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Occurrence and Molecular Characterization of Noroviruses in Korean Surface Water Between 2007 and 2010

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The occurrence of human norovirus (NoV) genogroup I (GI) and genogroup II (GII) strains was investigated in Korea. Between 2007 and 2010, 265 samples were collected from 89 Korean water source locations. NoV GI was detected in 4.5% and NoV GII in 1.5%. Samples collected in winter had the highest occurrence; 9.4% for NoV GI and 6.3% for NoV GII. NoV GI detection was highest in groundwater, with the next highest in river water and the lowest in lake water (5.9%, 5.4%, and 1.6%, respectively), and NoV GII was found only in river water. When three representative Korean basin systems (Han (H)-, Geum/Seom (G/S)-, and Nakdong (N)-river basins) were compared, both NoV genogroups were high in the G/S-, but absent in the H- river basin. The most prevalent genotypes within the GI and GII groups were GI.5 and GII.4, respectively. The NoVs found in surface water were identical to those found in patients and those found in groundwater. The NoVs appeared to be transmitted from the patient to the surface water, and then to the groundwater, suggesting a fecal-oral route of transmission. This is the first nationwide surveillance of NoV in major Korean water sources.

Keywords: Fecal-oral route transmission, food poisoning, genotyping, groundwater, norovirus, source water

Introduction

Food poisoning by noroviruses (NoVs) is a constant concern in Korea [2, 5, 10, 19]. Recent examples of large-scale outbreaks include 194 students on a field trip to Jeju Island, who contracted food poisoning in May 2004 [8], and about 3,000 people who were treated for food poisoning after eating at a food service institution in a metropolitan area in September 2006 [4]. The Korean government has therefore organized a "Waterborne and Foodborne Viruses" consortium made up of the Ministry of Environment, the Korean Centers for Disease Control and Prevention (KCDC), the Korean Food and Drug Administration (KFDA), and the Ministry of Land, Transport, and Maritime Affairs. This consortium manages and monitors NoV levels. The Ministry of Environment listed NoV levels as one of the factors to be determined when monitoring water quality. In addition, a government-certified NoV research institution conducts nationwide surveillance of NoVs in groundwater [2, 4, 13, 15, 17]. The KFDA also operates government-certified NoV surveillance laboratories, which conduct national NoV surveys on food products and water. The KCDC monitors cases of acute gastroenteritis, conducts screens for NoV in gastroenteritis patients, and manages the nucleotide databases of NoV sequences.

According to statistics released by the Ministry of Environment in 2010, the total Korean population is 51.43 million. Of these, 48.39 million people (94.1% of the total population) used surface water (river and lake water) or groundwater as drinking water sources [18]. The total annual water intake is 7.161 billion tons; 3.73 billion tons comes from rivers and 3.338 billion tons comes from lakes, meaning that surface water comprises 98.7% of the total water intake. However, there have been almost no investigations of NoV contamination of surface water sources. The only study reported was on a small partial region in Gyeonggi-do province, which investigated NoV levels in 58 samples [11]. In the present study, we investigated the occurrence of

NoV in 265 samples from 89 locations between February 2007 and September 2010. In addition, we performed a molecular characterization of the NoVs found in the surveillance.

Materials and Methods

Sampling

Between February 2007 and September 2010, 265 samples were collected from 89 Korean water source locations (Fig. 1). The viruses were sampled and concentrated following the United States Environmental Protection Agency (USEPA) guidelines [23], as previously described [12]. In brief, 200 L of raw water was collected in a device that included a flow meter, a pressure gauge, a cartridge containing a 1MDS positively charged filter (CUNO, Meriden, CT, USA), and tubing. The water pressure was controlled, and it did not exceed 2.11×10^4 Pa during the collection. If the water pH was over 8.0, the pH was adjusted to 6.5~7.5 with 0.1 M HCl. The flow rate was controlled to under 11.4 L/min.

Elution and Organic Flocculation of NoVs

The virus was concentrated and eluted using 1 L of sterilized beef extract solution (1.5% beef extract, pH 9.5, 0.375% glycine). Pressure was applied to the beef extract solution, resulting in its influx into the cartridge housing containing the sampling filter. The pressure was turned off; the filter was submerged completely into the beef extract solution and incubated for 1 min, thereby extracting the virus into the solution. This step was repeated twice. To concentrate the solution, the pH of the effluent was adjusted to 7.3 by adding 1 M HCl while stirring without forming bubbles. The volume of the effluent was measured, after which the pH was re-adjusted to 3.5 ± 0.1. The effluent was incubated for 30 min with stirring at room temperature followed by a centrifugation at 2,500 ×*g* at 4°C for 15 min. The supernatant was discarded, and the pellet was resuspended in 30 ml of 0.15 M sodium phosphate solution. The pH was adjusted to 9.3, followed by an incubation for 10 min at room temperature. The suspension was centrifuged at 4,000 ×*g* at 4°C for 10 min, after which the supernatant was collected. The pH of the concentrated effluent was adjusted to 7.3 ± 0.1.

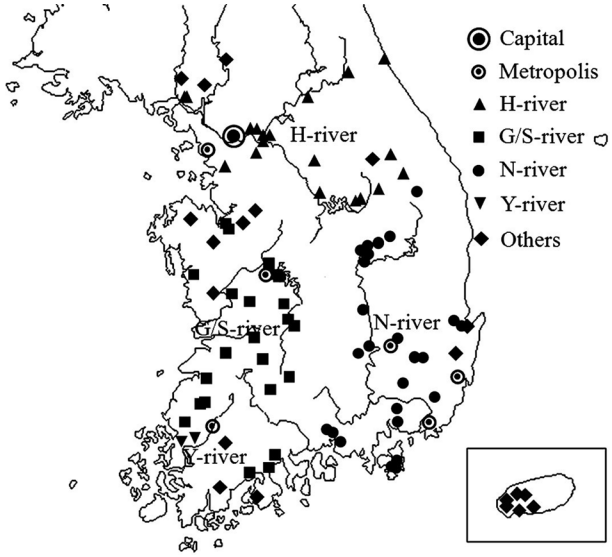

Fig. 1. Map of sampling locations.

RNA Extraction and RT-PCR

A QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used to extract viral RNA from 140 µl of the supernatant prepared as previously described [13, 17]. The extracted viral RNA was then resuspended in 50 µl of double-distilled, RNase-free water (Ambion, Austin, TX, USA). The extracted viral RNA was then subjected to reverse transcription polymerase chain reaction (RT-PCR) using NoV primers that have been previously published [13]. The RT-PCR mixture (30 µl) for the amplification of the NoV genomic RNA was composed of 5× Qiagen OneStep RT-PCR buffer (6 µl), 20 pmol primers (1 µl each), 10 mM dNTP mix (1 µl), enzyme mix (reverse transcriptase and Taq polymerase, 2 µl), 5× Q-solution (6 µl), RNase-free water (6 µl), and the extracted viral RNA template (7 µl). RT-PCR was conducted at 50°C for 30 min followed by 95°C for 15 min, after which the samples were subjected to 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. For nested PCR, the RT-PCR product (3 µl), 20 pmol primers (1 µl each), and RNase-free water (15 µl) were added to a Maxime PCR PreMix kit tube (iNtRON Biotechnology, Seongnam, Republic of Korea). The nested PCR conditions were an initial 5 min denaturation step at 94°C, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 90 sec, with a final extension at 72°C for 7 min. RT-PCR and nested PCR were conducted using the GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). The PCR products were electrophoresed on 1.5% pre-made agarose gels (iNtRON Biotechnology), stained with ethidium bromide, and visualized under UV light. The PCR products were then extracted from the agarose gel using a QIAquick Gel Extraction kit (Qiagen), and the nucleotide sequences were determined by Solgent Co. (Daejeon, Republic of Korea). Multiple negative controls were included to confirm that cross-contamination had not taken place during the nested PCR. The NoV RNA positive control was kindly provided by the Waterborne Virus Bank in the Republic of Korea (http://knrrb.knrrc.or.kr/english/index.jsp?rrb=wava).

Genotyping and Molecular Phylogenetic Analysis of NoVs

The newly isolated NoVs were genotyped using 68 NoV ORF2 nucleotide sequences previously proposed [24]. In addition, several nucleotide sequences from NoVs previously isolated from Korean groundwater and from the stool of gastroenteritis patients

were also added to the genotyping analysis of GII genogroup NoVs, providing evidence for fecal-oral transmission of NoVs. The nucleotide sequences were aligned using ClustalX (ver. 2.0) and evolutionary distances were calculated using the DNADIST program, based on the Kimura-two-parameter method, from the PHYLIP package (ver. 3.69, http://cmgm.stanford.edu/phylip/). The phylogenetic tree was established using the NEIGHBOR program based on the neighbor-joining method. Then, the phylogenetic tree was visualized with the Treeview program (ver. 1.6.6).

Nucleotide Accession Numbers

The accession numbers of the two NoVs that had been previously isolated from the stool of gastroenteritis patients in the Republic of Korea are FJ514242 and JQ622197. The accession numbers of GI NoVs that had been previously isolated from groundwater in the Republic of Korea are HM623464, HQ148227, HQ148296, JQ796694, and JQ796695. Accession numbers of GII NoVs that had been previously isolated from groundwater in the Republic of Korea are HM623465, HM623466, HM623467, HM623468, HQ148228, HQ148229, HQ148231, HQ148233, and HQ148236. The accession numbers of the NoV genogroup GI and GII nucleotide sequences obtained in the present study are JX488743–JX488754 and JX488755–JX488758, respectively.

Results

Prevalence of NoVs in Sampled Korean Water Sources

Out of 265 samples, 12 (4.5%) were NoV GI-positive and 4 (1.5%) were NoV GII-positive (Table 1). From a seasonal perspective, GI NoVs were found in five samples in the fall season (from September to November), representing the highest number of NoV-positive samples. However, the rate of occurrence of NoV GI was the highest in the winter season (3 out of 32 samples were positive; prevalence = 9.4%), and the prevalence of NoV GII was also highest in the winter season (6.3%) (Table 1). Comparing different water sources, the greatest number of positive samples was obtained from river water, with nine NoV GI-positive samples represented, but the prevalence of NoV GI in river water (5.4%) was comparable to that in groundwater (5.9%). Four positive GII NoV samples were also found in river water (Table 1). Comparing prevalence rates across the four representative Korean basin systems, no GI or GII NoVs were found in the Han (H)-river basin, and only one GI NoV was found in the N-river basin. The Geum/Seom-river had the highest NoV prevalence (GI = 6.9% and GII = 2.3%). The Yeongsan-river and other basins also showed a high NoV prevalence (GI = 7.8% and GII = 3.1%) (Table 1).

Genotyping of NoVs

Phylogenetic analysis was conducted in order to determine the genotypes of the detected NoVs. The NoV with nucleotide sequence JX488744, isolated in the present study, belonged to the genotype GI.2 cluster, along with WR96-GBR (AJ277610), SOV-GBR93 (L07418), and C59-USA (AF435807). Five of the presently isolated NoVs (JX488747–JX488751)

Table 1. Detection of noroviruses in Korean water: season, source, water basins, and year detected.

		Number of tested samples	Number of [a]NoV-positive samples		Prevalence of NoV (%)	
			GI	GII	GI	GII
Total		265	12	4	4.5	1.5
[b]Season	Spring	96	1	1	1.0	1.0
	Summer	62	3	1	4.8	1.6
	Autumn	75	5	0	6.7	0
	Winter	32	3	2	9.4	6.3
Source	River	166	9	4	5.4	2.4
	Lake	61	1	0	1.6	0
	Ground	34	2	0	5.9	0
	Mixed	4	0	0	0	0
[c]Basins	H	34	0	0	0	0
	G/S	87	6	2	6.9	2.3
	N	80	1	0	1.3	0
	Y/others	64	5	2	7.8	3.1

[a]NoV: norovirus.

[b]Season: Spring (March~May), Summer (June~August), Autumn (September~November), and Winter (December~February).

[c]Basins: H means Han-river basin; G/S, Geum/Seom; N, Nakdong; and Y, Yeongsan.

formed a genotype GI.5 cluster with SzUG1-JPN (AB039774), Musgrov-GBR00 (AJ277614), and AB318-USA (AF414406). Three other NoVs isolated in the present study, JX488743, JX488745, and JX488746, formed a genotype GI.6 cluster with Hesse-DEU98 (AF093797), WUG1-JPN (AB081723), and CS841-USA (AY502007). JX488752, JX488753, and JX48874 fell into the genotype GI.4 cluster with Chiba-JPN00 (AB042808), NO266-USA (AF414402), and Valetta-MLT (AJ277616) (Fig. 2A). Two of the NoV GII-positive nucleotide sequences (JX488755 and JX488757) belonged to the genotype GII.4 cluster, along with Bristol-GBR93 (X76716), DG4770-AUS (AF427115), and FMHill-USA (AY502023). JX488758 formed a genotype GII.17 cluster with CSE1-USA03 (AY502009), and JX488756 formed a GII.11 cluster with SW918-JPN01 (AB074893) (Fig. 2B). JX488755 and JX488757 fell within the same cluster as NoVs CUK-3 and CBNU2, which were collected from the stool of patients with gastroenteritis in the Republic of Korea. These four sequences clustered tightly in the phylogenetic tree (Fig. 2B).

Molecular Characterization of NoVs

BLAST searches using the 12 GI genogroup sequences were conducted, and these searches yielded 831 nucleotide sequences. Searches based on the four GII genogroup sequences yielded 223 nucleotide sequences. The total number of NoV sequences obtained in this way was 1,054. The query coverage was more than 95%, and the maximum identity was also more than 95%. The vast majority of these sequences had been collected from stool (691, 65.0%). Sequences from groundwater samples totaled 166 (15.7%), making up the second most important source. The remaining sequences were from wastewater sewage, clam, flood water, and other sources (Table 2). Regarding the countries of origin for these NoV sequences, 516 (49.0%), 178 (16.9%), 78 (7.4%), and 194 (18.4%) nucleotide sequences were from samples collected in Japan, Korea, China, and other Asian countries (including Singapore, Thailand, and Vietnam), respectively. Of these 1,054 NoVs, 966 sequences (91.7%) originated in Asian countries. Only 8.3% were collected from non-Asian countries (Table 2).

Discussion

Our research indicated prevalence rates for GI and GII NoVs of 4.5% and 1.5%, respectively, in major Korean water sources. This suggested that viral contamination in these water sources was not especially serious. It was reported that NoV GI was more prevalent (55.2%) than NoV GII (44.8%) in river water during 2002~2003 [11].

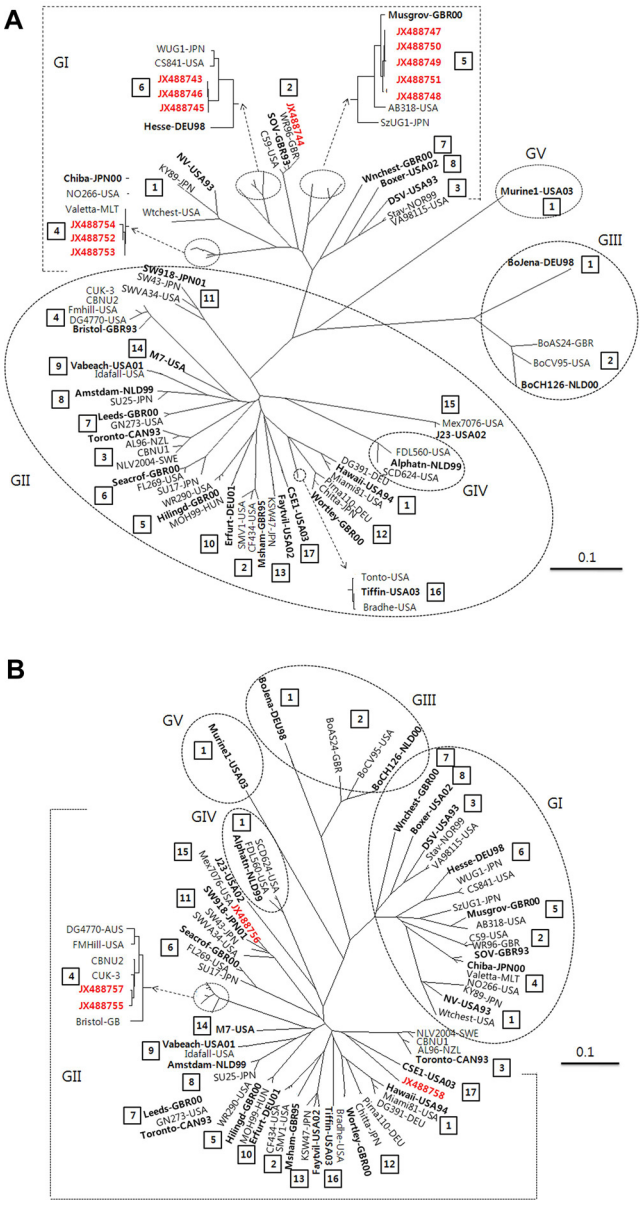

Fig. 2. Genotyping of NoVs by phylogenetic analysis based on the ORF2 nucleotide sequences of representative strains of genogroup GI (**A**) and GII (**B**) NoVs.
Phylogenetic relationships between the strains are visualized as unrooted trees. The strains can be grouped into five main clusters. The newly discovered sequences from this study are shown in red. Sequences in bold font are considered representative norovirus sequences for their genotypes.

Interestingly, the region they investigated was a portion of the H-river basin. However, our study, which investigated the overall area of the H-river basin, including river water and lake water, did not find any NoV in that region. This

Table 2. Isolation sources for NoV nucleotide sequences found by BLAST search using the newly discovered NoVs from this study as queries.

[a]NoVs	Accession numbers	*n*	Sources			Isolation from stool (%)	Countries					Isolation from Asian countries (%)
			Stool	Groundwater	Others		[b]CHN	[c]JPN	[d]KOR	Other Asian	Other non-Asian	
GI	JX488743	93	72	3	18	77.4	3	59	11	5	15	83.9
	JX488744	98	74	12	12	75.5	4	36	4	41	13	86.7
	JX488745	94	73	3	18	77.7	3	61	11	5	14	85.1
	JX488746	49	42	1	6	85.7	3	29	9	3	5	89.8
	JX488747	23	5	12	6	21.7	0	0	15	7	1	95.7
	JX488748	69	25	24	20	36.2	0	14	21	25	9	87.0
	JX488749	59	22	20	17	37.3	0	14	21	23	1	98.3
	JX488750	23	4	12	7	17.4	0	0	15	7	1	95.7
	JX488751	23	4	12	7	17.4	0	0	15	7	1	95.7
	JX488752	100	57	16	27	57.0	3	65	6	19	7	93.0
	JX488753	100	52	14	34	52.0	4	66	5	17	8	92.0
	JX488754	100	50	25	25	50.0	1	74	5	14	6	94.0
	Subtotal	831	480	154	197	57.8	21	418	138	173	81	90.3
GII	JX488755	100	99	1	0	99.0	28	48	15	6	3	97.0
	JX488756	0	0	0	0	0	0	0	0	0	0	0
	JX488757	100	97	3	0	97.0	28	49	16	4	3	97.0
	JX488758	23	15	8	0	65.2	1	1	9	11	1	95.7
	Subtotal	223	211	12	0	94.6	57	98	40	21	7	96.9
Total (GI + GII)		1054	691	166	197	65.0	78	516	178	194	88	91.7

[a]NoV, norovirus; [b]CHN, China; [c]JPN, Japan; [d]KOR, Korea.

either contradicts the previous report [11], or indicates that the contamination has for some reason been ameliorated during the intervening years. The detection of viruses as waterborne microorganisms may differ with different methods. However, contradictory results could also indicate true differences in the environmental microbiology at the different time points of each investigation [13]. There was a high protozoan and viral contamination level in the Han River prior to 2004 [16]. Then, the water pollution level decreased drastically after the implementation of ozone disinfection processes in the sewage disposal plant feeding this area of the river, in the summer of 2004 [16]. The fact that our research was conducted after the implementation of the ozone disinfection process may explain the difference in NoV occurrence between our study and that of Lee and Kim [11]. This may have important implications for public health measures that aim to reduce the prevalence of NoV in river water.

Lee and Lee [12] demonstrated that the prevalence and density of enteroviruses were low in most water sources. However, in groundwater, it was demonstrated that the NoV occurrence was 21.7% in the summer and 17.3% in the winter [17]. Then, Lee *et al*. [13] reported that the prevalence of NoVs was 8.7% in groundwater in Korea, which may represent a substantial drop. After comparing our present results with these collective findings, we concluded that the current state of NoV contamination of water sources was not especially serious. Contamination was probably relatively low because the major water sources in Korea that we investigated contain large amounts of water and are systematically managed by the government.

We were not able to determine the infectivity of the NoVs we detected, since our method of surveillance was RT-PCR. One of the reasons for the relative paucity of studies conducted on NoV contamination of water sources is our lack of knowledge about the real infectivity of NoVs from these sources and the potential degree of harm to people. However, it was reported that the exposure of NoV was associated with acute gastroenteritis incidence, in a study using RT-PCR for the detection of NoV [1]. Unlike groundwater, which can cause food poisoning, surface water goes through a water treatment process and,

importantly, a disinfection process. However, according to Keswick *et al*. [6], NoV is resistant to chlorine inactivation. On the other hand, a recent study showed that NoV can be inactivated by an adequate chlorine disinfection process [9, 20], indicating that, in areas shown by surveys such as the present study to be at risk for NoV contamination, a more stringent chlorine disinfection process may be warranted. A three-dimensional cell culture model and the use of ethidium monoazide RT-PCR to determine NoV infectivity has been reported recently [7, 21, 22]. More research on infectivity, as well as research quantifying viral levels, is needed in the future. Although there has been no report of the outbreaks caused by infectious NoVs in tap water, such studies represent important environmental and public health-related investigations that could help to secure clean drinking tap water for the people of Korea.

A recent study showed that the prevalent genotypes of NoV in Korean groundwater are GI.6 for the GI genogroup and GII.4 for the GII genogroup [13]. In the case of surface water, GI.5 was the most widely circulated, followed by GI.6 and GI.4. As for the GII genogroup, GII.4 circulated the most. These findings suggest that similar genotypes of NoV are circulating in both surface water and groundwater in Korea. NoVs are known to circulate *via* the fecal-oral route, and Lee *et al*. [13, 14] showed that nucleotide sequences of NoV from patients were identical to the sequences of NoV from groundwater in Korea, supporting the NoV fecal-oral route circulation theory. Our results showed that JX488755 and JX488757 fell within the GII.4 cluster, which also contains two NoVs, CUK-3 and CBNU2, isolated from Korean gastroenteritis patients. In addition, we demonstrated that the nucleotide sequences of the presently detected NoVs were highly comparable to the nucleotide sequences of NoVs from Asian countries such as Japan, Korea, and China. It is considered that NoVs have evolved into subclusters owing to regional specific circulation [3, 13]. The results mentioned above strongly support the hypothesis that NoVs are circulated both *via* the fecal-oral route and *via* regional specific circulation.

In conclusion, the present study represents the first nationwide investigation of NoV contamination in surface water in Korea. NoV GI was detected in 4.5% and NoV GII in 1.5%. The results suggested that the level of NoV contamination in Korea surface water surveyed in this study is not particularly serious. The most prevalent genotypes within the GI and GII groups were GI.5 and GII.4, respectively. The NoVs found in surface water were identical to those found in patients and those found in groundwater. The NoVs appeared to be transmitted from the patient to the surface water, and then to the groundwater, suggesting a fecal-oral route of transmission.

Acknowledgments

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