portions of final concentrate used in PCR amplification ranged from 0.04–10% in 11 reports (Borchardt *et al.*, 2003; Fout *et al.*, 2003; Parshionikar *et al.*, 2003; Bull *et al.*, 2006; La Rosa *et al.*, 2007, 2010; Miagostovich *et al.*, 2008; Rodríguez-Díaz *et al.*, 2008; Cheong *et al.*, 2009; Iwai *et al.*, 2009; Nakamura *et al.*, 2009). Thus, many reports on the pollution of certain waters with pathogenic viruses, all of which are supported by data produced from widely varied sample amounts, have been made public without differentiating their significance. While PCR problems related to false-positive results can be improved by using appropriate instruments and equipments and by the expertise of researchers, problems related to false-negative results originating from low numbers of target virus will be resolved, at least partly, by increasing the portion of water concentrate per reaction or the number of trials performed with freshly prepared RNA extracts. Traditional microbial indicators and newly studied candidates, including *Escherichia coli* and coliphages, may be used to supplement evaluations of PCR-negative waters although the correlation between waterborne virus pathogens and these indicators is inconclusive (Jofre, 2007). Thus, the development of a new assessment system must address all possible factors, including hydrogeochemical elements or various social aspects of residential settings and facilities with regard to human fecal pollution. Until then, we should use caution in our application of PCR-based methods, including that of RT-qPCR, as the "gold standard" to determine whether a given water source is truly free of non-culturable viruses.

Acknowledgements

This work was supported in part by the National Institute of Environmental Research (NIER, 2010). We thank professor Joong-Bok Lee, professor Kyung Lip Jang, Dr. Ok-Jae Rhee, and Dr. Gyu Cheol Lee for providing archived samples for this study.

References

Anbazhagi, S. and Kamatchiammal, S. 2010. A comparative study for the efficient detection of norovirus from drinking water by RT-PCR and real-time PCR. *Water Air Soil Pollut.* **213**, 71–84.

Arraj, A., Bohatier, J., Aumeran, C., Bailly, J.L., Laveran, H., and Traoré, O. 2008. An epidemiological study of enteric viruses in sewage with molecular characterization by RT-PCR and sequence analysis. *J. Water Health* **6**, 351–358.

Atmar, R.L. and Estes, M.K. 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. *Clin. Microbiol. Rev.* **14**, 15–37.

Aw, T.G. and Gin, K.Y.H. 2010. Environmental surveillance and molecular characterization of human enteric viruses in tropical urban wastewaters. *J. Appl. Microbiol.* **109**, 716–730.

Berg, G., Safferman, R.S., Dahling, D.R., Berman, D., and Hurst, C.J. 2001. Concentration and processing of waterborne viruses by positive charge 1MDS cartridge filters and organic flocculation, pp. 1–11. US EPA Manual of methods for virology, U.S. Environmental Protection Agency, Washington, D.C., USA.

Borchardt, M.A., Bertz, P.D., Spencer, S.K., and Battigelli, D.A. 2003. Incidence of enteric viruses in groundwater from household wells in Wisconsin. *Appl. Environ. Microbiol.* **69**, 1172–1180.

Bosch, A., Guix, S., Sano, D., and Pintó, R.M. 2008. New tools for the study and direct surveillance of viral pathogens in water. *Curr. Opin. Biotechnol.* **19**, 295–301.

Bull, R.A., Tu, E.T., McIver, C.J., Rawlinson, W.D., and White, P.A. 2006. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. *J. Clin. Microbiol.* **44**, 327–333.

Burkardt, H.J. 2000. Standardization and quality control of PCR analyses. *Clin. Chem. Lab. Med.* **38**, 87–91.

Carter, M.J. 2005. Enterically infecting viruses: pathogenicity, trans-

mission and significance for food and waterborne infection. *J. Appl. Microbiol.* **98**, 1354–1380.

Chan, M.C., Sung, J.J., Lam, R.K., Chan, P.K., Lee, N.L., Lai, R.W., and Leung, W.K. 2006. Fecal viral load and norovirus-associated gastroenteritis. *Emerg. Infect. Dis.* **12**, 1278–1280.

Cheong, S., Lee, C., Song, S.W., Choi, W.C., Lee, C.H., and Kim, S.J. 2009. Enteric viruses in raw vegetables and groundwater used for irrigation in Korea. *Appl. Environ. Microbiol.* **75**, 7745–7751.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P.G., and Estes, M.K. 2004. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* **85**, 79–87.

Fong, T.T. and Lipp, E.K. 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiol. Mol. Biol. Rev.* **69**, 357–371.

Fout, G.S., Brinkman, N.E., Cashdollar, J.L., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Karim, M.R., Grimm, A.G., Spencer, S.K., and *et al.* 2010. Method 1615. measurement of enterovirus and norovirus occurrence in water by culture and RT-qPCR, pp. 1–64. National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, USA.

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

Fout, G.S., Schaefer III, F.W., Messer, J.W., Dahling, D.R., and Stetler, R.E. 1996. ICR microbial laboratory manual. National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency.

Griffin, D.W., Donaldson, K.A., Paul, J.H., and Rose, J.B. 2003. Pathogenic human viruses in coastal waters. *Clin. Microbiol. Rev.* **16**, 129–143.

Haramoto, E., Katayama, H., Oguma, K., and Ohgaki, S. 2005. Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa river in Japan. *Appl. Environ. Microbiol.* **71**, 2403–2411.

Iwai, M., Hasegawa, S., Obara, M., Nakamura, K., Horimoto, E., Takizawa, T., Kurata, T., Sogen, S., and Shiraki, K. 2009. Continuous presence of noroviruses and sapoviruses in raw sewage reflects infections among inhabitants of toyama, Japan. *Appl. Environ. Microbiol.* **75**, 1264–1270.

Jofre, J. 2007. Indicators of waterborne enteric viruses, pp. 227–249. *In* Bosch, A. (ed.), Perspectives in Medical Virology, Elsevier, Amsterdam, The Netherlands.

Jung, J.H., Yoo, C.H., Koo, E.S., Kim, H.M., Na, Y., Jheong, W.H., and Jeong, Y.S. 2011. Occurrence of norovirus and other enteric viruses in untreated groundwaters of Korea. *J. Water Health* **9**, 544–555.

Katayama, H. 2008. Detection of microbial contamination in groundwater, pp. 151–169. *In* Takizawa, S. (ed.), Groundwater Management in Asian Cities, Springer, Japan.

Kitajima, M., Haramoto, E., Phanuwan, C., Katayama, H., and Ohgaki, S. 2009. Detection of genogroup IV norovirus in wastewater and river water in Japan. *Lett. Appl. Microbiol.* **49**, 655–658.

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* **339**, 237–238.

La Rosa, G., Fontana, S., Di Grazia, A., Iaconelli, M., Pourshaban, M., and Muscillo, M. 2007. Molecular identification and genetic analysis of norovirus genogroups I and II in water environments: comparative analysis of different reverse transcription-PCR assays. *Appl. Environ. Microbiol.* **73**, 4152–4161.

La Rosa, G., Iaconelli, M., Pourshaban, M., Fratini, M., and Muscillo, M. 2010. Molecular detection and genetic diversity of norovirus genogroup IV: a yearlong monitoring of sewage throughout Italy. *Arch. Virol.* **155**, 589–593.

Lambertini, E., Spencer, S.K., Bertz, P.D., Loge, F.J., and Borchardt, M.A. 2010. New mathematical approaches to quantify human infectious viruses from environmental media using integrated cell culture-qPCR. *J. Virol. Methods* **163**, 244–252.

Lee, G.C., Chong, C.K., Lee, C.H., and Lee, S.T. 2009. Cell-culture-based immunochromatography for rapid detection of group A human rotaviruses in aquatic environments. *Environ. Technol.* **30**, 37–43.

Lee, H.K. and Jeong, Y.S. 2004. Comparison of total culturable virus assay and multiplex integrated cell culture-PCR for reliability of waterborne virus detection. *Appl. Environ. Microbiol.* **70**, 3632–3636.

Lee, C.H. and Kim, S.J. 2008. The genetic diversity of human noroviruses detected in river water in Korea. *Water Res.* **42**, 4477–4484.

Mena, K.D. and Gerba, C.P. 2009. Waterborne adenovirus, pp. 1–35. *In* Whitacre, D.M. (ed.), Reviews of Environmental Contamination and Toxicology, Springer, New York, N.Y., USA.

Meng, X.J. 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet. Microbiol.* **140**, 256–265.

Miagostovich, M.P., Ferreira, F.F., Guimarães, F.R., Fumian, T.M., Diniz-Mendes, L., Luz, S.L., Silva, L.A., and Leite, J.P. 2008. Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of manaus, central amazonia, Brazil. *Appl. Environ. Microbiol.* **74**, 375–382.

Nakamura, K., Iwai, M., Zhang, J., Obara, M., Horimoto, E., Hasegawa, S., Kurata, T., and Takizawa, T. 2009. Detection of a novel recombinant norovirus from sewage water in toyama prefecture, Japan. *Jpn. J. Infect. Dis.* **62**, 394–398.

Ngazoa, E.S., Fliss, I., and Jean, J. 2008. Quantitative study of persistence of human norovirus genome in water using TaqMan real-time RT-PCR. *J. Appl. Microbiol.* **104**, 707–715.

Opel, K.L., Chung, D., and McCord, B.R. 2010. A study of PCR inhibition mechanisms using real time PCR. *J. Forensic Sci.* **55**, 25–33.

Park, K., Jeong, H., Baek, K., Lee, C., Park, S., Park, J., Choi, Y., Choi, H., and Cheon, D. 2010. Genetic analysis of norovirus GII.4 variants circulating in Korea in 2008. *Arch. Virol.* **155**, 635–641.

Parshionikar, S.U., Willian-True, S., Fout, G.S., Robbins, D.E., Seys, S.A., Cassady, J.D., and Harris, R. 2003. Waterborne outbreak of gastroenteritis associated with a norovirus. *Appl. Environ. Microbiol.* **69**, 5263–5268.

Powell, K.L., Taylor, R.G., Cronin, A.A., Barrett, M.H., Pedley, S., Sellwood, J., Trowsdale, S.A., and Lerner, D.N. 2003. Microbial contamination of two urban sandstone aquifers in the UK. *Water Res.* **37**, 339–352.

Pusch, D., Oh, D.Y., Wolf, S., Dumke, R., Schröter-Bobsin, U., Höhne, M., Röske, I., and Schreier, E. 2005. Detection of enteric viruses and bacterial indicators in German environmental waters. *Arch. Virol.* **150**, 929–947.

Rhoades, R.E., Tabor-Godwin, J.M., Tsueng, G., and Feuer, R. 2011. Enterovirus infections of the central nervous system. *Virology* **411**, 288–305.

Rodríguez-Díaz, J., Querales, L., Caraballo, L., Vizzi, E., Liprandi, F., Takiff, H., and Betancourt, W.Q. 2008. Detection and characterization of waterborne gastroenteritis viruses in urban sewage and sewage-polluted river waters in caracas, Venezuela. *Appl. Environ. Microbiol.* **75**, 387–394.

Rohayem, J., Bergmann, M., Gebhardt, J., Gould, E., Tucker, P., Mattevi, A., Unge, T., Hilgenfeld, R., and Neyts, J. 2010. Antiviral strategies to control calicivirus infections. *Antiviral Res.* **87**, 162–178.

Sánchez, G., Bosch, A., and Pintó, R.M. 2007. Hepatitis A virus detection in food: current and future prospects. *Lett. Appl. Microbiol.* **45**, 1–5.

Savolainen-Kopra, C. and Blomqvist, S. 2010. Mechanisms of ge-

netic variation in polioviruses. *Rev. Med. Virol.* **20**, 358–371.

Schwab, K. 2007. Waterborne gastroenteritis viruses, pp. 27–38. *In* Bosch, A. (ed.), Perspectives in Medical Virology, Elsevier, Amsterdam, The Netherlands.

Scipioni, A., Bourgot, I., Mauroy, A., Ziant, D., Saegerman, C., Daube, G., and Thiry, E. 2008. Detection and quantification of human and bovine noroviruses by a TaqMan RT-PCR assay with a control for inhibition. *Mol. Cell. Probes* **22**, 215–222.

Sobsey, M.D. and Meschke, J.S. 2003. Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin. Report for the World Health Organization. http://www.unc.du/courses/2008spring/envr/421/001/WHO_VirusSurvivalReport_21Aug2003.pdf. (accessed on June. 05. 2012)

Todd, S., Page, N.A., Steele, A.D., Peenze, I., and Cunliffe, N.A. 2010. Rotavirus strain types circulating in Africa: review of studies published during 1997–2006. *J. Infect. Dis.* **202**, 34–42.

Ventura, K.C., Hawkins, H., Smith, M.B., and Walker, D.H. 2000. Fatal neonatal echovirus 6 infection: autopsy case report and review of the literature. *Mod. Pathol.* **14**, 85–90.

Victoria, M., Rigotto, C., Moresco, V., De Abreu Corrêa, A., Kolesnikovas, C., Leite, J.P.G., Miagostovich, M.P., and Barardi, C.R.M. 2010. Assessment of norovirus contamination in environmental samples from Florianópolis city, southern Brazil. *J. Appl. Microbiol.* **109**, 231–238.

Wolf, S., Hewitt, J., and Greening, G.E. 2010. Viral multiplex quantitative PCR assays for tracking sources of fecal contamination. *Appl. Environ. Microbiol.* **76**, 1388–1394.

Wong, M., Xagoraraki, I., and Rose, J.B. 2007. Recent advances and future needs in environmental virology, pp. 259–284. *In* Bosch, A. (ed.), Perspectives in Medical Virology, Elsevier, Amsterdam, The Netherlands.

Wyn-Jones, P. 2007. The detection of waterborne viruses, pp. 177–204. *In* Bosch, A. (ed.), Perspectives in Medical Virology, Elsevier, Amsterdam, The Netherlands.