Occurrence of norovirus and other enteric viruses in untreated groundwaters of Korea

Ji Hee Jung, Chang Hoon Yoo, Eung Seo Koo, Hak Min Kim, Youjin Na, Weon Hwa Jheong and Yong Seok Jeong

ABSTRACT

A total of 39 water samples from 23 different groundwater wells in Korea were collected and analyzed in order to monitor the occurrence of norovirus (NoV) and other indicator microbes as the first part of a national survey of groundwater. More than 500 L of untreated groundwater were filtered through 1MDS filters. Following elution and concentration by organic flocculation, PCR and sequence analysis were employed to detect and identify NoV, enterovirus, rotavirus, hepatitis A virus and adenovirus (Adv). Somatic and F-specific phages, heterotrophic bacteria, total coliforms and *Escherichia coli* were also analyzed to infer possible fecal contamination. NoVs were detected in 18% of the 39 samples. Five out of seven NoV-positive samples (71%) were identified as GI while the other two (29%) were GII. Enteroviruses and Advs were detected in two and three samples, respectively. Rotavirus and hepatitis A virus were not detected. Total coliforms, *E. coli* and coliphages were detected in 49, 15 and 13% of the samples, respectively, but did not appear to be suitable indicators of enteric virus contamination in groundwater. These results suggest that additional treatment may be needed for a significant number of groundwaters prior to use as drinking water.

Key words | enteric viruses, fecal contamination, groundwater, norovirus

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INTRODUCTION

There are more than 140 enteric viruses identified from human feces (Leclerc *et al.* 2000) and many of them are present in various types of water source and which may infect the gastrointestinal tract and cause hundreds of diseases in humans (Lee & Jeong 2004; Pallansch & Roos 2007). These viruses can be transmitted via the fecal–oral route mainly through contaminated water, food and soil with high stability in the water environment (Moore 1993; Lee & Jeong 2004), especially in groundwater where biochemical disintegration can be minimized (Abbaszadegan *et al.* 1999). In particular, norovirus (NoV) is the leading cause of acute viral gastroenteritis in adults, causing numerous outbreaks worldwide (Inouye *et al.* 2000; Green 2007; Nordgren *et al.* 2009). NoVs belong to the family of Caliciviridae and are positive-sense RNA viruses with nonenveloped, icosahedral capsids. They are subdivided into five genogroups (GI–GV) based on the genetic diversity. While most strains infecting humans are members of the GI and GII genogroups (Zheng *et al.* 2006; Nordgren *et al.* 2009), GII strains are the most common agents, causing 75–90% of all NoV-related outbreaks (Fankhauser *et al.* 2002; Gallimore *et al.* 2007). Epidemiological studies indicate that the main seasonal occurrence of NoV is in the winter months (Duizer *et al.* 2004; KCDC 2008; Nordgren *et al.* 2009), but NoV gastroenteritis outbreaks may not be limited to winter (Kim *et al.* 2005). The health care/nursing home outbreaks caused by GII strains have strong winter seasonality, but outbreaks from other sources do not show this seasonality (Fankhauser *et al.* 2002; Lopman *et al.* 2003; Lysen *et al.* 2009). In addition, GI strains are very prevalent in outbreaks from other

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settings (Bon *et al.* 2005), especially in waterborne settings (Maunula & Von Bonsdorff 2005).

Following reports of enterovirus prevalence in groundwater (Marzouk *et al.* 1979), potentially pathogenic enteric viruses, such as human enteroviruses (EV) and NoV, have been subjected to systemic surveys in many countries as a common cause of epidemic waterborne viral gastroenteritis (Abbaszadegan *et al.* 1999; Dahling, 2002; Carducci *et al.* 2003; Fout *et al.* 2003; Powell *et al.* 2003; Borchardt *et al.* 2004; van Zyl *et al.* 2004). While drinking contaminated well water appeared responsible for 80% of the waterborne outbreaks associated with a viral agent in the United States for 1997 and 1998 (Barwick *et al.* 2000), approximately one-third of waterborne illnesses, including gastroenteritis, occurred in populations served by community or non-community groundwater systems from 1971 to 2002 (Reynolds *et al.* 2008). NoV and hepatitis A virus (HAV) have been recognized as major causative agents of groundwater-related outbreaks (De Serres *et al.* 1999; Parshionikar *et al.* 2003). The United States Centers for Disease Control and Prevention (CDC) reported that viruses, including NoV, were the etiologic agents responsible for 44.4% of nine drinking water-associated gastroenteritis outbreaks from 2005 to 2006 in the United States, suggesting viruses play a significant role in waterborne diseases (CDC 2008). A recent report from the Korea CDC (KCDC) noted that more than 20% of the total gastroenteritis that occurred between 2005 and 2007 was caused by rotavirus (RoV), NoV, astrovirus and human adenovirus (Adv) infections (KCDC 2008). Kim *et al.* (2005) also reported that NoVs were a likely cause of a groundwater-associated outbreak during 2004.

In 2007, the National Institute of Environmental Research of Korea established a 3-year monitoring program to determine the degree to which NoV contaminates groundwater in South Korea. This report describes the results of the first survey, which was conducted during wet and dry seasons in 2007. In this study, groundwater from public and private household facilities was tested for NoV contamination during the wet and dry seasons. Samples were also analyzed for four other enteric viruses, EV, RoV, HAV and Adv, for standard bacterial indicators, for somatic and male-specific coliphages, and for physicochemical parameters.

MATERIALS AND METHODS

Sample collection

Twenty sampling sites were initially chosen to investigate NoV occurrence in South Korean groundwater but three more sites were added later in order to adjust the sampling schedule. Four of the 23 sites are located in commercial food processing facilities, six sites are in schools or youth hostel facilities, five sites are in farming areas, and eight sites are located in residential buildings or public parks. More than 70% of the sites are utilized for drinking water in addition to other daily usages, such as showering, bathing or washing vegetables. The sites in this survey were selected based on previous periodical monitoring records with suspicion of fecal contamination.

Most of the groundwater samples were collected twice for each site during the 5 month period from July to November 2007. A total of 39 samples were collected; 20 samples during July–August (wet season) and 19 samples during October–November (dry season). Seven of 39 samples were collected only once owing to the permanent closure of the site after the first sampling.

Physicochemical parameters

The physicochemical parameters of groundwater samples were measured on site. Temperature (°C), conductivity (μS), pH, turbidity (NTU) and residual chlorine (ppm) were measured by using an Orion 4-star pH/conductivity meter (Thermo Fisher Scientific, Waltham, MA) and a chloridometer (Hanna Instruments, Italy).

Bacteriological analyses

Bacteriological analyses were performed according to the Standard Test Methods for Drinking Water Quality (Korea Ministry of Environment Bulletin 2007-146 2007). The total colony count method was used for detection of heterotrophic bacteria. Briefly, 1 mL of each groundwater sample was mixed with plate count agar medium, allowed to solidify, incubated at 35 °C for 48 h, and the resulting colonies

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enumerated. Total coliforms and *Escherichia coli* were detected in 100 mL of groundwater using Colilert® (IDEXX laboratories, Westbrook, ME) as described by the manufacturer.

Coliphage detection

EPA Method 1602, a single agar layer procedure (US EPA 2001), was used for detection of somatic and male-specific coliphages. In brief, log-phase *E. coli* CN-13 and *E. coli* F_{amp} host bacteria were prepared on the day of experiment and kept on ice until used. One hundred milliliters of each water sample was mixed with 0.5 mL of sterile 4 M magnesium chloride. The flask was placed in a 36 °C water bath for 5 min, followed by the addition of 10 mL of log-phase host bacteria. When the temperature of the sample and host bacteria mixture reached 43 °C in the water bath, 100 mL of 2 × tryptic soy agar with selective antibiotics (48 °C) was added to the mixture. After mixing, 20 mL portions were poured into a series of Petri dishes and solidified, incubated for over 16 h at 36 °C, and the resulting plaques were enumerated.

Virus concentration and nucleic acids extraction

The conventional adsorption–elution method was employed to concentrate virus particles present in the water samples based on the *Guide for Norovirus Detection in Groundwater* (Korea National Institute of Environmental Research 2007). Briefly, potential virus in the water samples was collected by passing at least 500 L of untreated groundwater through a 1MDS filter (CUNO, Meriden, CT) at a flow rate of 10 L per minute. The filters were transported back to the laboratory at 4 °C and processed within 72 h after completion of sample collection. The filters were eluted by submerging in 1.5% beef extract (BD Biosciences, San Jose, CA), containing 0.05 M glycine (pH 9.5), for 2–3 min in the filter housing, and the eluate transferred to a sterile 2-L beaker. After the elution process with the beef extract was repeated, the pH of eluate was adjusted to 7.0–7.5 by using 1 M HCl. The elution solution was gradually adjusted to pH 3.5 and stirred for 30 min. Then the eluate was centrifuged at 2,500 × *g* for 15 min at 4 °C, and the pellet was resuspended in 30 mL of 0.15 M Na_2HPO_4 buffer (pH 9.0–9.5). The solution was centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was filtered by using sterile, 0.22-μm pore size cellulose acetate filter. Final concentrated samples were stored at −70 °C until nucleic acid extraction. The efficiency of the concentration method was estimated as 80% on average.

Viral nucleic acid extraction was carried out using the QIAamp® DNA kit for Adv and the QIAamp® viral RNA kit for EV, HAV, RoV and NoV (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each extraction was performed using 140 μL of the final concentrated volume of groundwater sample, which represents approximately 3.5–5 L of the original groundwater collected through the 1MDS filters. The final nucleic acid extraction volume was 60 μl.

Reverse transcription and PCR conditions

For EV, HAV and RoV, cDNA synthesis was carried out using reverse transcription (RT) with random hexamers (Bionics, Seoul, Korea). First, 20 μL of nucleic acid extract was heated at 95 °C for 2 min in the presence of 25 pmol of the hexamers and then chilled on ice. Heat-denatured nucleic acid was then mixed with 2.5 mM of each dNTPs, reaction buffer (50 mM Tris–HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), and 200 U of MoMLV reverse transcriptase (Promega, Madison, WI). Finally, the reaction mixture (30 μL) was incubated at 25 °C for 20 min and 42 °C for 1 h, followed by the enzyme inactivation at 95 °C.

Sequence information for each virus-specific primer is described in Table 1. The first-round PCR condition for viral nucleic acids was as follows: 2 μL of each cDNA from RT reaction (EV, HAV, RoV) or Adv DNA was mixed with 18 μL of the PCR mixture, containing 10 pmol of each specific primer set. Initial amplification was carried out for three cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, followed by a subsequent 27 cycles of 94 °C for 15 s, 50 °C for 15 s, and 72 °C for 20 s. A final extension step was done at 72 °C for 2 min. For higher sensitivity and specificity, a second-round PCR was performed. Two microliters of the first-round PCR product was added to 18 μL of reaction mixture, which contained 10 pmol of each virus-specific semi-nested primer (Table 1). The PCR

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Table 1 | Primer sequence for virus detection in groundwater samples

Virus	Primer	Sequences (5′ → 3′)[a]	Polarity[b]	Product size[c] (bp)	Primer reference
Norovirus genogroup-I	GI-F1M	CGT CCC GAA TTY GTA AAT GAT GAT	F	313	Kim *et al.* (2005)
	GI-F2	ATG ATG ATG GCG TCT AAG GAC GC	SF		
	GI-R1M	CCA ACC CAR CCA TTR TAC ATY TG	R		
Norovirus genogroup-II	GII-F1M	GGG AGG GCG ATG GCA ATC T	F	310	Kim *et al.* (2005)
	GII-F3M	TTG TGA ATG AAG ATG GCG TCG ART	SF		
	GII-R1M	CCR CCI GCA TRI CCR TTR TAC AT	R		
Pan-enterovirus	EV 1	CAA GCA CTT CTG TTT CCC CGG	F	362	Puig *et al.* (1994); Lee & Jeong (2004)
	EV 2	ATT GTC ACC ATA AGC AGC CA	R		
	EV 3	CTT GCG CGT TAC GAC	SR		
Rotavirus	RV 1	GGC TTT AAA AGA GAG AAT TTC CGT CTG G	F	342	Le Guyader *et al.* (1994)
	RV 2	GAT CCT GTT GGC CAT CC	R		
	RV 3	GTA TGG TAT TGA ATA TAC CAC	SF		
Hepatitis A virus	HAV 1	GTT TTG CTC CTC TTT ATC ATG CTA TG	F	210	Le Guyader *et al.* (1994)
	HAV 2	GGA AAT GTC TCA GGT ACT TTC TTT G	R		
	HAV 3	TCC TCA ATT GTT GTG ATA GC	SR		
Adenovirus	AD 1	GCC GCA GTG GTC TTA CAT GCA CAT C	F	142	Puig *et al.* (1994)
	AD 2	CAG CAC GCC GCG GAT GTC AAA GT	R		
	AD 3	GCC ACC GAG ACG TAC TTC AGC CTG	SF		
	AD 4	TTG TAC GAC TAC GCG GTA TCC TCG CGG TC	SR		

[a]Y = C/T; R = A/G; I = A/T/G/C.
[b]F, forward primer; R, reverse primer; SF, semi-nested forward primer; SR, semi-nested reverse primer.
[c]Second round PCR product size.

conditions were identical to those of the first-round amplification.

For NoV, one-step RT–PCR reaction was performed by using Reverse-iT™ hot-start kit (ABgene, Rockford, IL) as described in the *Guide for Detection of Norovirus in Groundwater* (Korea National Institute of Environmental Research 2007) with minor modifications. In brief, five microliters of RNA extract was mixed with 20 μL of RT–PCR pre-mix, containing 50 pmol of each GI-F1M (or GII-F1M) and GI-R1M (or GII-R1M) primer pair (Table 1) for the first-round amplification. For cDNA synthesis, the final mixture was incubated for 30 min at 42 °C followed by 5 min RTase inactivation at 95 °C, and the subsequent amplification was carried out for 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s. A final extension step was done at 72 °C for 7 min. For a second-round semi-nested PCR, two microliters of the first-round PCR product was added to 18 μL of the second-round PCR mixture, which contained 20 pmol of each GI-F2 (or GII-F3M) and GIR1M (or GII-R1M) primer pair (Table 1). The PCR condition was identical to that of the first-round but with 25 cycles for amplification.

To avoid false-positive results due to cross-contamination of the first-round PCR product, quality control measures were taken throughout the PCR experiments as recommended (Kowk & Higuchi 1989). In short, one each of nucleic acids-free reagent control and negative control (without viral nucleic acid) were included in each amplification reaction. The product of the negative control of the first-round PCR was also included in the following

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semi-nested PCR as a second-round negative control. Poliovirus 3 RNA, Adv5 genomic DNA, and NoV RNA of GI (DQ 004614; Kim *et al*. 2005) or GII (DQ04654; Kim *et al*. 2005) from patient samples were used for positive controls for EV, Adv and NoV, respectively. We also tested whether any PCR inhibitor, such as humic acids, derived from environmental samples or the elution procedures involving beef extract, possibly affect the detection efficiency of viral nucleic acids. For the test, *in vitro* transcribed NoV RNA fragments from the cloned plasmid templates were quantified, serially diluted, and added to the PCR mixture with distilled water, final extract solution from the beef extract elution procedure, or final extract solution from field samples which had been confirmed as PCR-negative in this study. These three different mixtures were then subjected to the same RNA extraction steps and PCR experiment as described above. Approximately 10^3–10^6 viral RNA copies were used for each PCR. The detection efficiency of the PCR with the final dissolved solution from both beef extraction procedure and PCR-negative field samples was indistinguishable from the control solution of distilled water (data not shown), suggesting that the RNA extraction and subsequent procedures employed in this study were successfully removing PCR inhibitors.

Amplified products of the PCR were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Virus-positive results were determined by comparing with known band sizes of the semi-nested PCR (Table 1), and confirmed by sequencing analysis.

Sequences and phylogenetic analyses

NoV and enteric virus positive PCR products were purified with a RBC hiyield Gel/PCR DNA fragments extraction kit (Real Biotech, Taiwan) and their nucleotide sequences were determined by a commercial institute (Bionics, Seoul, Korea). All of the sequences from the PCR products and reference sequences were aligned using DNAMAN (lynnon, v. 6.0.3) and Clustal X (v. 1.81) in order to identify the genotypes of the detected viral nucleic acids. A phylogenetic tree for the detected NoV nucleic acids was constructed by the neighbor-joining (N-J) method, and Kimura's two parameter method using the MEGA (v. 4.0.2) software (Figure 1).

RESULTS

Detection of NoV and other enteric viruses in groundwater samples

Owing to the lack of available current methods to identify NoV infectious virions, the 39 final concentrated groundwater samples were analyzed for NoV by RT–PCR assay (Table 2). Seven of the 39 samples (18%) were shown to be positive for NoV. Six out of seven NoV-positive samples (85.7%) were collected during October and November, which are transitional months from fall to winter in Korea. GI (Accession No. EU249146 and AB354289) was detected in five out of seven positive samples (71%) and GII (Accession No. EU814451) was detected in two samples (29%). The GII samples were collected from the same sampling site but in different seasons.

In addition to the NoV analyses, the 39 groundwater samples were also examined for EV, HAV, RoV and Adv. EV-specific RNA sequence was detected in two samples (5%), one of which was identified as human coxsackievirus A1 (Accession No. AY603032) and the other as human poliovirus type 1 (Accession No. AM774341). In addition, these two samples were also shown to be positive for NoV GII and male-specific coliphage (Table 2). Adv-specific nucleic acid sequence was detected in three samples (8%) and they were all identified as Adv subgroup 2 based on the sequencing results of the PCR products (Accession No. FJ404725) while viral DNA of Adv subgroup 5 was employed as a positive control. None of the 39 groundwater samples was shown to be positive for HAV or RoV during this study (Table 2).

Figure 1 shows the phylogenetic analysis based on the NoV nucleic acid sequences of the PCR fragments of the capsid region (ORF2). One of two sequences of GII was clustered with genotype GII.17 and the other was with GII.4 Bristol. All five GI-related sequences were clustered with GI.2 Southampton and showed very limited sequence variations with each other (data not shown).

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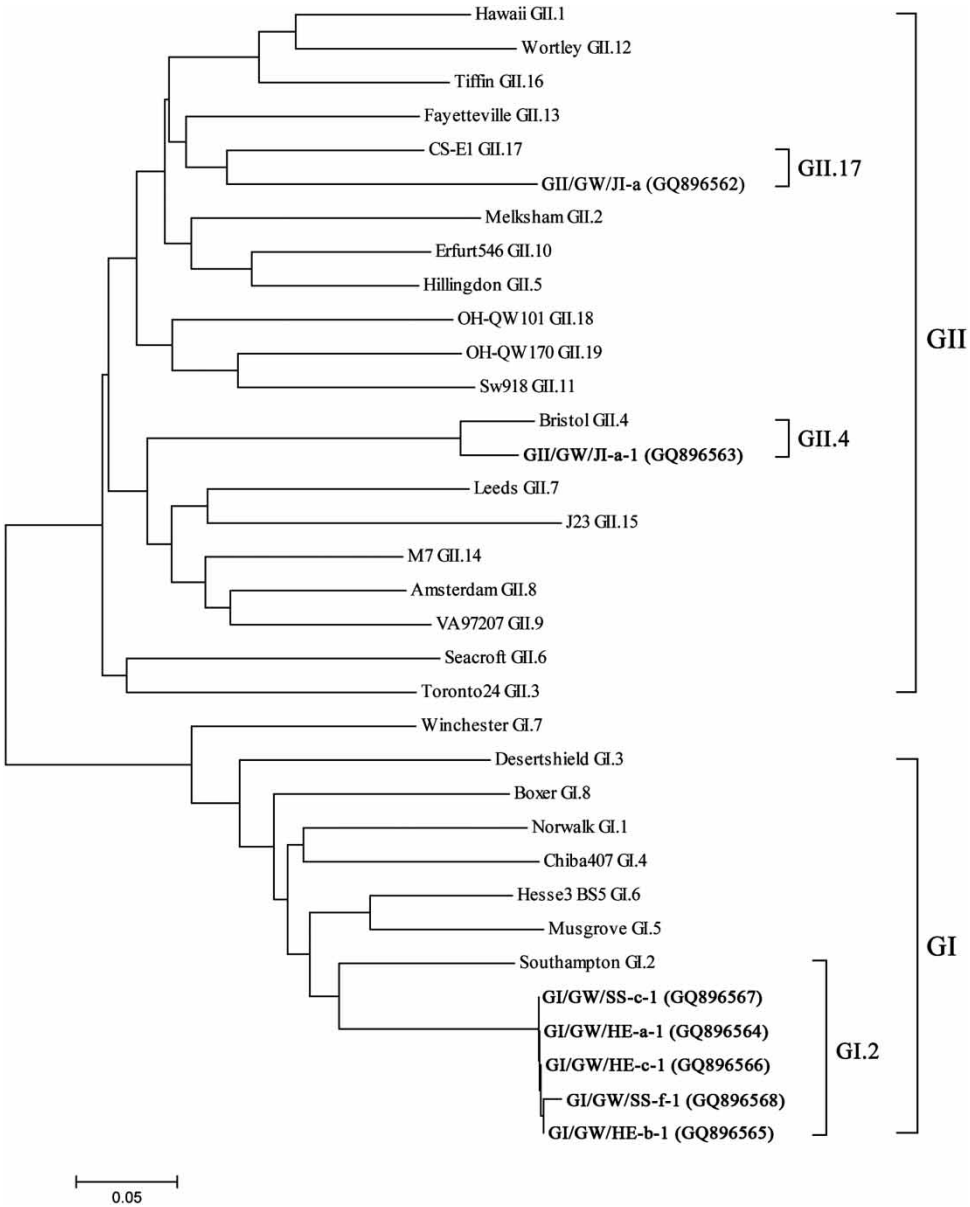

Figure 1 | Phylogenetic tree analysis of the NoVs detected in the groundwater samples during the primary nationwide survey in 2007. The NoV capsid region (314 nucleotides) was amplified by semi-nested RT–PCR. The phylogenetic tree was constructed by the neighbor-joining method and Kimura's two parameter method using the MEGA (v. 4.0.2) software. The reliability of the tree was tested by applying the bootstrap test with 1,000 bootstrap replication. Scaled bar indicates nucleotide substitution per site. Parentheses provide GenBank accession numbers.

Detection of indicator bacteria in groundwater

The number of heterotrophic bacteria (total colony count), and the presence of total coliform bacteria and *E. coli*, were measured to determine if the groundwater samples meet the drinking water standards of Korea (Table 2). Twelve of 39 water samples (31%) exceeded the standard for total colony counts of 100 cfu/mL. Total colony counts in samples not meeting the standards ranged from 106 to 912 cfu/mL, with nine having counts over 200 cfu/mL. Nineteen groundwater samples (49%) were determined to be positive for the presence of total

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Table 2 | Detection of viruses, indicator bacteria and coliphages in groundwater samples

			Bacteria			Coliphages	
Sampling periods	**Sample name**	**Viruses[a]**	**Total colony counts (cfu/mL)**	**Total coliforms (/100 mL)**	***E. coli* (/100 mL)**	**Somatic coliphages (pfu/100 mL)**	**Male-specific coliphages (pfu/100 mL)**
Jul–Aug	YP-a	–	58	+	+	32	0
	HS-a	–	4	–	–	0	0
	AS-a	–	3	+	+	0	0
	KP-a	–	8	+	+	0	0
	JS-a	–	0	–	–	0	0
	KH-a	–	13	+	–	0	0
	KH-aa	–	195	–	–	0	1
	BE-a	–	67	–	–	0	0
	CD-a	–	9	+	–	0	0
	JI-a	GII, CAV1	91	–	–	TMTC[b]	318
	JI-b	–	0	–	–	0	0
	JI-c	–	0	–	–	0	0
	HE-a	–	240	–	–	0	0
	HE-b	–	333	+	–	0	0
	HE-c	–	208	+	–	0	0
	SS-a	Adv2	106	+	–	0	0
	SS-b	Adv2	62	–	–	0	0
	SS-c	Adv2	7	–	–	0	0
	SS-d	–	10	+	–	0	0
	NS-a	–	0	–	–	0	0
Oct–Nov	YP-a-1	–	8	+	–	0	0
	AS-a-1	–	173	+	–	0	0
	KP-a-1	–	2	–	–	0	0
	JS-a-1	–	0	–	–	0	0
	KH-a-1	–	5	+	+	1	0
	KH-aa-1	–	379	+	–	0	0
	BE-a-1	–	66	–	–	0	0
	CD-a-1	–	5	–	–	0	0
	JI-a-1	GII, PV type 1	654	+	+	TMTC	70
	JI-b-1	–	48	–	–	0	0
	JI-d-1	–	241	–	–	0	0
	HE-a-1	GI	9	+	–	0	0
	HE-b-1	GI	1	–	–	0	0
	HE-c-1	GI	21	–	–	0	0
	SS-b-1	–	1	–	–	0	0
	SS-c-1	GI	912	+	–	0	0
	SS-e-1	–	303	+	+	129	0
	SS-f-1	GI	205	+	–	0	0
	NS-a-1	–	2	+	–	0	0

[a]GI/GII, NoV detection of norovirus genogroups I/II; CAV1/PV type 1, EV detection of pan-enterovirus (CAV 1, coxsackievirus A1; PV type 1, poliovirus type 1); Adv2, detection of adenovirus; –, PCR-negative.

[b]TMTC, too many to count.

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coliforms while six samples (15%) were positive for the presence of *E. coli*. On a per site basis 74% of the investigated groundwater wells did not meet the standards and thus are not suitable for drinking purposes without additional treatment.

Detection of coliphages in groundwater

In an attempt to see whether the presence of coliphages can be an additional indicator for fecal contamination in the groundwater, the amount of somatic coliphages and male-specific coliphages were determined by plaque assay as described in Materials and Methods. Six out of 39 samples were contaminated with these coliphages (15%), with two samples having both somatic and male-specific coliphages (Table 2). It should be noted that these two samples, JI-a and JI-a-1, independently collected from the same site in two different seasons, were also contaminated with NoV GII and EV (Table 2).

Basic physicochemical and other characteristics

The physicochemical parameters (temperature, pH, turbidity, conductivity and free chlorine) were measured on sites for all 39 groundwater samples according to the National Institute of Environmental Research guidelines as described in Materials and Methods (Table 3). The arithmetical mean and standard deviation of each parameter were: 17.4 ± 1.5 and 16.0 ± 1.6 for temperature (°C), 6.8 ± 0.6 and 6.9 ± 0.5 for pH, 2.7 ± 5.7 and 5.2 ± 11.9 for turbidity (NTU), and 359 ± 512 and 266 ± 116 for conductivity (μS) in the samples from the wet and dry seasons, respectively. In addition to high viral and bacterial contamination, the JI-a and JI-a-1 samples had significantly higher levels of turbidity and conductivity than the other samples. Average values for the JI-a and JI-a-1 samples were 33.0 ± 16.4 NTU and 435 ± 20 μS, compared with 2.4 ± 5.7 NTU and 244 ± 102 μS for all other samples, respectively. It was of interest that the arithmetical mean of conductivity for the samples with enteric virus contamination (340 ± 87 μS) was well above that of samples without virus (224 ± 99 μS). It would not be reasonable, however, to say that the physicochemical parameters determined in this study are related to the detection probability of the enteric viruses in groundwater samples.

The groundwater from 20 to 23 wells surveyed in this study was utilized as drinking water. Regarding well depth, 18–22 (no information available for one well) wells recorded more than 100 m in depth and two of those were 220 m and 367 m deep (data not shown). All remaining wells for which information was available were less than 50 m deep. Most of the wells (20/22) in this study were located beneath the bedrock layer while only two wells were at the alluvial layer (no information available for one well).

DISCUSSION

Eight (34%) of the 23 groundwater sites examined in this study were positive for human viruses. Viruses detected included NoV (G I and G II), coxsackievirus A1, poliovirus type 1, and human Adv 2. On a per-sample basis, ten of 39 samples (26%) were positive with one or more of those viruses. Of the ten virus-positive samples, seven turned out to be contaminated with NoV. Thus, there appears to be a wide distribution of NoV in the groundwaters studied. The finding that six out of seven NoV-positive samples (85.7%) were collected during the transitional months from fall to winter in Korea are in agreement with previous reports (Fankhauser *et al*. 2002; KCDC 2008). It was of interest that GI NoV was detected in 71% of the samples while GII NoV was detected in only 29%. This is discordant with a general recognition of GII dominance, but consistent with the findings of others (Bon *et al*. 2005; Maunula & Von Bonsdorff 2005). Since we detected viral nucleic acids by RT–PCR, virus-positive results may not reflect infectious NoV particles. Nonetheless, considering that the geometric mean inactivation rate of various enteric viruses is equal to or less than 0.1 $\log_{10}$/day at 15–20 °C (John & Rose 2005), the significance of NoV RNA detection should not be underestimated. The NoV detection rate in groundwater in this study is similar to that of other countries (Gerba 2007). However, given the recent outbreaks (Kim *et al*. 2005), it would be appropriate to develop an immediate action strategy for public health protection from NoV in groundwater in Korea.

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Table 3 | Basic physicochemical properties of the groundwater samples

	Wet season						Dry season				
Sample name	Temperature (°C)	pH	Turbidity (NTU)	Conductivity (μS)	Free chlorine (ppm)	Sample name	Temperature (°C)	pH	Turbidity (NTU)	Conductivity (μS)	Free chlorine (ppm)
YP-a	17.3	6.5	0.3	276	0.01	YP-a-1	16.5	6.7	0	300	0
AS-a	16.4	6.8	0	151	0.03	AS-a-1	15.7	7.1	0.6	146	0
KP-a	16.1	6.8	0	165	0	KP-a-1	14.2	6.8	1.6	155	0.11
JS-a	16	5.9	0	208	0	JS-a-1	14.5	7.0	0.8	154	0.02
KH-a	16.2	6.0	8.1	143	0	KH-a-1	16.9	6.1	0	160	0.08
KH-aa	16.5	6.9	0	151	0.01	KH-aa-1	16.2	6.8	4	122	0.01
BE-a	16.3	7.4	0.1	181	0	BE-a-1	15.6	8.0	0	178	0.01
CD-a	16.3	7.2	0	231	0.08	CD-a-1	17.9	6.3	0.8	205	0
JI-a	21	6.7	21.3	421	0.05	JI-a-1	19.5	6.8	44.6	449	0.06
JI-b	18.9	7.8	0.8	259	0.03	JI-b-1	17.2	7.5	3.4	288	0
JI-c	19.8	7.9	0	261	0	JI-d-1	16.4	7.1	1.2	366	0
HE-a	17.7	7.9	0.6	267	0.02	HE-a-1	14.2	8.0	0.7	279	0.02
HE-b	19.7	6.7	0.7	443	0.2	HE-b-1	15.9	6.8	1.2	426	0.03
HE-c	16.7	6.5	0	370	0.11	HE-c-1	15.9	6.9	1.8	336	0
SS-a	19.4	6.9	0	155	0	SS-b-1	14.2	6.3	1.4	355	0
SS-b	16.4	6.2	14.4	344	0.05	SS-c-1	15.5	6.8	2.7	312	0
SS-c	16.5	6.8	4.1	299	0.04	SS-e-1	18.2	6.7	31.8	387	0.06
SS-d	17.3	6.5	2.8	305	0	SS-f-1	15.4	6.7	1.5	376	0
NS-a	16	6.4	0	52	0.01	NS-a-1	13.2	6.9	0.7	53.6	0
HS-a	16.8	6.5	0	2,490	0.04						

In addition to NoVs, EV and Advs were found. EV have frequently been found in groundwater (Abbaszadegan *et al.* 1999, 2003; Dahling 2002; Fout *et al.* 2003), but this is one of the few reports of Advs in groundwater (Mena & Gerba 2009). While the human Adv type 2 is not classified as a causative agent of enteric diseases, it has been well recognized that most of the Adv members replicate in the human gastrointestinal tract and are frequently emitted through feces in high concentrations (Yates *et al.* 2006; Wold & Horwitz 2007). Because of the high concentrations seen in other types of water, Advs have been proposed as viral indicators of fecal contamination of waters (Mena & Gerba 2009).

Well depth and geological setting did not relate to NoV contamination in this study. Four of the six NoV-positive wells had depths greater than 100 m and five wells were below a bedrock layer. However, most aquifers in South Korea are in old, fractured rocks covered by a thin layer (about 6 m) of surface soil. Fractured bedrock can allow for rapid traveling of certain fecal contaminants on the soil surface or the leaks from septic tanks or sewage lines into the groundwater.

Detection of indicator bacteria

Currently, the maximum contaminant level (MCL) for total colony counts of the Drinking Water Standards of Korea is 100 cfu/mL. Applying this limit to the groundwater samples collected in this study, 31% of all groundwater samples failed to satisfy the requirement. However, four of seven NoV-positive samples (67%) were shown to be within the MCL of the total colony counts, suggesting that the concentration of heterotrophs may not be suitable to predict NoV contamination in groundwater. Total coliform bacteria and *E. coli*, which have been employed as standard indicator

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bacteria for fecal contamination worldwide for years (Bosch 1998; Clesceri *et al*. 1998; Miagostovich *et al*. 2008) were detected in 49% and in 15% of the analyzed groundwater samples, respectively. On the basis of these results, more than 53% of the collected groundwater samples, or 74% on a per-site basis, are not appropriate for drinking in terms of bacterial standards of Korea. However, even though the detection frequencies of bacterial contamination were high due to the site selection criteria, only 5 (26%) of 19 total coliform-positive samples and only one of six *E. coli*-positive samples was associated with human enteric viruses (Table 2). These observations are in agreement with previous reports that standard bacterial indicators of fecal contamination are not statistically associated with the presence of human enteric viruses in groundwater (Marzouk *et al*. 1979; Abbaszadegan *et al*. 2003; Borchardt *et al*. 2004).

Implication of the presence of coliphages

Somatic and male-specific coliphages, which are bacterial viruses that are infective for *E. coli*, have been used as viral indicators of fecal pollution (Gerba 1987) because their morphology and transportation dynamics through soils resemble that of the human enteric viruses (Puig *et al*. 1994). Male-specific coliphages, which more closely resemble the human viruses than do the somatic coliphages, have been reported to be more specific for enteric viruses from fecal origin by some (Leclerc *et al*. 2000; Borchardt *et al*. 2003), but not all (Havelaar *et al*. 1993) researchers. In this study, more than one type of coliphage was identified in 15% of the samples and 22% of the sites, but only 33.3% of the coliphage-positive samples and 4% (one) of the sites were associated with the presence of human enteric viruses (Table 2). These results suggest that neither of the coliphages was able to predict the presence of human enteric viruses in the groundwater samples analyzed in this study. However, there was co-occurrence of *E. coli* with somatic coliphage in four of five somatic coliphage-positive samples, but only one out of three male-specific coliphage-positive samples (Table 2). Therefore, although the number of samples analyzed here may not be sufficient to have a statistical association, the co-occurrence of *E. coli* and somatic coliphage seems to indicate possible fecal contamination as has been reported (Leclerc *et al*. 2000).

In conclusion, we found that a significant portion (25%) of the groundwater wells we monitored in this study was contaminated with NoVs, EVs and Adv. Because of the deliberate selection of the study sites, the higher detection rate of bacterial indicators, including *E. coli*, was rather expected and should not be generalized in an interpretation of groundwater quality in South Korea. Nonetheless, the detection of human enteric viruses in addition to fecal indicator bacteria calls for immediate public attention to the biological safety of groundwater systems. Neither the indicator bacteria nor somatic and male-specific coliphages showed meaningful correlations to the presence of the enteric viruses. Geographically, five of the eight virus-positive sites (63%) are in a residential area that includes schools, hotels and public parks. The other three virus-positive sites are in rural area used for agriculture for decades. Furthermore, three of the five virus-positive sites in the residential areas are the emergency water supply facilities which have been officially maintained by local governments. Despite a couple of drawbacks of this study, including insufficient sample numbers and hydrogeological data, these results present a concern that virus contamination in groundwater in Korea could occur regardless of well depth, land use, geological features or the system of facility maintenance.

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REFERENCES

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

Abbaszadegan, M., LeChevallier, M. & Gerba, C. 2003 Occurrence of viruses in US groundwaters. *J. Am. Water Works Assoc*. **95**, 107–120.

Barwick, R. S., Levy, D. A., Craun, G. F., Beach, M. J. & Calderon, R. L. 2000 Surveillance for waterborne-disease outbreaks: United States, 1997–1998. *Morb. Mortal. Wkly Rep. CDC Surveill. Summ*. **49**, 1–21.

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Bon, F., Ambert-Balay, K., Giraudon, H., Kaplon, J., Le Guyader, S., Pommepuy, M., Gallay, A., Vaillant, V., de Valk, H., Chikhi-Brachet, R., Flahaut, A., Pothier, P. & Kohli, E. 2005 Molecular epidemiology of caliciviruses detected in sporadic and outbreak cases of gastroenteritis in France from December 1998 to February 2004. *J. Clin. Microbiol.* **43**, 4659–4664.

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

Borchardt, M. A., Haas, N. L. & Hunt, R. J. 2004 Vulnerability of drinking-water wells in La Crosse, Wisconsin, to enteric-virus contamination from surface water contributions. *Appl. Environ. Microbiol.* **70**, 5937–5946.

Bosch, A. 1998 Human enteric viruses in the water environment: a mini review. *Int. Microbiol.* **3**, 191–196.

Carducci, A., Casini, B., Bani, A., Rovini, E., Verani, M., Mazzoni, F. & Giuntini, A. 2003 Virological control of groundwater quality using biomolecular tests. *Water Sci. Technol.* **47**, 261–266.

Centers for Disease Control and Prevention (CDC) 2008 Surveillance for waterborne disease and outbreaks associated with recreational water use and other aquatic facility-associated health events: United States, 2005–2006. *MMWR.* **57**, 1–38.

Clesceri, L. S., Greenberg, A. E. & Eaton, A. D. 1998 *Standard methods for the examination of water and wastewater*, 20th edition, p. 906. American Public Health Association, Washington, DC.

Dahling, D. R. 2002 An improved filter elution and cell culture assay procedure for evaluating public groundwater systems for culturable enteroviruses. *Water Environ. Res.* **74**, 564–568.

De Serres, G., Cromeans, T. L., Levesques, B., Brassard, N., Barthe, C., Dionne, M., Prud'homme, H., Paradis, D., Shapiro, C. N., Nainan, O. V. & Margolis, H. S. 1999 Molecular confirmation of hepatitis A virus from well water: epidemiology and public health implications. *J. Infect. Dis.* **179**, 37–43.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F. & Koopmans, M. 2004 Inactivation of caliciviruses. *Appl. Environ. Microbiol.* **70**, 4538–4543.

Fankhauser, R. L., Monroe, S. S., Noel, J. S., Humphrey, C. D., Bresee, J. S., Parashar, U. D., Ando, T. & Glass, R. I. 2002 Epidemiologic and molecular trends of 'Norwalk-like viruses' associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* **186**, 1–7.

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

Gallimore, C. I., Iturriza-Gomara, M., Xerry, J., Adigwe, J. & Gray, J. J. 2007 Inter-seasonal diversity of norovirus genotypes: emergence and selection of virus variants. *Arch. Virol.* **152**, 1295–1303.

Gerba, C. P. 1987 Distribution of coliphages in the environment: General considerations. In: *Phage Ecology* (S. M. Goyal, C. P. Gerba & G. Bitton, eds.). Wiley-Interscience, New York, pp. 87–123.

Gerba, C. P. 2007 Virus occurrence and survival in the environmental waters. In: *Human Viruses in Water* (A. Bosch, ed.). Elsevier, Oxford, pp. 91–108.

Green, K. Y. 2007 Caliciviridae: The noroviruses. In: *Virology* (B. N. Fields, ed.). Lippincott Williams & Wilkins, Philadelphia, PA, pp. 949–979.

Havelaar, A. H., Van Olphen, M. & Drost, Y. C. 1993 F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl. Environ. Microbiol.* **59**, 2956–2962.

Inouye, S., Yamashita, K., Yamadera, S., Yoshikawa, M., Kato, N. & Okabe, N. 2000 Surveillance of viral gastroenteritis in Japan: pediatric cases and outbreak incidents. *J. Infect. Dis.* **181** (Suppl. 2), S270–S274.

John, D. E. & Rose, J. B. 2005 A review of factors affecting microbial survival in ground water. *Environ. Sci. Technol.* **39**, 7345–7356.

Korea Centers for Disease Control and Prevention (KCDC) 2008 Molecular epidemiology of group A rotavirus infection in Korea. *Public Health Wkly Rep.* **1**, 325–327.

Kim, S. H., Cheon, D. S., Kim, J. H., Lee, D. H., Jheong, W. H., Heo, Y. J., Chung, H. M., Jee, Y. & Lee, J. S. 2005 Outbreaks of gastroenteritis that occurred during school excursions in Korea were associated with several waterborne strains of norovirus. *J. Clin. Microbiol.* **43**, 4836–4839.

Korea Ministry of Environment Bulletin 2007-146 2007 *The Standard Test Methods for Drinking Water Quality*. Korea Ministry of Environment, Gyeonggi-do, pp. 434–456.

Korea National Institute of Environmental Research 2007 *Guide for Norovirus Detection in Groundwater*. Korea National Institute of Environmental Research, Incheon, Korea.

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

Leclerc, H., Edberg, S., Pierzo, V. & Delattre, J. M. 2000 Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *J. Appl. Microbiol.* **88**, 5–21.

Lee, H. K. & 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.

Le Guyader, F., Dubois, E., Menard, D. & Pommepuy, M. 1994 Detection of hepatitis A virus, rotavirus, and enterovirus in naturally contaminated shellfish and sediment by reverse transcription-seminested PCR. *Appl. Environ. Microbiol.* **60**, 3665–3671.

Lopman, B. A., Adak, G. K., Reacher, M. H. & Brown, D. W. 2003 Two epidemiologic patterns of norovirus outbreaks: surveillance in England and Wales, 1992-2000. *Emerg. Infect. Dis.* **9**, 71–77.

Lysen, M., Thorhagen, M., Brytting, M., Hjertqvist, M., Anderson, Y. & Hedlund, K-O. 2009 Genetic diversity among food-

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borne and waterborne norovirus outbreaks in Sweden. *J. Clin. Microbiol.* **47**, 2411–2418.

Marzouk, Y., Goyal, S. M. & Gerba, C. P. 1979 Prevalence of enteroviruses in ground water of Israel. *Ground Water*. **17**, 487–491.

Maunula, L. & Von Bonsdorff, C.-H. 2005 Norovirus genotypes causing gastroenteritis outbreaks in Finland 1998–2002. *J. Clin. Virol*. **34**, 186–194.

Mena, K. D. & Gerba, C. P. 2009 Waterborne adenovirus. *Rev. Environ. Contam. Toxicol*. **198**, 133–167.

Miagostovich, M. P., Ferreira, F. F., Guimaraes, F. R., Fumian, T. M., Diniz-Mendes, L., Luz, S. L., Silva, L. A. & 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.

Moore, B. E. 1993 Survival of human immunodeficiency virus (HIV), HIV-infected lymphocytes, and poliovirus in water. *Appl. Environ. Microbiol*. **59**, 1437–1443.

Nordgren, J., Matussek, A., Mattsson, A., Svensson, L. & Lindgren, P. E. 2009 Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant. *Water Res*. **43**, 1117–1125.

Pallansch, M. A. & Roos, R. P. 2007 Enteroviruses: Polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: *Virology* (B. N. Fields, ed.). Lippincott Williams & Wilkins, Philadelphia, PA, pp. 840–893.

Parshionikar, S. U., Willian-True, S., Fout, G. S., Robbins, D. E., Seys, S. A., Cassady, J. D. & 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. & Lerner, D. N. 2003 Microbial contamination of two urban sandstone aquifers in the UK. *Water Res*. **37**, 339–352.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G. & Girones, R. 1994 Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl. Environ. Microbiol*. **60**, 2963–2970.

Reynolds, K. A., Mena, K. D. & Gerba, C. P. 2008 Risk of waterborne illness via drinking water in the United States. *Rev. Environ. Contam. Toxicol*. **192**, 117–158.

US Environmental Protection Agency (US EPA) 2001 *Method 1602: Male-specific (F^+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure*. EPA 821-R-01–029, Office of Water, USEPA, Washington, DC.

Van Zyl, W. B., Williams, P. J., Grabow, W. O. & Taylor, M. B. 2004 Application of molecular method for the detection of group A rotavirus in raw and treated water. *Water Sci. Technol*. **50**, 223–228.

Wold, W. S. M. & Horwitz, M. S. 2007 Adenoviruses. In: *Fields Virology*, 5th edition (D. Knipe, ed.). Lippincott, Williams & Wilkins, PA, Philadelphia, PA, pp. 2395–2436.

Yates, M. V., Malley, J., Rochelle, P. & Hoffman, R. 2006 Effect of adenovirus resistance on UV disinfection requirements: a report on the state of adenovirus science. *J. Am. Water Works Assoc*. **98**, 93–106.

Zheng, D. P., Ando, T., Fankhauser, R. L., Beard, R. S., Glass, R. I. & Monroe, S. S. 2006 Norovirus classification and proposed strain nomenclature. *Virology* **346**, 312–323.

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