TABLE 1. Viruses detected from feces of healthy children in Toyama, Japan, 2006 to 2008

Date of sample	Age of subject (yr)	No. of stool samples	No. of samples (%) positive for[a]:		
			NoV GII.4	NoV GII.7	NoV GII.13
September 2006	<1	12	1		
January 2007	<1	2	1		
	1	10	1	5	2
	2	2			
	3	21			
	4	0			
	5	5			
	6	21		1	6
September 2007	<1	10			
January 2008	1	15			
	2	3			
	3	9			
	4	8			
	5	3			
	6	13			
Total		134	3 (2.2)	6 (4.5)	8 (6.0)

[a] No samples were positive for either NoV GI or SaV.

2006 and GII.13 in December 2007 (Fig. 3A). Genotypes were not determined in two instances. No outbreak caused by SaV occurred.

In 2006 and 2007, the total number of patients with gastroenteritis reported from pediatric clinics sharply increased in early November and then decreased in January (Fig. 3B). Gastroenteritis occurred in the winter season of 2006 to 2007 more frequently than in the season of 2007 to 2008. NoV GII.4 was detected from six patients diagnosed as sporadic gastroenteritis (Fig. 3B). NoV GII.6 and SaV GII.3 and GIV.1 were also observed from one patient each (Fig. 3B). For the other viruses, rotavirus group A (seven children), astrovirus (one child), adenovirus (two children), and parechovirus type 1 (one child) were also detected is samples from children with gastroenteritis (data not shown).

Thus, while NoV GII.4 was the main cause of outbreaks, NoV GII.4 and rotavirus group A were predominant among children with gastroenteritis in Toyama Prefecture from 2006 to 2008.

NoVs and SaVs detected from healthy children. Since the viruses detected from raw sewage were supposed to be of human origin, we investigated whether they existed in healthy individuals. For this purpose, we examined 134 available stool samples from healthy children and found that NoVs were detected in 17 stools (12.7%) (Table 1). Whereas NoV GII.4 was observed in three samples (2.2%), NoV GII.7 and GII.13 were detected in six (4.5%) and eight (6.0%) samples, respectively. Most NoVs derived from healthy children were observed in January 2007 when NoV GII.4 was prevalent in gastroenteritis cases (Fig. 3). Further investigation throughout the year will be needed to verify the presence of these viruses among overall healthy inhabitants.

These results indicate that there were certain healthy children shedding at least NoV GII.4, GII.7, and GII.13 in the winter of 2006 to 2007.

Phylogenetic analysis of NoVs detected from raw sewage and human specimens. The genetic variations of NoV GI.8 and GII.4, -7, and -13 strains detected from raw sewage were compared with those from human clinical cases by phylogenetic analysis (Fig. 4). NoV GII.4 strains were divided into three clusters: the types of 2006a, 2006b, and Chiba-4e (Fig. 4A) (6, 35, 41, 42). While a strain detected from raw sewage in May 2006 and the strains in July and August 2006 belonged to GII.4 strain 2006a (GII.4/2006a) and GII.4/Chiba-4e, respectively, most of the GII.4 strains from raw sewage belonged to the 2006b cluster. All GII.4 strains derived from patients with gastroenteritis and from healthy children also belonged to the 2006b cluster, except for two GII.4/2006a strains from outbreaks in May 2006 and one GII.4/Chiba-4e strain from a healthy child in September 2006 (Fig. 4B). In addition, the NoV GII.7, GII.13, and GI.8 strains detected from raw sewage formed a cluster with strains detected from healthy children or clinical cases. The identities of nucleotide sequences in 302 bases of the partial capsid regions among these strains were 96.4 to 97.4%, 98.0 to 99.3%, and 99.3 to 100%, respectively (Fig. 4A). Thus, the genotypes of NoVs detected from raw sewage showed a close relationship with those from human cases.

DISCUSSION

In this study, we compared NoVs and SaVs detected from raw sewage with those from human specimens. From 2006 to 2008, especially in winter, a large number of sporadic gastroenteritis cases and many outbreaks caused by NoV GII.4 occurred in Toyama, Japan (Fig. 3). NoV GII.4 was also predominantly detected from raw sewage in winter. In addition, the copy number of NoV GII in raw sewage of the winter season of 2006 to 2007 was higher than that of 2007 to 2008 (Fig. 2B), a result that correlates well with the prevalence of gastroenteritis and the number of outbreaks caused by NoV GII.4 infection. Clinical outbreaks preceded the high counts of NoV GII in raw sewage. Phylogenetic analysis showed that the nucleotide sequences of these NoV GII.4 strains were closely related to each other. Therefore, NoVs GII in raw sewage are thought to reflect mainly NoV GII.4 derived from clinical cases.

At least three clusters of NoV GII.4, 2006a, 2006b, and Chiba-4e, appeared to exist in Toyama Prefecture from 2006 to 2008. In Japan including Toyama, NoV GII.4/2006b has been dominantly prevalent since 2006, whereas a few NoV GII.4/2006a strains were detected from patients with gastroenteritis (20, 33). It is uncertain whether NoV GII.4/2006a and GII.4/Chiba-4e were locally extinct or persisted at low levels in Toyama Prefecture. Because NoV GII.4/2006a and GII.4/2006b epidemics had occurred in European countries beginning in December 2005 (22), these three clusters of NoV GII.4 might have migrated from Europe although migration routes have not been clarified.

NoV GI.8 and GII.13 were less frequently observed in outbreaks and were also detected from raw sewage, indicating that raw sewage contained minor genotypes of NoVs in the environment. Because GI.8 was still detected in sewage more than 1 year after the outbreak, GI.8 seemed to circulate over a long period of time in the community.

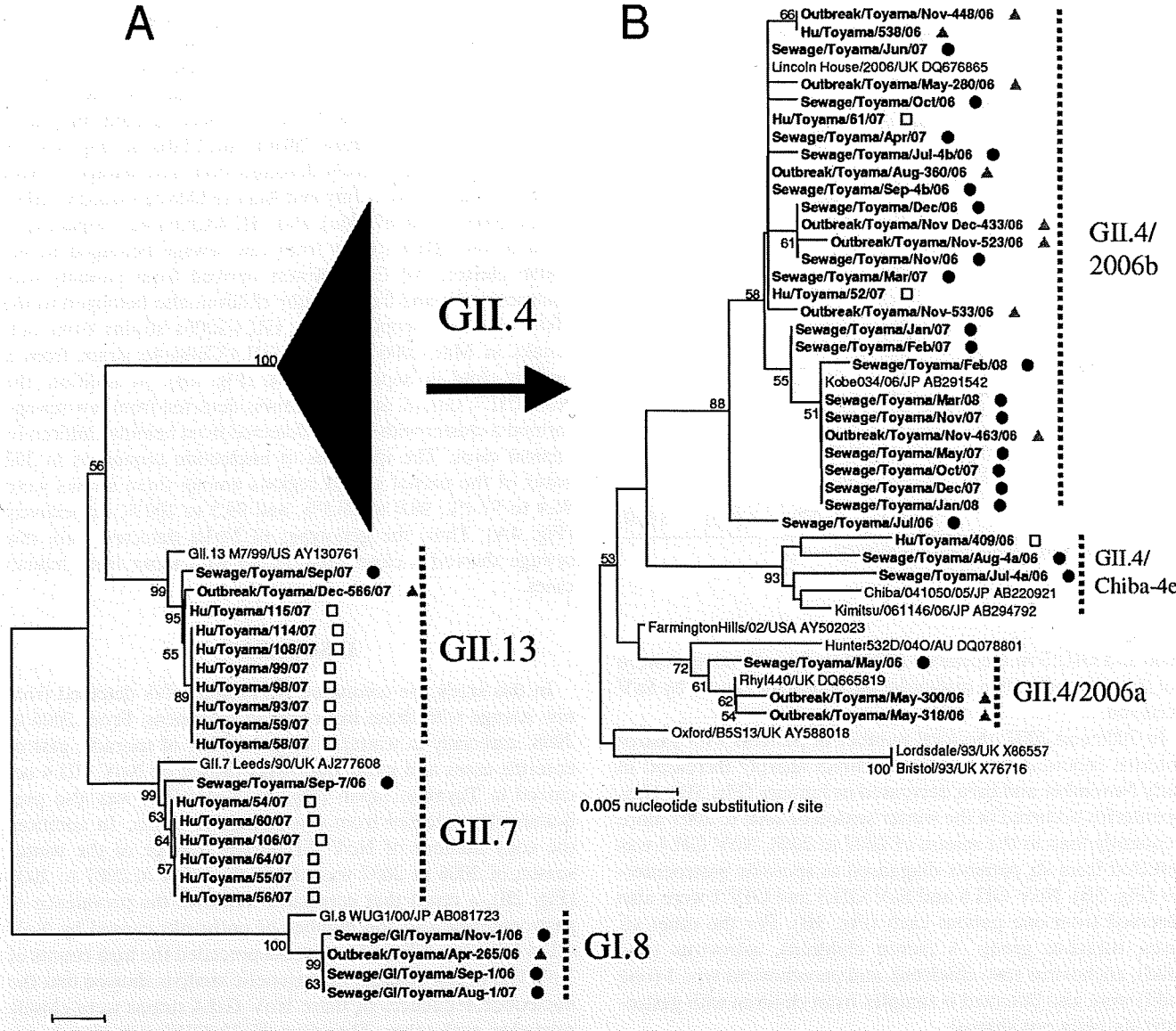

FIG. 4. Phylogenetic tree for NoV (GII.4, GII.7, GII.13, and GI.8) using about 300 nt from the 3′ end of ORF1 to the beginning of the capsid region; the tree was generated by the neighbor-joining method with Toyama strains derived from raw sewage and reference strains. (A) Phylogenetic tree of the strains of NoV GII.4, GII.7, GII.13, and GI.8. (B) Part of the phylogenetic tree for the strains of NoV GII.4 was magnified with a modification of branch indexes. Filled circles (●) and open squares (□) indicate the Toyama strains derived from raw sewage and asymptomatic healthy children, respectively. Filled triangles (▲) indicate the strains detected from the patients with gastroenteritis in hospitals or outbreaks from April 2006 to March 2008.

Various genotypes of NoV (GI.1, -2, -4, -5, -8, -11, -12, and -14; GII.3, -4, -6, -7, -10, -13, -14, and -15) and SaV (GI.1, GI.2, GII.1, and GIV.1) found in raw sewage are predicted to have originated from infected subjects. Among them, NoV GII.7, which was not detected in clinical cases, was found in raw sewage in September 2006 and in six stool specimens of healthy children in January 2007. Moreover, GII.7 and GII.13 were found more frequently than GII.4 in healthy children. These findings suggest that certain NoVs are shed from healthy children and that the population retains these viruses.

Although some other genotypes of NoV and SaV in raw sewage did not correlate with those from clinical cases, our findings suggest that they are also circulating in the environment throughout the year. NoV GI.4 was consistently detected in raw sewage but was not detected from fecal specimens of patients with gastroenteritis or from healthy children. The origin of NoV GI.4 remains to be clarified. Recent work by Okabayashi et al. showed that NoV GII.2, GII.3, GII.8, and GII.12 were detected from asymptomatic food handlers in 2005 and 2006 but not NoV GII.4, despite many outbreaks (34). Healthy adults may be infected with various genotypes of viruses that differ from the prevalent ones causing gastroenteritis. However, these viruses have the potential to be a source of an endemic or epidemic.

In a Mexican study by García et al. (7), nine different genotypes of NoV (GI.1, -3, -5, -7, and -14; GII.1, -2, -7, and -17) were detected in 48 out of 161 stool specimens (29.8%) from asymptomatic children under 2 years of age in June to August 1998. In an Indian study by Monica et al. (31), SaVs (GI.1, -2, and -3; GII.1 and -2) and NoVs (GI.3; GII.2, -3, and -4) were positive in 6 (3.5%) and 7 (4.0%) out of 173 asymptomatic children, respectively, under 3 years old living in an urban slum community in 2001 to 2004. On the other hand, a study in Australia by Marshall et al. (27) showed that NoV was not detected from 399 asymptomatic individuals aged between 5 months and 52 years in July to August 1997. Variation in the detection rate may depend on the differences in sanitation, such as the distribution of the sewage system, age groups of examinees, and methods of viral detection. Generally, improvement of waterworks or sewage facilities is necessary to prevent the transmission of enteric pathogens that infect humans by the fecal-oral route. However, even though the sewage facilities are widely maintained in the Toyama area (86.2% of the population was provided sewage facilities, and most of the rest treated wastewater individually, according to the Toyama Prefectural government in March 2006), the influence of the wastewater that bypasses the treatment system on viral prevalence could not be eliminated. Furthermore, Ueki et al. (44) reported that a few NoV genomes were also detected from treated wastewater of a sewage facility because of the difficulty to inactivate NoV thoroughly by present sewage treatments. The leakage of the NoVs from the sewage treatment system might be an additional cause of the viral prevalence. Continuous existence of NoVs is also probably due to their genetic and antigenic diversity that result from the high mutation rate (9, 25, 41). Another reason seems to be the physical stability of virions in the environment (3, 4, 19) and refractoriness against serum antibodies (2, 15, 38). Moreover, small numbers of NoV virions are reportedly able to establish infection in humans (3, 12), resulting in the easy expansion of viruses in the community. Thus, some genotypes of NoVs may infect healthy children, such as Mexican and Indian children, and outbreaks of gastroenteritis occur every year (7, 31). This study suggests that certain NoVs continuously exist in the community though certain NoVs can become locally extinct. Therefore, surveillance of circulating viruses in the inhabitants is necessary to control and prevent infection by NoVs and SaVs. The above concept correlates with our previous reports showing environmental surveillance of polioviruses and echoviruses that are mostly asymptomatic (14, 30). It is important to inform public health officials about the continuous existence of NoVs and SaVs in the community to prevent outbreaks among inhabitants.

In conclusion, NoVs and SaVs detected from raw sewage reflect their prevalence and circulation in the inhabitants, regardless of symptoms. A combination of the surveys of raw sewage with those of clinical cases helps us to understand the relationship between infection with these viruses and gastroenteritis.

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Short Communication

Detection of a Novel Recombinant Norovirus from Sewage Water in Toyama Prefecture, Japan

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SUMMARY: Recently, the recombination event of norovirus (NoV) has been reported with high frequency, suggesting that RNA recombination is a major driving force in NoV evolution. To assess the incidence of NoV recombination in a residential area, we conducted a molecular biological survey of NoVs existing in sewage water in Toyama Prefecture, Japan. Although GII/4 was predominantly detected in sewage water that was associated with a high frequency of outbreaks caused by this genotype, other genotypes, including two types of recombinant strain, were identified during the survey period. One of the recombinants is the WUG1 type, which was first detected in Saitama Prefecture in 2000. The other recombinant is a novel type derived from two parent strains of genogroup II, GII/7 for the RNA-dependent RNA polymerase and GII/13 for the capsid. This suggests that certain NoVs circulating in the area are occasionally changing their genetic properties by recombination events.

Norovirus (NoV), a member of the *Caliciviridae* family, is a major causative agent of acute gastroenteritis in human. Over the last decade, NoV-associated gastroenteritis has vigorously expanded worldwide and has been recognized as a public health problem (1,2). Recent developments in molecular biological techniques to detect the NoV genome contributed to an acquisition of large numbers of NoV strains (3-5). These NoVs are now classified into five genogroups (GI to GV) by molecular characterization based on the partial or complete capsid, or according to their RNA-dependent RNA polymerase (RdRp) sequences (3,6-8). GI, GII and GIV are known to infect humans (9). GII, the most prevalent causal genogroup in recent acute gastroenteritis, contains porcine strains as well as human strains. GIII and GV are composed of bovine and murine strains, respectively (5). GI and GII are further divided into many genotypes, and this classification reflects constant evolution with the discovery of new strains.

For most NoV strains, sequence information on the RdRp region obtained by diagnostic reverse transcription (RT)-PCR correlates with clustering based on the capsid sequence. However, several studies have shown that the clusters to which strains belong seem to depend on either the RdRp or capsid gene analyzed. This suggests the occurrence of recombination among NoV strains (4,6,10-13). A growing number of reports on NoV recombinant strains suggest that RNA recombination is a critical driving force in NoV evolution.

Raw sewage water is a suitable material for capturing NoVs circulating in the environment, because such water could contain enteric viruses shed by infected people. Especially, analyses of raw sewage show that they can detect NoVs shed by asymptomatic populations that have not exhibited clinically recognized NoV infections (14-16). In the present study, we conducted a molecular biological survey of NoVs existing in sewage water in Toyama Prefecture, Japan, to learn the incidence of NoV recombination in this area.

Raw sewage samples collected monthly from October 2006 to December 2007 were used in this examination. Raw sewage water was collected at a point of influx to a primary treatment tank at a sewage disposal facility in the western Toyama Prefecture. This facility covers an area having about 300,000 inhabitants. Two liters of collected raw sewage was centrifuged at 3,000 rpm for 30 min, and 1 liter of each supernatant was subsequently 100-fold concentrated to a 10-ml volume by the filter adsorption/elution method or polyethylene glycol (PEG) precipitation method, as described elsewhere (16-18). Viral RNA was extracted from 140 μl of concentrated raw sewage using a QIAamp viral RNA Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. Extracted RNA was treated with 5U of DNase I (Takara Bio Inc., Shiga, Japan), and cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen Japan K.K., Tokyo, Japan) with a random hexamer according to the manufacturer's instructions. The cDNA obtained was used for the following PCR. To screen the recombinant NoV, primers 1421f (5′-ATA CCACTATGATGCAGAYTA-3′), 1364f (5′-YTCYTTCTAT GGYGATGATGA-3′), G1SKR (5′-CCAACCCARCCAT TRTACA-3′), G2SKR (5′-CCRCCNGCATRHCCRTTRTA CAT-3′) and NV2oR (5′-GTRAACGCRTTYCCMGC-3′) (1,15,19) were chosen. 1421f and 1364f are positioned at nt 4279-4299 and 4585-4605 on the RdRp gene of the NoV GII Loadsdale strain (GenBank accession no. X86557), respectively. Our preliminary study confirmed the cross-reactivity of these primers with corresponding sites of NoV GI strains. G1SKR binds as a negative sense at nt 5653-5671 on the capsid gene of the NoV GI Norwalk virus (GenBank accession no. M87661). G2SKR and NV2oR bind as a negative sense at nt 5367-5389 and 5412-5428 in the capsid gene of the NoV GII Loadsdale strain, respectively. A primer pair of 1421f and G1SKR was used for the first PCR, and a pair of 1364f and G1SKR was used for semi-nested PCR to detect NoV GI strains. To detect NoV GII, the first round of PCR

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using a primer pair of 1421f and NV2oR, followed by nested PCR using a primer pair of 1364f and G2SKR, was performed. The obtained PCR products, composed of the 3′ end of RdRp and the 5′ part of capsid genes, were cloned into pGEM-T Easy Vector (Promega K.K., Tokyo, Japan) according to the manufacturer's instructions. Subsequently, nucleotide sequences of amplified fragments were determined. The nucleotide sequences of the 3′ end of the RdRp gene and the 5′ part of the capsid gene of the analyzed strains were separately applied for the construction of the phylogenetic trees to screen the recombinant type of NoV. Multiple sequence alignments of NoVs were generated by Clustal W, and the bootstrapped phylogenetic trees were constructed by the neighbor-joining method, with 1,000 bootstrap replicates in the Molecular Evolutionary Genetics Analysis version 3.1 (MEGA 3.1) software. The genetic distances among NoV strains were calculated by Kimura's 2-parameter method (20). The genotypes of NoV strains detected in this study were primarily determined according to the classification based on the partial capsid gene as previously proposed by Katayama et al. (4). Since a certain number of reference strains lacked sequence information for the RdRp gene, additional reference strains described in a recent report (13) were quoted to determine the genotypes based on the RdRp gene. The designation GIIb, GIIc and GIId were used to describe the genetic clusters composed of genetically diverse RdRp genes (13,21) in the phylogenetic tree constructed in this study.

We previously reported that NoVs could be detected in raw sewage water throughout the year (16). That report also indicated the existence of multiple NoV strains in samples collected at the same time. On the basis of our previous report, the present study adopted the molecular cloning strategy of the PCR-amplified NoV gene to distinguish multiple strains existing in the samples. As a result, GI/8 strains were detected from the samples collected in September 2006 and in January, March, May and August 2007 (shown as sewage/Toyama/SW0609-7/06/JP, sewage/Toyama/SW0701-2/07/JP, sewage/Toyama/SW0703-18/07/JP, sewage/Toyama/SW0705-9/07/JP and sewage/Toyama/SW0708-1/07/JP in Fig. 1, respectively) besides the GI/4 strains previously identified (16). Further genetic analysis indicated that these GI/8 strains were a WUG1-type recombinant, which was first detected in Saitama Prefecture in 2000, and composed of the GI/8 capsid gene and the GI/2 RdRp gene (Fig. 1) (4). This WUG1-type recombinant has also been detected in Hiroshima Prefecture as a cause of the outbreak (22). Our study revealed that the WUG1-type recombinant has existed in Toyama Prefecture. These findings suggest that the genetic combination of the GI/2 RdRp and GI/8 capsid genes was stable in the environment and widely prevalent in Japan.

One strain forming a new branch and two strains clustering with the GI/7 Winchester strain were also detected in February and March 2007 (shown as sewage/Toyama/SW0702-1/07/JP, sewage/Toyama/SW0702-11/07/JP and sewage/Toyama/SW0703-10/07/JP in Fig. 1, respectively). These strains probably possessed the indigenous RdRp gene, although this gene was not clearly identified because of insufficient reference data concerning the RdRp gene of NoV GI (Fig. 1).

As for the GII strains, GII/4 was dominantly detected. This seemed to correlate with the high frequency of outbreaks caused by this genotype. GII/6 strains were also detected in January and May 2007 (shown as sewage/Toyama/SW0701-35/07/JP and sewage/Toyama/SW0705-2/07/JP in Fig. 2, respectively). These strains were not recombinant, since the clustering based on the RdRp gene was concordant with the capsid sequences (Fig. 2). Notably, GII/13 strains were detected in March and September 2007 (shown as sewage/Toyama/SW0703-6/07/JP and sewage/Toyama/SW0709-11/07/JP in Fig. 2, respectively). Construction of the phylogenetic tree based on the capsid gene revealed that both strains belonged to the same cluster. However, another phylogenetic

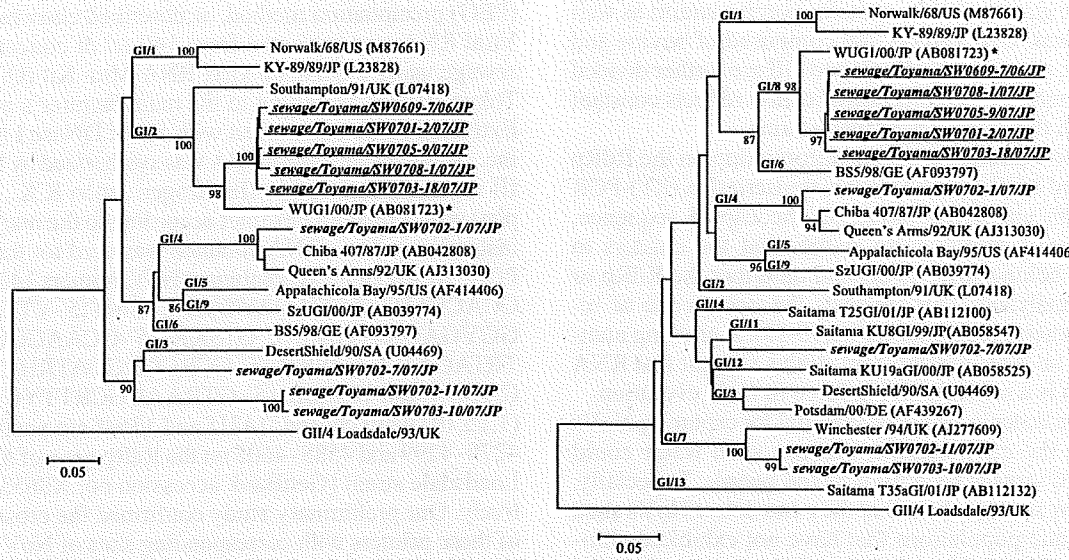

Fig. 1. Phylogenetic analysis of NoV GI strains. The left tree is reconstructed for the 3′ end of the RdRp region. The right tree is reconstructed for the 5′ part of the capsid region. The DDBJ accession numbers of each reference strain are shown in the parentheses. Asterisk indicates a recombinant strain previously reported (4). More than 80% of bootstrap values are shown on the branches. Newly sequenced strains are shown as an italic face, and suspected recombinant strains are underlined. The nucleotide sequence data of sewage/Toyama/SW0609-7/06/JP, sewage/Toyama/SW0701-2/07/JP, sewage/Toyama/SW0702-1/07/JP, sewage/Toyama/SW0702-7/07/JP, sewage/Toyama/SW0702-11/07/JP, sewage/Toyama/SW0703-10/07/JP, sewage/Toyama/SW0703-18/07/JP, sewage/Toyama/SW0705-9/07/JP and sewage/Toyama/SW0708-1/07/JP are deposited in the DDBJ database under accession numbers of AB504688, AB504692, AB504695, AB504697, AB504698, AB504701, AB504702, AB504705 and AB504708, respectively.

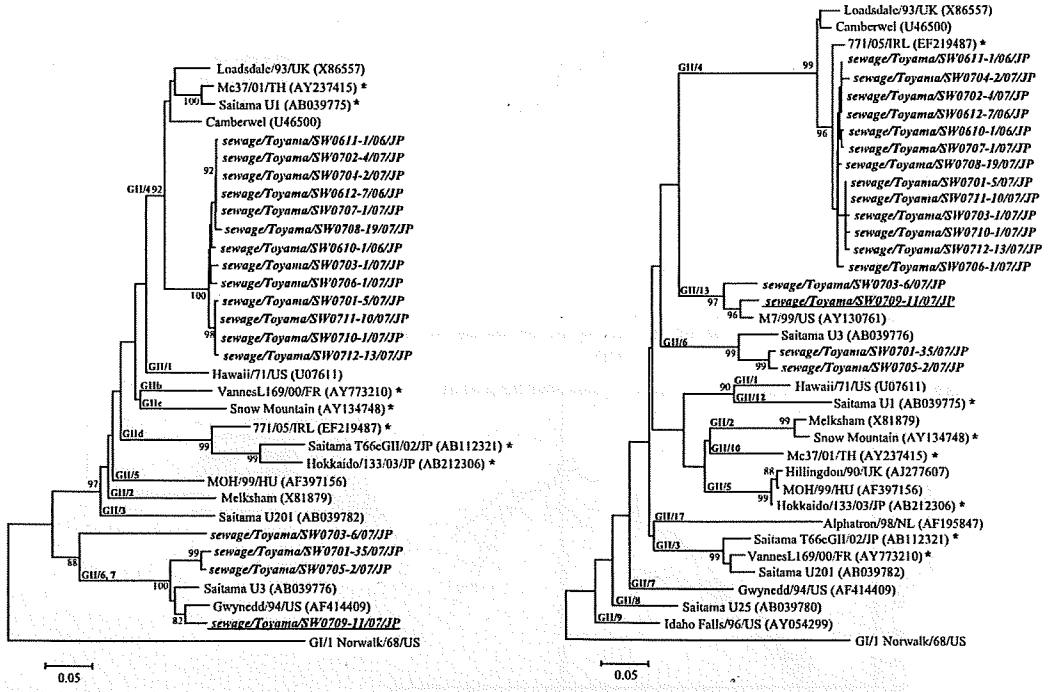

Fig. 2. Phylogenetic analysis of NoV GII strains. The left tree is reconstructed for the 3′ end of the RdRp region. The right tree is reconstructed for the 5′ part of the capsid region. The DDBJ accession numbers of each reference strain are shown in the parentheses. Asterisk indicates a recombinant strain previously reported (13). More than 80% of bootstrap values are shown on the branches. Newly sequenced strains are shown as an italic face, and suspected recombinant strains are underlined. The nucleotide sequence data of sewage/Toyama/SW0610-1/06/JP, sewage/Toyama/SW0611-1/06/JP, sewage/Toyama/SW0612-7/06/JP, sewage/Toyama/SW0701-5/07/JP, sewage/Toyama/SW0701-35/07/JP, sewage/Toyama/SW0702-4/07/JP, sewage/Toyama/SW0703-1/07/JP, sewage/Toyama/SW0703-6/07/JP, sewage/Toyama/SW0704-2/07/JP, sewage/Toyama/SW0705-2/07/JP, sewage/Toyama/SW0706-1/07/JP, sewage/Toyama/SW0707-1/07/JP, sewage/Toyama/SW0708-19/07/JP, sewage/Toyama/SW0709-11/07/JP, sewage/Toyama/SW0710-1/07/JP, sewage/Toyama/SW0711-10/07/JP and sewage/Toyama/SW0712-13/07/JP are deposited in the DDBJ database under accession numbers of AB504689, AB504690, AB504691, AB504693, AB504694, AB504696, AB504699, AB504700, AB504703, AB504704, AB504706, AB504707, AB504709, AB504710, AB504711, AB504712 and AB504713, respectively.

tree, based on their RdRp gene, showed that sewage/Toyama/SW0709-11/07/JP formed a cluster with the GII/7 Gwynedd strain, whereas sewage/Toyama/SW0703-6/07/JP branched outside of the cluster containing GII/6 and GII/7 strains (Fig. 2). SimPlot (ver. 3.5.1, provided by SCRoftware website) (23) analysis showed more than 90% similarity between sewage/Toyama/SW0709-11/07/JP and the GII/7 Gwynedd strain located at the 3′ end of the RdRp gene, whereas the similarity was drastically reduced rearward of the junction site between the *orf1* and *orf2* genes, which had been reported as the most common breakpoint among recombinant NoVs (24) (Fig. 3A). In addition, the similarity between the sewage/Toyama/SW0709-11/07/JP and GII/13 M7/99/US strains at the 5′ part of the capsid gene was more than 95%. These results indicate that sewage/Toyama/SW0709-11/07/JP was the recombinant type derived from the GII/7 ancestor for the RdRp gene and the GII/13 ancestor for the capsid gene. The derivation of sewage/Toyama/SW0703-6/07/JP RdRp gene remains obscure because referential sequence information for the GII/13 RdRp gene is not available. However, the sewage/Toyama/SW0703-6/07/JP RdRp gene has low similarity to that of the GII/6 and GII/7 strains, in contrast with the sewage/Toyama/SW0709-11/07/JP RdRp gene (Figs. 3A and B). The pairwise distance between the GII/6 and sewage/Toyama/SW0703-6/07/JP RdRp genes (0.268 - 0.280) was correlated to that of capsid genes (0.248 - 0.261). Therefore, sewage/Toyama/SW0703-6/07/JP seemed to possess an indigenous GII/13 RdRp gene that is thought to be distant from the GII/6 RdRp gene.

Our previous report revealed that healthy infants frequently harbor the GII/7 and GII/13 strains without apparent clinical symptoms, suggesting the virulence of these strains was relatively mild (16). These harbored viruses could be parental strains of the novel recombinant type NoV reported here; such a recombination event potentially influenced their pathogenicity. Indeed, in the winter of 2007 we experienced an outbreak caused by this novel recombinant type NoV at a nursery school (our laboratory's investigation). Various NoVs including GII/7 have been frequently detected from asymptomatic children in Mexico (25). Moreover, a recombinant strain showing a genetic combination similar to that of sewage/Toyama/SW0709-11/07/JP was detected from a hospitalized child with acute gastroenteritis in China (26). These facts suggest that infants and young children are a major reservoir for the genetic and biological diversification of certain NoVs.

Recent epidemiological surveys have revealed that outbreaks associated with recombinant NoVs have sometimes occurred (22,27,28). Recombinant strains having characteristic RdRp have been noted as a cause of outbreaks (27,28). GIIb RdRp, which is genetically distinct from conventional RdRp genes (21), has been shown to recombine with a variety of capsid genes (27). It is noteworthy that different recombinant strains having a common RdRp gene resulted in significant numbers of outbreaks. This fact suggests that a certain type of RdRp may play crucial roles in NoV pathogenicity. Epidemiological and comparative studies of the recombinant NoV strains retrospectively and currently identified may elucidate the significance of genetic recombination.

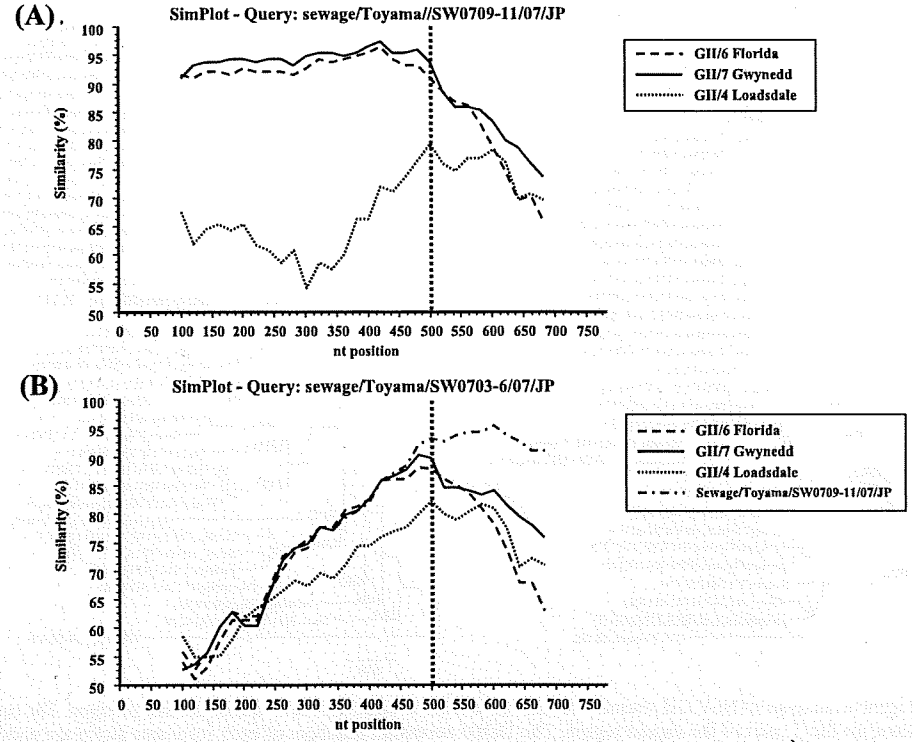

Fig. 3. SimPlot analysis for partial RdRp and capsid sequences. The window size was 100 bp with a step size of 10 bp. The query sequences are (A) sewage/Toyama/SW0709-11/07/JP, (B) sewage/Toyama/SW0703-6/07/JP, respectively. The vertical axis indicates the nucleotide identities between the query sequence and reference strains, expressed as percentages. The horizontal axis indicates the nucleotide positions of analyzed fragment. Vertical lines indicate the beginning of the *orf2* gene. Dashed line, GII/6 Frolida; bold line, GII/7 Gwynedd; dotted line, GII/4 (a representative genotype observed in clinical cases) Loadsdale; broken line, GII/13 recombinant sewage/Toyama/SW0709-11/07/JP.

Recently, a recombinant strain derived from different genogroups, GI/3 for the RdRp gene and GII/4 for the capsid gene, were detected from a patient with an acute watery diarrhea in India (29). This finding indicates the potential for further diversification of NoVs by the intergenogroup recombination event. Our study also aimed to examine whether or not intergenogroup recombinants exist in this area. The PCR performed in this study was designed to detect intergenogroup recombinant strains by the cross-binding ability of primers 1421f and 1364f on both GI and GII RdRp genes. Although no evidence of intergenogroup recombination events in this area has been observed, our study does not exclude the possibility that the intergenogroup recombination will occur, since the co-circulation of GI and GII in the environment was revealed by the simultaneous detection of both genogroups from sewage water.

In conclusion, this study detected recombinant NoVs from sewage water, although at a low frequency than that of GII/4, which was a major cause of outbreaks. Certain NoVs were circulating in the area without clinical detection and occasionally changing their genetic properties by recombination events. The molecular cloning strategy adopted in this study encouraged the detection of multiple NoV strains from samples collected at the same time. Genetic analysis of the longer fragment, including the 3′ end of the RdRp gene and the 5′ part of the capsid gene, enabled us to find a recombinant type of NoV. Continuous surveillance with a view toward genetic recombination will promote a precise understanding of the molecular evolution and pathogenicity of NoVs.

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Humoral Immune Responses Against Norovirus Infections of Children

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In 2 infants with gastroenteritis associated with Norovirus (NoV), serum immunoglobulin (Ig) G, IgM, IgA, and fecal IgA antibody responses against NoV were examined by enzyme-linked immunosorbent assay using 11 different antigenic and genetic types of NoV virus-like particles expressed in insect cells. These two cases were putative primary single NoV infections, because antibodies against NoVs were not detected in acute-phase serums. In one of two cases, long-term excretion of virus RNA for 33 days was observed. Serum IgG responses demonstrated strong seroresponse to the homologous type, and weak seroresponse to the heterologous types within the genogroup. After more than 2 years, the IgG antibody titer remained high to the homologous type and low to the heterologous type within the genogroup. IgM and IgA were specific to the homologous type. IgM was short lived and the serum IgA antibody titer remained low to the homologous type for a long period. These results improve our understanding of the humoral immune response to NoV infection.
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KEY WORDS: norovirus; primary infection; humoral immune response; ELISA; virus-like particle

INTRODUCTION

Noroviruses (NoVs) in the family of *Calicviridae* are the major cause of acute nonbacterial gastroenteritis in all age groups, and NoV infections have occurred worldwide as outbreaks and sporadic cases [Green et al., 2001].

NoVs are a genetically and antigenically diverse group. Genetic analysis of the RNA polymerase and capsid region revealed that human NoVs can be divided into two genogroups, genogroup I (GI) and genogroup II (GII) [Green et al., 1994; Wang et al., 1994]. Recent studies of genotyping on the basis of capsid *N*-terminal/shell (N/S) domain classified NoVs into at least 31 genotypes (14 genotypes in GI and 17 genotypes in GII) [Katayama et al., 2002; Kageyama et al., 2004]. These viruses cannot be grown in cell culture, but the expression of the major capsid protein (VP1) in insect cells resulted in the formation of virus-like particles (VLPs) that are morphologically and antigenically similar to native NoV [Jiang et al., 1992; Lew et al., 1994]. Antigenic analysis using enzyme-linked immunosorbent assay (ELISA) with VLPs and hyperimmune antisera showed that the genetic and antigenic relationship corresponded well [Kobayashi et al., 2000a,b,c; Katayama et al., 2002; Kamata et al., 2005; Hansman et al., 2006], but there were unusual cross-reactivities between certain genogroups and/or genotypes based on the antibody ELISA [Hansman et al., 2006]. Serological studies using these recombinant VLP (rVLPs) have shown a high prevalence and broad responses of NoV-specific antibodies both in children and adults [Parker et al., 1994a, 1995; Noel et al., 1997; Farkas et al., 2003]. IgM, IgA, and IgG serologic responses in adult volunteers and patients also reported [Treanor et al., 1993;

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Gray et al., 1994; Rockx et al., 2005a]. But almost all sera used in previous studies had pre-existing antibodies against many types of NoV. Therefore, it was not clear whether the production of cross-reactivity antibodies emerged after primary infection or by multiple infections during their lifetime. In addition, there is little information about the immune response to NoV infections in infants, including primary infection. To interpret the cross-reactivity of antibodies to NoVs, it is necessary to investigate the immune response to primary NoV infection.

In the present study, serum and fecal samples from infants infected with NoV were examined for specific IgM, IgA, and IgG using ELISA with 11 different genotypes of VLPs. This study describes the cross-reactivity and specific antibody responses in putative primary NoV infection.

MATERIALS AND METHODS

Patients

Fecal or serum samples were collected from 2 infants suffering from nonbacterial gastroenteritis at their medical examination and follow-up in the hospital. After the ethical discussion in this working group, we concluded that this study was ethically acceptable. Informed consent for this study was obtained from their parents. The histo-blood group antigen (HBGA) type of the two infants was unknown. Their development was normal. After recovery from diarrhea, their prognosis was favorable. These two cases were sporadic and not related to each other.

Patient A (male): He had acute gastroenteritis at 2 years in December. The major symptoms were diarrhea and vomiting. The diarrhea was sometimes accompanied by benign afebrile convulsions. He had mild diarrhea for about 6 weeks. During the diarrhea, five fecal and serum samples on the 5th, 12th, 18th, 33rd, and 40th days post-onset were collected. After recovery from diarrhea, one fecal and one serum sample on the 60th day post-onset, and one serum sample at 2 years and 10 months post-onset were collected. Re-infection of NoVs during this long period was unknown.

Patient B (male): He had acute gastroenteritis at 1 year in January. The major symptom was diarrhea. The diarrhea was sometimes accompanied by benign afebrile convulsions. He had mild diarrhea for about 1 week. During the diarrhea, one fecal and one serum sample on the 2nd day post-onset were collected. After recovery from diarrhea, one serum sample on the 105th day post-onset was collected. He did not have gastroenteritis again during the period studied.

Detection of NoVs and Other Viruses in Fecal Specimens

NoVs were detected using RT-PCR with primer pairs, Ando's G1 (SR33, SR48, SR50, and SR52) and G2 (SR33 and SR46) primer sets [Ando et al., 1995] amplifying a 123-bp RNA polymerase region, mon381/mon383 [Noel et al., 1997] amplifying a 322-bp capsid region, and SK primers [Kojima et al., 2002] amplifying a 344-bp capsid N/S region as previously described [Iritani et al., 2000; Seto et al., 2005]. Other gastroenteritis viruses were detected with our laboratory method [Iritani et al., 2003]. Briefly, antigens of group A rotavirus and enteric adenoviruses (serotypes 40 and 41) were tested using commercially available antigen ELISA kits, ROTACLONE, and ADENOCLONE-E, respectively, according to the instructions (Meridian Bioscience, Inc., Cincinnati, OH). Enteroviruses and adenoviruses were tested using cell cultures with Vero and RD-18S cells. The virus-negative samples for group A rotavirus, adenoviruses, enteroviruses, and NoVs were tested using electron microscopy (EM) [Iritani et al., 2000], to directly detect virus particles with a negative stain.

Genetic Analysis of NoVs

Sequencing of RT-PCR products and phylogenetic analysis were performed as previously described [Iritani et al., 2000; Seto et al., 2005]. Genotyping based on the Capsid N/S domain was performed as described by Katayama et al. [2002] and Kageyama et al. [2004].

Expression of VLPs

Eleven VLPs (four genotypes in GI and seven genotypes in GII), expressed in insect cells infected with recombinant baculoviruses carrying the capsid gene, were used for ELISA (Table I). These VLPs were produced in the Department of Virology II, National Institute of Infectious Disease. The expressed capsid antigens were purified by a sucrose gradient followed by CsCl gradient centrifugation and confirmed by EM, as previously described [Kobayashi et al., 2000a,b,c; Tamura et al., 2000; Kamata et al., 2005].

Antibody ELISA for Serum Samples

The wells of 96-well flat-bottom microtiter plates (IMMULON2 HB, Dynex Technologies, Inc., Chantilly, VA) were coated with 100 μl of each VLP (0.5 μg/ml in 0.05 M carbonate–bicarbonate buffer (pH 9.6)), and incubated at 4°C overnight. The wells were then washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T; pH 7.2) using a micro plate washer (S8/12J model, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and were blocked at room temperature for 1 hr with 200 μl of Block Ace (Dainippon Pharmaceutical Co., Ltd.). The wells were washed twice and twofold serial dilutions in PBS-T containing 25% Block Ace (25% BA/PBS-T) of serum samples, starting at a 1:50 dilution, were added to antigen-coated plates. After incubation for 1 hr at 37°C, the wells were washed five times, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (heavy and light chains), IgM (μ-chain), or IgA (α-chain) -specific antibodies (Zymed Laboratories, Inc., South San Francisco, CA) at a dilution of 1:4,000 in 25% BA/PBS-T were added, and the plates were incubated for 1 hr at 37°C. After washing the wells five times, 100 μl of substrate, 0.4 mg/ml of *O*-phenylenediamine (Sigma Chemical Co.

TABLE I. Description of 11 VLPs Used in This Study

Genogroup	Genotype	VLP	Strain	Accession no.	References
GI	GI/1	rSeto	Aichi124/1989/JP (Seto)	AB031013	Kobayashi et al. [2000b]
	GI/2	rFUV	Funabashi 258/1996/JP	AB078335	Tamura et al. [2000]
	GI/3	r645	Kashiwa645/1999/JP	BD011871	Kamata et al. [2005]
	GI/4	rChiba	Chiba407/1987/JP	AB022679	Kobayashi et al. [2000a]
GII	GII/3	r809	Sanbu809/1998/JP	BD011876	Kamata et al. [2005]
	GII/4	rNAV	Narita104/1997/JP	AB078336	Kitamoto et al. [2002]
	GII/5	r745	Ichikawa745/1998/JP	BD011877	Kamata et al. [2005]
	GII/6	rUEV	Ueno7k/1994/JP	AB078337	Tamura et al. [2000]
	GII/7	r10–25	Osaka10-25/1999/JP	BD011881	Kamata et al. [2005]
	GII/12	rChitta	Chitta76/1996/JP	AB032758	Kobayashi et al. [2000c]
	GII/14	rKAV	Kashiwa47/2000/JP	AB078334	Kitamoto et al. [2002]

Ltd., St. Louis, MO) was added and the plates were incubated for 30 min at room temperature. The reaction was stopped with 50 μl of 4N H_2SO_4. Absorbance at 492 nm (A492) was measured with a microplate reader (Multickan MS-UV model, Labsystems OY, Helsinki, Finland). IgG, IgM, or IgA titers were defined as the highest dilution of serum given an A492 that was threefold higher than the A492 of the corresponding antigen control well.

Antibody ELISA for Fecal IgA

Five fecal samples on the 5th, 12th, 18th, 40th, and 60th days from Patient A were used for ELISA to detect VLP-specific IgA. There was not sufficient volume of the other samples for ELISA. Fecal samples were prepared as a 10% (wt/vol) suspension in PBS containing 10% fetal bovine serum (FBS). Each fecal suspension was homogenized, and centrifuged at 12,000*g* for 10 min. The supernatant was used for ELISA to detect VLP-specific IgA.

The plates were coated with VLPs as described above. Twofold serial dilutions of fecal suspensions were made in PBS containing 10% FBS. The diluted suspensions were added to antigen-coated plates; thereafter, the ELISA protocol was performed as described above. Virus-negative fecal samples were included on each plate as a negative control. The sample was considered positive for VLP-specific IgA when the absorbance of the well containing fecal samples from patient A was threefold higher than that of the negative control well.

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study were deposited in DDBJ with the following accession numbers: AB089871, OCS980730 (patient A); AB262773, OCS000564 (patient B).

RESULTS

Detection of the Viruses in Stool Specimens From Two Infants With Gastroenteritis

NoVs were detected in two infants by RT-PCR (Table II). In patient A, who had mild diarrhea for about 6 weeks, four fecal samples on the 5th, 12th, 18th, and 33rd day were NoV-positive, although the sample on the 33rd day produced a thin band (data not shown). Those four RT-PCR products had identical nucleotide sequences. In patients B, who had mild diarrhea, fecal samples on the 2nd were NoV-positive, respectively. These stool specimens were negative to other etiological agents of gastroenteritis. NoV strains from the two infants were classified as GII/4 genotype in the capsid N/S region (Fig. 1). These two NoV strains were closely related, with 100% amino acid identity and 98.8% nucleotide identity in the RNA polymerase region, and 100% amino acid identity and 98.5% nucleotide identities in the capsid region. Pairwise comparison of the capsid N/S region showed that these two NoV strains had 100% amino acid identity and 98.9% nucleotide identities to NAV (GII/4), 73.4–78.7% amino acid identities to the other 6 GII VLP strains, and 60.2% amino acid identities to the 4 GI VLP strains.

Detection of Specific Antibodies to 11 VLPs in the Two Infants

The specific IgG antibody titer to the 11 VLPs is shown in Table II. In two acute-phase serum samples from patient A on the 5th day and patient B on the 2nd day, none of the specific IgG antibodies to all VLPs were detected (<1:50). IgG antibodies to rNAV in patient A showed that the first detection was the 12th day post-onset, and the highest titer was observed on the 33rd to 60th day (1:25,600). IgG antibodies to other five GII VLPs excluding r809 appeared from the 18th to 40th day post-onset and their appearance had a time lag. In a serum sample from patient A at 2 years and 10 months, high IgG titers (1:6,400) to rNAV have persisted, and those IgG titers to the other six GII VLPs were the same or higher than other convalescent-phase serum. In patient B, IgG antibodies to rNAV and other six GII VLPs including r809 were detected with high titer (1:51,200) and low (1:100 – 1:200), respectively. There were no specific IgG antibodies to the four GI VLPs in any serum samples.

Specific IgM antibodies to rNAV were detected in four serum samples on the 12th, 18th, 33rd, and 40th days from patient A (Table III). Two serum samples on the 12th and 18th days had a high titer (1:1,600), and later

TABLE II. Detection of IgG to the 11 Kinds of VLPs in Serum Samples Collected From Infantile Patients by ELISA

Patient (age)	Time post-onset of illness	Symptoms[a]	RT-PCR[b]	Reciprocal of serum dilution										
				Genogroup I				Genogroup II						
				rSeto (GI/1)	rFUV (GI/2)	r645 (GI/3)	rChiba (GI/4)	r809 (GII/3)	rNAV (GII/4)	r745 (GII/5)	rUEV (GII/6)	r10-25 (GII/7)	rChitta (GII/12)	rKAV (GII14)
A (2 years)	5th day	D, V, AC	+	<50	<50	<50	<50	<50	**<50**	<50	<50	<50	<50	<50
	12th day	D	+	<50	<50	<50	<50	<50	**800**	<50	<50	<50	<50	<50
	18th day	D, AC	+	<50	<50	<50	<50	<50	**12,800**	50	<50	50	<50	<50
	33rd day	D, AC	+w	<50	<50	<50	<50	<50	**25,600**	200	<50	100	50	100
	40th day	D, AC	−	<50	<50	<50	<50	<50	**25,600**	200	50	100	50	100
	60th day	Recovered	−	<50	<50	<50	<50	<50	**25,600**	200	50	100	100	100
	2 years 10 months	Normal	NT	<50	<50	<50	<50	400	**6,400**	400	200	200	200	400
B (1 year)	2nd day	D, AC	+	<50	<50	<50	<50	<50	**<50**	<50	<50	<50	<50	<50
	105th day	Recovered	NT	<50	<50	<50	<50	100	**51,200**	100	100	200	100	100

Bold: homologous type.
[a]D, Diarrhea; V, vomiting; AC, afebrile convulsions.
[b]+, Positive; +w, positive but weak band; −, negative; NT, not tested.

samples had a reduction in the IgM titer. There were no specific IgM antibodies to rNAV in serum samples from patient B. IgM antibodies to the other 10 VLPs were not detected in any serum samples.

Specific IgA antibodies to rNAV were detected in all serum samples excluding two acute-phase sera on the 5th day of patient A and the 2nd day of patient B (Table III). For patient A, three serum samples on the 18th, 33rd, and 40th days had a high IgA antibody titer (1:800–1:1,600) and a serum sample at 2 years and 10 months had a low titer (1:100) to rNAV. IgA antibodies to the other 10 VLPs were not detected in any serum samples excluding a serum sample from patient A at 2 years and 10 months to r809 (Table III).

In the five fecal samples of patient A, specific IgA antibodies to rNAV were detected in three samples collected after the 18th day post-onset (Table III). IgA antibodies to the other 10 VLPs were not detected in any fecal samples.

DISCUSSION

From the detection of NoV-specific antibodies in the two patient sera, it appeared that these two patients were putative primary single infections of NoV because antibodies against NoVs were not detected in acute-phase sera. These two cases showed three distinct features compared to the usual NoV-associated gastroenteritis; obstinate mild diarrhea for 1 and 6 weeks, long-term excretion of virus RNAs from patient A for 33 days, and benign afebrile convulsions. The detection of NoVs in patient A indicated that his diarrhea was related to NoV infection up to 33 days, but the direct relation between diarrhea and NoV infection is then unclear, because NoV was not detected from fecal samples of diarrhea on the 40th day post-onset. In a recent study, Rockx et al. [2002] showed that children under 1 year had a tendency toward long-term duration of diarrhea up to the 28th day and excretion of the virus gene up to the 22nd day. Some reports also showed the long-term duration of diarrhea in NoV infections in patients under 2 years [Sakai et al., 2001; Tsugawa et al., 2006]. Our previous study showed that benign afebrile convulsion-associated diarrhea in NoV infections was observed in 6.7% of children under 2 years [Iritani et al., 2003]. Primary NoV infection or age under 2 years may be related to those three distinct features, persistent diarrhea, viral excretion, and afebrile convulsion. Therefore, these two cases in this study were considered general infection cases with NoVs.

Our data showed that the kinetics of serum antibody responses for NoV infection as follows, the peak of IgM in 5th to 12th day, decrease in 18th to 33rd day, and disappearance in 40th to 60th day; the peak of IgA in 12th to 18th day, decrease in 40th to 60th day, and persistence for a long period with low titer; the peak of IgG in >18th day, and persistence for a long period with high titer. This kinetics of serum antibody responses is similar to those seen in previous studies [Erdman et al., 1989; Gray et al., 1994; Brinker et al., 1998, 1999]. Fecal

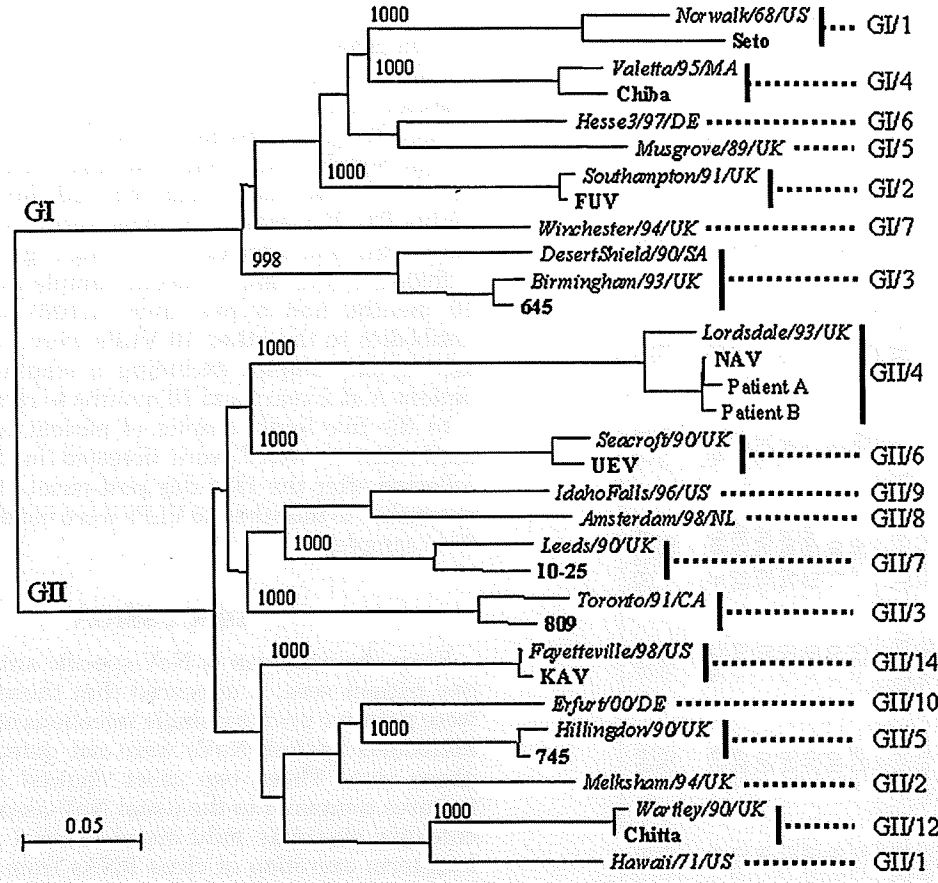

Fig. 1. Phylogenetic tree based on the capsid N/S domain region (GI, 294 nt; GII, 282 nt) constructed using neighbor-joining method. NoV strains for VLP and reference strains are represented in boldface and italics, respectively. Bar indicates the number of substitutions per site. The numbers on each branch indicate the bootstrap value of clusters including NoV strains for VLP. GenBank accession numbers for reference strains used in this analysis are as follows: Amsterdam/98/NL, AF195848; Birmingham/93/UK, AJ277612; DesertShield/90/SA, U04469; Erfurt/00/DE, AF427118; Fayetteville/98/US, AY113106; Hawaii/71/US, U07611; Hesse3/97/DE, AF093797; Hillingdon/90/UK, AJ277607; IdahoFalls/96/US, AY054299; Leeds/90/UK, AJ277608; Lordsdale/93/UK, X86557; Melksham/94/UK, X81879; Musgrove/89/UK, AJ277614; Norwalk/68/US, M87661; Seacroft/90/UK, AJ277620; Southampton/91/UK, L07418; Toronto/91/CA, U02030; Valetta/95/MA, AJ277616; Wartley/90/UK, AJ277618; Winchester/94/UK, AJ277809.

IgA antibodies specific to rNAV were detected from the 18th day post-onset. This specific fecal IgA seems to be followed by the reduction of PCR product or virus excretion, suggesting that fecal IgA was effective in the excretion of NoV.

The cross-reactivity of IgG in this study showed lower reaction to the heterologous type within the genogroup. Many serological studies in adults or children also demonstrated cross-reactivity within and between the genogroups [Treanor et al., 1993; Noel et al., 1997; Hale et al., 1998; Smit et al., 1999; Farkas et al., 2003; Rockx et al., 2005a]. Some reports showed that cross-reactivity was stronger or limited to the genogroups [Noel et al., 1997; Hale et al., 1998; Farkas et al., 2003]. In antibody ELISA using VLPs and their hyperimmune serums, low levels of cross-reaction were observed [Kamata et al., 2005; Hansman et al., 2006]. These findings indicated that NoV strains had a common epitope to stimulate the production of IgG. The common epitopes within and between the genogroups were previously described [Kitamoto et al., 2002; Yoda et al., 2003; Parker et al., 2005]. Yoda et al. [2003] described that the common epitope between genogroups may be located in the *N*-terminus of capsid protein. This region is highly conserved and located in the inner part of capsid protein surrounding the RNA genome [Prasad et al., 1999]. From the above results, cross-reactivity between genogroups may be difficult to produce.

As described in previous reports [Parker et al., 1994a, 1995; Smit et al., 1999], antibodies to NoVs were acquired at a young age and had cross-reactivity; therefore, it is unclear whether cross-reactive antibodies occurred after single infection, or after multiple infections with different genotypes of NoV. Our study demonstrated that cross-reactive antibodies to heterologous types were produced after primary single infection. We could observe that the production of IgG to the heterologous type was later and lower than the homologous type, and had a time lag in patient A. The time lag of IgG production may be related to antigenic differences among these GII NoV strains. The seroconverted against r809 of IgG and IgA in a serum of patient A at 2 years and 10 months are uncertain because the serum was obtained too long after the last serum sample was negative.

TABLE III. Detection of Serum IgM, IgA, and Fecal IgA to the 11 VLPs in Infantile Patients by ELISA

Patient (age)	Time post-onset of illness	RT-PCR[a]	Reciprocal of serum dilution				Existence of specific fecal IgA[a]	
			IgM		IgA			
			rNAV (GII/4)	Other VLPs	r809 (GII/3)	rNAV (GII/4)	Other VLPs	rNAV (GII/4)	Other VLPs
A (2 years)	5th day	+	**<50**	<50	<50	**<50**	<50	−	−
	12th day	+	**1,600**	<50	<50	**50**	<50	−	−
	18th day	+	**1,600**	<50	<50	**1,600**	<50	+	−
	33rd day	+w	**200**	<50	<50	**800**	<50	**NT**	NT
	40th day	−	**200**	<50	<50	**1,600**	<50	+	−
	60th day	−	**<50**	<50	<50	**50**	<50	+	−
	2 years 10 months	NT	**<50**	<50	50	**100**	<50	**NT**	NT
B (1 year)	2nd day	+	**<50**	<50	<50	**<50**	<50	**NT**	NT
	105th day	NT	**<50**	<50	<50	**400**	<50	**NT**	NT

Bold: homologous type.
[a]+, Positive; +w, positive but weak band; −, negative; NT, not tested.

A serum sample from patient A at 2 years and 10 months had IgG antibodies to the homologous type with a high titer and the heterologous type within the genogroup with a low titer. The existence of IgG antibodies to VLPs may have been maintained without re-infection or emerged from re-infection. Patient A might have been re-infected with GII/4 NoV or other GII NoV excluding these seven genotypes in GII, because the IgG titer to the heterologous type was ≥twofold higher and IgM responses were not observed. The data of IgG analysis suggested that the cross-reactivity of IgG is produced in the primary infection and then the level of IgG against NoVs will be raised each time NoV infection occurs throughout life.

IgM and IgA antibody responses were specific to the homologous genotype. Previous studies using adult sera also suggested that serum IgM and IgA antibodies might be more specific for the homologous type than the heterologous type [Parker and Cubitt, 1994b; Brinker et al., 1998, 1999; Hale et al., 1998; Rockx et al., 2005a; Tsugawa et al., 2006]. To interpret the cross-reactivity of IgM and IgA, further investigations are needed based on cases of NoV primary infection.

Recent studies have shown that HBGA may function as receptors of NoV through outbreak investigation [Hennessy et al., 2003; Rockx et al., 2005b] and volunteer challenge studies [Hutson et al., 2002; Lindesmith et al., 2003]. The relation between HBGA and immune response to NoV infection was unknown, because the HBGA type of these two patients was unknown, but these are cases, which were naturally infected with NoV and the typical symptoms appeared, and will represent general immune response to primary NoV infection. This is the first study on humoral immune response in putative primary NoV infection using 11 different antigenic and genetic types of VLPs. Our data will improve understanding of the humoral immune response to NoV infection. However, only two cases were examined in this study. To interpret the immunity for NoV infection, further investigations at the human level are needed.

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Epidemic of Genotype GII.2 Noroviruses during Spring 2004 in Osaka City, Japan[▽]

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Between March and May 2004, a GII.2 genotype norovirus strain caused an epidemic of acute gastroenteritis in Osaka, Japan. Phylogenetic analysis showed that this strain was distinct from all other GII.2 strains detected in Osaka City between April 1996 and March 2005.

Noroviruses (NoVs) are a major cause of acute gastroenteritis worldwide. Their transmission modes are food, person-to-person contact, and environmental contamination (5). In many countries, cold weather seasonality of NoV infections has been observed (9, 13, 14). The human NoVs are divided into three genogroups (GI, GII, and GIV), of which GI and GII strains are the most commonly found (2, 21). Within a genogroup, strains can be further divided into genotypes based on >80%

TABLE 1. Description of outbreaks in which NoVs were detected in Osaka City, Japan, between March and May 2004[a]

Outbreak no.	Day(s)/mo	Place	Source	Age group	Attack rate (no. ill/no. at risk)	No. of specimens		Genotype(s) (capsid)
						Total	NoV positive	
04032	3/March	Restaurant	Oysters	Adults	9/12	7	5	GI.1, GII.5
04034[b]	8/March	Restaurant	Oysters	Adults	3/3	2	1	GI.1, GII.8
04037[b]	6/March	Restaurant	Oysters	Adults	2/2	2	1	GII.12[c]
04038	**11/March**	**Restaurant**	**UK[d]**	**Children**	**29/60**	**29**	**22**	**GII.2**
04039[b]	**15/March**	**Home**	**UK**	**Children**	**2/UK**	**2**	**1**	**GII.2**
04041	14/May	Restaurant	Food	Adults	2/UK	2	2	GII.5
04042	**14/March**	**Restaurant**	**Food**	**Adults**	**40/71**	**10**	**9**	**GII.2**
04043	**17/March**	**Kindergarten**	**PP[e]**	**Children**	**20/UK**	**2**	**2**	**GII.2**
04047[f]	3/April	Hotel	Food	Adults	162/565	3	3	GII.4
04048[f]	7/April	Restaurant	Oysters	Adults	6/14	1	1	GII.8
04056	**12–13/April**	**Kindergarten**	**PP**	**Children**	**114/UK**	**60**	**50**	**GII.2**
04057[f]	23/April	Hotel	UK	Adults	325/796	1	1	GII.4
04059	**18–30/April**	**School**	**PP**	**Children**	**268/UK**	**84**	**74**	**GII.2**
04062[f]	1/May	Restaurant	UK	Adults	72/176	2	2	GII.6
04067	**10–15/May**	**School**	**PP**	**Children**	**154/UK**	**41**	**26**	**GII.2**
04071	**16–23/May**	**Kindergarten**	**PP**	**Children**	**95/UK**	**56**	**49**	**GII.2**
04073	**22/May**	**Restaurant**	**UK**	**Adults**	**4/5**	**2**	**2**	**GII.2**
04075	**25/May**	**School**	**PP**	**Children**	**41/UK**	**22**	**19**	**GII.2**
04076	**25–26/May**	**School**	**PP**	**Children**	**11/UK**	**9**	**9**	**GII.2**

[a] GII.2-cap NoV-associated outbreaks are indicated in boldface.
[b] Only one sample tested positive for NoV, but the outbreak was confirmed based on epidemiological data.
[c] Kageyama et al. (10).
[d] UK, unknown route.
[e] PP, person-to-person contact.
[f] The outbreaks occurred in other cities and had other NoV-positive patients.

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sequence identity in the complete capsid protein VP1 (5, 23). However, for molecular epidemiological investigations, tentative genotyping methods based on partial genomic sequencing of the RNA-dependent RNA polymerase (RdRp) and capsid genes are commonly used (3, 10, 19, 20). Between March and May 2004, an unusual increase in NoV-associated outbreaks was observed in Osaka City, Japan.

In Osaka City, with a population of approximately 2.6 million, NoV surveillance is conducted by collecting a basic set of epidemiological data (age range of patients, setting of outbreak, mode of transmission, date of onset, and attack rate) and testing stool specimens. An outbreak of acute gastroenteritis is defined as two or more patients with diarrhea and or vomiting who are linked by place and time. Patients with acute gastroenteritis attending sentinel pediatric clinics in Osaka are included as sporadic cases (8). Stool specimens were tested for NoV by reverse transcription-PCR (RT-PCR) using primers targeting the RdRp region until April 2001 (9) and by real-time RT-PCR since that time (18). All GII.2 strains were characterized by both partial RdRp and capsid gene sequencing as follows. RT-PCR assays were developed to amplify long genomic fragments using different sets of primers: (i) primer pair LV4282-99F (5′-YCAY TATGATGCWGAYTA-3′)/N235Rex (5′-GCWANRAAAGCT CCWGCCAT-3′) for the partial RdRp and the complete capsid genes (2,451 bp) and (ii) LV4282-99F/G2SKR (12) for the partial RdRp and the capsid N-terminal/shell (N/S) genes (1,108 bp). The amplified fragments were sequenced in both orientations with the primers. Phylogenetic analysis and genotyping based on the capsid N/S domain were performed as described by Katayama et al. (11). Assignment of genotype was based on the complete VP1 sequence according to Zheng et al. (23) and expressed as "genotype number-cap" (for example, "GII.2-cap"). Genotyping based on the RdRp region was performed using the criteria described by Vinjé et al. (19). The RdRp genotype was expressed as "genotype number-pol" (for example, "GII.2-pol").

A total of 238 NoV-positive outbreaks and 300 positive sporadic cases were detected between April 1996 and March 2005. Most (91.6%) of the NoV-positive outbreaks occurred between November and March of each year, whereas 85.0% of the NoV-positive sporadic cases occurred between October and February of each year. Between March and May 2004, 11 GII.2-cap NoV-associated outbreaks were observed (Table 1). In other years, a total of eight genetically different GII.2-cap strains, found on a separate branch on the phylogenetic tree (Fig. 1A), were detected. Thus, the number of the GII.2-cap NoV-associated outbreaks in the spring of 2004 was unusual compared with those for other seasons and higher than in all previous years (Poisson distribution, $P < 0.0001$). No NoV-associated outbreaks were observed between June and October 2004.

Of the 11 GII.2-cap NoV-associated outbreaks in the spring of 2004, nine occurred in children (81.8%), whose most common transmission mode was person-to-person contact (63.6%) (Table 1). In both children and adults, symptoms in GII.2-cap NoV-associated outbreaks were similar to those in outbreaks caused by other NoV genotypes. No epidemiological links were found among the outbreaks that could explain their spring emergence. In contrast, the eight genetically different GII.2-cap strains observed during our

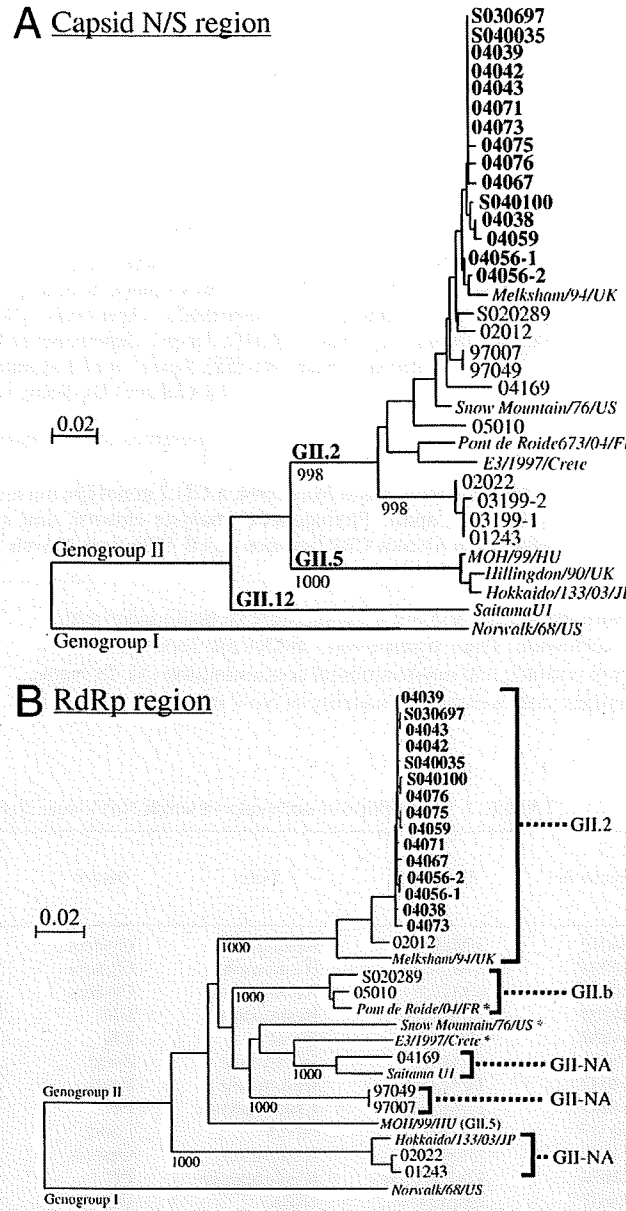

FIG. 1. Phylogenetic analysis of the capsid N/S (278 nucleotides) (A) and the partial RdRp (B) regions of the GII.2-cap strains detected in Osaka City. The GII.2-cap strains detected between March and May 2004 (04spring strains) are represented in boldface. Reference strains of NoV used in this study are represented in italics. The bootstrap values are indicated on each branch. The scale indicates the number of substitutions per site. (A) In outbreaks 03199 and 04056, there were two kinds of sequences, whereas all other outbreaks had only one type of sequence. (B) The tree was constructed with 720 nucleotides of the 3′ end of ORF1. Strains 03199-1 and -2 could not be amplified in the RdRp gene. The asterisks indicate the GII.2-cap NoVs, which have been reported as the GII.2-capsid sequences associated with other RdRp sequences (1, 3, 7). The genotypes at the RdRp region, which are not assigned numbers, are represented as GII-NA. The GenBank accession numbers for the reference strains of NoV used in this study are as follows, E3/97/Crete, AY682552; Hillingdon/90/UK, AJ277607; Hokkaido/133/03/JP, AB212306; Melksham/94/UK, X81879; MOH/99/HU, AF397256; Norwalk/68/US, M87661; Pont de Roide 673/04/FR, AY682549; Saitama U1, AB039775; Snow Mountain/76/US, AY134748.

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9-year NoV surveillance were found mainly in December or January, mostly in adults, with transmission by the consumption of contaminated foods. Among sporadic cases, three GII.2-cap strains were detected in the spring of 2004. These cases seemed to be epidemiologically unrelated to the 11 outbreaks of the same period. From the genetic analysis, all GII.2-cap strains detected during the spring of 2004 (04spring strains) were classified into the GII.2 genotype at the RdRp region and were closely related to one another (≥99.1% nucleotide and ≥98.5% nucleotide identities in RdRp and capsid N/S regions, respectively). The eight genetically distinct GII.2-cap strains from other seasons were segregated into GII.2 (strain 02012) and other four other genotypes (one GII.b and three GII-NA) at the RdRp region, suggesting that these four were recombinant strains (Fig. 1B). Comparison of the amino acid sequences of the complete capsid genes revealed no common difference between the 04spring strains and the other GII2-cap strains (data not shown).

In this study, we focused on an unusual cluster of GII.2 NoV-associated outbreaks in spring 2004 in Osaka City. These GII.2-cap strains were rare in Osaka City in the previous 9 years of our surveillance. The spring 2004 outbreaks were distinct from the other GII.2-cap NoV-associated outbreaks in seasonality (spring versus winter), age of patients (children versus adults), and transmission mode (contact versus food). These occurrences could be explained by the rarity of GII.2 strains in the population. Since the strains were rare, children in Osaka City most likely did not have antibodies to the 04spring strains. The genetic characterization of these strains showed that they formed a distinct cluster that suddenly appeared, spread in Osaka City for a few months, and disappeared. Their disappearance may reflect acquisition of immunity to the 04spring strains in the population. Previous reports described the sudden emergence and disappearance of certain genotypes of NoV (6, 8, 9, 17) in a limited region. For GII.4 strains, this phenomenon has been observed globally (13, 15, 16, 22). It is unclear why differences in behavior exist among NoVs belonging to different genotypes. The emergence of a GII.2 strain with matching RdRp and capsid genotypes as the dominant cause of a cluster of outbreaks suggests that recombination may affect the behavior of NoV strains. Most other GII.2 viruses found throughout the surveillance period were recombinant strains detected in isolated outbreaks. Gallimore et al. (4) likewise suggest that variants differ in their impact on public health according to the accumulation of point mutations and recombinants. Future studies using structured surveillance are needed to address this hypothesis and improve our understanding of NoV epidemiology. Such insight is essential to design evidence-based strategies for NoV control and prevention.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in DDBJ with the following accession numbers: AB089882 and AB279553 to AB279576.

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