W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html). A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method. The distance of the nucleotide substitutions per site was calculated by Kimura's two-parameter method and illustrated using NJPlot software. The nucleotide and amino acid sequences were analyzed with GENETYX Mac Software, version 12.2.6 (Genetyx Corp., Tokyo, Japan).

We recently reported two SaV strains detected in gastroenteritis outbreaks in 2000 and 2005 in Hokkaido, and determined the partial nucleotide sequences of the polymerase and capsid gene (10). To further characterize these strains, we amplified the 3′ terminus 2.3-kb fragment spanning the capsid start to the genome end using the stool specimens containing the highest viral load, 2.78×10^{10} (Yakumo8/2000/JP) and 5.41×10^{10} (Nayoro4/2005/JP) copies per gram of stool, as described above. Two forward primers, SV-F13 (6) and SV-F14 (6), and the reverse primer TX30SXN were used for the first PCR, and SV F11 (6) and TX30SXN primers were used for the second PCR. The PCR products were purified and sequenced as described above.

Phylogenetic analysis based on the entire capsid nucleotide sequences indicated Kushiro5/2007/JP and Nayoro4/2005/JP strains clustered into GII, whereas Yakumo8/2000/JP was clustered into GIV (Fig. 1). The nucleotide identity was 97% between Kushiro5/2007/JP and Nayoro4/2005/JP, and one and three amino acid differences were found in the capsid VP1

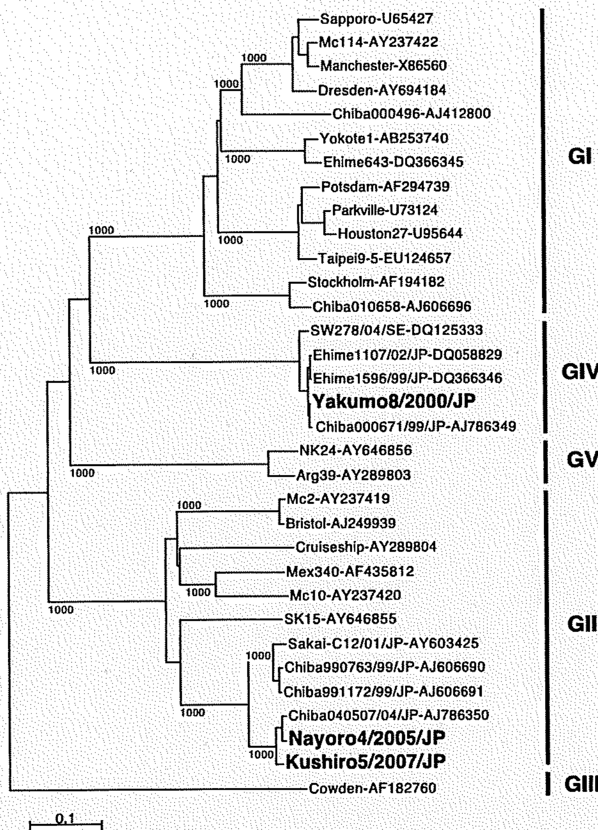

Fig. 1. Phylogenetic tree of sapovirus (SaV) based on complete capsid nucleotide sequences. GenBank/EMBL/DDBJ accession numbers for Kushiro5/2007/JP (AB455793), Nayoro4/2005/JP (AB455794), and Yakumo8/2000/JP (AB455795) are shown in bold letters in the tree. The number on each branch indicates the bootstrap value, where a value of 950 or higher is considered statistically significant for the grouping. The scale represents nucleotide substitutions per site.

and ORF2 proteins, respectively (data not shown). These two strains were close to Chiba040507/04/JP (AJ786350), which was detected in a 1-year-old female in 2004 in Chiba (3), with 97 and 98% nucleotide identities, respectively. These strains were also close to Sakai C12/01/JP (AY603425) (11), Chiba990763/99/JP (AJ606690) (3), and Chiba991172/99/JP (AJ606691) (3), with 90% nucleotide identity over the 2.3-kb fragment. In addition, Kushiro5/2007/JP and Nayoro4/2005/JP were close to Ishikawa04-721/04/JP (AM049951), with 96 and 98% nucleotide identities, respectively, when compared with the partial 400-nt capsid gene (data not shown), indicating that a genetically similar GII SaV likely persisted or circulated between 1999 and 2007 in Japan. GIV Yakumo8/2000/JP was close to Chiba000671/99/JP (AJ786349) detected in 1999 in Chiba (3), to Ehime1596/99/JP (DQ366346) and Ehime1107/02/JP (DQ058829) (12) detected in 1999 and 2002, respectively, in Ehime, and to SW278/04/SE (DQ125333) detected in 2004 in Sweden (12,13), with 99, 98, 99, and 97% nucleotide identities over the 2.3-kb fragment, respectively. In addition, GIV Yakumo8/2000/JP was identical to Tokyo18/DCC/43/00/JP (AB236378) and close to Osaka19-098/07/JP (AB327282), Yokohama16/07/JP (AB305049), and Osaka07-767/08/JP (AB433785) detected in Japan, CMH044/03/THA (EF600796) detected in Thailand, and CU050202/05/HK (DQ155647) detected in Hong Kong with 97 - 98% nucleotide identities when compared with the partial 300 nt of the capsid gene (data not shown). These findings indicate that genetically similar GIV SaV strains were widely spread and caused gastroenteritis between 1999 and 2008 in Japan and other countries, although the mode of transmission is unknown.

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Sapovirus in Water, Japan

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Sapoviruses are etiologic agents of human gastroenteritis. We detected sapovirus in untreated wastewater, treated wastewater, and a river in Japan. A total of 7 of 69 water samples were positive by reverse transcription–PCR. Phylogenetic analysis of the viral capsid gene grouped these strains into 4 genetic clusters.

The family *Caliciviridae* contains 4 genera, *Sapovirus*, *Norovirus*, *Lagovirus*, and *Vesivirus*, which include sapovirus (SaV), norovirus (NoV), rabbit hemorrhagic disease virus, and feline calicivirus strains, respectively. SaV and NoV are agents of human gastroenteritis. The most widely used method of detection is reverse transcription–PCR (RT-PCR), which has a high sensitivity and can also be used for genetic analysis. Only a limited number of SaV studies have been conducted, although most studies have shown that SaV infections are more frequent in young children than in adults and that nearly all children are infected by 5 years of age.

NoVs have been detected in oysters (and other shellfish), water from drinking fountains, ice, and community drinking water (*1–4*). Environmental studies of SaV have not been conducted. SaV strains can be divided into 5 genogroups (GI–GV), among which GI, GII, GIV, and GV infect humans; GIII infects porcine species. Phylogenetic studies have also designated SaV clusters or genotypes to further describe strains that differ by ≈10% in nucleotide or amino acid sequences. The purpose of this study was to identify and describe SaV strains in environmental samples, namely, untreated wastewater, treated wastewater, a river, and seawater, in Japan.

The Study

Water samples were obtained at different locations once a month in Miyagi Prefecture, Japan, from March 14, 2004, through February 16, 2005 (*5*). A total of 69 samples were obtained, which included 12 untreated wastewater samples, 12 treated wastewater samples, 23 river samples (2 different locations), and 22 seawater samples (2 different locations) (Figure 1). Untreated wastewater and treated wastewater were obtained from a wastewater treatment plant that processes domestic wastewater from residents living in a nearby city (Matsushima City). The treated wastewater is chlorinated at the wastewater treatment plant and then discharged into the Takagi River. The river runs directly into Matsushima Bay and then into the Pacific Ocean. River water was obtained from 2 locations upstream from the wastewater treatment plant, and seawater was obtained from 2 locations outside Matsushima Bay in the Pacific Ocean.

The methods of viral concentration were different for each location, as previously described (*5*). For untreated wastewater, 1 L was centrifuged for 15 min at 9,000 × *g* and concentrated with polyethylene glycol (resuspended in 4 mL distilled water). For treated wastewater and river water, 1 L was directly concentrated with polyethylene glycol. For seawater, 10 L was filtered, viruses were absorbed to a filter (type HA negatively charged membrane with a 0.45-μm pore size, Nihon Millipore, Tokyo, Japan) and eluted in 40 mL alkali buffer, and 40 mL buffer was further concentrated by ultracentrifugation to give a final volume of 500 μL (*6*).

RNA was extracted as previously described (*7*). Nested RT-PCR was used to detect all human genogroups (*8*). For the first PCR, primers F13, F14, R13, and R14 were used. For the nested PCR, primers F22 and R2 were used. All RT-PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. RT-PCR products were excised from

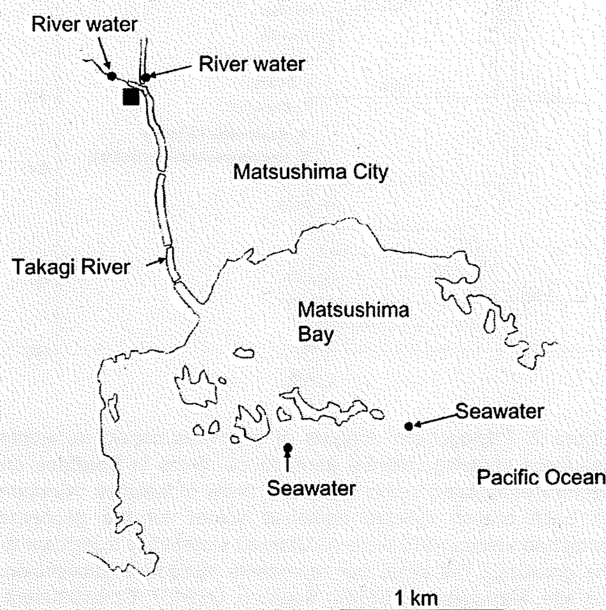

Figure 1. Locations in Miyagi Prefecture, Japan, from which water was isolated. The solid square shows the location of the wastewater treatment plant (sampling site of untreated and treated wastewater).

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the gel and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Nucleotide sequences were determined with the terminator cycle sequence kit (version 3.1) and the ABI 3130 Avant sequencer (PerkinElmer Biosystems, Wellesley, MA, USA). Sequences were aligned with Clustal X (*9*), and distances were calculated by using the Kimura 2-parameter method as previously described (*10*). Nucleotide sequence data from this study have been deposited in GenBank under accession nos. DQ915088–DQ915094.

SaV was detected in 7 (10%) of 69 concentrated water samples. Negative controls were included in the RT-PCR and showed negative results (data not shown). Genetic analysis of the positive samples showed 4 distinct genetic clusters, which included 3 GI clusters and 1 GV cluster (Figure 2). Three GI sequences were identical (strains 16, 24, and 42), 2 of which were obtained from treated wastewater 3 months apart (strains 24 and 42), and 1 was obtained from the river water (strain 16). The other 2 GI sequences grouped into 2 different clusters (strains 29 and 64) and were isolated from untreated wastewater. The 2 GV sequences were identical (strains 5 and 6). These 2 GV-positive samples were obtained on the same day, although they were obtained from different locations, i.e., untreated wastewater and treated wastewater (Figure 1). Comparison of SaV sequences detected in this study with sequences in GenBank indicated that all 7 isolates closely matched previously reported SaV sequences (Figure 2). Positive SaV samples were obtained in both hot (summer) and cold (winter) months.

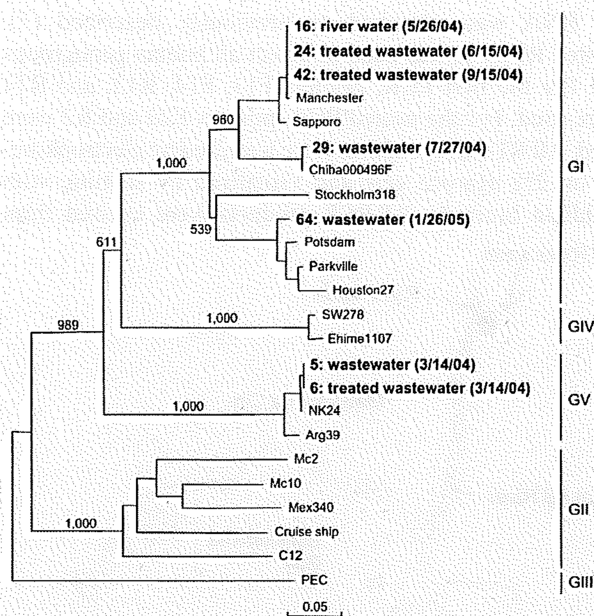

Figure 2. Phylogenetic analysis of sapovirus capsid nucleotide sequence showing different genogroups. Items in **boldface** are sequences isolated in this study and dates of isolation. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values ≥950 were considered statistically significant for the grouping. The scale bar represents nucleotide substitutions per site. Manchester, X86560; Sapporo, U65427; Chiba000496F, AJ412800; Stockholm318, AF194182; Potsdam, AF294739; Parkville, U73124; Houston27, U95644; SW278; DQ125333; Ehime1107, DQ058829; NK24, AY646856; Arg39, AY289803; Mc2, AY237419; Mc10, AY237420; Mex340, AF435812; cruise ship, AY289804; C12, AY603425; PEC, AF182760.

Conclusions

Human SaVs infections are being detected more often worldwide (*7,11,12*). These novel results have shown that like NoV (*5*), SaV can also be detected in water samples. Most sequences detected in water samples (5 of 7) belonged to GI. This genogroup likely represents the dominant genogroup worldwide (*7,10,13*). Two sequences (strains 5 and 6) belonged to GV, which has not yet been reported in Japan.

In a similar study, NoV was detected from water samples from the same research locations (*5*). Detection of SaV in river water samples upstream from the wastewater treatment plant suggests human fecal contamination in the river and that SaVs persist in freshwater. Screening for SaV may be worthwhile in oyster samples because NoVs were detected in oysters from local oyster farms (*5*). However, the failure to detect SaV in seawater samples may indicate that the sampling sites were not affected by human fecal contamination or that SaVs do not survive in marine waters. Nevertheless, further environmental studies are clearly needed to address this issue.

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Dr Hansman is a scientist at the National Institute of Infectious Diseases in Tokyo, Japan. His research interests include the epidemiology, expression, and cross-reactivity of sapoviruses and noroviruses that cause gastroenteritis in humans.

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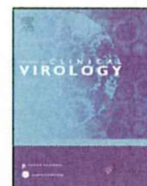

Characterization of sapoviruses detected in gastroenteritis outbreaks and identification of asymptomatic adults with high viral load

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ABSTRACT

Background: Sapovirus (SaV) is a pathogen of acute gastroenteritis, and contains a positive-sense single-stranded 7.5 kb RNA genome. Characterization of SaV has been mainly performed with short nucleotide sequences, and the viral load has not been widely determined.
Objectives: To characterize SaV strains from two outbreaks in Nagano Prefecture, Japan, using long nucleotide sequences and to measure the viral load in feces.
Study design: Approximately 2.3 kb of the 3′ terminus of the genome corresponding to the entire capsid gene, ORF2 gene, and 3′ untranslated region were amplified with semi-nested RT-PCR followed by sequencing analysis. The copy numbers of the SaV genome were determined with real-time RT-PCR.
Results: In Outbreak 1, SaV strains belonging to genogroup I (GI) were detected from seven symptomatic nursery children, in which six SaV isolates had identical nucleotide sequences while one had a single synonymous nucleotide substitution. In Outbreak 2, two similar GIV SaV sequences were detected, in which three nucleotide differences accompanying two non-synonymous substitutions were observed between symptomatic high school students and asymptomatic food handlers at a hotel. The cDNA copies were 1.36×10^7 to 1.05×10^{11} ($n=7$), and 5.05×10^6 to 1.27×10^{10} ($n=6$) per gram of stool specimens in the two outbreaks.
Conclusions: The nucleotide sequence covering the 3′ terminal 2.3 kb of the genome is useful for better characterization of the SaV strains. In addition, we found for the first time adults who secreted SaV with high viral loads without gastroenteritis symptoms.

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1. Introduction

Sapovirus (SaV), a member of the family *Caliciviridae*, is a causative agent of acute gastroenteritis and associated with outbreaks among young children and adults.[1–9] The SaV genome is a linear, positive-sense, nonsegmented single-strand RNA molecule of approximately 7.5 kb that is polyadenylated at the 3′ terminus. The SaV genome contains two or three open reading frames. The ORF1 encodes non-structural proteins and the capsid protein (VP1), and ORF2 and ORF3 encode proteins of yet unknown functions.[10] Phylogenetic analysis using the nucleotide sequences encoding the capsid protein revealed at least five genetic groups, genogroup I (GI) to GV. The human SaVs are classified into GI, GII, GIV, and GV, whereas porcine SaV belongs to GIII.[11] Human SaV strains are not cultivable, and single-round and/or nested RT-PCR are widely used for SaV detection.[12–17] A quantitative real-time RT-PCR for SaV capable of detecting all human genogroups was recently developed.[18]

Detection and characterization of SaV strains have been mainly done with short sequences of the capsid or polymerase of SaV.[12–17] In addition, the viral loads of SaV in feces have been poorly analyzed.

The aims of this study were to characterize the SaV strains from the gastroenteritis outbreaks in Nagano Prefecture, Japan, by using the nucleotide sequences of the 2.3-kb 3′ end of the genome covering the entire capsid, ORF2, and untranslated region to better characterize the strains, and to determine the SaV viral loads in feces using quantitative real-time RT-PCR.

In this study, we report for the first time the presence of asymptomatic adults with high viral loads in feces.

2. Methods

2.1. Stool specimens

Stool specimens were collected from two gastroenteritis outbreaks occurring in Nagano Prefecture, Japan. From these samples,

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Table 1
Fecal specimens collected from two outbreaks.

Outbreak	Specimen no.	Characteristics of examined person				Date of specimen collection	RT-PCR	Viral load (copies/g stool)	Accession no.
		Occupation	Sex	Age (years)	Onset				
1	Nagano18-1	Food handler	F	Adult	–	June 19, 2004	–	nt[a]	·
	Nagano18-2	Food handler	F	Adult	–	June 19, 2004	–	nt	·
	Nagano18-3	Food handler	F	Adult	–	June 19, 2004	–	nt	·
	Nagano18-4	Nursery child	M	5	June 16, 2004	June 19, 2004	+	8.20×10^9	AB436383
	Nagano18-5	Nursery child	M	3	June 17, 2004	June 19, 2004	–	nt	·
	Nagano18-6	Nursery child	M	3	June 17, 2004	June 19, 2004	+	1.05×10^{11}	Same as AB436383
	Nagano18-7	Nursery child	F	5	June 17, 2004	June 19, 2004	–	nt	·
	Nagano18-8	Nursery child	M	2	June 18, 2004	June 19, 2004	+	1.85×10^8	Same as AB436383
	Nagano18-9	Nursery child	M	5	June 16, 2004	June 19, 2004	+	8.04×10^{10}	AB436384
	Nagano18-10	Nursery child	M	4	June 17, 2004	June 19, 2004	+	1.21×10^8	Same as AB436383
	Nagano18-11	Nursery child	M	5	June 16, 2004	June 21, 2004	+	5.98×10^9	Same as AB436383
	Nagano18-12	Nursery child	M	5	June 17, 2004	June 21, 2004	+	1.36×10^7	Same as AB436383
	Nagano18-13	Nursery child	F	3	June 15, 2004	June 21, 2004	+	nt	·
2	07-1109	Student	M	17	July 22, 2007	July 31, 2007	+	2.13×10^7	Same as AB36385
	Nagano10-1	Student	M	16	July 24, 2007	July 27, 2007	+	1.06×10^{10}	AB436385
	Nagano10-2	Student	M	16	July 24, 2007	July 27, 2007	+	1.22×10^{10}	Same as AB36385
	07-1082	Student	M	15	July 25, 2007	July 30, 2007	+	5.05×10^6	Same as AB436385 and AB436386
	Nagano10-3	Food handler	F	Adult	–	July 27, 2007	+	8.04×10^7	AB436386
	Nagano10-4	Food handler	F	Adult	–	July 27, 2007	–	nt	·
	Nagano10-5	Hotel staff	M	Adult	–	July 27, 2007	–	nt	·
	Nagano10-6	Food handler	M	Adult	–	July 27, 2007	+	1.27×10^{10}	Same as AB436386

[a] nt, not tested.

10% (wt/wt) suspensions with phosphate-buffered saline were prepared and stored at −85 °C until use.

2.2. RNA extraction and cDNA synthesis

Each 10% stool suspension was clarified by centrifugation at 10,000 rpm for 20 min. Viral RNA was extracted from the supernatant using QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. For reverse transcription (RT)-PCR and real-time RT-PCR, random hexamers were used to prepare the cDNA as previously described.[18] For semi-nested RT-PCR targeting the 3′ terminus of the genome, the TX30SXN primer was used to synthesize the cDNA as described previously.[7,19]

2.3. PCR

RT-PCR was performed using the forward primer SLV5317 and reverse primer SLV5749 as previously described.[16] Quantitative real-time RT-PCR was performed as described.[18] Semi-nested RT-PCR to amplify the 2.3-kb 3′ end of the genome was performed with forward primers SV-F13 and SV-F14[13] and the reverse primer TX30SXN for the first PCR, and the forward primer SV F11[12] and reverse primer TX30SXN for the second PCR as previously described.[7]

2.4. DNA sequencing and phylogenetic analysis

The 2.3 kb PCR products generated by semi-nested RT-PCR were separated with 1% agarose gel electrophoresis, purified with QIAquick PCR purification Kit (Qiagen), and directly sequenced with the Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer, 3130 genetic analyzer (Applied Biosystems). Default values of BLAST (Basic Local Alignment Search Tool, http://blast.ddbj.nig.ac.jp/top-j.html) were used to find homologous hits. The nucleotide sequences were aligned with Clustal W version 1.83. (http://clustalw.ddbj.nig.ac.jp/top-j.html). A phylogenetic tree with 1000 bootstrap replications was constructed by the neighbor-joining method. The distance of nucleotide substitutions per site was calculated by Kimura's two-parameter method,[20] and illustrated by the NJPlot software (http://pbil.univ-lyon1.fr/software/njplot.html).[21] The SaV nucleotide sequences determined in this study have been deposited in GenBank/EMBL/DDBJ under the accession numbers AB436383–AB436386.

3. Results

Outbreak 1 occurred at a nursery school from June 15 to 18, 2004 with a peak on June 16 (Fig. 1A). Seventeen (12.2%) of 139 nursery children (age 2–5) had clinical symptoms, whereas none of the 21 adult staff including food handlers reported gastroenteri-

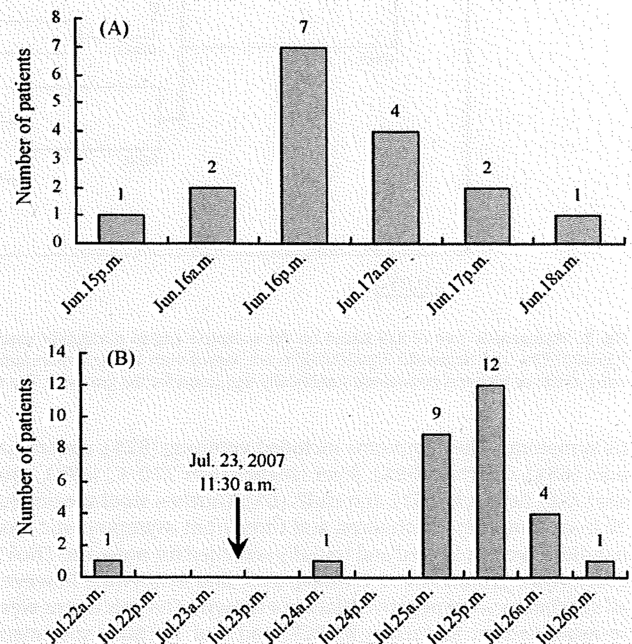

Fig. 1. (A) Number of patients in Outbreak 1. Onset times of 17 patients with gastroenteritis from 15 to 18 June, 2004 are indicated. (B) Number of patients in Outbreak 2. Onset times of 28 patients with gastroenteritis from 22 to 26 July, 2007 are indicated. The arrow indicates the arrival time of the patient group to the hotel.

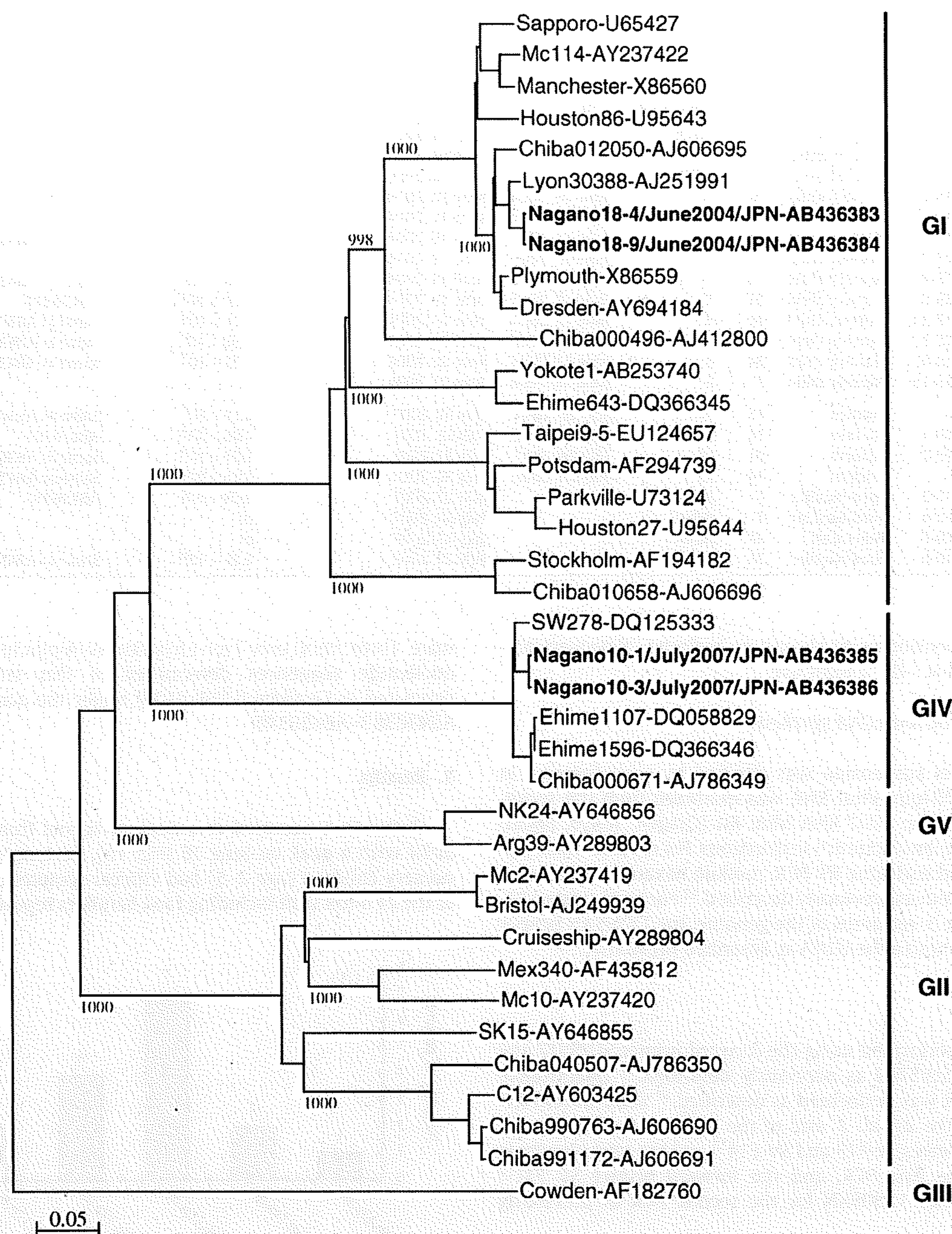

Fig. 2. Phylogenetic tree of SaV based on the complete capsid nucleotide sequences. Four SaV strains, Nagano18-4/Jun2004/JPN, Nagano18-9/Jun2004/JPN, Nagano10-1/Jul2007/JPN, and Nagano10-3/Jul2007/JPN, are shown with the accession number in bold letters. The number on each branch indicates the bootstrap value, where a value of 950 or higher is considered statistically significant for the grouping. The scale represents nucleotide substitutions per site.

tis symptoms. The symptoms included vomiting (82%), abdominal pain (65%), diarrhea (59%), fever (higher than 37.0 °C) (47%), nausea (35%), headache (6%), and chills (6%). Thirteen stool specimens, 10 from symptomatic children and three from asymptomatic food handlers, were collected and initially screened for norovirus (NoV), adenovirus, groups A and C rotaviruses, and astrovirus; however, all specimens were negative (data not shown). The specimens were re-examined for SaV using RT-PCR, and 8 of 10 (80%) specimens from the symptomatic children were positive for SaV, whereas none of the specimens from the food handlers were positive for SaV (Table 1). To confirm the RT-PCR results and to determine the cDNA copies in stools, real-time RT-PCR was performed for 7 of the 8 SaV-positive specimens. The number of cDNA copies ranged between 1.36×10^7 and 1.05×10^{11} per gram of stool with a median of 5.98×10^9 copies per gram stool ($n = 7$) (Table 1).

Sequence analysis of the 2261-nt of the 3′ terminus of the genome showed that 6 out of 7 specimens from the outbreak had 100% nucleotide identity, while one had a synonymous substitution (T to C) in VP1 at nucleotide position 549. The representative strains, Nagano18-4/Jun2004/JPN and Nagano18-9/Jun2004/JPN (containing one nucleotide difference) were registered as accession nos. AB436383 and AB436384, respectively. These strains were clustered into genogroup I/genotype 1 based on the complete capsid nucleotide sequences[10] (Fig. 2).

Outbreak 2 occurred at a hotel from July 22 to 26, 2007, with a peak on July 25 (Fig. 1B). Twenty-eight (62.2%) of 45 soccer training camp participants including 41 high school students (age 15–17), and 4 accompanying teachers reported symptoms. The symptoms included fever (higher than 37.0 °C) (79%), nausea (64%), headache (50%), vomiting (43%), chills (36%), abdominal pain (25%), diarrhea (18%), and cramping (4%). Eight stool specimens (4 from symptomatic students and 4 from asymptomatic adult staff) were collected (Table 1). All of these specimens were negative for norovirus, *Campylobacter*, diarrhogenic *Eshelichia coli*, *Salmonella*, and *Shigella* (data not shown), and were re-examined for SaV using RT-PCR. Four of 4 (100%) from symptomatic students, and 2 of 4 (50%) from asymptomatic food handlers were positive for SaV; the number of cDNA copies were 5.05×10^6 to 1.22×10^{10} per gram of stool for symptomatic students, and 8.04×10^7 and 1.27×10^{10} per gram of stool for two asymptomatic food handlers (Table 1). Direct sequence analysis of the 2264-nt of the 3′ terminus of the genome revealed the presence of two types of SaV sequence. SaV from 4 students had an identical nucleotide sequence (AB436385), whereas those from the two food handlers had another identical sequence (AB436386). Three nucleotide differences were observed in these two strains, one synonymous T to C change in VP1 at nt 1077, one nonsynonymous change at nt 1239 in VP1 accompanying an I to T change at aa 413, and one nonsynonymous A to G change at nt 2109 in ORF2-encoded protein accompanying an R to G change at aa 153. One of the four symptomatic students had microheterogeneities in these three nucleotides, which were recognized by the superimposed signals in the sequencing reaction (data not shown). These strains were clustered into GIV based on the complete capsid nucleotide sequences (Fig. 2).

4. Discussion

Detection and characterization of SaV have generally been performed with a relatively short region corresponding to the partial polymerase or capsid.[12–17] In this study, the 2.3-kb 3′ end of the genome including the capsid gene, ORF2 gene, and 3′ untranslated region was amplified, and subjected to direct sequence analysis for better characterization of the outbreak strains.

In Outbreak 1, six of seven SaV isolates had 100% nucleotide identity, whereas one had a single synonymous nucleotide substitution, suggesting that two very similar strains were co-circulated at the same time, or that the nucleotide substitution occurred during the transmission. Although, there is no direct evidence, we speculated that SaV spread via a contaminated lavatory because the patients were mainly found in two classrooms located near a lavatory used only by children; the adult staff including food handlers who used a different lavatory were all negative for SaV. The SaV strains detected in Outbreak 1 were close to Lyon/30388/98/F (AJ251991) detected in 1998 in France with 97% nucleotide identities over the 2.3-kb fragment. In addition, these strains were identical to Chiba 040506/2004 (AM049928) detected in 2004 in Japan when compared with a 390-nt part of the capsid gene (data not shown), indicating that a genetically similar GI SaV likely persisted or circulated between 1998 and 2004 in Japan and other countries.

In Outbreak 2, one student (specimen no. 07-1109) with fever and abdominal pain in the morning on July 22, 2007 before reaching the hotel (Fig. 1B) was likely to be the index case of this outbreak. SaV infection had probably occurred among the students via shared sports drinks during the training, although there is no direct evidence. Slight differences in the nucleotide sequences were observed between the strains detected in the students and food handlers (accession nos. AB436385 and AB436386) when the 2.3-kb 3′ end of the genome was compared. However, the number of available specimens was too small to suppose two independent transmission events. The possibility of the nucleotide changes being due to mutations resulting in quasi-species needs to be considered. The SaV strains detected in Outbreak 2 were close to Ehime1107/02/JP (DQ058829) detected in 2002 in Japan, and to SW278/04/SE (DQ125333) detected in 2004 in Sweden, with 97% nucleotide identities over the 2.3-kb fragment, respectively. In addition, these strains were close to CMH044/03/THA (EF600796) detected in 2003 in Thailand; CU050202/05/HK (DQ155647) detected in 2005 in Hong Kong; Yokohama16/07/JP (AB305049), Osaka 8345/07/JP (FJ445107), and Saga8151/07/JP (FJ445102), detected in 2007 in Japan; and Sapporo 8411/08/JP (FJ445097), Maizuru8240/08/JP (FJ445108) and Osaka07-767/08/JP (AB433785) detected in 2008 in Japan with 99% nucleotide identities when the 300-nt partial capsid genes were compared (data not shown). These findings indicate that genetically similar GIV SaV strains were a widespread cause of gastroenteritis between 2002 and 2008 in Japan and other countries.

Diarrhea and vomiting have been reported as the typical symptoms for SaV infection.[1–6,22] In fact, vomiting and diarrhea were major symptoms (82% and 59%, respectively) in Outbreak 1, whereas fever (higher than 37.0 °C) was the major symptom (79%) in Outbreak 2, with vomiting and diarrhea (43% and 18%, respectively) also present. The number of cDNA copies per gram of stool was consistent to those from 4 other recent outbreak cases.[3–5,7] One of the novel findings of this study was the high SaV viral loads, i.e., 8.04×10^7 and 1.27×10^{10} copies per gram stool found in asymptomatic food handlers, much higher than those observed in symptomatic students in Outbreak 2 (Table 1). The finding of high viral loads in the asymptomatic food handlers is important, because these individuals could serve as a source of infection in the community. To support this idea, a very similar SaV strain, Yokohama/16/2007 (Accession No. AB305049) was recently identified in a SaV outbreak in May–June 2007 in Japan.[23] Usuku et al. reported asymptomatic food handlers at a hotel restaurant were speculated as a source of this outbreak by demonstrating the identity of short nucleotide sequences in the polymerase and capsid among asymptomatic food handlers and the symptomatic students.[23]

More attention to SaV infection is required because of the high virus load in feces in the case of asymptomatic infections. Characterization of SaV detected not only in human specimens but also in specimens from animals, environment, and foods may be also necessary to understand the transmission route of SaV. Furthermore, little is known about immunity to SaVs and in future investigations, it would be interesting to obtain data on antibody prevalence in exposed individuals and correlate exposure with the presence or absence of symptoms and with viral load.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgements

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Single Base Substitutions in the Capsid Region of the Norovirus Genome during Viral Shedding in Cases of Infection in Areas Where Norovirus Infection Is Endemic▽

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Norovirus (NoV) infections are the major cause of food- and waterborne nonbacterial gastroenteritis in Japan. Some individuals showed long-term excretion of the virus into feces in 29 outbreaks of acute nonbacterial gastroenteritis that occurred in Toyama Prefecture, Japan, in fiscal year 2006. In one of these cases, single base substitutions from A to G in the capsid region of the NoV genome were commonly detected in two individuals during virus shedding by direct sequencing of PCR products. The A-to-G substitution was accompanied by an N-to-S amino acid change. The population of clones that possessed A at the corresponding site was gradually replaced by those with G during the infectious course. Although other substitutions were observed in the complete open reading frame 2 sequence, they were not common in these two individuals. NoVs are capable of evolving in the gastroenteric tract.

Noroviruses (NoVs), previously known as Norwalk-like virus or small round structured virus, belong to the family *Caliciviridae*. The NoV genome is a single-stranded, positive-sense RNA molecule of about 7.5 kb that comprises three open reading frames (ORFs) (6). NoV infections are the major cause of acute nonbacterial gastroenteritis worldwide, and the illness occurs in people of all ages. NoVs are transmitted not only by the fecal-oral route but also by direct person-to-person contact. Numerous outbreaks due to NoV-contaminated food and water have been reported (8, 11, 15, 26).

Since no in vitro culture system for NoVs has been established, electron microscopy (EM) or immuno-EM is routinely used to diagnose infections and to detect NoV particles in stool specimens. After the cloning and sequencing of representative NoV strains, Norwalk/68/US (14) and Southampton/91/UK (20), a reverse transcription-PCR (RT-PCR) assay was developed to target the RNA-dependent RNA polymerase gene of ORF1 of the NoV genome (2, 3, 13). Based on sequence information obtained from the polymerase region, human NoV strains can be divided into three genogroups: genogroup I (GI), GII, and GIV (23). NoV GI and GII each comprise a large number of genetically diverse strains (4, 12). A recent study indicated that NoV GI and GII strains consist of at least 14 and 17 genotypes, respectively (17, 23).

ORF2 encodes a major structural capsid protein, including a shell (S) domain and a protruding (P) domain (1, 24). Several reports have suggested a correlation between the genetic clustering of ORF2 and antigenicity confirmed by the patient's immune response (7, 22). It has been suggested that considerable genetic and antigenic divergence is a major cause of the repetitive global prevalence of this virus (9, 27). Despite the significance of the variation, the origin of the diversity and evolutionary mechanisms by which new and possibly more virulent strains evolve remain unclear. Recent studies have shown that the virus can be excreted from a person for much longer than previously thought (25), and nucleotide as well as amino acid changes accumulate, especially in the P2 domain of the capsid region, suggesting immune-mediated positive selection (21). Here, we report an NoV-infected group, in which two employees shed viruses in feces for up to 2 months. During excretion of the virus, a certain amino acid change resulting from a single nucleotide substitution in the capsid region was observed, suggesting evolution of the virus in the gastrointestinal tract.

MATERIALS AND METHODS

Outbreaks and specimens. Between April 2006 and March 2007, 29 outbreaks of acute nonbacterial gastroenteritis occurred in Toyama Prefecture, Japan. NoV GII and NoV GI genotypes were detected in 28 cases and 1 case, respectively, by real-time PCR. The outbreak described in the present study occurred in a local hotel in May 2006. Clinical information and stool specimens were collected from 30 individuals, and the samples were examined for microorganisms.

RNA extraction and RT. A 10% stool suspension was prepared as described previously (16). Viral RNA was extracted from 140 μl of the suspension with a QIAamp Viral RNA minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was eluted with 60 μl of elution buffer. Viral RNA (12 μl) was treated at 37°C for 30 min and then 75°C for 5 min in 3 μl of DNase mixture (1 U of DNase I, 125 mM Tris-HCl [pH 8.3], 187.5 mM KCl, and 7.5 mM $MgCl_2$). This was followed by the addition of 15 μl of a mixture containing 75 mM Tris-HCl (pH 8.3), 112.5 mM KCl, 4.5 mM $MgCl_2$, a 1 mM concentration of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 0.75 μg of random hexamer [$pd(N)_6$; GE Healthcare, Piscataway, NJ], 33 U of RNase inhibitor (Takara Bio Inc., Otsu, Japan), and 300 U of SuperScript II RNase H^- transcriptase XL (Invitrogen Corp., Carlsbad, CA). RT was performed at 42°C for 60 min, and the enzyme was inactivated at 99°C for 5 min.

Real-time PCR. Real-time PCR was carried out as described by Kageyama et al. (16). Real-time quantitative PCR was carried out in 50-μl reaction mixtures containing 5 μl of cDNA; 25 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ); 20 pmol each of the primers COG2F (5′-CAR

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TABLE 1. Detection of NoVs from employees

Employee and sample no.	Date of sampling	Real-time PCR		PCR result[a]
		Result[a]	Log_{10} copy no. of NoVs/g[b]	
Employee a (symptomatic)				
a-1	13 May	–		+
a-2	18 May	+	1.13E + 07	+
a-3	23 May	+	1.53E + 06	+
a-4	6 June	+	4.10E + 05	+
a-5	20 June	+	6.74E + 06	+
a-6	5 July	+	4.70E + 06	+
a-7	19 July	–		–
Employee b (asymptomatic)				
b-1	24 May	+	1.07E + 09	+
b-2	5 June	+	9.24E + 06	+
b-3	20 June	+	2.11E + 04	–
b-4	5 July	–		–

[a] +, virus was detected; –, virus was not detected.
[b] For example, "1.13E + 07" indicates 1.13 multiplied by 10^7 copies.

GAR BCN ATG TTY AGR TGG ATG AG), ALPF (5′-TTT GAG TCC ATG TAC AAG TGG ATG CG), and COG2R (5′-TCG ACG CCA TCT TCA TTC ACA); and 11.4 pmol of probe RING2AL-TP (5′-VIC-TGG GAG GGS GAT CGC RAT CT-TAMRA) for the detection of GII NoV. PCR amplification was performed with an ABI 7000 sequence detector (Applied Biosystems) under the following conditions: 2 min at 50°C and 10 min at 95°C and then 45 cycles at 95°C for 15 s and 56°C for 1 min. The data were corrected by using internal standards as described by Kageyama et al. (16).

PCR. To amplify the N-terminal/shell (N/S) domain of the capsid region, PCR was carried out with puRe *Taq* Ready-To-Go PCR beads (GE Healthcare) and the primers G2-SKF (5′-CNT GGG AGG GCG ATC GCA A) and G2-SKR (5′-CCR CCN GCA TRH CCR TTR TAC AT) for GII NoV strains as described by Kojima et al. (19). The mixture containing 2 μl of cDNA was incubated at 95°C for 10 min, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final incubation of 72°C for 15 min.

To amplify ORF2, PCR was carried out with 50 pmol primers LV6717 (5′-AGT ACC TTG TTC CGC TCC A) and LV4922G10A (5′-CAC GGC CCA ACA TTC TAC) modified from the reference (21). A 50-μl PCR mixture contained 2 μl of cDNA, 2.5 U of *TaKaRa LA Taq* (Takara Bio), 10× LA PCR buffer II 5 μl, 2.5 mM $MgCl_2$, and a 0.4 mM concentration of each deoxynucleoside triphosphate. Amplification was performed by using 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. Second PCR was performed in the same way.

Sequencing analysis. After purification of the amplicon, the nucleotide sequences were determined with the BigDye Terminator v3.1 cycle sequencing kit and an ABI 3100 or 3130 sequencer (Applied Biosystems).

Cloning analysis. PCR products of the N/S domain were cloned by using a TOPO10 PCR cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was purified by using a mini-plasmid purification kit (Promega Corp., Madison, WI) according to the manufacturer's recommendations. Plasmids containing an insert of appropriate length were sequenced as described above with the M13 universal primer.

Phylogenetic analysis. Capsid sequences of the reference strains of NoV were obtained from GenBank. Phylogenetic analysis was performed as described by Katayama et al. (17). Briefly, 302-bp sequences of the N/S domain of the capsid region were aligned by using CLUSTAL W (version 1.83). A phylogenetic tree was constructed by the neighbor-joining method, and genetic distances were calculated according to the Kimura two-parameter method (18). The reliability of the tree was estimated by performing 1,000 bootstrap replications, and bootstrap values of 950 or higher were considered statistically significant for a grouping (10).

RESULTS

Outbreak. An outbreak of gastroenteritis occurred in a local hotel in Toyama prefecture. Fecal specimens from thirty individuals, including patients and employees, were collected and examined for microorganisms. Real-time PCR analysis identified the GII sequence in specimens from fourteen individuals. Consecutive surveys showed that two employees excreted the NoV gene for one and two months, respectively, even after their symptoms had disappeared (Table 1 and Fig. 1). The copy number of the genome in one employee showed two characteristic peaks, followed by a rapid decrease to below the limit of detection ("a" terms in Fig. 1). The other asymptomatic employee showed a continuous decrease in the copy number of the NoV genome to an undetectable level ("b" terms in Fig. 1).

Sequence analysis. Sequencing revealed that the NoV causing this outbreak belonged to GII genotype 4, which is the most predominant type in recent outbreaks in Japan (5). Phylogenetic analysis showed that recently identified sequences, including this one, formed a distinct cluster from past-identified sequences of genotype 4 (Fig. 2). Direct sequence analyses of consecutive specimens from these two employees revealed that two single base substitutions occurred: (i) A (nucleotide 50 counting from the first initiation codon of the capsid gene) changed to G and (ii) C (nucleotide 279) to T. The C-to-T change was detected only in subject "a" between the first and second consecutive specimens, while the A-to-G change was observed in both subjects a and b, between the third and fourth and between the first and second specimens, respectively. Although no signal from "a-1" was detected by real-time PCR (Table 1), the PCR product was not regarded as a false-positive reaction due to contamination, since patient a was already symptomatic on May 10. The sequences of specimens from another 11 of 12 individuals in this outbreak had A (nucleotide 50) and T (nucleotide 279). The remaining individual could not be analyzed.

To examine whether additional nucleotide change existed in another area of ORF2, especially in the hypervariable region, we determined the complete sequence of ORF2 in these specimens. The ORF2s in the specimens from a-2, a-6, b-1, and b-2 were amplified by PCR and directly sequenced. Alignment of the sequences of ORF2 (1,623 bp) revealed that eight single base substitutions occurred (including the A-to-G change at

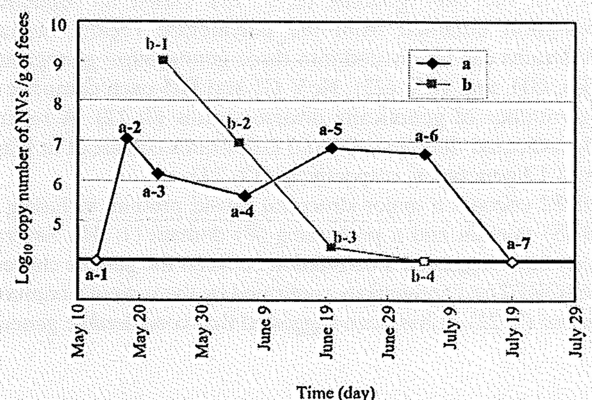

FIG. 1. Change in copy numbers of NoVs in feces obtained from employees. The copy number of NoV was measured by real-time PCR, and represented by the log_{10} copy number/g of feces. Open symbols indicates NoV negativity. The horizontal bold line indicates the limit of detection.

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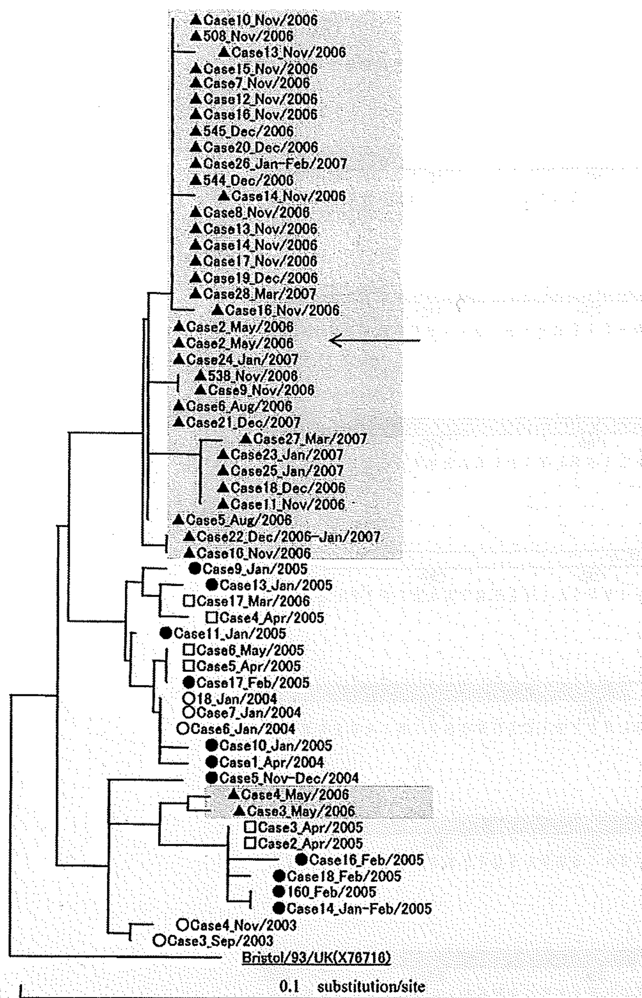

FIG. 2. Phylogenetic tree of NoV GII genotype 4 sequences obtained from outbreaks in Toyama prefecture from April 2003 to March 2007. Symbols indicate the following seasons: ○, 2003/2004; ●, 2004/2005; □, 2005/2006; ▲, 2006/2007. The shaded area indicates outbreaks that occurred in the 2006/2007 season. Samples except those from cases 3 and 4 in the 2006/2007 season form a unique cluster. Samples from cases 3 and 4 form another unique cluster. Samples are showed as "case no._month/year" (mass outbreaks) or as "sample no._month/year." The case that involves employees a and b is indicated by an arrow. Reference strains are underlined and presented as "strain (accession no.)".

nucleotide 50) and one microheterogeneity occurred in a-2 at 209, which was recognized by the superimposed signals in the sequencing reaction (Table 2). Only the substitution at nucleotide 50 was common in the two employees. A different nucleotide between a-2 and b-1 already existed at nucleotide 516. All of these nucleotide changes result in amino acid changes. One amino acid change at position 17 is located in the N-terminal domain, four changes at positions 70, 108, 172 and 193 are located in the S domain, two changes at positions 291 and 297 are located in the P2 domain, and another two at positions 460 and 483 are located in the P1 domain (Fig. 3); thus, the amino acid changes were not restricted to some distinct region.

Cloning analysis. To assess whether clonal change of the viral genome occurred in these cases, the PCR products were each cloned into plasmid DNA, and 20 to 30 clones were isolated and sequenced. As for the A-to-G change, while all a-2 and a-3 clones had A at this position, 9 and 15 clones of a-4 had A and G, respectively. One and twenty-four clones of a-5 had A and G, respectively, and all clones of a-6 had G (Fig. 4A and Table 3). In the case of subject b, all clones of b-1 had A, whereas 1 and 28 clones of b-2 had A and G, respectively (Table 3). Thus, nucleotide 50 in the capsid region seemed to gradually change from A to G during the infectious course of both individuals.

As for the C-to-T change, all clones of a-1 possessed a C residue, whereas those of a-2 possessed T (Table 3); thus, no gradual change was observed.

Although other nucleotide variations or deletions among the clones were observed in the capsid region, none was fixed to a unique sequence (Fig. 4B). The A-to-G substitution changes amino acid number 17 of the capsid from N to S; however, the N-to-S substitution may not result in a significant structural alteration of the capsid protein, because the amino acid sequence surrounding 17N (N-L-V) does not match the consensus sequence of N-linked glycosylation (N-X-S/T), and capsid protein has not been reported to be glycosylated. The C-to-T change does not cause an amino acid change.

DISCUSSION

In this report, we examined the sequence of the capsid region of NoV and found nucleotide and amino acid substitutions in consecutive specimens of two individuals that shed the virus into feces for a relatively long period of time.

Since an A-to-G change occurring in two individuals at the same time is unlikely, the change likely occurred in one of them, and then the mutated NoV was transmitted to the other. The proportion of b-2 (on June 5) clones with G (A:G = 1:28) was higher than that of a-4 (A:G = 9:15) (on June 6). The second increase in the copy number of the NoV genome in employee a on June 20 also indicates that the NoV in employee b with very high copy numbers was transmitted to employee a at this time.

NoV with a T residue might have been transmitted from subject b to subject a on around 10 May to cause the first increase in the copy number, and took over the NoV with C in subject a within a short period. Since all other NoV genomes detected in

TABLE 2. Alignment of the sequence of the complete ORF2 region of NoV GII from employees a and b

Nucleotide no. in the capsid region	Nucleotide in sample no.:			
	a-2	a-6	b-1	b-2
50	A	G	A	G
209	A or G	A	A	A
322	A	C	A	A
516	A	T	T	T
579	A	A	A	C
871	A	G	A	A
889	C	T	C	C
1378	C	C	C	T
1447	G	A	G	G

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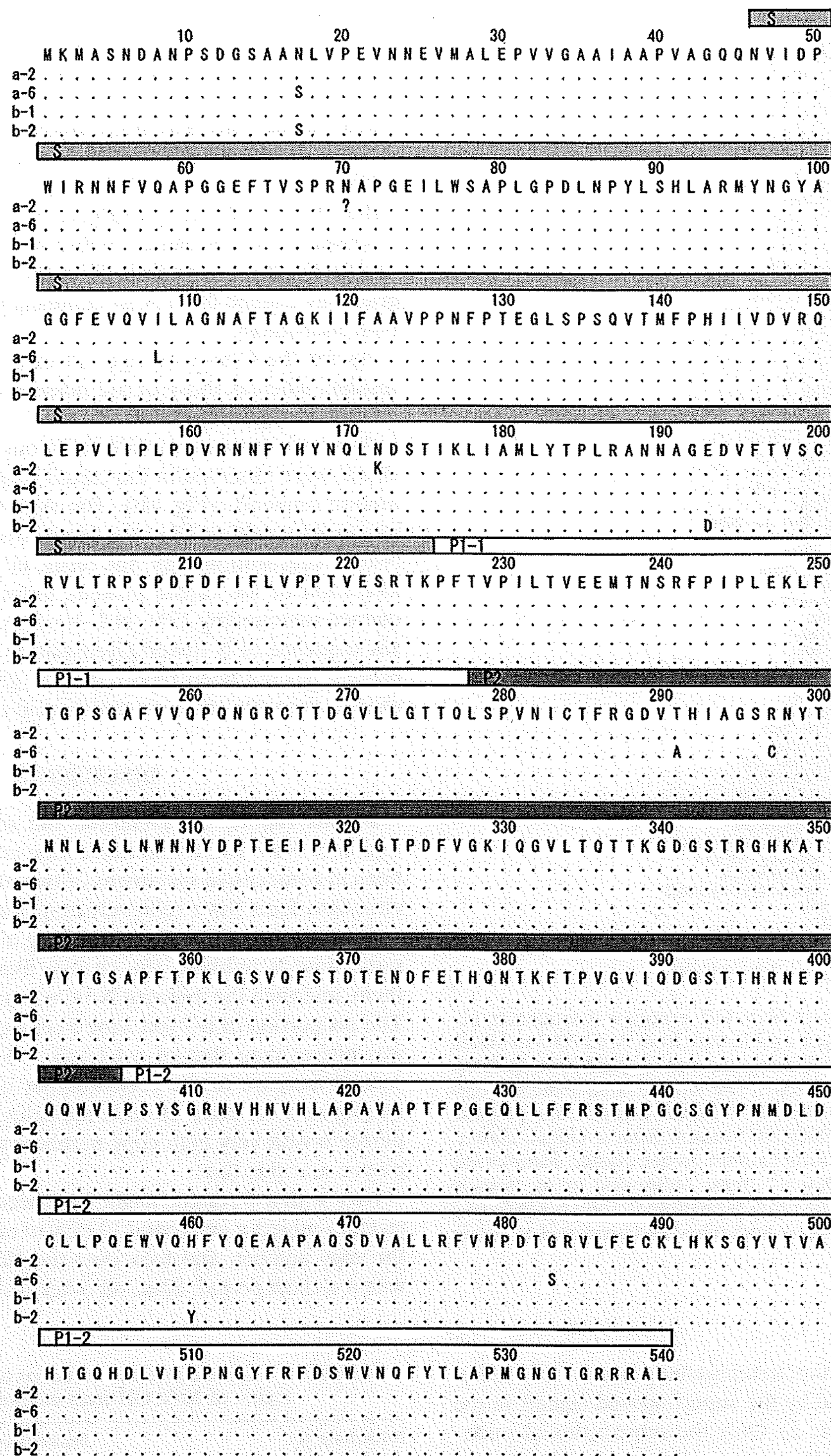

FIG. 3. Alignment of the amino acid sequence of ORF2. Amino acids identical to the consensus sequence are indicated by periods, and only the different sequences are included. The positions of S, P1, and P2 domains are estimated (1, 24) and shown above amino acid number. The codon at amino acid 70 of a-2 is N (AAC) or S (AGC) and is indicated by "?".

Toyama prefecture in 2005 and 2006 had a C residue at this site, the NoV with T is unique in recent outbreaks. That subject b demonstrated extremely high copy numbers of NoV, especially in the early phase, indicates that multiple replications of NoV occurred in this individual; therefore, the C-to-T change might have occurred in subject b and been transmitted to subject a.

Since the outbreak occurred in an isolated area in Toyama prefecture, the possibility that different NoV was introduced from

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(A) employee "a"

(nucleotide No. in the capsid region)

a-3

clone No.	CCGCAGCCAA (50)	CCTCGTCCCA (60)
3-01	----------	----------
3-02	----------	----------
3-03	----------	----------
3-05	----------	----------
3-06	----------	----------
3-07	----------	----------
3-08	----------	----------
3-09	----------	----------
3-10	----------	----------
3-11	----------	----------
3-12	----------	----------
3-13	----------	----------
3-14	----------	----------
3-15	----------	----------
3-16	----------	----------
3-17	----------	----------
3-18	----------	----------
3-19	----------	----------
3-20	----------	----------
3-22	----------	----------
3-23	----------	----------
3-24	----------	----------
3-25	----------	----------

a-4

clone No.	CCGCAGCCAA (50)	CCTCGTCCCA (60)
4-01	----------	----------
4-02	----------	----------
4-03	----------	----------
4-04	----------	----------
4-05	----------	----------
4-06	---------G	----------
4-07	---------G	----------
4-08	---------G	----------
4-09	---------G	----------
4-10	---------G	----------
4-11	----------	----------
4-12	----------	----------
4-13	---------G	----------
4-14	----G----G	----------
4-15	---------G	----------
4-16	---------G	----------
4-17	---------G	----------
4-19	---------G	----------
4-20	---------G	----------
4-21	---------G	----------
4-22	----------	----------
4-23	---------G	----------
4-24	----------	----------
4-25	----------	----------

a-5

clone No.	CCGCAGCCAA (50)	CCTCGTCCCA (60)
5-01	---------G	----------
5-02	---------G	----------
5-03	---------G	----------
5-04	---------G	----------
5-05	---------G	----------
5-06	---------G	----------
5-07	---------G	----------
5-08	---------G	----------
5-09	---------G	----------
5-10	---------G	----------
5-11	---------G	----------
5-12	---------G	----------
5-13	---------G	----------
5-14	---------G	----------
5-15	---------G	----------
5-16	---------G	----------
5-17	---------G	----------
5-18	---------G	----------
5-19	---------G	----------
5-20	---------G	----------
5-21	---------G	----------
5-22	---------G	----------
5-23	----------	----------
5-24	---------G	----------
5-25	---------G	----------

a-6

clone No.	CCGCAGCCAA (50)	CCTCGTCCCA (60)
6-02	---------G	----------
6-03	----G----G	----------
6-05	---------G	----------
6-06	---------G	----------
6-07	---------G	----------
6-08	---------G	----------
6-11	---------G	----------
6-12	---------G	----------
6-13	---------G	----------
6-14	----G----G	----------
6-15	---------G	----------
6-16	---------G	----------
6-17	---------G	----------
6-18	---------G	----------
6-19	---------G	----------
6-20	---------G	----------
6-21	---------G	----------
6-22	---------G	----------
6-23	---------G	----------
6-24	---------G	----------

(B) employee "a"

(nucleotide No. in the capsid region)

a-3

clone No.	GAGGTCAACA (70)	ATGAGGTTAT (80)
3-01	----------	----------
3-02	----------	----------
3-03	----------	----------
3-05	----------	----------
3-06	----------	----------
3-07	----------	----------
3-08	----------	----------
3-09	----------	----------
3-10	----------	----------
3-11	----------	----------
3-12	----------	----------
3-13	----------	----------
3-14	----------	----------
3-15	----------	----------
3-16	----------	----------
3-17	----------	----------
3-18	----------	----------
3-19	----------	----------
3-20	----------	----------
3-22	----------	----------
3-23	----------	------C---
3-24	----------	----------
3-25	----------	----------

a-4

clone No.	GAGGTCAACA (70)	ATGAGGTTAT (80)
4-01	----------	----------
4-02	-G--------	----------
4-03	-G--------	----------
4-04	-G--------	----------
4-05	----------	----------
4-06	----------	----------
4-07	----------	----------
4-08	----------	----------
4-09	--A-------	----------
4-10	----------	----------
4-11	-G--------	----------
4-12	----------	----------
4-13	----------	----------
4-14	----------	----------
4-15	----------	----------
4-16	----------	----------
4-17	----------	----------
4-19	----------	----------
4-20	----------	----------
4-21	----------	--A-------
4-22	-G--------	----------
4-23	----------	----------
4-24	-G--------	----------
4-25	-G--------	----------

a-5

clone No.	GAGGTCAACA (70)	ATGAGGTTAT (80)
5-01	----------	----------
5-02	----------	----------
5-03	----------	----------
5-04	----------	----------
5-05	----------	------C---
5-06	----------	----------
5-07	----------	---G------
5-08	----------	----------
5-09	----------	----------
5-10	----------	----------
5-11	----------	----------
5-12	----------	----------
5-13	----------	----------
5-14	----------	----------
5-15	----------	----------
5-16	----------	----------
5-17	----------	----------
5-18	----------	----------
5-19	----------	----------
5-20	----------	----------
5-21	----------	----------
5-22	----------	----------
5-23	----------	----------
5-24	----------	------C---
5-25	----------	----------

FIG. 4. Nucleotide sequences of the cloned capsid region. (A) Alignment of cloned nucleotide sequences from positions 41 to 60 of consecutive specimens of employee a. PCR products were cloned into plasmid DNA and sequenced. The consensus sequence is shown at the top of the figure. Only nucleotides different from the consensus sequence are included. (B) Alignment of cloned sequences from positions 61 to 80 of consecutive specimens of employee a as a representative of nucleotide variations.

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TABLE 3. Numbers of clones containing an A or G residue at nucleotide 50 and C or T residue at nucleotide 279 of employees a and b

Employee and sample no.	Date of sampling	No. of clones at nucleotide[a]:			
		50		279	
		A	G	C	T
Employee a (symptomatic)					
a-1	13 May	27	0	27	0
a-2	18 May	24	0	0	24
a-3	23 May	23	0	0	23
a-4	6 June	9	15	0	24
a-5	20 June	1	24	0	25
a-6	5 July	0	20	0	20
a-7	19 July	–	–	–	–
Employee b (asymptomatic)					
b-1	24 May	27	0	0	27
b-2	5 June	1	28	0	29
b-3	20 June	–	–	–	–
b-4	5 July	–	–	–	–

[a] –, PCR negative and could not be sequenced.

another area seems to be extremely low. Although the NoVs in subjects a and b were possibly transmitted to each other, only one with a high copy number seems to have affected the other with a low copy number.

Other different nucleotides found in a-6 and b-2 may be the consequence of independent evolution in these individuals. The higher number of changes in a-6 than those in b-2 may reflect a longer incubation time in the gastroenteric tract. Other sporadic variations observed in both the capsid and the polymerase region (data not shown) did not persist during the infectious course. Although the possibility remains that a small population of NoV with G at the corresponding position already existed at the first exposure, our observations suggest that NoV really evolves in the gastrointestinal tract in a single individual.

An accumulation of mutations in the capsid region was reported in a persistently NoV-infected patient who excreted the virus for more than 2 years (21). Most of the mutations were accumulated in the hypervariable domain (P2 domain), which is the most exposed part of the structure and has been proposed to contain determinants for strain specificity (24). Hence, these mutations were speculated to be a result of immune pressure. The N-to-S change in the capsid protein caused by the A-to-G nucleotide change in this report is located at amino acid 17, as counted from the N terminus of the protein, where an apparent antigenic epitope has not been observed (28). Consistently, other changes found in ORF2 were not restricted to the hypervariable P2 domain. Thus, these changes seem not to be caused by immune-driven pressure. Mechanisms other than immune pressure, such as interaction with receptors, or a relation to other changes elsewhere in the genome of NoV, might be associated with amino acid substitution. Alternatively, these changes might be only footprints that reflect the viral evolution occurring in the gastroenteric tract.

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Continuous Presence of Noroviruses and Sapoviruses in Raw Sewage Reflects Infections among Inhabitants of Toyama, Japan (2006 to 2008)▽

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Various genotypes of norovirus (NoV) (genogroup I genotype 1 [GI.1], -2, -4, -5, -8, -11, -12, and -14; GII.3, -4, -6, -7, -10, -13, -14, and -15), and sapovirus (SaV) (GI.1 and GI.2, GII.1, and GIV.1) were detected from raw sewage from April 2006 to March 2008, while limited numbers of genotypes of NoV (GI.8, GII.4, GII.6, and GII.13) and SaV (GII.3 and GIV.1) and of NoV (GII.4, GII.7, and GII.13) were detected from clinical cases and healthy children, respectively. During the winter 2006 to 2008, a large number of sporadic gastroenteritis outbreaks and many outbreaks caused by NoV GII.4 occurred among inhabitants in Toyama, Japan. The copy number of genomes of NoV GII detected from raw sewage changed in relation to the number of outbreaks. NoV strains of the same genotypes observed in both raw sewage and human specimens belonged to the same cluster by phylogenetic analysis and had almost identical nucleotide sequences among each genotype. These data suggest that NoVs and SaVs detected from raw sewage reflect the viruses circulating in the community, irrespective of symptoms, and that subclinical infections of NoV are common in Japan. Combined surveys of raw sewage with those of clinical cases help us to understand the relationship between infection of these viruses and gastroenteritis.

Norovirus (NoV) and sapovirus (SaV), members of the *Caliciviridae* family, are considered to be a major cause of acute gastroenteritis in humans. Both NoV and SaV infect humans via the fecal-oral route and cause family or community-wide outbreaks, mainly in the winter season. NoVs are shed in feces at a level of 10^5 to 10^9 virus particles per gram during the symptomatic phase (32, 37), and viruses are continuously shed from patients after cessation of the symptoms (28, 37, 40). In addition, recent reports showed relatively high levels of shedding of the viruses from asymptomatic individuals (7, 8, 32, 37).

NoVs and SaVs show high diversity in their genomes (5, 9). According to such a genetic diversity, they are classified into several genogroups (genogroup I [GI], GII, and GIV for human NoV and GI, GII, GIV, GV for human SaV) and further divided into many genotypes (NoV GI genotypes 1 to 14 [GI.1-14] and GII.1-17 and SaV GI.1-5, GII.1-6, GIV.1, and GV.1) (10, 17, 18). In 2006 to 2007, NoV GII.4 caused a large number of outbreaks of acute gastroenteritis worldwide (1, 11, 35, 43, 45). However, the other genotypes of NoV and SaV may infect humans asymptomatically and persist in the environment.

Raw sewage could contain enteric viruses shed from affected people, and therefore, detectable viruses in raw sewage would reflect the actual state of the circulating viruses in the area. We previously reported that polioviruses in raw sewage and river water were isolated at the same time as oral vaccination in babies, and these isolates were derived from vaccine strains (13, 30). We also showed that the nucleotide sequences of echovirus type 13 isolated from river water were closely related to those from patients with aseptic meningitis during the outbreak in 2002 (14). For NoVs and SaVs, many epidemiological surveys have been conducted to determine the prevalence and virological properties of these viruses (42). Previous reports have shown that the nucleotide sequences of NoV strains from stools of outbreaks in nursing homes and from sewage were identical for an individual outbreak (26), and NoVs detected from gastroenteritis patients, domestic sewage, river water, and cultivated oysters in the area were related to each other (44). However, less is known about infection of the viruses with minor genotypes that are silently circulating in the population.

In this study, we investigated NoVs and SaVs in raw sewage from 2006 to 2008 in Japan and compared the results with the viruses detected from clinical cases as well as healthy individuals to show the comprehensive prevalence of these viruses in the community.

MATERIALS AND METHODS

Samples and preparation of viral suspension. (i) Raw sewage. Raw sewage was collected monthly from April 2006 to March 2008 at the threshold point of a waste tank in the sewage disposal plant located in Toyama Prefecture, Japan. This facility covers an area with about 300,000 inhabitants, which is the largest group served by 29 sewage disposal plants for a population of 1,100,000 in Toyama Prefecture. The raw sewage from each household in the area reaches the facility within 4 to 8 h. The temperature of raw sewage ranged from 13.8°C to 25.0°C during the year. The average inflows of raw sewage per day in the fiscal year 2007 were 46,063 m^3 (70.9%), 2,535 m^3 (3.9%), and 16,323 m^3 (25.2%) derived from household sewage, industrial wastewater, and unidentified wastewater, respectively.

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Virus		2006 Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	2007 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	2008 Jan	Feb	Mar	Frequency of virus detection*	χ^2 p value
NoV	GI1					■																				1/24	<0.001
	GI2		■																							1/24	
	GI4	■	■	■							■	■	■		■		■	■	■		■	■	■	■	■	15/24	
	GI5															■										1/24	
	GI8						■		■									■								3/24	
	GI11								■																	1/24	
	GI12				■																					1/24	
	GI14	■												■												2/24	
	GII3			■	■		■										■	■								5/24	<0.001
	GII4		■		■	■	■	■	■	■	■	■	■	■	■	■				■	■	■	■	■	■	19/24	
	GII6					■																				1/24	
	GII7						■																			1/24	
	GII10							■																		1/24	
	GII13																		■							1/24	
	GII14							■																		1/24	
	GII15	■		■																						2/24	
SaV	GI1	■	■	■						■	■	■		■	■	■	■				■	■	■			13/24	<0.001
	GI2								■																	1/24	
	GII1												■											■		2/24	
	GIV.1	■																								1/24	

FIG. 1. Genotypes of NoV and SaV detected from raw sewage (2006 to 2008). Shading indicates times of virus detection. Frequency of virus detection (*) is defined as the ratio of the number of months when viruses were detected to total number of months in the investigation period.

Two liters of raw sewage was centrifuged at 3,000 rpm for 30 min (4°C), and the supernatants were applied for subsequent concentration of viruses using the filter adsorption and elution method and the polyethylene glycol (PEG) precipitation method, as described previously (24, 29).

For the filter adsorption and elution method, $MgCl_2$ (final concentration, 0.05 M) was added to 1 liter of the supernatant of the raw sewage, and the pH was adjusted to 3.5 with HCl. The supernatant was filtered through a mixed cellulose ester-type membrane to adsorb viruses. Then, the membranes were soaked in 10 ml of 3% beef extract solution, and the viruses were eluted by sonication.

For the PEG precipitation method, 80 g of PEG 6000 (WAKO) was added to 1 liter of the supernatant of raw sewage, and the supernatant was stirred for 2 h at 4°C to suspend PEG 6000. After the suspension was centrifuged at 10,000 rpm for 30 min (4°C), the pellet was collected and dissolved in 4 ml of 0.15 M Na_2HPO_4 (pH 9.0). The solution was recentrifuged at 10,000 rpm for 30 min (4°C) to recover the supernatant. The eluted solution or the recovered supernatant was used for viral RNA extraction.

(ii) Stool specimens. A total of 805 fecal specimens were collected from hospital patients with gastroenteritis or from outbreaks from April 2006 to March 2008. An outbreak was defined as occurring when at least three patients from the same area came down with similar clinical symptoms at roughly the same time. A total of 780 of the 805 samples could be assigned to 59 outbreaks according to the above definition. The remaining 27 samples were considered cases of sporadic gastroenteritis diagnosed at pediatric clinics.

With permission from guardians, 134 fecal specimens were collected from healthy children from 2006 to 2008. The ages of the healthy children ranged from <1 to 6 years old. The specimens were originally collected for a survey of poliovirus in September and January in 2006 to 2008, which is at least 2 months after the vaccination for poliovirus. Personal information of donors was disconnected and deidentified from the samples.

A 10% (wt/vol) suspension of stool was prepared by mixing with phosphate-buffered saline, followed by centrifugation at 13,000 rpm for 30 min (4°C). The supernatant fluids were used for viral RNA extraction.

Reverse transcription-PCR and genotyping. Viral RNA was extracted from 140 μl of concentrated raw sewage or supernatant of stool suspension, using a QIAamp viral minikit (Qiagen) according to the manufacturer's procedure. Extracted RNA was treated with 5 U of DNase I (TaKaRa), and cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) with a random hexamer, according to the manufacturer's instructions. The cDNA was used for PCR and real-time PCR.

For NoV PCR, we used the primers SK1F (sense; 5'-CTG CCC GAA TTY GTA AAT GA-3'), SK1R (antisense; 5'-CCA ACC CAR CCA TTR TAC A-3'), SK2F (sense; 5'-CNT GGG AGG GCG ATC GCA A-3'), and SK2R (antisense; 5'-CCR CCN GCA TRH CCR TTR TAC AT-3'), which amplify 330 bp corresponding to nucleotides (nt) 5342 to 5671 of Norwalk/68/US (GenBank accession number M87661) for GI or 344 bp (nt 5046 to 5389) of Lordsdale/93/UK (accession no. X86557) for GII, encompassing the 3' end of ORF1 to the beginning of the capsid region, as described by Kojima et al. (21). For SaV PCR, we used the primers SV-F11 (sense; 5'-GCY TGG TTY ATA GGT GGT AC-3') and SV-R1 (antisense; 5'-CWG GTG AMA CCA TTK TCC AT-3'), which amplify about 780 bp (nt 5098 to 5878 of Manchester virus; accession number X86560) of the N-terminal of VP1 region, as described by Okada et al. (36). To determine the genotypes of these viruses, the PCR products were directly applied for sequence analysis using an ABI Prism BigDye Terminator, version 3.1, cycle sequencing kit and an ABI Prism 3100 DNA sequencer (Applied Biosystems). TA cloning (TOPO TA cloning kit; Invitrogen) of the NoV GII PCR products derived from raw sewage from July to October in 2006 was also performed to detect mixed populations of NoV GII.4 because the predominant genotype of NoV GII.4 had changed from 2006a and Chiba-4e to 2006b during that time, as described in the Results section. We sequenced 25 amplicons per month. The genotypes were determined by comparing sequences with those of reference strains in the GenBank (6, 10, 17, 18, 25, 35, 41, 42). The genetic relationship between the strains in this study (Toyama strains) and reference strains was analyzed by MEGA, version 3.1, software (23), using the genomic regions described above. The phylogenetic tree was constructed by the neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method. A bootstrapping test was performed 1,000 times.

Real-time PCR. The cDNA samples of NoV were used for the TaqMan-based real-time PCR as previously described by Kageyama et al. (16). NoV probes labeled with the TaqMan dye VIC were synthesized at Applied Biosystems Japan, Ltd.

Counting of coliforms. The amounts of coliforms in raw sewage were measured using the pour plate method with desoxycholate agar (39). Briefly, each 1 ml of a 10-fold dilution of raw sewage was spread into the 90-mm-diameter dishes and then mixed with 20 ml of desoxycholate agar medium. The dishes were incubated at 35°C overnight. The number of colonies of coliforms was counted and defined as the number of CFU.

Statistical number of pediatric patients with gastroenteritis. The number of patients with gastroenteritis was counted according to the reports from 29 out of 141 pediatric clinics (20.6%) in Toyama Prefecture, from April 2006 to March 2008.

Nucleotide sequence accession numbers. Nucleotide sequences determined in this study were deposited in the GenBank database under accession numbers AB437141 to AB437235.

RESULTS

NoVs and SaVs in raw sewage. NoVs and SaVs detected from raw sewage from April 2006 to March 2008 are summarized in Fig. 1. Both the GI and GII genogroups of NoVs were observed almost every month during the survey period. They included eight genotypes of GI (GI.1, -2, -4, -5, -8, -11, -12, and -14) and eight genotypes of GII (GII.3, -4, -6, -7, -10, -13, -14, and -15). NoV GII.4 was the most frequently detected among the genotypes of NoVs, especially in winter and spring. NoV GI.4 was detected with the second highest frequency. Four

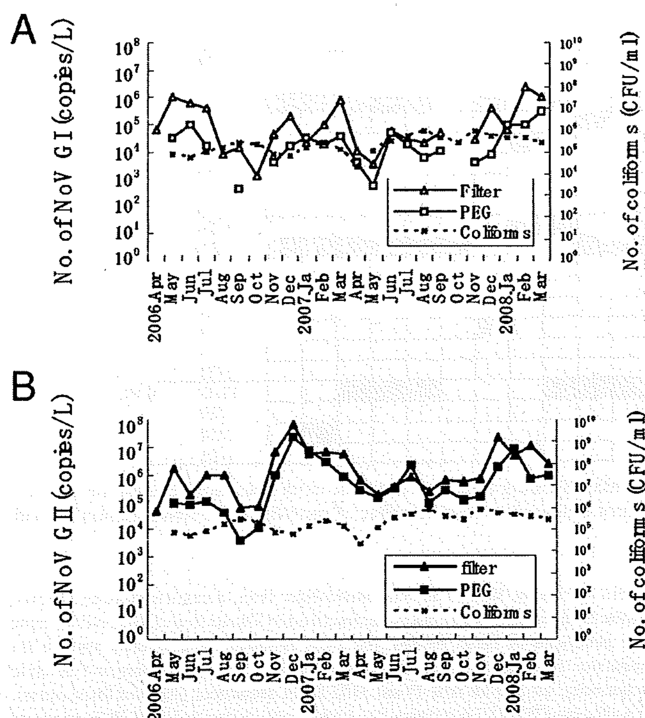

FIG. 2. Quantification of NoV in raw sewage. (A) Values indicate the number of NoV GI genome copies per liter of raw sewage concentrated by the filter absorption and elution method (filter) and the PEG precipitation method. (B) Values are the number of NoV GII genome copies per liter of raw sewage concentrated by each of the indicated methods. The dotted lines indicate the amounts of coliforms per milliliter of raw sewage.

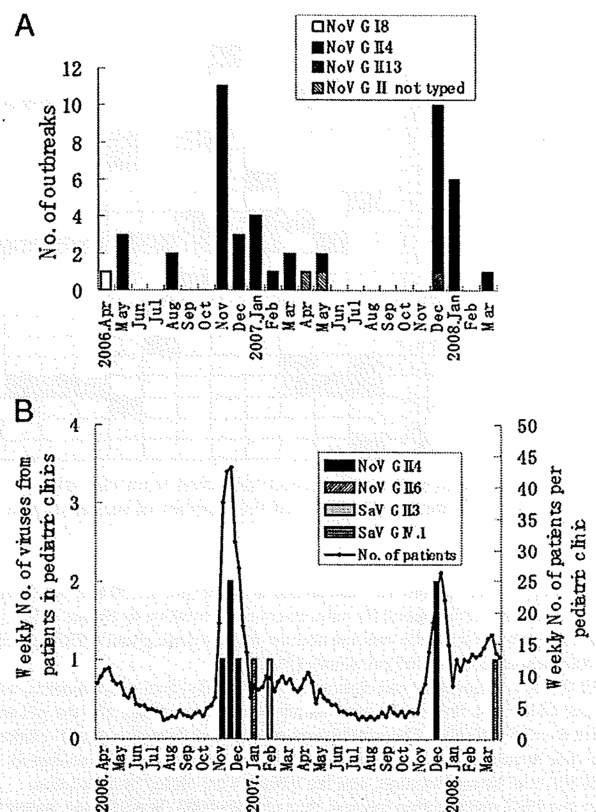

FIG. 3. (A) Numbers of outbreaks caused by NoVs in Toyama Prefecture (2006 to 2008). Values are the instances caused by NoV GI.8, GII.4, and GII.13 and instances caused by infection with NoV II agents whose genotypes were not determined, as indicated on the figure. (B) Weekly number of viruses detected from children with gastroenteritis in pediatric clinics and weekly number of patients with gastroenteritis per pediatric clinic in Toyama Prefecture, Japan (2006 to 2008). Data represent patients with NoV GII.4, NoV GII.6, SaV GII.3, and SaV GIV.1, as indicated. The dots indicate the weekly mean number of patients with gastroenteritis from 29 pediatric clinics from April 2006 to March 2008.

genotypes of SaVs, GI.1, GI.2, GII.1, and GIV.1, were observed, and GI.1 was the most frequently detected among four genotypes. The frequencies of detection of NoVs GI.4, GII.4, and SaV GI.1 were significantly high between each genotype of NoV GI, NoV GII, and SaV, respectively (χ^2 test, $P < 0.001$).

Thus, various genotypes of NoV and SaV were detected from raw sewage, while NoV GII.4 was predominantly detected in winter and spring.

Quantification of NoVs in raw sewage. We quantified the NoVs in raw sewage using real time PCR to examine the seasonal changes. To concentrate viruses, both the filter adsorption and elution method and the PEG precipitation method were employed since efficiency or preference of virus detection is believed to be different between these two methods. Although the filter adsorption-elution method was found to be more sensitive than the PEG precipitation method, both methods showed similar profiles of seasonal change (Fig. 2).

The raw sewage contained not only human excrement but also drainage, such as from a factory; therefore, the degree of dilution of raw sewage might differ every day. Therefore, we measured the number of coliforms as an index for the dilution level of raw sewage. The number of coliforms in raw sewage ranged from 2.0×10^4 to 8.1×10^5 CFU/ml, and the geometric mean titer was 2.0×10^5 CFU/ml (Fig. 2). The number of coliforms showed only a small dispersion (coefficient of variation [CV], 0.076) during the survey period. It indicates that the input of raw sewage into the sewage disposal plant is almost uniform. Furthermore, there was no correlation between levels of coliforms and either NoV GI or NoV GII ($r = -0.0751$ or $r = -0.0331$, respectively; $P < 0.05$).

The copy number of NoV GI in raw sewage ranged from 4.6×10^2 to 2.3×10^6 copies/liter (CV, 0.17) during the survey period, except for October 2006, when NoV GI was not detected. Although smaller amounts of NoV GI in raw sewage were observed than NoV GII, as described later, sewage tended to contain larger amounts of NoV GI in the period from winter to spring than from summer to fall (Fig. 2A).

The copy number of NoV GII ranged from 3.8×10^3 to 7.1×10^7 copies/liter (CV, 0.14). The amount of NoV GII was 10^4 to 10^6 copies/liter in July to November, and 10^6 to 10^8 copies/liter in December to June, indicating a clear correlation between the amount of NoV GII and the number of outbreaks (Fig. 3). Raw sewage contained a higher amount of NoV GII genome than NoV GI during the survey period.

NoVs and SaVs detected from clinical cases. Fifty-nine outbreaks of gastroenteritis occurred, mainly in November to January, during the survey period. Most outbreaks were caused by NoV GII.4, except for two instances caused by GI.8 in April